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**Evaluation of fungal diversity in the rhizosphere of
Alkanna tinctoria and impact of selected fungi on growth
and production of secondary metabolites in *Alkanna
tinctoria* and *Echium vulgare***

Thèse de doctorat présentée par

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Table of content

Table of content

Table of content	I
Acknowledgements.....	V
List of abbreviations	VII
Glossary	XI
Summary	XIII
Outline of the thesis	XV
Author's contribution.....	XIX
INTRODUCTION.....	1
STATE OF THE ART.....	5
I. Medicinal plants	5
I.1. Importance as producers of natural biological compounds	5
I.2. Major secondary metabolites and their metabolism	5
I.3. The Boraginaceae family	8
I.3.1. <i>Alkanna tinctoria</i>	8
1.3.1.1. Origin of the plant.....	8
I.3.1.2. Botanical description	8
I.3.1.3. Uses of the plant	9
I.3.2. <i>Echium vulgare</i>	10
I.3.2.1. Origin of the plant.....	10
I.3.2.2. Botanical description	10
I.3.2.3. Uses of the plant	11
I.4. Methods of production of alkannin/shikonin and their derivatives	11
I.5. Alkannin/shikonin and their derivatives biosynthesis pathway	12
I.6. Secondary metabolites quantification and identification methods	14
II. Arbuscular mycorrhiza fungi (AMF)	16

Table of content

II.1. AMF life cycle	17
II.2. Phylogeny of arbuscular mycorrhizal fungi	21
II.3. General benefits of arbuscular mycorrhizal fungi	26
II.4. Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants	27
III. Endophytic fungi.....	79
III.1. Phylogeny of endophytic fungi	79
III.2. General benefits of endophytic fungi	82
III.3. Endophytic fungi and production of secondary metabolites in medicinal plants.....	83
RESEARCH QUESTIONS	89
MATERIALS AND METHODS	91
I. Biological Materials	91
I.1. Arbuscular mycorrhizal fungi	91
I.2. Endophytic fungi isolated from <i>A. tinctoria</i> in the wild	98
I.3. Plant species.....	101
II. Endophytic fungal community study	103
III. qPCR to study gene expression regulating A/Sd biosynthesis	109
IV. Evaluation of root colonization by AMF.....	115
RESEARCH RESULTS.....	119
PART I <i>ALKANNA TINCTORIA</i> STUDIES	121
Chapter I Metabolite production in <i>Alkanna tinctoria</i> links plant development with the recruitment of individual members of microbiome thriving at the root soil interface	125
Chapter II Arbuscular mycorrhizal fungi impact the production of alkannin/shikonin and their derivatives in <i>Alkanna tinctoria</i> Tausch. grown in semi-hydroponic and pot cultivation systems	163
Chapter III Impact of arbuscular mycorrhiza fungi on the production of alkannin/shikonin and their derivatives in the hairy roots of <i>Alkanna tinctoria</i>	207
PART II <i>ECHIUUM VULGARE</i> STUDY	223

Table of content

Chapter IV Effects of fungal endophytes and arbuscular mycorrhizal fungi on growth of <i>Echium vulgare</i> and alkannin/shikonin and their derivatives production in roots	227
GENERAL DISCUSSIONS.....	255
CONCLUSIONS AND PERSPECTIVES	265
ANNEXES	269
OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS	319
REFERENCES	321

Table of content

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List of abbreviations

List of abbreviations

ACC	1-Aminocyclopropane-1-Carboxylate
ACN	Acetonitrile
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
AOS	Allene Oxidase Synthase
A/S	Alkannin/Shikonin
A/Sd	Alkannin/Shikonin and their derivatives
ASV	Amplicon Sequence Variant
BAP	6-Benzylaminopurine
CTAB	Cetyltrimethylammonium Bromide
CHS	Chalcone Synthase
DEPC	Diethylpyrocarbonate
DMAPP	Dimethylallyl Pyrophosphate
DNA	Deoxyribose Nucleic Acid
DXR	1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase
DXS	1-Deoxy-D-xylulose 5-Phosphate Synthase
EDTA	Ethylenediaminetetraacetic Acid
ERM	Extracellular Mycelium
FA	Formic Acid
GA3	Gibberellic Acid
GAs	Gibberellins
GHQ	Geranylhydroquinone
GHQ-3''-OH	3''-Hydroxy-Geranylhydroquinone

List of abbreviations

GHQH	Geranylhydroquinone 3''-Hydroxylase
GINCO	Glomeromycota IN vitro COllection
HAM-P	Half-Closed Arbuscular Mycorrhizal-Plant
HAO	Hellenic Agricultural Organization
HC	Hyphal Compartment
HNQs	Hydroxynaphthoquinones
HPLC	High Performance Liquid Chromatography
HPLC-PDA	High Performance Liquid Chromatography Photodiode Array
HRC	Hariy Root Culture
HRMS	High-Resolution Mass Spectrometry
IPP	Isopentenyl Pyrophosphate
JA	Jasmonic Acid
LC-MS	Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LSU	Large SubUnit (gene)
MAFFT	Multiple Alignment using Fast Fourier Transform
MDP	Mycorrhizal Donor-Plant
MEP	Methyletriphosphate
MS	Mass Spectrometry
MS medium	Murashige Skoog Medium
MSR medium	Modified Strullu-Romand Medium
MUCL	Mycothèque de l'Université Catholique de Louvain
MVA	Mevalonic Acid

List of abbreviations

NGS	Next Generation Sequencing
PAL	L-Phenylalanine Ammonia Lyase
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
PGT	P-Hydroxybenzoate Geranyltransferase
PPFD	Photosynthetic Photon Flux Density
PVP	Polyvinylpyrrolidone
RC	Root Compartment
Ri T-DNA	Ri Plasmid Transferred DNA
RNA	Ribonucleic Acid
ROC	Root Organ Culture
RT	Retention Time
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
S-H	Semi-Hydroponic
SMs	Secondary Metabolites
SBS	Sequencing by Synthesis
SPE	Solid-Phase Extraction
TA	Tropane Alkaloids
TFA	Trifluoroacetic Acid
TPS	Terpene Synthase
UHPLC	Ultra-High Performance Liquid Chromatography

List of abbreviations

Glossary

Alpha diversity (α -diversity): refers to diversity on a local scale, describing the species diversity (richness) within a functional community (Andermann et al., 2022).

Arbuscular mycorrhiza fungi: symbiotic biotrophs that form close relationships with a host plant via intracellular fungal structures, namely arbuscules, in root cortical cells (Rillig et al., 2002).

Beta diversity (β -diversity): describes the amount of differentiation between species communities (Andermann et al., 2022).

Core microbiome: any set of microbial taxa, as well as the associated genomic or functional attributes characteristic of a specific host or environment (Neu et al., 2021).

Elicitation: refers to the induction of secondary metabolite production by the molecules or treatments called elicitors (Halder et al., 2019).

Endophytes: an endosymbiotic group of microorganisms – often bacteria or fungi – that colonize the inter- and/or intracellular locations of plants (Pimentel et al., 2011; Singh and Dubey, 2015).

Hairy root culture: a type of plant tissue culture that raised after the infection of explants/cultures by the gram negative soil bacterium *Rhizobium rhizogenes* (previously referred to as *Agrobacterium rhizogenes*) (Gutierrez-Valdes et al., 2020). It is also known as transformed root culture and used to study plant metabolic processes, producing valuable secondary metabolites or recombinant proteins often with plant genetic engineering (Sévon and Oksman, 2002).

Library preparation: a molecular biology protocol that converts a genomic DNA sample (or cDNA sample) into a sequencing library, which can then be sequenced on an NGS instrument. The first step in library preparation is random fragmentation of the DNA sample, followed by ligation of 5' and 3' adapters to each DNA fragment. Alternatively, “tagmentation” combines the fragmentation and ligation reactions into a single step and greatly increases the efficiency of the library preparation process (<https://emea.illumina.com/science/technology/next-generation-sequencing/beginners/glossary.html>).

Microbiome: the collective genomes of the micro-organisms in a particular environment (Valdes et al., 2018).

Glossary

Microbiota: *the community of micro-organisms themselves* (Valdes et al., 2018).

Metabolome: *all the small molecules that can be found in a cell, organ, or organism and contains the downstream products of genomics, transcriptomics, and proteomics, as well as the results from genome-environment interactions* (Chin and Slupsky, 2013).

Metabolomics: *large-scale study of small molecules, commonly known as metabolites, within cells, biofluids, tissues or organisms* (<https://www.ebi.ac.uk/training/online/courses/metabolomics-introduction/what-is/>).

Medicinal plants: *any plant that in one or more of its organs, contains substances that can be used for therapeutic purposes or are precursors for the synthesis of useful drugs* (Sofowora et al., 2013).

Secondary metabolites: *natural products synthesized by an organism that are not essential to support growth and life but serve survival functions for the organisms producing them* (Pyne et al., 2019; Demain and Fang, 2000).

Summary

Plants roots are associated with very diverse microbial communities among which endophytic bacteria and fungi and arbuscular mycorrhizal fungi (AMF). These microorganisms play key roles in plant health, development and productivity and may as well influence plant metabolome, thus impacting the production of secondary metabolites (SMs).

Alkanna tinctoria L. and *Echium vulgare* are plant species belonging to the Boraginaceae family, with well-known medicinal virtues. Their roots are rich in naphthoquinones, especially alkannin, shikonin (A/S) and their derivatives (A/Sd), whose pharmaceutical uses are widely documented. However, if and how microorganisms associated to the roots of these plants influence the production of SMs (e.g. A/Sd) are practically unknown.

In a first step, *A. tinctoria* was grown in three distinct soils having thus different microbiomes. An in-deep assessment of the bacterial and fungal root-associated communities was made at four distinct developmental stages (vegetative growth, blooming, fruiting and decay stages) of the plant and primary and secondary metabolites analyzed. The most important driver influencing the plant metabolites content was the developmental stage, with the highest contents of A/Sd measured at flowering. Root microbial diversity was influenced both by soil origin and to a lesser extent by the developmental stage. **In a second step**, AMF and endophytic root fungi isolated from *A. tinctoria* plants grown in the wild in Greece were isolated and identified. The AMF isolates (the native *Rhizophagus irregularis* and *Septoglomus viscosum*) and one AMF strains from the Glomeromycota *in vitro* collection (*Rhizophagus irregularis* MUCL 41833) were tested in a semi-hydroponic cultivation system and in pots on plant growth, genes regulating the SMs biosynthesis and SMs production. The plants inoculated with the native *R. irregularis* had a significantly higher production of total A/Sd content in both systems compared with those inoculated with the other AMF strains or with the non-inoculated controls. In addition, a significantly higher production of shikonin content was observed in *A. tinctoria* plants inoculated with the native *R. irregularis*, in pots as compared with the other treatments. **In a third step**, hairy root cultures (HRCs) of *A. tinctoria* were associated with the native *R. irregularis* and with *R. irregularis* MUCL 41833 to evaluate their effects on A/Sd production in roots. None of the strains impacted

Summary

the content of A/Sd in the hairy roots. Moreover, the antifungal effect of *A. tinctoria* hairy roots on AMF (via the possible release of A/Sd) was evaluated by incubating spores of 3 different *R. irregularis* strains (native, MUCL 41833 and MUCL 43194) in the vicinity of *A. tinctoria* hairy roots. Spore germination of the native and MUCL 43194 strains was not impacted. This was also the case for *R. irregularis* MUCL 41833, though a significantly lower germination rate was observed as compared with the two other strains. **In a last step**, 10 endophytic fungi isolated from *A. tinctoria* in the wild and 5 AMF from GINCO (*Rhizophagus clarus* MUCL 46238, *Rhizophagus irregularis* MUCL 41833, *Glomus aggregatum* MUCL 49408, *Rhizophagus intraradices* MUCL 49410 and *Glomus hoi* MUCL 45686) were tested in the greenhouse on growth of *E. vulgare* and A/Sd production in roots. Some of the endophytic fungi tested increased plant growth parameters, with *Trichoderma hispanicum* being the most promising. However, none impacted A/Sd production. Conversely, none of the AMF strains tested increased plant growth but those inoculated with *R. intraradices* MUCL 49410 had a significantly higher concentration of alkannin/shikonin (A/S), acetyl-A/S, β , β – dimethylacryl-A/S, isovaleryl-A/S and total A/Sd compared to the control plants.

In conclusion, these studies indicate the importance of applying endophytic fungi and AMF in the production of SMs in medicinal plants. Further studies are needed to investigate the mechanisms involved in the production of SMs, especially A/Sd in plants associated to specific endophytic fungi /AMF and on the cultivation conditions needed for optimal production.

Outline of the thesis

Within this thesis, different experiments were conducted to address the following four main objectives:

Investigate the relationship between, on the one hand, the microorganisms associated with the roots of *A. tinctoria* and, on the other hand, the type of soil or the growth stage and production of A/Sd of the plant.

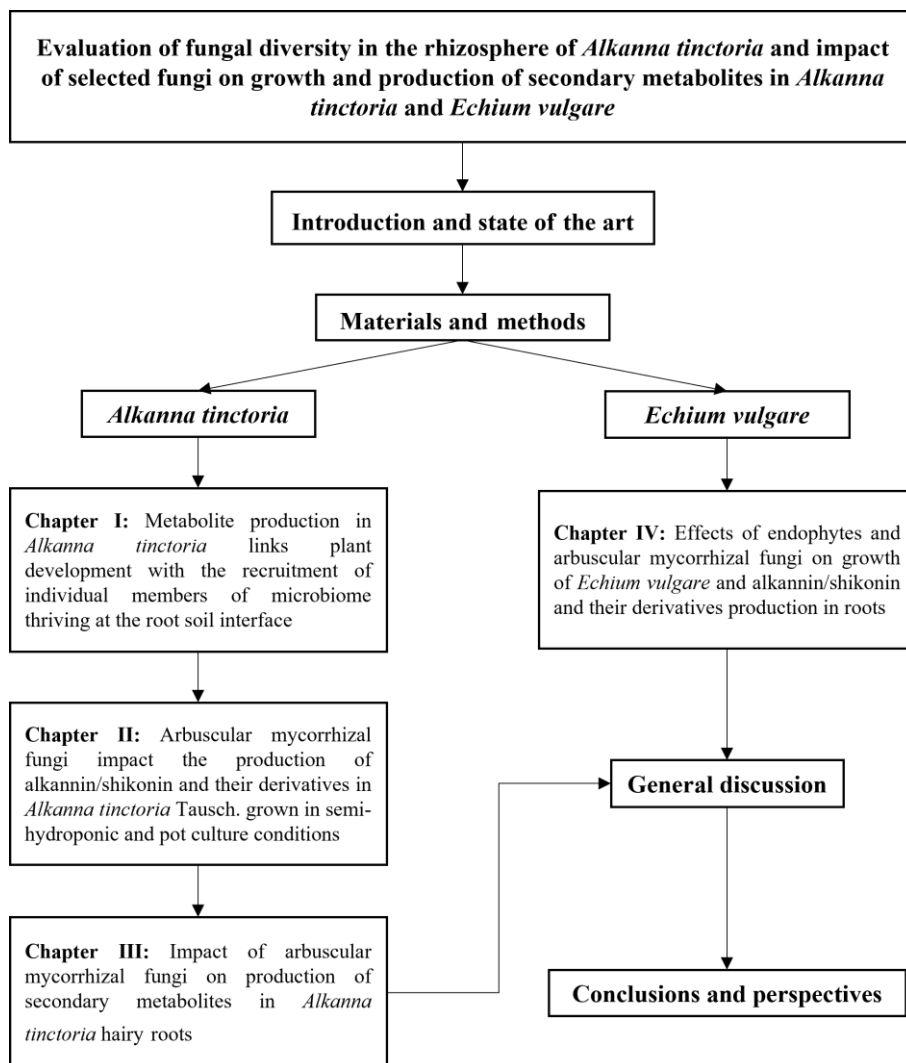
Isolate, identify and characterize AMF from roots of *A. tinctoria* collected from the wild in Greece and test their effects on growth and production of SMs (with focus on A/Sd) of *A. tinctoria*.

Evaluate the effect of the AMF *R. irregularis* isolated from roots of *A. tinctoria* on the production of A/Sd in transformed hairy roots of this plant.

Isolate, identify and characterize fungal endophytes from roots of *A. tinctoria* collected from the wild in Greece and test their effects on growth and production of SMs (with focus on A/Sd) of *E. vulgare*.

Outline of the thesis

The successive steps undertaken to answer these objectives are schematised below.



In the **Introduction** section, the context and the general objectives of the thesis were presented.

In the **State of the Art** section, an overview on medicinal plants and the major bioactive compounds they produce was presented. We further described the Boraginaceae family with focus on *A. tinctoria* and *E. vulgare*, two major medicinal plants. We continued by describing the biosynthetic pathway of A/Sd and the methods used for the production of these metabolites. An exhaustive bibliography was also made on

Outline of the thesis

AMF and endophytic fungi with focus on their phylogeny, classification, ecological importance and relationships with medicinal plants and SMs production. This section was concluded with a detailed and in-depth review of the literature on the effects of AMF on the production of SMs in medicinal plants, which was published as a review paper in *Mycorrhiza* (2022).

In the **Materials and Methods** section, we detailed the biological material and provided a brief description of the most important methods and techniques used throughout the thesis.

The **Research results** section was divided in two parts focusing on *Alkanna tinctoria* Tausch. (Part I) and *Echium vulgare* L. (Part II), respectively.

Part I was divided in three chapters:

Chapter I aimed at analyzing the microbiome and metabolome of *A. tinctoria* plants at different developmental stage and grown in different soils. The bacterial and fungal microorganisms were detected by amplicon sequencing of 16S rRNA and ITS markers, respectively. The metabolites were identified via a combination of ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) and high-performance liquid chromatography with UV/Vis (HPLC-UV/Vis) detection. Correlation analyses were conducted on the metabolites content and abundance of individual bacterial and fungal taxa. The results showed that the plant developmental stage was the most important driver influencing the plant metabolite content, revealing peak contents of A/Sd at the fruiting stage. Root microbial diversity was both influenced by soil origin and to a small extent by the developmental stage. The bacterial genera *Labrys* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, as well as four species of the fungal genus *Penicillium* were found to be positively correlated with higher content of alkannins.

This work was published in journal *mSystem* (2022): Metabolite production in *Alkanna tinctoria* links plant development with the recruitment of individual members of microbiome thriving at the root-soil interface.

Chapter II aimed at comparing native AMF species isolated from *A. tinctoria* plants collected from the wild in Greece with AMF species from the Glomeromycota IN vitro Collection (GINCO) on growth and A/Sd production of *A. tinctoria*. The experiment was conducted in pots

Outline of the thesis

and in a semi-hydroponic cultivation system under greenhouse conditions and SMs were analyzed by HPLC-PDA and HPLC-HRMS/MS. In parallel, the expression of genes regulating the shikonin and their derivatives biosynthesis was investigated by qPCR. Two AMF species from the wild (*R. irregularis* and *Septoglomus viscosum*) were identified and compared with *R. irregularis* MUCL 41833 in pots and *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408 in the semi-hydroponic cultivation system. *Alkanna tinctoria* inoculated with the native AMF *R. irregularis* showed a significantly higher production of A/Sd production in both the S-H and pot cultivation systems. Different gene expressions were observed in these three experiments.

This work has been submitted to *Frontiers in Plant Science*.

Chapter III aimed at comparing the effects of the native AMF *R. irregularis* with *R. irregularis* MUCL 41833 (from GINCO) on A/Sd production of *A. tinctoria* hairy roots. The experiment was conducted in an *in vitro* bi-compartmental Petri plate system and SMs analyzed by LC-MS. In parallel, a spore germination test was conducted with the three AMF strains inoculated in the vicinity of hairy roots of *A. tinctoria* and *Daucus carota* L. clone 1E. No significant impact was noticed on A/Sd production between the native AMF *R. irregularis*, *R. irregularis* MUCL 41833 and the non-AMF inoculated hairy root. Also, no significant impact of hairy root type was noticed on spore germination of the three AMF.

Part II contained a single chapter:

Chapter IV aimed at investigating the effects of endophytic fungi on growth and production of A/Sd of *E. vulgare* plants grown in the greenhouse. The fungal endophytes tested were isolated and identified from field collected *A. tinctoria* plants. In addition, 5 AMF strains selected from GINCO were tested on growth and production of A/Sd. No significant impact was noticed on growth and A/Sd production suggesting a strain-host specificity between plant and fungi.

In the **General Discussion** section, the major findings of the thesis were summarized and discussed.

Finally, in the **Conclusion and Perspectives** section, the major outcomes of the thesis were reviewed and commented. Further studies were proposed to improve our knowledge on the mechanism involved in the production of SMs in medicinal plants inoculated with AMF or endophytic fungi.

Author's contribution

The section Introduction as well as the General Discussion, Conclusions and Perspectives have not been published. They were written by myself. Part of the State of the Art was published as a review paper and was fully written by myself. Co-authors of the review have been involved in revising the manuscript.

For the chapters below, all the experimental designs and research questions were done with discussion of the supervisors Prof. Stéphane Declerck and Dr. Ismahen Lalaymia (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology).

Chapter I is a research paper published in *mSystem* (2022). My contribution to this paper was approximately 50%. The experiment set up and sample collection were performed by myself with the help of Dr. Ismahen Lalaymia (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) and Cintia Csorba (PhD at AIT Austrian Institute of Technology GmbH, Center for Health & Bioresources, Bioresources Unit). Microbiome related laboratory molecular work was done by myself and Cintia Csorba. Nebojša Rodić (PhD) and Dr. Angeliki Vlachou (Aristotle University of Thessaloniki, School of Chemical Engineering, Laboratory of Organic Chemistry and Center of Interdisciplinary Research and Innovation of AUTH, Natural Products Research Centre of Excellence (NatPro-AUTH), Thessaloniki, Greece) performed the metabolomics analysis. Dr. Livio Antonielli (AIT Austrian Institute of Technology GmbH, Center for Health & Bioresources, Bioresources Unit) performed the bioinformatical pipeline on the raw Illumina MiSeq datasets and provided valuable insights for the microbial ecology data analysis. Cintia Csorba (PhD) performed the microbial ecology data analysis and microbiome-metabolome correlations. Evangelia Tsiokanos (PhD at Department of Pharmacognosy and Natural Product Chemistry of the National and Kapodistrian University of Athens) processed aboveground plant material for UHPLC-HRMS analysis, which due-to unfortunate sample loss generated design imbalance, and could not be used in the present study, but her tremendous work was acknowledged in the form of authorship. Cintia Csorba, Nebojša Rodić, Yanyan Zhao and Livio Antonielli wrote the manuscript. All authors read and approved the final manuscript.

Chapter II is a research paper published in *Fontiers in Microbiology*.

Author's contribution

My contribution to this paper was approximately 50%. Isolation of AMF was performed by myself with the help of Dr. Ismahen Lalaymia (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology). Identification of AMF was done by myself with the help of Dr. Mónica Garcés-Ruiz (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology) on the phylogenetic tree making of AMF. The greenhouse experiment setup, data collection on AMF growth parameters and plant harvest were conducted by myself with the help of Ph.D. Annalisa Cartabia (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology). Extraction of the metabolites in the pot experiment, A/Sd production and genes expression was conducted by myself. LC/MS analysis were done with the help of Dr. Sergio Ortiz and Dr. Marie-France Herent (Faculty of Pharmacy and Biomedical Sciences Pharmacognosy lab, Louvain Drug Research Institute, UCLouvain) under the supervision of Prof. J. Quetin-Leclercq. Data analysis (plant growth parameter, root colonization and gene expression) was done by Dr. Ismahen Lalaymia and Ph.D. Annalisa Cartabia. Ph.D. Annalisa Cartabia and I wrote the manuscript. Co-authors of the chapter have been involved in revising the manuscript.

Chapter III is a research experiment conducted under *in vitro* culture condition. My contribution to this chapter was estimated at 80%. The *A. tictoria* hairy roots were provided by Angélique Rat (PhD at the University of Ghent, Belgium). The experimental set up was done by myself with the help of Dr. Ismahen Lalaymia. Harvest of plants and medium and extraction of bioactive compounds from both were conducted by myself. Anthony Arguelles Arias (pHd at the Microbial Processes and Interactions (MiPI), University of Liege) performed the analysis of A/Sd by LC-MS and data analysis. The chapter was written by myself. The authors above have been involved in revising the chapter.

Chapter IV is a research paper submitted to the journal of Fungal biology. The experiments were conducted in the greenhouse. My contribution to this paper was approximately 80%. The isolation and identification of endophytic fungi from *A. tinctoria* roots, the experimental set up and the sample collection were done by myself with the help of Dr. Ismahen Lalaymia (UCLouvain Earth and Life Institute - Applied Microbiology - Laboratory of Mycology). The follow up of the experiment, plant growth parameter measurements, estimation of AMF root colonization and root sampling were done by myself.

Author's contribution

Nebojša Rodić (PhD at Aristotle University of Thessaloniki, School of Chemical Engineering, Laboratory of Organic Chemistry and Center of Interdisciplinary Research and Innovation of AUTH, Natural Products Research Centre of Excellence (NatPro-AUTH), Thessaloniki, Greece) performed the analysis of A/Sd and data analysis. The statistics were done with the help of Dr. Ismahen Lalaymia. The manuscript was fully written by myself. Co-authors of the chapter have been involved in revising the manuscript.

Author's contribution

INTRODUCTION

INTRODCTION

This PhD study was part of the innovative training networks (ITN) project MICROMETABOLITE funded by the European Union's Horizon 2020 research and innovative programme, under the Marie Skłodowska-Curie grant agreement (No 721635). The project was aimed to explore the plant-associated microflora for improving the production of bioactive secondary plant metabolites that are of high value for industrial applications. The project focused on members of the Boraginaceae plant family including *Lithospermum*, *Alkanna* and *Echium* species, which produce the naphthoquinones alkannin/shikonin and their derivatives (A/Sd).

Secondary metabolites (SMs) are small molecules produced by any lifeform (e.g., plants, animals, bacteria, fungi). In plants, they play essential roles in defence mechanisms and interactions with the environment. Secondary metabolites also constitute a huge reservoir of chemical substances representing active ingredients for several pharmaceuticals, cosmetics and food supplements.

As one of the most important groups of SMs, the naturally occurring naphthoquinone compound alkannin, its enantiomer shikonin (thereafter A/S) and its derivatives (thereafter A/Sd). These compounds are produced in the roots of medicinal plants (e.g., *Alkanna tinctoria*, *Arnebia euchroma*, *Echium lycoris*, *Echium vulgare*, *Eritrichium sericeum*, *Lithospermum erythrorhizon*, and *Onosma armeniacum*), belonging to the Boraginaceae family.

Alkanna tinctoria L. Tausch. (also called Dyer's Bugloss) native to the Mediterranean region is the most documented plant producing alkannin, mainly in the root bark with a dark red color (Mita et al., 1994). Shikonin, with red-purple color, is a major compound in the roots of *L. erythrorhizon* (Sieb. et Zucc.), a perennial herb native to Japan, Korea, and China (Yazaki et al., 2017), while *Echium vulgare* roots show strong fluctuation in alkannin and shikonin concentration within plant populations.

Alkannin and shikonin have traditionally been used as natural dyes in textile, food and cosmetic industries (Alihosseini and Sun 2011). Besides their use as pigments, A/Sd possess a broad spectrum of biological activities such as tissue regeneration, wound healing (Nikita et al., 2015), antimicrobial (Sasaki et al., 2002), anti-inflammatory (Yang et al., 2022) , tumor-inhibiting (Chen et al., 2011), anti-

INTRODUCTION

angiogenic (Hisa et al., 1998), anti-topoisomerase (Ahn et al., 1995) anti-HIV-1 (Chen et al., 2003) and anticancer (Andujar et al., 2013).

In nature, active compounds such as shikonin (Yazaki, 2017) are often present at concentration below 1% of the plant dry weight and are therefore not very profitable for commercial exploitation. To increase their production, growing plants under controlled conditions is an option but can also have disadvantages. For example, the cultivation of *L. erythrorhizon* is very difficult due to low seed germination, seedlings susceptibility to infection by viruses and sensitivity to disinfectants. It takes more than three years of culture to obtain mature plants producing sufficient quantities of crude drugs. Similarly, the culture of *Alkanna* sp. is difficult to establish under greenhouse conditions. The cultivation of plant cells and tissues/organs offers an alternative source of these metabolites. For example, shikonin has been produced industrially in Japan since 1983 by dedifferentiated cell cultures, but was limited to supplying the cosmetic industry (Fujita et al., 1983). The chemical synthesis of A/S has also been attempted but yield remained insufficient (0.7%) for industrial production (Terada et al., 1983). There is thus a need for innovative technologies based on novel approaches of plant cultivation.

Many microorganisms have been reported to influence SMs production by plants. Among these are the arbuscular mycorrhizal fungi (AMF) which colonize the roots of almost 70% of terrestrial plants (Stoyke and Currah, 1993). They provide numerous benefits to their hosts such as improved nutrition (mostly phosphorus) and growth, increased drought tolerance and resistance to pest and diseases (Smith and Read, 1997). They also modify plant metabolism and thus the production either qualitatively or quantitatively of metabolites (Smith and Read, 1997). For instance, *Castanospermum australe* (Abu-Zeyad et al., 1999), *Catharanthus roseus* (Andrade et al., 2013), *Phellodendron amurense* (Fan et al., 2006) and *Camptotheca acuminata* (Zhao et al., 2006) inoculated with AMF had higher contents in alkaloids.

Similarly to AMF, root endophytes (bacteria or fungi) colonizing healthy plant tissues intercellularly and/or intracellularly without causing any apparent symptoms of disease (Wilson, 1995), have been reported to promote the growth and increase the production of SMs in plants (Strobel et al., 2004). Jia et al. (2016) in a review of the past 30 years noticed that certain endophytes significantly influence the production of SMs in plants, affecting both the quality and quantity of

INTRODUCTION

crude drugs used as pharmaceuticals. Similarly, Venieraki et al. (2017) reported that endophytic fungi residing in medicinal plants are able to synthesize pharmaceutical active SMs similar or identical to those produced by their hosts. Selection and monitoring of appropriate microbial strains (AMF and endophytic fungi/bacteria) with cultivated medicinal plants could therefore be particularly useful in improving the quality and quantity of SMs produced (Khaosaad et al., 2006; Muthukumar et al., 2006).

In this perspective, the present thesis aims to study the fungal community associated with the roots of *A. tinctoria* during the different stages of plant development. In parallel, AMF from GINCO and fungal endophytes and AMF isolated from the roots of *A. tinctoria* grown in the wild in Greece were tested on growth and A/Sd production of *A. tinctoria* and *E. vulgare*. The experiments were conducted in pots, in a semi-hydroponic cultivation system or with hairy roots *in vitro* and A/Sd analysed by high-performance liquid chromatography coupled with photodiode array detection (HPLC-PDA) and ultra-high-performance liquid chromatography (UHPLC) or HPLC coupled with high-resolution mass spectrometry detection (UHPLC/HPLC-HRMS/MS).

INTRODUCTION

STATE OF THE ART

I. Medicinal plants

I.1. Importance as producers of natural biological compounds

Medicinal plants have been a valuable source of therapeutic agents to treat many infectious diseases, such as diarrhea, fever or cold, birth control and dental hygiene, since ancient times (Hossain et al., 2014; Khan, 2014). These plants initially took the form of crude drugs, such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004). Numerous modern methods including synthetic chemistry, combinatorial chemistry, and molecular modeling have been utilized for drug discovery (Ley and Baxendale, 2002; Geysen et al., 2003; Lombardino and Lowe, 2004). However, isolation from plants and other natural sources, remain an essential source of new drugs, new drug leads, and new chemical entities (NCEs) (Katiyar et al., 2012; Najmi et al., 2022), because of their better cultural acceptability, higher compatibility and adaptability with the human body compared with the synthetic ones (Garg et al., 2021).

In total between 50,000 and 80,000 flowering plant species are used for medicinal purposes according to the International Union for Conservation of Nature and the World Wildlife Fund (Chen et al., 2016). Up to 80% of people in developing countries are totally dependent on herbal drugs for their primary healthcare, and over 25% of prescribed medicines in developed countries are derived from wild plant species (Hamilton, 2004). The presence and richness of active compounds, mostly belonging to the secondary metabolism of medicinal plants, contribute to their therapeutic values.

I.2. Major secondary metabolites and their metabolism

Secondary metabolites (SM) are compounds that are not required for the growth or reproduction of an organism but are produced to confer a selective advantage to the organism (Craney et al., 2013). Thousands of structurally different SMs evolved during plant evolution as a defense against herbivores and pathogenic bacteria, fungi and viruses (Schmeller et al., 1997; Teuscher et al., 2010; Wink, 2003). Some SMs also serve as signal compounds to attract pollinating and seed-dispersing animals, or as antioxidants signaling and UV protectants. From the perspective of evolutionary pharmacology, plant SMs represent an exciting library of bioactive compounds filtered by natural

STATE OF THE ART

selection, which have been used by humans to treat infections and health disorders, or as spices, perfumes, poisons of arrows, toxins and pesticides (Wink, 2010; Perrine, 1996; Rättsch, 2005; Russo, 2001).

Among these SMs of medicinal plants, different chemical groups can be found, such as alkaloids, terpenoids, phenolics, saponines and naphthaquinones (Hussein and El-Anssary, 2018).

Alkaloids – Alkaloids are a large class of naturally occurring compounds which typically comprise at least one nitrogen atom and having a restricted distribution (Dey et al., 2020). More than 20,000 different alkaloids were already identified (Rajput et al., 2022).

Alkaloids are produced by plants either constitutively or in response to pests, diseases or other external stimuli (War et al., 2012) and are found in different organs. Many of these compounds possess potent pharmacological effects such as the well-known narcotic analgesics morphine, apomorphine and codeine, the muscle relaxant papaverine, the antimicrobial agents sanguinarine, berberine and quinine, and anticancer agent paclitaxel (Bribi, 2018). Other pharmacological activities have been reported such as anti-inflammatory, anti-malarial, anti-hypertensive, anti-diabetic, antioxidant,... (Bribi, 2018; Rajput et al., 2022).

Terpenoids – Terpenoids (also known as isoprenoids) are a large and diverse class of naturally occurring compounds derived from five carbon isoprene units (Reyes et al., 2018). They are synthesized in nature by two distinct biochemical pathways: (1) the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway and (2) the mevalonic acid (MVA) pathway (Banerjee and Sharkey, 2014; Frank and Groll, 2017; Phillips et al., 2008).

Terpenoids are essential for plant growth and development (Tholl, 2015). They also contribute to the flavor, scents, and color of leaves, flowers, and fruits and to the protection against insects, herbivores, fungal and bacterial diseases (Reyes et al., 2018). They are major constituents of essential oils produced by aromatic plants (Agatonovic-Kustrin et al., 2019). Essential oils are volatile lipophilic mixtures of SMs, consisting mostly of monoterpenes, sesquiterpenes, and phenylpropanoids, which often are used as flavors and fragrances, as antimicrobials and antioxidants, and as medicines (Hay and Waterman 1993). For example, many essential oils have been evaluated for anti-inflammatory and analgesic activity in a variety of cellular and animal

STATE OF THE ART

models (Leja et al., 2019; Sarmiento-Neto et al., 2015). Small clinical trials carried out with essential oils have demonstrated positive effects against dysmenorrhea (menstrual pain), infantile colic, traumatic or surgical joint-related pain, headaches, postherpetic neuralgia, and irritable bowel syndrome (Agatonovic-Kustrin and Morton, 2018).

Saponins are characterized by a polycyclic aglycone moiety with either a steroid (steroidal saponins) or triterpenoid (triterpenoidal saponins) attached to one or more carbohydrate unit(s) (Hussein and El-Anssary, 2018).

Saponins exhibit a wide range of biological and medicinal activities such as anti-inflammatory, antibacterial, antifungal, antiviral, insecticidal, anticancer (Just et al., 1998; Sparg et al., 2004; Sindambiwe et al., 1998; Simões et al., 1999; Geyter et al., 2007; Cheng et al., 2011). In addition, they are reported to exhibit cholesterol-lowering action in animals and humans (Oboh and Omofoma, 2008; Moghimipour and Handali, 2015). Saponins have been considered as a starting precursor for the semi-synthesis of steroidal drugs in the pharmaceutical industry (El Aziz et al., 2019).

Phenolics and derivatives— Phenolic compounds are among the most widely distributed SMs in plants consisting of one or more aromatic rings, with one or more hydroxyl groups attached (Cartea et al., 2011).

Currently, more than 8000 phenolic compound structures are known (Lourenço Neto et al., 2018) among which are flavonoids, curcuminoids, coumarins, tannins, stilbenes, lignans, phenolic acids and quinones (Cosme et al., 2020). They are synthesized through two metabolic pathways: (1) the shikimic acid pathway where phenylpropanoids are formed, and (2) the acetic acid pathway, in which the main products are the simple phenols and anthraquinones (Reis Giada, 2013). The combination between both pathways leads to the synthesis of flavonoids, the most abundant group of phenolic compounds in nature (Reis Giada, 2013).

Phenolics correlate with the flavor and color characteristics of fruits and vegetables (Cartea et al., 2011). In addition, they possess a remarkable spectrum of biological activities such as antimicrobial, antiallergic, anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic and anticancer activities (Rao et al., 2018; Tsao, 2010; Ferguson, 2001; Dewick, 2002; Lamoral-Theys et al., 2010).

Naphthoquinones – Naphthoquinones are structurally related to

STATE OF THE ART

naphthalene (Kollef et al., 2011) and characterized by two carbonyl groups in the 1,4 positions, named 1,4-naphthoquinones (Hughes et al., 2011) and in the 1,2 position, named 1,2-naphthoquinones.

Naphthoquinones are highly reactive organic compounds, used as natural or synthetic dyes whose colors range from yellow to red. As SMs of plants and microorganisms, they confer activity in various biological oxidative processes and represent a chemical defense used by many plants (López et al., 2014). Naphthoquinones and their derivatives have been shown to have antibacterial, antifungal, antiviral, antitumor and antimalarial activities (Liu et al., 2023; Wellington et al., 2019; Tandon et al., 2004; Liu et al., 2019; Schuck et al., 2013).

1.3. The Boraginaceae family

The Boraginaceae family include around 150 genera and more than 2700 plant species found worldwide (Moustafa, 2002; Berry, 2014). Plants in this family are most often herbaceous and hairy and can be annuals or perennials. Some are vines or trees, and a few are obligate parasites.

Some plants in the Boraginaceae family are important for pharmacology and cosmetology. For example, *Alkanna tinctoria* and *L. erythrorhizon* have been used for burns and wounds healing in folk medicine around the world (Assimopoulou and Papageorgiou, 2013). The therapeutic effect of these plants is related to the content of many biologically active compounds, including naphthaquinones, flavonoids, terpenoids and phenols, or purine derivative – allantoin – which exhibit antimicrobial, antitumor, antiviral, anti-inflammatory, cardiogenic, contraceptive and antiplatelet activities (Sharma et al., 2009; Papp et al., 2011; Taravati et al., 2014).

1.3.1. *Alkanna tinctoria*

1.3.1.1. Origin of the plant

Alkanna tinctoria L. (Tausch) is found across southern Europe, northern Africa, and southwestern Asia, with a central distribution across the Mediterranean region (Valdés, 2011). In particular, it grows wild in arid maritime areas of southern Europe. Although native to southern Europe, it also grows and is imported from Albania, India, Egypt, and Turkey (Bisset et al., 1994; Roeder et al., 1995).

1.3.1.2. Botanical description

Alkanna tinctoria (L. Taush) is an herbaceous plant belonging to the

STATE OF THE ART

genus *Alkanna* that contains about 50 species (Salimikia et al., 2015). It is a perennial plant growing 30 to 60 cm height with pubescent lanceolate leaves. The stem is rough and coarse with stiff hairs. It produces small blue-purple flowers from May to July (**Figure 1**) (Zannou and Koca, 2020). The roots are surrounded by large cortex scales where a dark reddish-brown color pigment (i.e., alkannin) is produced (Jaradat et al., 2018; Papageorgiou et al., 1999) (**Figure 1**).



Figure 1: Whole *Alkanna tinctoria* plant with flowers (left) growing in the wild (Copyright Efeetherios Dariotis (Liberto Dario), horticulturist and botanist from Athens, Greece: <https://davesgarden.com/guides/pf/showimage/448562/#b>) and *A. tinctoria* roots after washing with water (right)

1.3.1.3. Uses of the plant

Due to the red-purple alkannin produced in the roots, *A. tinctoria* has been used as a natural source of colorant for cosmetics (lipstick in particular for its sharp red colour), food (i.e., oils, butters, wines, confectionery, and sweets) and textiles (i.e., wool, silk, and cotton) since antiquity (Jaradat et al., 2018; Tung et al., 2013). Besides, alkanna roots have been utilized for centuries as crude sources of traditional medicines for curing ulcerous sores, wounds, burns, inflammations and infection diseases (Papageorgiou et al., 1999). The first recorded use of alkanna roots is attributed to the Greek doctor and philosopher Hippocrates (460-370 BC) who used the plant roots in the treatment of ulcers

(<http://webatomics.com/Classics/Hippocrates/ulcers.10.10.html>). Later shikonin, the r-enantiomer of alkannin (**Figure 2**) was also extracted from the plant (Chen et al., 2002). This compound is the representative compound in *L. erythrorhizon*. Both compounds contain double ring structure that conveys the intense red pigments. Many other derivatives of alkannin were detected in *Alkanna* sp., including acetylalkannin, propionylalkannin, isobutylalkannin, angelylalkannin, β , β -

STATE OF THE ART

dimethylacryl alkannin, isovalerylalkannin, α -methyl-n-butylalkannin, and β -hydroxy-isovalerylalkannin (Assimopoulou et al., 2006). These derivatives and their proportion were different within the same species grown in different regions and altitudes and between different plant species (Assimopoulou et al., 2006).

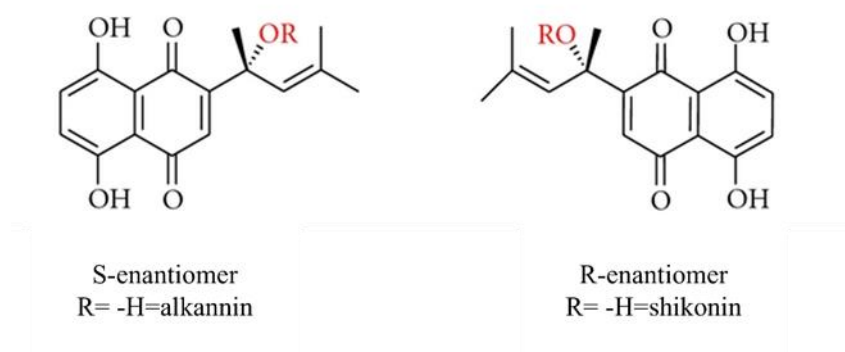


Figure 2: Structure of alkannin and shikonin (Haghbeen et al., 2011)

1.3.2. *Echium vulgare*

1.3.2.1. Origin of the plant

Echium vulgare L. is native to most of Europe as well as western and central Asia (Kapusterynska et al., 2020). It has naturalized over much of North America (except the far southeast and southwest) where it is commonly found in pastures, fields, disturbed sites, waste places and roadsides.

1.3.2.2. Botanical description

Echium vulgare (blueweed or viper's bugloss), belongs to the genus *Echium* containing 67 described species (Jin et al., 2020). It can be biennial or short-lived perennial (Kapusterynska et al., 2020). The stems of mature plants are straight with height of more than 70 cm. The leaves are alternate, drying up during flowering. There can be up to 50 cymes per stem, and each cyme bears up to 20 flowers on top. The showy flowers vary in size from 1 to 2 cm. The funnel-shaped, five-lobed flowers are typically bright blue, but may also be purple, pink or rarely, white (**Figure 3**). Roots consist of thick, sparsely branched, contractile taproots that may exceed 1 m in depth (Albertson, 1921). The seeds of *E. vulgare* are called nutlets. They are brown or gray with a rounded pyramid shape and are quite small (Kapusterynska et al., 2020).

STATE OF THE ART



Figure 3: *Echium vulgare* plant in rosette stage (left) (<http://www.zermattflora.net/content/echium-vulgare-vipers-bugloss-1/>) and flowering stage (right) growing in the wild (<https://strictlymedicalseeds.com/product/vipers-bugloss-echium-vulgare-seeds-organic-mt-wa-no/>)

I.3.2.3. Uses of the plant

Echium vulgare is known as an important wild honey plant (Klemow et al., 2002) and a good source of unique fatty acids, i.e., alpha-linolenic acid and stearidonic acid (Velasco and Goffman 1999; Krol and Kowalski, 2004; Mhamdi et al., 2009). Horticulturally, it is used most extensively as an annual or biennial border plant, in wildflower and rock gardens (von Miklos and Fiore, 1969; Hellyer, 1971). However, this species may spread beyond where it is planted (Aiken, 1969). Moreover, this plant species is particularly rich in pyrrolizidine alkaloids, flavonoids, phenolcarboxylic acids, sterones and naphthoquinones (Szabó, 2005) and has been commonly used in folk medicine in the Mediterranean region. The roots have been used in folk medicine for treatment of wound healing, bruising, pulled muscles, ligaments, and sprains in both Turkey and Germany (Eruygur et al., 2016; Sezik et al., 1997; Eruygur et al., 2012). Leaves, and flowers can be used as diuretic and cough medicine (Arroyo et al., 2017). Various plant species of *Echium* have been reported to possess antibacterial, antiinflammatory, antiproliferative, antidepressant, antioxidant, antiviral, anxiolytic and cytotoxic activities (Farahani et al., 2013).

I.4. Methods of production of alkannin/shikonin and their derivatives

Due to the increasing commercial importance of alkannin/shikonin and

STATE OF THE ART

their derivatives in cosmetics and pharmaceuticals, several cells/tissues culture methods of Boraginaceae plants that produce large amounts of A/Sd have been studied and developed (Tabata et al., 1974; Fukui et al., 1983; Wang et al., 2014; Malik et al., 2016) (**See section II.4**). For instance, callus induction of *L. erythrorhizon*, *A. euchroma*, *Echium lycopsis*, *Echium italicum* and *Onosma bulbotrichom* (Mizukami et al., 1977; Fu and Lu, 1999; Fukui et al., 1983; Zare et al., 2011; Bagheri et al., 2018), shoot culture, cell suspension culture and protoplast culture of *L. erythrorhizon* (Chung et al., 2006; Yazaki, 2017; Touno et al., 2005), as well as cell suspension culture of *A. euchroma* (Kumar et al., 2011; Damianakos et al., 2012) have been developed. This resulted in the 1980s in the production of shikonin by dedifferentiated cell cultures of *L. erythrorhizon* for the industrial production of cosmetics, such as lipsticks, lotions, and soaps (Yazaki, 2017). Shikonin production by plant cell culture was far more economical than by chemical synthesis (Terada et al., 1983; 1987).

In addition to plant cell cultures, research has focused on using transformed roots (the so-named hairy roots) to increase the production of shikonin (Chaudhury and Paul, 2010; Pal et al., 2019) (See section II.4). Several Boraginaceous plant species (e.g., *L. erythrorhizon* and *Lithospermum canescens*) have been transformed with *Rhizobium rhizogenes*² for the production of shikonin derivatives (Sim and Chang, 1993; Sykłowska-Baranek et al., 2012). For example, in *L. canescens*, different lines of transgenic roots have been selected for their high potential for production of shikonin derivatives. Moreover, the optimal growth stage to achieve the highest red pigment production with the treatment of methyl jasmonate (MJ) was also investigated (Syklowska-Baranek et al., 2012).

I.5. Alkannin/shikonin and their derivatives biosynthesis pathway

In the past 35 years, many studies have focused on the biosynthesis pathway of A/Sd to understand and better manipulate A/Sd production. Alkannin/Shikonin biosynthesis is achieved via the phenylpropanoid and mevalonate pathways (Takanashi et al., 2019). Two intermediates, *p*-hydroxybenzoic acid and geranyl diphosphate, which are derived from the phenylpropanoid and mevalonate pathways respectively, are coupled to give geranylhydroquinone (**Figure 4**). The 3'' position of

² it was formerly called *Agrobacterium rhizogenes*.

STATE OF THE ART

the geranyl side chain is hydroxylated, giving the intermediate, 3''-hydroxygeranylhydroquinone (GHQ 3''-OH). Cyclization of the GHQ 3''-OH gives a naphthoquinone structure which is then followed by several oxidation steps to give deoxyshikonin (**Figure 4**).

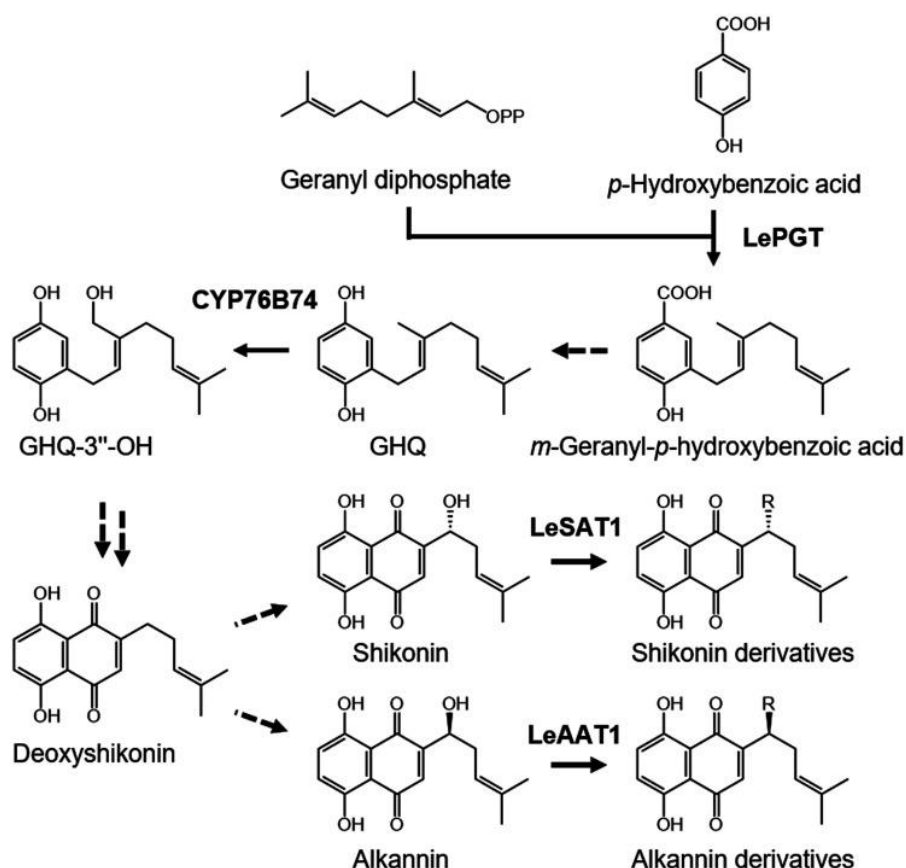


Figure 4: Pathways involved in the biosynthesis of alkannin/shikonin and their derivatives (Oshikiri et al., 2020).

Although the complete A/Sd biosynthesis pathway remains obscure, several genes encoding enzymes directly involved in shikonin biosynthesis, have been identified (Wang et al., 2014; Zhu et al., 2017; Wu et al., 2017; Takanashi et al., 2019). For instance, the phenylalanine ammonia lyase (PAL) gene coding for the enzyme in the phenylpropanoid pathway converting L-phenylalanine to trans-cinnamic acid and the hydroxymethylglutaryl-CoA reductase gene (HMGR) coding the rate-controlling enzyme of the mevalonate pathway for the conversion of HMG-CoA to mevalonic acid have been cloned and characterized in *L. erythrorhizon* and *Arnebia euchroma*.

STATE OF THE ART

(Yazaki et al., 1997a; Singh et al., 2010). Moreover p-hydroxybenzoate geranyltransferase gene (*PGT*) (**Figure 4**) coding for the enzyme involved in catalysing the coupling of p-hydroxybenzoic acid and geranyl diphosphate to produce m-geranyl-p-hydroxybenzoic acid, which is the first step in forming the basic carbon skeleton leading to A/Sd, have been cloned and characterized in *L. erythrorhizon* or *Arnebia euchroma* in cell cultures (Yazaki et al., 2002; Singh et al., 2010; Yazaki et al., 1999). In addition, a new enzyme CYP76B74 has been reported, which is a cytochrome P450 in *A. euchroma*, hydroxylating the 3'' position of geranylhydroquinone (GHQ) to give 3''-hydroxygeranylhydroquinone (GHQ-3''-OH;) (Song et al., 2020) (**Figure 4**). Although several studies have attempted to identify the enzymes catalyzing the reactions between GHQ 3''-OH and deoxyshikonin, no enzymes have been identified to date (Yazaki, 2017). Furthermore, two BAHD acyltransferases catalysing the last step in the A/Sd biosynthetic pathway have been identified and characterized from *L. erythrorhizon* by Oshikiri et al. (2020), in which shikonin O-acyltransferase (LeSAT1) was specific to shikonin and alkannin O-acyltransferase (LeAAT1) to alkannin (**Figure 4**). Both enzymes recognized acetyl-CoA, isobutyryl-CoA, and isovaleryl-CoA as acyl donors to produce their corresponding A/S derivatives (Oshikiri et al., 2020).

In addition, several genes indirectly related to shikonin biosynthesis such as *L. erythrorhizon* dark-inducible gene 1, 2, 3, 4, 5a/b/c (LeDI-1, 2, 3, 4, 5a/b/c) and *L. erythrorhizon* 1-aminocyclopropane-1-carboxylate synthase gene (LeACS) have been discovered and cloned in the past 30 years (Yazaki et al., 2001; Fang et al., 2016). Moreover, environmental factors, developmental stage, light intensity, soil composition, or various abiotic stress factors have been shown to qualitatively and quantitatively influence primary and secondary metabolism of plants (Chaparro et al., 2013; Li, 2020). For instance, light, methyl jasmonate, nitric oxide, ethylene and fungal elicitor have been found to be crucial regulators of shikonin biosynthesis during the cell cultures of *L. erythrorhizon*, and other plants from the Boraginaceae (Yazaki et al., 1999; Yazaki et al., 1997b; Hao et al., 2014; Wu et al., 2009; Touno et al., 2005).

I.6. Secondary metabolites quantification and identification methods

Several techniques exist to quantify and identify SMs. Among these,

STATE OF THE ART

high-performance liquid chromatography (HPLC) or ultra high-performance liquid chromatography (UHPLC) with different detectors as UV/DAD or mass spectroscopy (MS) are widely used.

I.6.1. High performance liquid chromatography

High-performance liquid chromatography (HPLC) is a specific form of column chromatography generally used to separate, identify, and quantify active compounds (Martin and Guiochon, 2005). It includes two types of systems, reversed-phase high performance liquid chromatography (RP-HPLC) which means the stationary phase is non-polar and the mobile phase is polar and normal phase liquid chromatography (NPC or NPLC or NP-HPLC) which means the stationary phase is polar and the mobile phase is non-polar (Shabir, 2010).

The sampler introduces the sample mixture into the mobile phase (pressurized mixture of any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile used in RP-HPLC with water) (Abidi, 1991)) which transfers it to the column. The solubility of samples is ruled by the polarity of solvent used, by the degree of polymerization and by interactions of target metabolites with other constituents of the sample (Sbrana et al., 2014). Depending on the composition of the mobile phase, two different modes: isocratic mode and gradient mode are generally applicable. If the makeup of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system, and if the composition of the mobile phase is changed during separation, the HPLC system is defined as a gradient one (Abidi, 1991) system. The pumps deliver the desired flow and composition of the mobile phase through the column, which is filled with the adsorbent (made from grainy solid particles such as silica or polymers). The separation efficiency correlates with the length of the column and the type and particle size of the column packing material. It separates each compound on the basis mainly of its polarity, solubility, and size. The detector then produces a signal proportionate to the amount of sample constituent eluting from the column. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time (RT) (Malviya et al., 2010) which is used for the identification of specific compound comparing to the reference standard compound.

UHPLC can also be used. The main difference is the use of smaller size particules (<2 μ m) allowing a better interaction of the analytes with the

STATE OF THE ART

stationary phase, so better separations, but needing a higher pressure to allow the mobile phase to pass through the column.

The most used detectors are ultraviolet (UV), Visible (Vis), and photo diode array (DAD) detectors and more recently MS detectors.

I.6.2. Liquid chromatography-mass spectroscopy

Coupling MS to chromatographic techniques has always been desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors (Pitt, 2009). The high performance liquid chromatography coupled to mass spectrometry combines the separation power of HPLC with the detection power of mass spectrometry (MS) (Nikalje and Ramesh, 2018).

The sample extract is first inserted into the column of the HPLC. This column retains sample metabolites based on their physical-chemical characters, and the different metabolites flow to the mass spectrometer at different time intervals. MS can be used to determine the molecular mass and elemental composition of an analyte as well as to elucidate the structure of the analyte (United States Pharmacopeia, 2022). First, an ion source (electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)) transforms liquid phase samples into a charged (ionised) state. Fragmentation of mother ions may also occur or be promoted in a collision cell. Then ions are transferred to a mass analyzer grouping the ions by their mass to charge ratio (m/z) (Pitt, 2009) using electromagnetic fields. Finally, a detector quantifies the ions and delivers data on abundance of each ion and fragment present in the sample. The optimal conditions are highly dependent on the nature of the analyte and the LC separation, making it difficult to give generic conditions. Each analyte/mixture requires individual optimisation. Although published methods are a valuable starting point, assay performance and optimal conditions can vary greatly between different instruments and sample matrices (Pitt, 2009).

II. Arbuscular mycorrhiza fungi (AMF)

Arbuscular mycorrhizal fungi belonging to the phylum Glomeromycota (Schüßler et al., 2001) are soilborne root obligate biotrophs associated with 70–90% of the world's vascular land plants (Smith and Read, 2008; Brundrett, 2009). They are characterized by their obligate nature and by the formation of arbuscules (i.e., tree-shaped structures) within root cells (**Figure 5**) (Lewis et al., 2016). These fungi are among the oldest

STATE OF THE ART

living, supposedly, asexual eukaryotes (Carr and Baldauf, 2011). Indeed, arbuscules resembling those of extant AMF species have been detected in the subterranean organs of fossil plants dated 400 million-year-old (Ma) in the Rhynie Chert formation (Stürmer et al., 2018).

In this symbiotic association, AMF develop hyphae structures within plant roots and an extended hyphal network in the soil. They receive up to 20% of total photosynthates (Parniske, 2008; López-Gutiérrez et al., 2008) and lipids (Keymer et al., 2017; Kameoka and Gutjahr, 2022) from the host plant in rule of minerals (e.g., phosphate, nitrogen, sulphur, zinc and copper) and water (Smith and Read, 2008; Bowles et al., 2016).

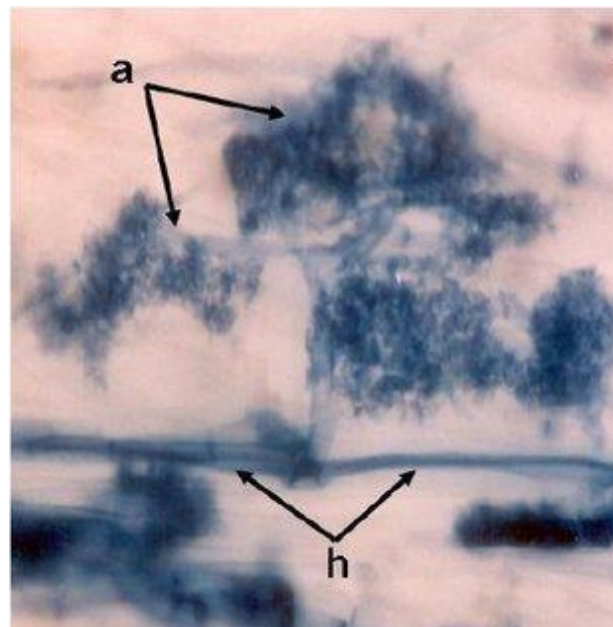


Figure 5: Arbuscular mycorrhizal fungal structures in basil plant roots. (a) arbuscules and (h) fungal hyphae (Rasouli-Sadaghiani et al., 2010).

II.1. AMF life cycle

In nature, AMF colonised root fragments (containing spores and/or vesicles) and spores (both termed propagules) are the primary sources of inoculum for root colonization. In addition, AMF hyphae extending from root systems represent another important source of inoculum capable of colonizing new plants. These propagules exhibit different abilities to colonize roots between genera. For example, Klironomos and Hart (2002) demonstrated that *Glomus* and *Acaulospora* were able

STATE OF THE ART

to colonize roots from all three sources of inoculum, while *Scutellospora* and *Gigaspora* appear to depend mostly on spores. The life cycle of AMF is separated in three phases: asymbiotic, pre-symbiotic and symbiotic (Bücking et al., 2012).

Asymbiotic phase:

The asymbiotic phase is characterized by the absence of signals emitted by the host plant to trigger spores¹ germination or hyphal growth (Breuninger and Requena, 2004).

In this phase, spores are able to germinate. However, germinating hyphae stop growing after a few days before consumption of spore reserves if a host root is not present in the environment (Bécard and Piché, 1989) and cytoplasm, nuclei and cellular organelles retract as a result of sequential septa formation in hyphae (Giovannetti, 2002).

Pre-symbiotic phase:

In proximity to a host plant and upon detection of plant signaling molecules called ‘branching factors’ (bfs) (strigolactones), AMF activate oxidative metabolism, which leads to increased growth and branching of hyphae, thereby increasing the chance of physical contact with a root (Akiyama and Hayashi, 2006; Besserer et al., 2008). The recent discovery of a transporter, NO PERCEPTION1 (NOPE1) in maize (*Zea mays*) and rice (*Oryza sativa*) suggests that strigolactones are not the only important signal molecules during the pre-symbiotic phase (Nadal et al., 2017).

Upon receiving signals from the host root, AMF release bioactive molecules that trigger the signaling transduction pathways in the plant involving perception, transmission and transcription required for symbiotic establishment (Bonfante and Requena, 2011). A mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs) secreted by AMF have been identified as signaling molecules (the so-called ‘Myc factors’) to communicate with their host plants (Maillet et al., 2011; Rush et al., 2020). However, the failure of Myc-LCOs to elicit a transcriptional response associated with AM symbiosis in rice raises the possibility that these signaling molecules may not be recognized ubiquitously by all plants (Miyata et al., 2014).

After reciprocal recognition between the two partners, the AMF hyphae enters into contact with the host root and forms a hyphopodium (Genre

¹ We used spores to describe the life cycle.

STATE OF THE ART

et al., 2005) at the surface of the epidermic cells. The molecular and developmental mechanisms mediating the switch from linear apical growth to the swollen and branched form of the hyphopodium are still obscure (Bonfante and Genre, 2010).

Symbiotic phase:

The hyphae emerging from the hyphopodium then passes through a prepenetration apparatus (PPA) which guides the fungal hyphae through the root epidermal cells, colonizing the root cortex (Bücking et al., 2012). Extensive intercellular hyphal growth and the terminal intracellular structures called arbuscules subsequently form. The arbuscule, which is the nutrient exchange interface in the symbiosis, is an ephemeral structure with an estimated lifespan of 4–5 days. It is highly branched and surrounded by plant plasma membrane. Some genes that affect arbuscule development have been identified by reverse genetics. For instance, the RNA interference knockdown of the Vapyrin gene, coding for a cytoplasmic protein with unknown function, has been shown to induce a marked decrease in epidermal penetration and a total block of arbuscule formation and only intercellular hyphae develop between cortical cells. (Pumplin et al., 2010). Within root cortex, two types of AMF hyphal colonization have been detected: Arum-type and Paris-type. In the Arum-type, the hyphae spreads intercellularly between cortical root cells and penetrates cells to differentiate arbuscules while in the Paris-type, the fungus grows directly from one cell to the next within the cortex and forms intracellular hyphal coils and intercalary arbuscules along the coils (**Figure 6**). The Paris and Arum type development is primarily determined by the host plant family, and some families or species are even capable of both types (Yamato 2005; Matekwor Ahulu et al., 2005). Most cultivated plants produce Arum-type mycorrhizal colonization whereas many trees and forest herbs form Paris-type colonization (Dickson 2004).

STATE OF THE ART

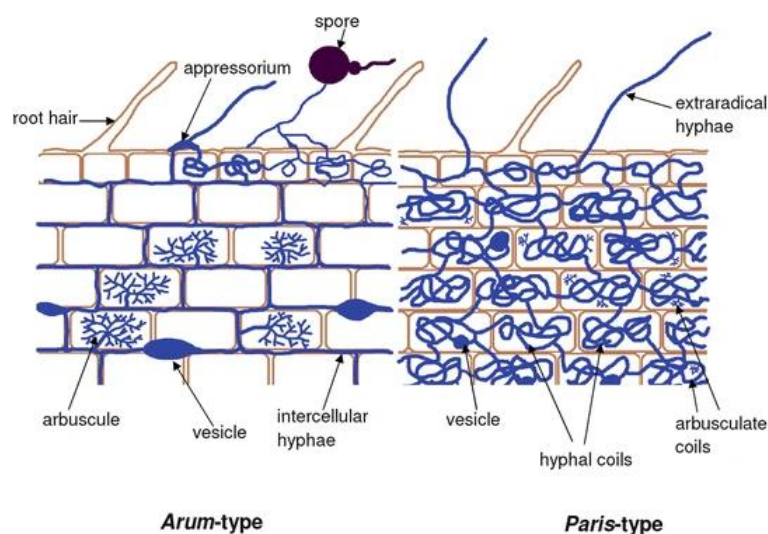


Figure 6: Arbuscular mycorrhizal fungal structures showing *Arum*- and *Paris*-types (Priyadharsini and Muthukumar, 2015).

In parallel to arbuscules, a number of AMF genera (i.e. *Rhizophagus*, *Glomus*, *Acaulospora*) develop inter- or intracellular vesicles or spores in the roots, which serve as storage organs or reproductive structures (Declerck et al., 1998; Smith and Read, 2008). Vesicles are thin-walled and balloon-like structures that lack occlusion, whereas intraradical spores are wall thickening or have occlusion from the subtending hypha. Some AMF genera (e.g., *Gigaspora* and *Scutellospora*) do not form vesicles at all, while *Acaulospora* spp. do not form spores in roots. However, it is difficult to distinguish these two AMF structures (Walker et al., 2018).

Once the AMF symbiosis has been established, fungal hyphae exit from roots spreading into the soil into a dense extraradical mycelium (ERM) network mainly consisting of AMF hyphae and spores. The ERM take up nutrients from the soil and translocate these nutrients to the host root (Bücking et al., 2012).

Arbuscular mycorrhizal fungi form spores singly or in clusters and in some species (*Glomus clavisporum*, *Glomus sinuosum* and *Glomus taiwanensis* - Panna and Highland 2010), spore clusters are surrounded by a ring of hyphae forming a fruiting body called sporocarp. The ERM is coenocytic with unlimited growth and forms “branched absorbing structures” (BAS) on short terminal hyphae (Bago et al., 1998). In some AMF species such as *Gigaspora rosea*, *Cetranspora gilmorei* and *Scutellospora calospora*, the ERM produce auxiliary cells (ACs). These

STATE OF THE ART

ACs are usually clusters of small ($\sim 10\text{--}20\ \mu\text{m}$), spiny, knobbed or smooth structures (Walker et al., 2018). Their exact function is still unknown but have been assumed to serve as carbon storage organs playing a role in the production of extraradical spores (Declerck et al., 2004).

The life cycle of AMF is summarized in **Figure 7**.

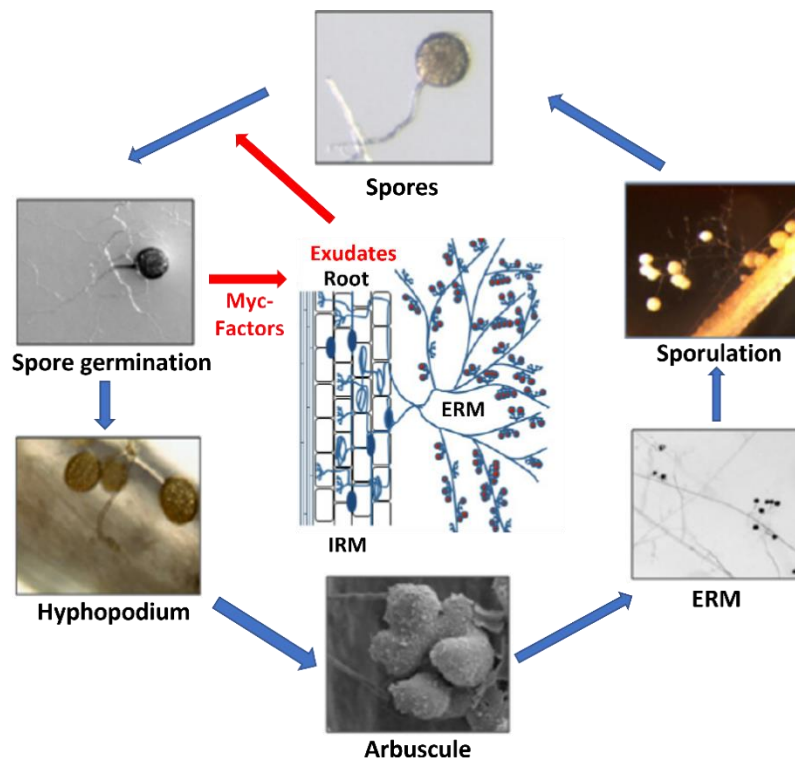


Figure 7: Life cycle of AMF and the different steps during AMF development (Bücking et al., 2012)

II.2. Phylogeny of arbuscular mycorrhizal fungi

Classification of AMF based on morphological features – Early descriptions of AMF species relied only on sporocarp-forming organisms that could be recognized macroscopically (Stürmer, 2012). The development of the wet sieving method allowed to extract sporocarps and non-sporocarpic spores from soils (Gerdemann and Nicolson, 1963), increasing the number of species identified. The first key to AMF classification was based on descriptive characters and color of spores (Mosse and Bowen, 1968) allowing the delineation of seven

STATE OF THE ART

genera. Because of the superficial similarities of AMF spores with those of Endogone, the species belonging to the genus *Glomus* were first classified within the Endogonaceae (Zygomycota) (Gerdemann and Trappe, 1974).

Later, spore wall layers, size, colour and ontogeny were used and adapted for AMF species identification (Harbans et al., 2018). For example, Walker and Sanders (1986) erected a new genus *Scutellospora* from the *Gigaspora* of Gerdemann and Trappe (1974). This separation was based on the presence of a germination shield found in the innermost layer of *Scutellospora* and not in *Gigaspora*. Only 12 years after the monograph by Gerdemann and Trappe (1974), which proposed a classification including 30 species forming arbuscular mycorrhizas, the number of described glomeromycotan species jumped to 77 (Trappe 1982), and 6 years later, Schenck and Perez (1988) listed 126 species. All the descriptions of AMF species at that time were based on morphological characters and subcellular structures of spores. However, the lack of standardized terminology to describe the diversity of spore subcellular structures has hampered attempts to identify new species. In order to overcome this limitation, Walker (1983) proposed a new terminology for the description of species based on the types of “walls” formed by glomeromycotan spores and which can be grouped into “wall groups”. In addition, he also proposed a “murograph” that consists of a graphic representation to depict the different wall types and groups found in a spore. As new species were described, additional spore wall types were introduced: such as expanding (Berch and Koske, 1986), amorphous (Morton, 1986), coriaceous (Walker, 1986), notched (Koske and Gemma, 1995), and germinal (Spain et al., 1989). The terminology proposed by Walker (1983) is still used in species description, although sometimes it is embedded within an ontogenetic framework. Various morphological characteristics used for the identification of AMF are summarized in **Table 1** (Muthukumar and Prakash, 2009).

Table 1: Various morphological characters of spores used for identification of AMF (Muthukumar and Prakash, 2009).

Morphological characters	
Sporocarp morphology	Size, Shape, Peridium
Spore morphology	Colour, Shape, Size, Content
Subtending hyphae	Shape, Width, Pore Occlusion
Auxiliary cells	Ornamentation
Mycorrhizal anatomy	Hyphal characters, Intraradical spores

STATE OF THE ART

Spore wall structure	Colour, Dimension, Number, Type, Ornamentation, Reaction
Spore germination	Direct, Indirect

However, in nature, there can be great variations in spore morphology even within one single AMF species (Walker and Vestberg, 1998). For example, in some species of *Glomus* or *Acaulospora*, identification is particularly complicated because of the presence of dimorphic spores (i.e., that shows two types of morphologies) (Rosendahl, 2008). Moreover, *Scutellospora* species often have several inner membranous walls and a complex germination shield making them difficult to identify. Finally, many AMF species may reproduce only vegetatively without producing spores (Helgason et al., 2002).

Identification and classification of AMF species based on morphological characteristics has resulted in the description of many AMF species. However, individual species forming different spore morphs may be described as members of different orders or phylogenetically different orders of AMF may be placed in the same genus (Krüger et al., 2009). Therefore, new methods were needed for accurate classification of AMF species.

Molecular classification of AMF – Since the last two decades, molecular techniques have been used in addition to morphological methods for AMF species identification and classification. For example, Redecker et al. (2000) used both morphological and molecular data (small subunit rRNA (SSU) gene sequencing) and transferred *Sclerocystis coremioides* to the genus *Glomus*. In 2001, Schüßler et al. (2001) proposed the phylum Glomeromycota for all the species belonging to AMF, based on the SSU rRNA gene sequences, and in 2010, a revision of the Glomerales was published by Schüßler and Walker (2010), resulting in the modification of names. For example, '*Glomus versiforme*' was renamed *Diversispora epigaea*. Spatafora et al. (2016) proposed a new classification of AMF and placed these fungi in a sub-phylum (i.e., Glomeromycotina) in the Mucoromycota phylum. However, this classification has been challenged by a more inclusive study of Tedersoo et al. (2018) and the Glomeromycota phylum was resurrected.

The current classification (**Table 2**) of AMF comprises one phylum, 4 orders, 12 families, 44 genera for a total of 346 validly described species (Schüßler and Walker, 2010; Redecker et al., 2013;

STATE OF THE ART

Wijayawardene et al., 2018) according to DNA-barcoding on the LSU D2 region and the SSU-ITS-LSU DNA fragment updated on the 14 January 2023 (<https://docs.google.com/spreadsheets/d/1vHHj9XtfYj9B60L7UP4rUci8bJeolUP/edit#gid=1484552018>).

STATE OF THE ART

Table 2: Classification of the Glomeromycota updated on the 14 January 2023 (<https://docs.google.com/spreadsheets/d/1vHHj9XtfIYj9B60L7UP4rUci8bJelUP/edit#gid=1484552018>)

Orders	Families	Genus	Number of described species
Archaeosporales	Ambisporaceae	<i>Ambispora</i>	10
	Archaeosporaceae	<i>Archaeospora</i>	7
	Geosiphonaceae	<i>Geosiphon</i>	1
	Polonosporaceae	<i>Polonospora</i>	1
Glomerales	Glomeraceae	<i>Dominikia</i>	14
		<i>Epigeocarpum</i>	1
		<i>Funneliformis</i>	14
		<i>Funneliglomus</i>	1
		<i>Glomus sensu stricto</i>	7
		<i>Glomus sensu lato</i>	46
		<i>Halonatospora</i>	1
		<i>Kamienskia</i>	1
		<i>Microdominikia</i>	1
		<i>Microkamienskia</i>	3
		<i>Nanoglomus</i>	1
		<i>Oehlia</i>	1
		<i>Orientoglomus</i>	1
		<i>Rhizophagus</i>	24
		<i>Sclerocarpum</i>	1
		<i>Sclerocystis</i>	2
		<i>Sclerocystis "sensu lato"</i>	8
		<i>Septoglomus</i>	15
		<i>Silvaspora</i>	1
Diversisporales	Acaulosporaceae	<i>Acaulospora</i>	58

STATE OF THE ART

	Diversisporaceae	<i>Corymbioglomus</i>	3
		<i>Diversispora</i>	21
		<i>Desertispora</i>	1
		<i>Otospora</i>	1
		<i>Redeckera</i>	6
		<i>Sieverdingia</i>	1
	Gigasporaceae	<i>Bulbospora</i>	1
		<i>Cetraspora</i>	7
		<i>Dentiscutata</i>	9
		<i>Intraornatospora</i>	1
		<i>Gigaspora</i>	9
		<i>Racocetra</i>	13
		<i>Scutellospora</i>	18
	Pacisporaceae	<i>Pacispora</i>	6
	Sacculosporaceae	<i>Sacculospora</i>	2
	unknown	<i>Paradentiscutata</i>	2
Paraglomerales	Paraglomeraceae	<i>Innospora</i>	1
		<i>Paraglomus</i>	9
	Pervetustaceae	<i>Pervetustus</i>	1
Entrophosporales	Entrophosporaceae	<i>Entrophospora</i>	13
unclear	unclear	<i>Tricispora</i>	1
Total	12	43	346

II.3. General benefits of arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi develop within the plant roots and spread into the surrounding soil as an extensive extraradical mycelium (ERM), representing 9 to 55% of the total soil microbial biomass (Olsson et al., 1999). This dense ERM considerably enhances the access of roots to water and mineral nutrients (e.g., P, N, K, Ca, S, Zn, Cu), often increasing plant biomass (Smith and Read 2008; Bowles et al. 2016). In addition, it changes soil structure (Chen et al., 2018a), thereby

STATE OF THE ART

improving soil quality and fertility (Zou et al. 2016; Thirkell et al. 2017). These fungi are also well known to improve resistance and tolerance of plants to stress conditions, such as drought, salinity, nutrients deprivation, extreme temperatures, heavy metals, pests and diseases (Ahanger et al., 2014; Salam et al., 2017; Porcel et al., 2012; Cicatelli et al., 2014). Besides these benefits, AMF could also impact quantitatively and qualitatively the production of SMs by their hosts, potentially improving the nutraceutical value of horticultural products and pharmaceutical value of medicinal plants (Giovannetti et al., 2012a, b). For example, AMF-colonized soybean (*Glycine max*) and strawberry (*Fragaria ananassa*) exhibited increased levels of SMs (isoflavonoids and phenolic compounds) resulting in enhanced antioxidant properties (Morandi and Bailey, 1984; Castellanos-Morales et al., 2010).

II.4. Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants

Adapted from a review paper published in *Mycorrhiza*: 32:221–256 (2022).

Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants

(YanYan Zhao, Annalisa Cartabia, Ismahen Lalaymia, Stéphane Declerck)

II.4.1. Effect of AMF on growth and secondary metabolites production of medicinal plants

Since the pioneer work of Wei and Wang (1989, 1991), reporting the positive effect of AMF inoculation of *Datura stramonium* and *Schizonepeta tenuifolia* on the production of active compounds, numerous studies have been conducted. The literature focusing on AMF in medicinal plants involves 81 plant species belonging to 28 families (**Table 3**). These medicinal plants present different characteristics to be studied with AMF: important medicinal herbs to treat certain disease such as *Artemisia annua* producing artemisin to treat malaria in developing countries (Domokos et al., 2018); important condiment plants such as *Allium sativum* in India (Borde et al., 2009); rare plant species difficult to culture such as *Arnica montana* (Jurkiewicz et al.,

STATE OF THE ART

2010); aromatic plants to produce essential oil widely used in the pharmaceutical, cosmetic, and food industries such as most plant species from the Apiaceae [*Anethum graveolens* and *Coriandrum sativum* (Rydlová et al., 2015)] and the Lamiaceae [*Origanum vulgare* (Karagiannidis et al., 2012)]; and health foods such as *Dioscorea spp.* yam (Lu et al., 2015). For the majority of these plants, studies were focused on the effects of AMF on biomass increase and production of bioactive compounds simultaneously (45 studies) or only focused on bioactive compounds production (30 studies). For a few other studies, the attention was focused on AMF community composition (four studies) or on the effects of AMF on plant growth under different conditions (15 studies) (**Table 3**). Among the AMF species tested, *Funneliformis mosseae*² is the most investigated species (25 studies), followed by *Rhizophagus intraradices* (16), *Claroideoglossum etunicatum* (14), *Rhizophagus fasciculatus* (14), *Rhizophagus irregularis* and *Rhizophagus clarus* (six studies each) and *Gigaspora margarita* (five studies) (**Table 3**). Only a few medicinal plant species were inoculated with AMF present in the soil native to those plants, while the vast majority were inoculated with commercial inoculants.

A direct relationship has been highlighted between the biomass of AMF-colonized plants and the concentration of secondary metabolites for several medicinal plants, such as *Chlorophytum borivillianum*, *Dioscorea spp.*, *Gymnema sylvestre*, *Glycyrrhiza uralensis*, *Libidibia ferrea*, *Ocimum basilicum*, *Satureja macrostema* and *Salvia miltiorrhiza* (Dave et al., 2011; Lu et al., 2015; Zimare et al., 2013; Chen et al., 2017; Silvia et al., 2014; Zolfaghari et al., 2013; Carreón-Abud et al., 2015; Yang et al., 2017). Conversely, in *Cynara cardunculus* colonized by *R. intraradices* and *F. mosseae*, a significant increase in yield was noticed, but the concentrations of phenolics decreased (Colonna et al., 2016). Other studies conducted with *Hypericum perforatum* inoculated with *R. intraradices* or a mixture of *Funneliformis constrictum*, *Funneliformis geosporum*, *F. mosseae*, and *R. intraradices* reported no increase in shoot biomass, while in *Valeriana officinalis* inoculated with *R. intraradices* or a mixture of six AMF species (*F. mosseae*, *R. intraradices*, *Glomus cladoideum*, *Rhizoglossum microaggregatum*, *Funneliformis caledonum* and *C. etunicatum*) a negative effect on rhizome and root biomass was noticed

² The species names of AMF shown in this thesis study follow the current nomenclature and not that of the original publications.

STATE OF THE ART

(Zubek et al., 2012; Nell et al., 2010). However, an increased concentration of active compounds (e.g., hypericin and pseudohypericin and sesquiterpenic acids, respectively) was noticed for both plants (Zubek et al., 2012; Nell et al., 2010).

Another beneficial aspect of AMF is their capability to improve plant nutrient uptake (Bowles et al., 2016), influencing directly or indirectly the concentration of secondary metabolites (Yamawaki et al., 2013). For instance, *F. mosseae* improved shoot and root biomass, root system architecture and flavonoids accumulation in *Glycyrrhiza uralensis* growing under P deficient nutrient conditions (Chen et al., 2017).

A number of studies also have reported enhanced survival and increased growth of micropropagated medicinal plants at the transfer stage from *in vitro* to *ex vivo* conditions (Rai, 2001). For instance, with *F. mosseae*, the survival rate of micropropagated *Spilanthes acmella* and *Glycyrrhiza glabra* plantlets was 100%, and plant growth and development were improved under glasshouse and greenhouse conditions (Yadav et al., 2012; Yadav et al., 2013) while in the absence of AMF, the survival rate was only 60%-70%. Similarly, height, fresh weight of shoots, roots, and seeds of *Scutellaria integrifolia* seedlings inoculated with *C. etunicatum* were significantly increased in pots following micropropagation (Joshee et al., 2007).

These studies clearly evidenced the potential of using AMF inoculants for improving the yield of raw materials (e.g., roots, shoots) of medicinal plants, thus potentially increasing the quantity of active compounds. Different groups of secondary metabolites whose production was enhanced by AMF inoculation are detailed below.

Alkaloids – Since Wei and Wang (1989) first observed that AMF symbiosis can increase the total content of hyoscyamine and scopolamine in *Datura stramonium*, numerous studies have reported a positive role of AMF in the accumulation of alkaloids. For example, a positive correlation was found between AMF colonization (a mixture of *R. intraradices* and *G. margarita*) of *Castanospermum australe* tree and the castanospermine content (which was reported to inhibit the HIV virus) of leaves (Abu-Zeyad et al., 1999). The content of some commonly used “heat-clearing” herbs compounds, such as berberine, jatrorrhizine and palmatine, were increased in seedlings of *Phellodendron amurense* inoculated with AMF (Fan et al., 2006). Other active compounds were increased in the presence of AMF: trigonelline in roots and leaves of *Prosopis laevigata* colonized by *Gigaspora rosea*

STATE OF THE ART

under *in vitro* conditions; colchicine in tubers of *Gloriosa superba* colonized by *F. mosseae* growing under glasshouse conditions, and scopolamine in leaves of *Eclipta prostrata* colonized by a mixture of *C. etunicatum*, *Claroideoglomus claroideum*, *F. mosseae*, *F. geosporum*, *R. irregularis* and *Rhizogloium microaggregatum* growing in climate chamber conditions (Rojas-Andrade et al., 2003; Pandey et al., 2014; Vo et al., 2019).

Terpenoids – Several studies have reported an AMF impact on the production of essential oils by medicinal and aromatic plants (**Table 3**). For instance, the production of these compounds was increased in *Corianderum sativum*, *Trachyspermum ammi*, *Atractylodes lancea*, *Inula ensifolia*, *Artemisia umbelliformis*, *Plectranthus amboinicus*, *Satureja macrostema*, *Salvia officinalis*, *Origanum vulgare* and *Origanum onites*, *Thymus daenensis*, *Thymus vulgaris* and *Foeniculum vulgare* colonized by AMF (Rydlová et al., 2016; Kapoor et al., 2002; Liang et al., 2018; Zubek et al., 2010; Binet et al., 2011; Merlin et al., 2020; Carreón-Abud et al., 2014; Sete da Cruz et al., 2019; Karagiannidis et al., 2012; Arpanahi et al., 2020; Machiani et al., 2021; Kapoor et al., 2004). The content of artemisinin, an important sesquiterpene lactone compound found in *Artemisia annua* and well known for its effects on malaria and more recently on cancer (Krishna et al., 2008) was increased in leaves of plants colonized by *F. mosseae* or a combination of *Glomus macrocarpum* and *R. fasciculatus* or *Diversispora epigaea* and *R. irregularis* grown in pots or under field conditions (Huang et al., 2011; Chaudhary et al., 2008; Domokos et al., 2018). The forskolin content, a diterpene extensively used to treat heart diseases, glaucoma, asthma, and certain types of cancers (Kavitha et al., 2010), was significantly increased in roots of *Coleus forskohlii* inoculated with *Glomus bagyarajii* growing under greenhouse conditions (Sailo and Bagyaraj, 2005). Similarly, Singh et al. (2013) reported an increased content of forskolin in tubers of *Coleus forskohlii* associated with *R. fasciculatus* growing under organic field conditions.

Researchers also have studied the impact of AMF symbiosis on medicinal plants derived from tissue cultures. An example is the increased content of the essential oil carvacrol, a phenolic monoterpenoid with antimicrobial, antioxidant, and anticancer activities (Sharifi-Rad et al., 2018) in micropropagated *Origanum vulgare* subsp. *hirtum* after association with the AMF *Septogloium viscosum* (Morone Fortunato and Avato, 2008).

STATE OF THE ART

Phenolics – Arbuscular mycorrhizal fungi have been shown to increase the content of phenols in medicinal plants (**Table 3**). For instance, the production of formononetin (an antimicrobial, antioxidant, antilipidemic, antidiabetic, antitumor and neuroprotective compound) (Vishnuvathan et al., 2016), was increased in *Medicago sativa* grown in the presence of *R. intraradices* (Volpin et al., 1994). The production of curcumin (an anti-inflammatory, antioxidant, anticancer, antiseptic, antiplasmodial, astringent, digestive, diuretic compound) was increased by circa 26% in *Curcuma longa* colonized by AMF species belonging to the genera *Glomus/Rhizophagus*, *Gigaspora* and *Acaulospora* sp, under greenhouse conditions (Dutta and Neog, 2016). The concentration of total tannins, used to treat tonsillitis, pharyngitis, hemorrhoids and skin eruptions (Britannica, 2021), was increased by 40% in the fruits of *Libidibia ferrea* inoculated with *Acaulospora longula* under field conditions (Santos et al., 2020). Additionally, the concentrations of cichoric acid in *Echinacea purpurea* colonized by *R. intraradices* (Araim et al., 2009) and p-hydroxybenzoic acid and rutin in *Viola tricolor* colonized by *R. irregularis* (Zubek et al., 2015), and the total content of flavonoids in *Libidibia ferrea* colonized by *Gigaspora albida* and gallic acid in *Valeriana jatamansi* colonized by a consortium of three different isolates of *R. intraradices* spp (Silvia et al., 2014; Jugran et al., 2015) were increased by the AMF symbiosis.

Saponins – Arbuscular mycorrhizal fungi were reported to enhance the production of saponins in medicinal plants (**Table 3**). For instance, the content of glycyrrhizic acid, a triterpenoid saponin used to alleviate bronchitis, gastritis, and jaundice (Pastorino et al., 2018) was increased by 0.38-1.07 fold and by 1.34-1.43 fold after four and 30 months, respectively, in *Glycyrrhiza glabra* (liquorice) plants colonized by *F. mosseae* and *D. epigaea* alone or in combination, grown in sand under greenhouse conditions (Liu et al., 2007). Similarly, Johny et al. (2021) reported an increase of glycyrrhizic acid concentration in *Glycyrrhiza glabra* inoculated with *C. etunicatum* under greenhouse conditions.

Other chemical compounds – Hypericin and pseudohypericin are naphthodianthrone (anthraquinone derivatives) mainly extracted from *Hypericum* species (Ayan et al., 2008). They have many pharmaceutical properties, such as sedatives, antiseptics, and antispasmodics (Baytop, 1999). Zubek et al. (2012) reported an increased content of hypericin and pseudohypericin in *Hypericum perforatum* colonized by *R. intraradices* alone or by a mixture of *F. constrictum*, *F. geosporum*, *F. mosseae* and *R. intraradices*, under greenhouse conditions.

STATE OF THE ART

Withaferin-A, a steroidal lactone, traditionally used in ayurvedic medicine (Mirjalili et al., 2009), has a wide range of pharmacological activities including cardioprotective, anti-inflammatory, immuno-modulatory, anti-angiogenesis, anti-metastasis and anti-carcinogenic properties. Johny et al. (2021) reported that association between the medicinal plant *Withania somnifera* and *R. irregularis* increased the concentration of withaferin-A as compared to non-inoculated plants under greenhouse conditions.

It should be noted, however, that AMF showed a neutral or decreased effect on the production of certain secondary metabolites. For example, Nell et al. (2010) found that *F. mosseae* has no effect on the total concentrations of phenolic and rosmarinic acid in the roots of *Salvia officinalis*; and Geneva et al. (2010) showed that *R. intraradices* decreased total phenols and flavonoids contents in the leaves of *Salvia officinalis*. Similarly, Zubek et al. (2010) reported significant differences in the effectiveness of different AMF species tested in *Inula ensifolia*. An increased production of thymol derivatives was found in plant roots inoculated with *Rhizophagus clarus*, while a decreased production of these metabolites was reported in roots inoculated with *R. intraradices* under greenhouse conditions (Zubek et al., 2010). Moreover, changes in secondary metabolites composition have been observed in medicinal plants inoculated with AMF. For instance, Geneva et al. (2010) observed a modified composition of essential oils and promotion of the relative quantities of bornylacetate, 1,8-cineole, α -thujones and β -thujones in *Salvia officinalis* associated with *R. intraradices*. Similarly, *Artemisia umbelliformis* inoculated with an alpine microbial community containing *Planticonsortium tenue* (formerly *Glomus tenue*), *R. intraradices*, *G. claroideum/etunicatum* and a new *Acaulospora* species showed a significant increase of E-ocimene concomitant with a decrease of E-2-decenal and (E, E)-2-4-decadienal (Binet et al., 2011). Therefore, the selection of the most effective AMF strains for improving the accumulation of desirable active compounds needs to be taken into account.

II.4.2. Effect of AMF on biomass and production of secondary metabolites in medicinal plants under biotic and abiotic stress conditions

Drought, salinity, heavy metals, pests and diseases can impact plant growth, reducing their biomass (Hashem et al., 2014; Alwhibi et al., 2017) and consequently affecting the production of secondary

STATE OF THE ART

metabolites. Arbuscular mycorrhizal fungi can increase the tolerance/resistance of plants against those abiotic and biotic stresses, potentially influencing secondary metabolites production (Hashem et al., 2018).

Several studies have shown that AMF symbiosis can improve the growth and secondary metabolites production of medicinal plants under water deficit conditions. For example, a recent study by Machiani et al. (2021) showed that inoculation with *F. mosseae* significantly improved biomass and essential oil content (mainly thymol, p-cymene and γ -terpinene) of *Thymus vulgaris* plants grown in a 2-year field experiment in intercropping with soybean under water deficit conditions. Similarly, Mirzaie et al. (2020) reported that inoculation with *F. mosseae* significantly increased geranial and β -pinene (both belong to oxygenated monoterpenes essential oils) yields of *Cymbopogon citratus* grown in a greenhouse pot experiment under moderate water stress conditions (50% field capacity).

Salt stress stimulates the accumulation of phenolic compounds in plants as a general defense mechanism to stress (Parvaiz and Satyawati, 2008). Intriguingly, this abiotic stress is a principal elicitor influencing synthesis of compounds in many herbs (e.g., cinnamic, gallic and rosmarinic acids in *Thymus vulgaris*; glycyrrhizin in *Glycyrrhiza glabra*; quinic, gallic, and protocatechuic acids in *Polygonum equisetiforme*) (Bistgani et al., 2019; Behdad et al., 2020; Boughalleb et al., 2020). A recent study by Amanifar and Toghranegar (2020) reported that moderate salt stress stimulated higher production of valerenic acid in *Valeriana officinalis* than a situation without salt stress. Interestingly, this increase was significant when the plants were colonized by *F. mosseae*. Duc et al. (2021) found that a mixture of six AMF species (*C. etunicatum*, *C. claroideum*, *F. mosseae*, *F. geosporum*, *Rhizoglosum microaggregatum* and *R. intraradices*) increased the tolerance of *Eclipta prostrata* under moderate salt stress in a pot experiment under controlled conditions, inducing major changes in its polyphenols profile.

Minerals, such as cadmium (Cd) and zinc (Zn), also were reported to impact secondary metabolites production in medicinal plants colonized by AMF. For instance, Hashem et al. (2016) observed that an AMF mixture comprising *C. etunicatum*, *F. mosseae* and *R. intraradices* enhanced the chlorophyll and protein content and considerably reduced lipid peroxidation in *Cassia italica* plants under Cd stress in a pot

STATE OF THE ART

experiment. Moreover, AMF inoculation caused a further increase in proline and phenols content ensuring improved plant growth under stress conditions.

Arbuscular mycorrhizal fungi symbiosis improved the disease tolerance of medicinal plants through the mediation of secondary metabolites. For instance, Jaiti et al. (2007), reported that a complex of native AMF species increased the tolerance of *Phoenix dactylifera* [a plant characterized by high nutritional and therapeutic value of its fruits (Al-Alawi et al., 2017)] against bayoud disease (the most damaging vascular disease of date palm caused by *Fusarium oxysporum* f. sp. *albedinis*) by increasing the enzymatic activities of peroxidases and polyphenoloxidases, which are associated with an increase of phenolic compounds in the cell wall.

II.4.3. Mechanisms by which AMF symbiosis promotes secondary metabolism in medicinal plants

It is often considered that the increased concentrations of various secondary metabolite groups (e.g., flavonoids, phenolics) in AMF-colonized plants are a result of the elicitation of several defense response pathways as reviewed by Zeng et al. (2013). For instance, terpenoids in the carotenoid pathway, flavonoids, phenolic compounds and some alkaloids (such as hyoscyamine and scopolamine) in the phenylpropanoid pathway are often increased in AMF-colonized plants (Kaur and Suseela, 2020). These pathways play different roles in the plant-AMF symbiosis, such as signaling, stress tolerance, nutrient uptake, and resistance against biotic and abiotic stresses. However, it is still not totally clear how AMF trigger changes in the concentrations of phytochemicals in plant tissues (Toussaint et al., 2007).

Many studies have focused on the mechanisms by which AMF modulates the production of terpenoids, phenolic compounds and alkaloids in plants. Terpenoids are synthesized from isoprene units in the methylerythrophosphate (MEP) and the mevalonic acid (MVA) pathways (Zhi et al., 2007). Phenolic compounds (e.g., phenols, flavonoids, protanthocyanidins, tannins) are synthesized in the shikimic acid pathway where phenylpropanoids are formed and in the malonic acid pathway (Oksana et al., 2012). Most of the alkaloids are synthesized from various biological precursors (most amino acids) such as tyrosine and tryptophane in the shikimic acid pathway (Facchini 2001) (**Figure 8**).

STATE OF THE ART

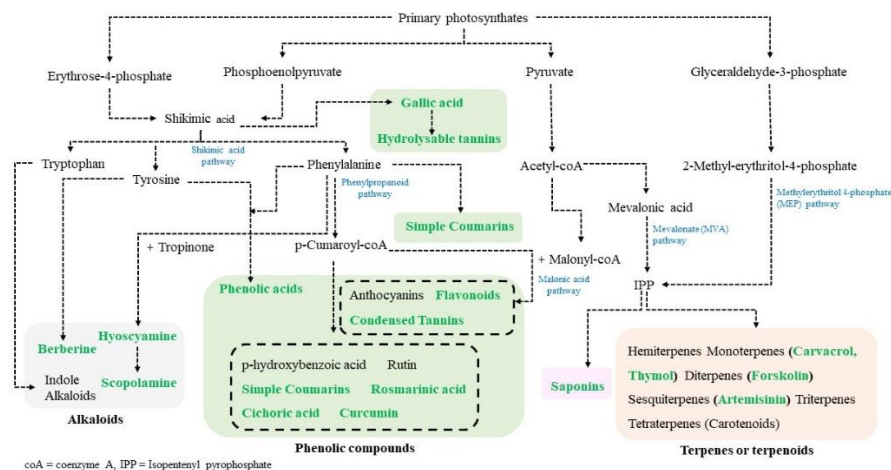


Figure 8: Main pathways of secondary plant metabolism resulting in the production of alkaloids, phenolics, saponins and terpenes (in grey, green, pink, and brown shaded portions, respectively) mentioned in this review. Examples of up-regulated compounds or classes of compounds in medicinal plants associated with AMF are highlighted with green type. This figure is modified from Dos Santos et al. (2021).

Several common nutritional and non-nutritional factors have been proposed to explain the increased production of secondary metabolites in AMF-colonized plants (Kapoor et al., 2017; Sharma et al., 2017; Dos Santos et al., 2021) (**Figure 9**).

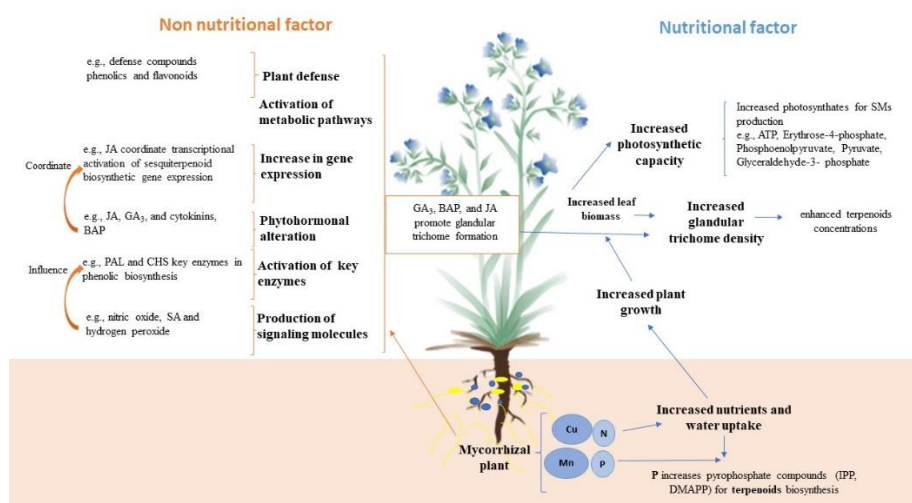


Figure 9: Non-nutritional and nutritional factors influencing the production of

STATE OF THE ART

secondary metabolites (i.e., terpenoids, phenolics, and flavonoids) in AMF-colonized plants.

Non-nutritional factors (leftside in orange): AMF-colonization results in the activation of plant defence mechanisms with the production of phenolics and flavonoids. Change in phytohormone levels, such as jasmonic acid (JA), gibberellic acid (GA₃) and 6-benzylaminopurine (BAP) increases the number and size of glandular trichomes and leads to transcriptional activation of sesquiterpenoid biosynthetic gene expression. AMF induce the production of signaling molecules, such as nitric oxide, salicylic acid (SA) and hydrogen peroxide, which influence the activation of key enzymes such as L-phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), for the biosynthesis of phenolic compounds.

Nutritional factors (rightside in blue): AMF-colonization increases plant nutrients and water uptake leading to increased plant growth and leaf biomass. This result in enhanced plant photosynthetic capacity and increased production of photosynthates which are precursors of different secondary metabolites. Increased leaf biomass leads to a increased density of glandular trichomes in which terpenoids are synthesized and stored.

This figure is adapted with permission from [Springer Nature Customer Service Centre GmbH: Springer Nature, Phytochemistry Reviews. Insight into the mechanisms of enhanced production of valuable terpenoids by arbuscular mycorrhiza. Kapoor et al. (2017)]. We thank Evangelia Tsiokanou (National and Kapodistrian University of Athens, Greece) for graciously providing the picture of the plant used in this figure.

Regarding nutritional factors, the increase was first attributed to the enhanced uptake of nutrients by AMF-colonized plants (Lima et al., 2015; Oliveira et al., 2015; Riter et al., 2014). For example, the role of phosphorus in the synthesis of terpenoids precursors via the MVA (acetyl-CoA, ATP and NADPH) as well as the MEP (glyceraldehyde phosphate and pyruvate) pathways is widely recognized (Kapoor et al., 2017). Phosphorus enhances terpenoid biosynthesis by increasing the concentration of pyrophosphate compounds, such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Kapoor et al., 2002; 2004; Zubek et al., 2010), which contain high-energy phosphate bonds. However, Khaosaad et al. (2006) found that the concentration of essential oils significantly increased in two *Origanum* sp. genotypes colonized by *F. mosseae*, while the levels of essential oils in plants treated with P did not change. This suggests that the increased concentration of essential oils in AMF-colonized *Origanum* sp. plants may directly depend on the association with the

STATE OF THE ART

fungus. In another study by Zubek et al. (2012), AMF colonization improved hypericin and pseudohypericin concentrations in *Hypericum perforatum*, probably because of an improved plant P and/or N nutrition in presence of the fungi. The increased growth through improved nutrients and water uptake of AMF-colonized plants also explains the enhanced production of these compounds in plants. It is well known that the AMF symbiosis increases shoot biomass, shoot length and number of nodes in *Ocimum basilicum* (Gupta et al., 2002; Khaosaad et al., 2008; Rasouli-Sadaghiani et al., 2010; Copetta et al., 2006). Elevated leaf biomass results in increased photosynthetic capacity (Dave et al., 2011; Zubek et al., 2010), thus increasing the production of total photosynthates (e.g., ATP, carbon substrate, glyceraldehyde-3-phosphate, pyruvate, phosphoenolpyruvate or erythrose-4-phosphate) required for terpenoids, phenolics and alkaloid biosynthesis (Cao et al., 2008; Hofmeyer et al., 2010; Niinemets et al., 2002).

Regarding non-nutritional factors, alterations in the levels of phytohormones in AMF-colonized plants may reflect their enhanced production (Mandal et al., 2013; Mandal et al., 2015a; Zubek et al., 2012). Indeed, it has been shown that the AMF symbiosis changes the concentrations of phytohormones, such as jasmonic acid (JA), gibberellic acid (GA₃) and cytokinins (Allen et al., 1980, 1982; Hause et al., 2002; Shaul-Keinan et al., 2002) in plants. Moreover, it has been reported that phytohormones play a role in the secondary metabolism of plants (An et al., 2011; Maes and Goossens, 2010). For instance, JA has been reported to coordinate transcriptional activation of sesquiterpenoid biosynthetic gene expression in *Artemisia annua* (Maes et al., 2011). Furthermore, the phytohormonal alterations of GA₃, BAP (6-benzylaminopurine), and JA have been reported to promote the formation of glandular trichomes (Maes et al., 2011) which is positively correlated with an enhanced concentration of terpenoids in plant leaves. Glandular trichomes are the epidermal secretory structures which can synthesize, store, and secrete large amounts of bioactive metabolites, such as terpenoids, phenylpropanoids, flavonoids and alkaloids (Schilmiller et al., 2008). The enhanced concentration of terpenoids (essential oils) and increased glandular trichome density has been observed in a number of plants (e.g., *Mentha x piperita*, *Phaseolus lunatus*, and *Lavendula angustifolia*) (Ringer et al., 2005; Bartram et al., 2006; Behnam et al., 2006). Thus, an increase in trichome density upon mycorrhization often has been linked with an enhanced concentration of terpenoids (Copetta et al., 2006; Kapoor et al., 2007;

STATE OF THE ART

Morone-Fortunato and Avato, 2008). The modification of these secondary metabolite concentrations in AMF-plants also may be due to signaling mechanisms between host plants and the fungi (Rojas-Andrade et al., 2003; Xie et al., 2018). For example, Zhang et al. (2013a) have reported that *F. mosseae* associated with *Trifolium repens* promoted changes in the concentration of signaling molecules, such as nitric oxide, salicylic acid (SA) and hydrogen peroxide, which influence the activation of key enzymes in phenolics biosynthesis [e.g., L-phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS)]. Moreover, AMF may increase the expression of genes encoding enzymes leading to the biosynthesis of these compounds in mycorrhizal plants (Andrade et al., 2013; Battini et al., 2016; Mandal et al., 2015a, b; Xie et al., 2018). For example, induction of terpene synthase (TPS) family genes TPS31, TPS32 and TPS33 has been observed in AMF-colonized tomato plants and probably can explain the changes in their terpenoid profile (Zouari et al., 2014). Mandal et al. (2015a) reported the increase of artemisinin in leaves of *Artemisia annua* inoculated with *R. intraradices*. This result was correlated with a higher expression of key biosynthesis genes (such as an allene oxidase synthase gene encoding one of the key enzymes for JA production) via enhanced JA levels. In addition, AMF may enhance the biosynthesis of these compounds either by increasing the production of precursors through the induction of metabolic biosynthetic pathways (Zimare et al., 2013; Dos Santos et al., 2021) and/or by induction of key synthase enzymes (Mandal et al., 2013; Shrivastava et al., 2015; Sharma et al., 2017; Dos Santos et al., 2021). For example, mycorrhizal colonization (*R. intraradices*) has been found to elevate the transcript levels of two of the pivotal enzymes of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in wheat roots (Walter et al., 2000). DXS is an enzyme that catalyzes the initial step of the MEP pathway, where many isoprenoids are biosynthesized, and DXR is an enzyme that is immediately downstream from DXS in the MEP pathway (Walter et al., 2000). In another study by Walter et al. (2002), DXS2 transcript levels were strongly stimulated in *Medicago truncatula* roots upon colonization by AMF (a mixture of *F. mosseae* and *R. intraradices*), and were correlated with the accumulation of carotenoids and apocarotenoids. Finally, alterations in these secondary metabolites' production also can result from plant defense responses to AMF colonization (Mechri et al., 2015; Zubek et al., 2012, 2015; Torres et al., 2015).

STATE OF THE ART

Various studies have reported increased production of Alkannin/Shikonin and their derivatives (A/Sd) in cell cultures of boragenaceous plants (e.g., *L. erythrorhizon*, *A. tinctoria*, and *Arnebia euchroma*) after applying exogenous jasmonate (Gaisser and Heide, 1996; Urbanek et al., 1996; Bychkova et al., 1993). Alkannin/Shikonin are naphthoquinone compounds with a broad spectrum of biological activities, such as wound healing, anti-inflammatory and anticancer (Kheiri et al., 2017; Kourounakis et al., 2002; Andújar et al., 2013). Interestingly, AMF-colonization of various other plants, such as *Hordeum vulgare*, *Cucumis sativus*, *Medicago truncatula*, and *Glycine max* have resulted in the increase of endogenous levels of jasmonates within roots (Hause et al., 2002; Vierheilig and Piché, 2002; Stumpe et al., 2005; Meixner et al., 2005). Jasmonic acid and its derivatives, commonly termed jasmonates, are hormonal regulators involved in plant responses to abiotic and biotic stresses as well as in plant development (Creelman and Mullet, 1997; Wasternack, 2007). The level of endogenous jasmonate was shown to increase after wounding or pathogen attack. There is no direct study on the effects of AMF on A/Sd production in these Boragenaceous medicinal plants. However, these findings suggest that AMF could be a potential factor enhancing A/Sd production in mycorrhizal Boraginaceae plants through the regulation of jasmonate.

II.4.4. Cultivation techniques for secondary metabolites production in mycorrhiza-associated medicinal plants

Plant secondary metabolites are often extracted from individuals grown in nature. For instance, around 95% of the medicinal plants used in the Indian herbal industry today are collected from the wild (Lakshman, 2016). However, the quantity and quality of secondary metabolites from plants grown in nature are erratic, often influenced by abiotic and biotic factors, such as extreme temperatures, drought, alkalinity, salinity and plant pathogens, impacting the metabolic pathways responsible for the accumulation of bioactive substances (Dayani and Sabzalian, 2017; Giurgiu et al., 2017; Ramakrishna and Ravishankar, 2011). Furthermore, overharvesting of medicinal plant species in nature could place them at a high risk of extinction (Roberson, 2008). Finally, growing medicinal plants under field conditions may be time consuming, especially for woody plants (e.g., *Taxus brevifolia* and *L. erythrorhizon*) and slow-growing perennial plants (e.g., *Panax ginseng*), which can take several years to reach the desired metabolites production (Malik et al., 2011; Chandran et al., 2020; Yazaki, 2017;

STATE OF THE ART

Murthy et al., 2014). Therefore, there is a need for alternative production systems.

Production of medicinal herbs in controlled environments provides opportunities for improving the quality, purity, consistency, bioactivity, and biomass production of the raw material (Hayden, 2006). In order to secure the commercial production of secondary metabolites, several cultivation techniques have been developed, potentially compatible with AMF application.

4.4.1 Substrate-based cultivation systems

Greenhouse cultivation – Greenhouses are widely used for crop production all-year round. Environmental parameters (e.g., temperature, humidity) are controlled, providing optimal growth conditions to the target crop or plant, favoring development and thus safeguarding the yield and consistent production of high-quality bioactive compounds (Panwar et al., 2003).

Many medicinal plant species, such as *Echinacea angustifolia*, *Echinacea purpurea*, *Ocimum basilicum*, *Withania somnifera*, and *Psoralea corylifolia* have been grown under greenhouse conditions (Zheng et al., 2006; Panwar et al., 2003). Similarly, many such as *Artemisia annua*, *Curcuma longa*, *Coleus forskohlii*, *Glycyrrhiza glabra*, *Gloriosa superba*, have been associated with AMF under greenhouse conditions with high production of bioactive compounds reported (Huang et al., 2011; Dutta and Neog, 2016; Sailo and Bagyaraj, 2005; Liu et al., 2007; Pandey et al., 2014). Therefore, growing medicinal plants in association with AMF under greenhouse conditions could represent a suitable method for improving the quality and production of bioactive compounds at large scale.

4.4.2 Substrate-free cultivation systems

Aeroponics – In the aeroponics cultivation system, the roots of plants are hung inside a sealed container in darkness and exposed to a water nutrient-rich spray through atomizers (Lakhari et al., 2018) (**Figure 10a**). This technique has been developed for the cultivation of many different plants, such as horticultural crops (e.g., *Lactuca sativa*, *Cucumis sativus*, and *Solanum lycopersicum*) (Movahedi and Rostami, 2020), medicinal herbs (e.g., *Anemopsis californica*, *Crocus sativus*, and *Valeriana officinalis*) (Hayden, 2006; Souret and Weathers, 2000; Tabatabaei, 2008), and medicinal crops (e.g., *Arctium lappa* and *Zingiber officinale*) used to extract secondary metabolites from their

STATE OF THE ART

roots (Hayden et al., 2004a, b). It has been reported that *Ocimum basilicum* grown under aeroponic conditions had a higher yield, comparable phenolics and flavonoids contents, and antioxidant properties compared to plants grown in a solid substrate (Chandra et al., 2014). Similarly, *Cichorium intybus*, *Withania coagulans* and *Echinacea sp.* grown in an aeroponic system had higher yields compared to the same plants grown in soil (Movahedi and Rostami, 2020). This system was efficient for the production of bioactive molecules from roots of medicinal crops, such as chlorogenic acid in *A. lappa* and β -sitosterol in *Cannabis sativa* (Hayden et al., 2006; Ferrini et al., 2021). For various medicinal plants, root apices constitute the main sites where active substances are produced and stored (Watson et al., 2015). However, these active substances are almost impossible to harvest through conventional farming methods. By using the Plant Milking Technology (Plant milking[®]) (<https://www.plantadvanced.com/home>) for *Morus alba* (an emblematic tree of traditional Chinese medicine, rich in alkaloids and flavonoids), Chajra et al. (2020) obtained an extract enriched in prenylated flavonoids that was 18-fold higher than commercial root extracts (**Figure 10a and b**).

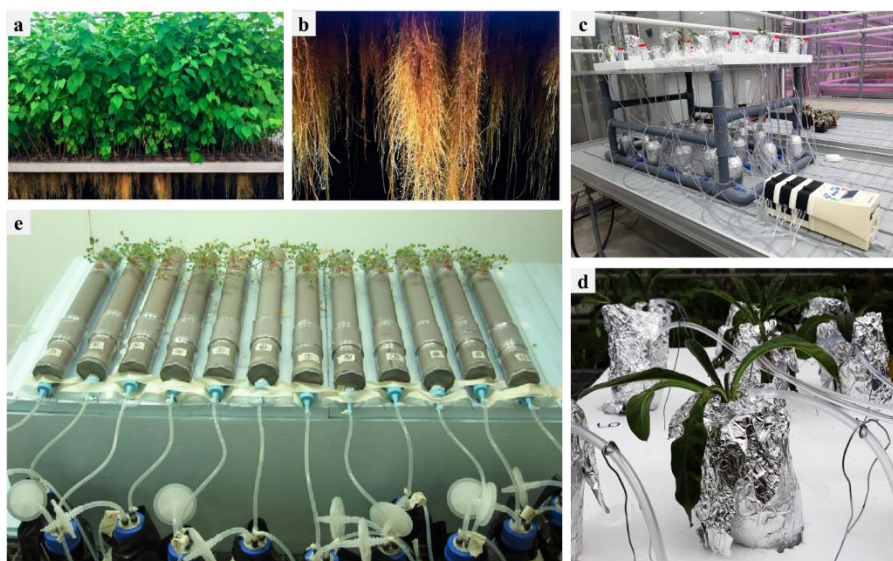


Figure 10: (a) *Morus alba* trees cultivated in aeroponic conditions and (b) close-up view of *Morus alba* roots grown aeroponically (Chajra et al., 2020); (c) *Anchusa officinalis* associated with *Rhizophagus irregularis* MUCL 41833 growing in a semi-hydroponic cultivation system and (d) close-up view of a plant (UCLouvain, greenhouse); (e) Plant-based bioreactor system for the

STATE OF THE ART

mass production of AMF as described in Declerck et al. 2009 (WO/2009/090220).

Interestingly, aeroponic cultivation systems also have been developed and used for the production of AMF inoculum in which roots (and AMF) were bathed in a nutrient solution mist (Zobel et al., 1976; Hung and Sylvia, 1988). For the production of AMF, plants are precolonized prior to their introduction into the system, through preculturing plant seedlings and AMF propagules (both preferably surface-sterilized) in pots containing a substrate (e.g., mixture of sand and perlite). Then the precolonized plants are transferred to the aeroponic container where the roots (and AMF) develop. The container is usually protected from light to prevent the development of algae (Jarstfer and Sylvia, 1995). The mist can be applied by various techniques that differ mainly in the size of the fine droplets produced (e.g., atomizing disk, pressurized spray through a microirrigated nozzle, an ultrasonically generated fog of nutrient solution with droplets of 3–10 µm diameter, and ultrasonic nebulizer technology resulting into microdroplets of 1 µm in diameter) (IJdo et al., 2011; Jarstfer and Sylvia, 1995; Mohammad et al., 2000). Mohammad et al. (2000) reported a high number of viable AMF propagules obtained in aeroponic culture, and such inoculum was used in a field experiment (Mohammad et al., 2004). Thus, aeroponic systems could potentially be used for growing medicinal plants associated with their AMF partners in order to obtain substantial biomass and production of secondary metabolites.

Hydroponics – Hydroponic systems include all systems that deliver nutrients in liquid, with or without a solid medium to anchor plant roots (Hayden, 2006) (**Figure 10c**). Such systems have been applied to several medicinal plants, such as *Echinacea angustifolia*, *Ocimum basilicum*, *Leonurus quinquelobatus*, *Mentha piperita*, *Salvia officinalis*, *Achillea millefolium*, *Bidens tripartite*, *Leonurus sibiricus*, *Linum usitatissimum*, *Hypericum perforatum*, *Tanacetum parthenium* (Maggini et al., 2012; Mairapetyan et al., 2018; Simeunovic, 2002). Thanks to these systems, the biosynthesis of active compounds, such as tropane alkaloids in *Datura innoxia*, total phenols and rosmarinic acid in *Ocimum basilicum* and oil production in *Valeriana officinalis* have been obtained (Gontier et al., 2002; Sgherri et al., 2010; Tabatabaei, 2008).

Different hydroponic culture systems also exist for the mass production of AMF. They mainly differ in the mode of aeration and application of the nutrient solution (IJdo et al., 2011). For instance, in the static

STATE OF THE ART

hydroponic culture system, the nutrient solution is not flowing and needs to be aerated via an aeration pump to prevent roots of mycorrhizal plants from suffering oxygen deprivation (IJdo et al., 2011). Via this system, Dugassa et al. (1995) obtained large quantities of mycorrhizal *Linum usitatissimum* plant roots as well as extramatrical mycelium and chlamydospores free of residues from solid substrate components. In another nutrient film technique (NFT) hydroponic system, a thin nutrient solution (i.e., film) flows into inclined channels (also called gullies) where the plant roots and AMF develop (IJdo et al., 2011). This technique has been used to culture AMF since the early nineteen-eighties with the production of many sporocarps by *F. mosseae* (Elmes and Mosse, 1984). Later, IJdo et al. (2011) developed an innovative low-cost *in vitro* plant-based bioreactor system for the mass production of AMF. In this system, *Medicago truncatula* roots and AMF (*Glomus* sp.) were grown in a sterilized tube connected at both extremities to a reservoir containing sterilized liquid culture medium. This nutrient solution circulates across the mycorrhizal root system, feeding the plant/fungus associates, while the plant shoot develops in open-air conditions inside a controlled growth chamber (**Figure 10e**). The hydroponic system also has been developed for studying the effect of the AMF symbiosis (e.g., P uptake) on maize plants (Garcés-Ruiz et al., 2017). Therefore, hydroponic or semi- hydroponic systems could potentially be combined with medicinal plants and AMF in order to obtain increased production of secondary metabolites. In a recent study, Cartabia et al. (2021) showed how the arbuscular mycorrhizal fungus *R. irregularis* modified the primary and secondary metabolism and the root exudates of the medicinal plant *Anchusa officinalis* growing under a semi-hydroponic cultivation system (**Figure 10c**). Moreover, permeabilization treatments can be conducted in these cultivation systems, in order to extract the compounds exuded by roots in a non-destructive process that “milks” the same plants several times a year. For example, in the study by Gontier et al. (2002), *Datura innoxia* plants were cultivated in hydroponic conditions (no AMF were involved) and the plant roots subsequently permeabilized with Tween 20. As a result, a high concentration of tropane alkaloids (TA) (e.g., hyoscyamine and scopolamine) was detected in the nutrient solution. Interestingly, all the plants were able to survive after being rinsed and replaced in the hydroponic system. This approach allows the permeabilization of the plant multiple times without loss of viability (Gontier et al., 2002). Moreover, different permeabilization treatments (e.g., doses and duration of Tween 20, addition of TA precursors) can

STATE OF THE ART

be chosen to release additional bioactive compounds in the nutrient solution [i.e., TA precursors (phenylalanine and ornithine) leading to 10-80 mg/l TA in the nutrient solution] (Gontier et al., 2002). This study, however, did not include association with AMF.

4.4.3 *In vitro* production systems

Micropropagation or *in vitro* propagation is the clonal propagation of plants by tissues, cells or organs. It involves the aseptic culture of explants of tissues or organs in closed vessels using defined culture media in a controlled environment (Debnath and Arigundam, 2020).

Whole plant *in vitro* culture – *In vitro* cultivation of whole plants is widely used for mass propagation, conservation of germplasm, production of bioactive compounds and for genetic improvement of a large number of medicinal plant species (Nalawade and Tsay, 2004). For instance, protocols have been developed for the *in vitro* mass propagation of *Limonium wrightii*, *Adenophora triphylla*, *Gentiana davidii*, *Anoectochilus formosanus*, *Scrophularia yoshimurae*, *Pinellia ternata*, *Bupleurum falcatum*, *Zingiber zerumbet*, *Dendrobium linawianum*, and *Fritillaria hupehensis* via shoot morphogenesis, for *Angelica sinensis* and *Corydalis yanhusuo* via somatic embryogenesis, and for *Taxus mairei*, *Angelica dahurica*, *Angelica sinensis*, *Dioscorea doryophora*, *Gentiana davidii*, and *Bupleurum falcatum* via cell suspension cultures (Nalawade and Tsay, 2004).

The association of AMF with whole plants *in vitro* has been described in several studies (e.g., Dupré de Boulois et al., 2006; Voets et al., 2005; Lalaymia and Declerck, 2020). For instance, using the mycorrhizal donor-plant *in vitro* cultivation system, Voets et al. (2009) obtained fast and homogenous mycorrhization of *Medicago truncatula* seedlings by placing the plantlets in an actively growing mycelial network arising from a mycorrhizal donor plant (**Figure 11a**). In another system, called the half-closed arbuscular mycorrhizal-plant *in vitro* culture system, roots of micropropagated potato plantlets were associated with actively growing AMF propagules, while the shoots developed in open air conditions (Voets et al., 2005) (**Figure 11c**). Applying both systems, several thousand spores of *R. intraradices* were produced on an extensive extraradical mycelium and abundant root colonization has been obtained. Hence, they could be extended to medicinal plants to enhance secondary metabolites production (**Figure 11**).

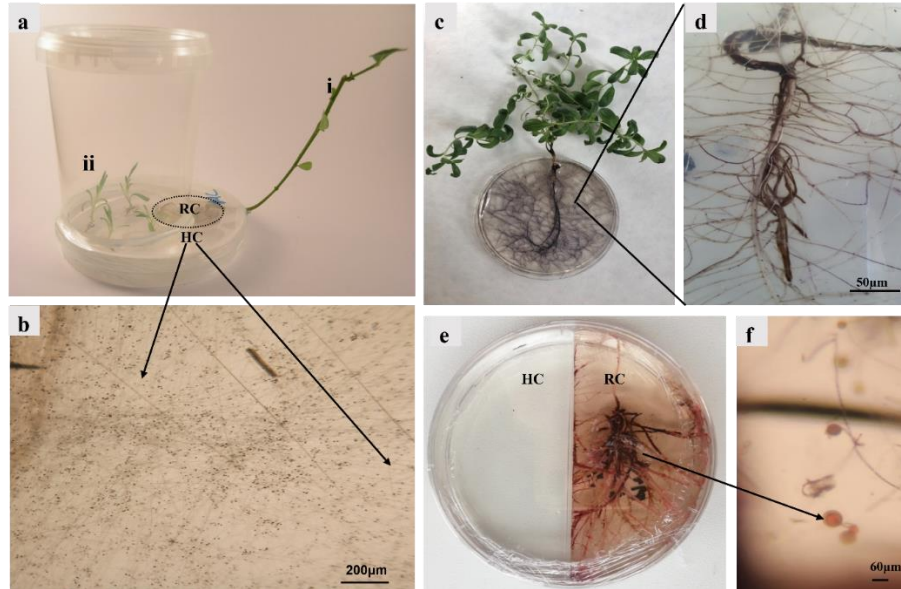


Figure 11: (a) 145 mm mycorrhizal donor plant *in vitro* culture system. (i) The donor plant is *Crotalaria spectabilis* growing in a root compartment (RC) in close association with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 and (ii) the receiver plants are *Alkanna tinctoria* growing under a lid in a hyphal compartement (HC) in which only a profuse, active extraradical mycelium network proliferates; (b) close-up view of extensive development of extraradical mycelium and spores in the HC; (c) 90 mm half-closed arbuscular mycorrhizal plant *in vitro* culture system allowing the growth of the roots of *L. erythrorhizon* in close association with *R. irregularis* MUCL 41833; (d) close-up view of the reddish roots due to shikonin production; (e) 90 mm root organ culture *in vitro* system allowing the growth of Ri T-DNA transformed *A. tinctoria* hairy root (Rat et al., 2021) in association with *R. irregularis* MUCL 41833 in the RC; (f) close-up view of the red AMF spores produced in the RC (arrows). We thank Alicia Varela Alonso (Institut für Pflanzenkultur, Germany) for graciously providing the picture c and d and Angélique Rat (Ghent University, Belgium) for providing the *Alkanna tinctoria* hairy roots used in this figure.

The system (a) starts with a donor plant (*Crotalaria spectabilis*) introduced into the RC of a bi-compartmented system [a small Petri plate indicated by a dashed circle (RC) (90 mm diameter)] placed in a large Petri plate (HC) (145 mm diameter). A hole is made in both Petri plates allowing the shoot to extend outside the system. Approximately 500 spores from an AMF *in vitro* culture are placed in contact with the roots. The roots and AMF are kept in the dark during the whole growth period, while shoots remain under light. Once the

STATE OF THE ART

donor plant is well colonized, the extraradical mycelium starts to cross the partition wall separating the RC from the HC, developing profusely in the HC. At that time, one or several receiver micropropagated plants (*Alkanna tinctoria*) are placed in the HC with their roots in contact with the extraradical mycelium. The plants are planted inside the HC under a lid. Briefly, the base of a cylinder (150 mm high 100 mm diameter) matches a hole made in the lid of the 145 mm Petri plate. The cylinder top is glued to a 100 mm Petri plate lid. The culture plates containing the *A. tinctoria* plants are sealed and covered, up to the base of the cylinder, by black plastic bags. with black plastic bags. The systems are incubated in a growth chamber to allow plant and AMF growth [Detailed procedures of this system can be found in Lalaymia and Declerck (2020)].

For system (c), homogenously chopped agar containing AMF propagules from an AMF *in vitro* culture is inoculated to the newly growing root of a micropropagated seedling of *L. erythrorhizon*. After a few days, the new hyphae growing from the spores colonizes the roots of *L. erythrorhizon*.

In system (e), fine root structures of Ri T-DNA transformed *Alkanna tinctoria* hairy roots are cut and placed in the RC part of a bi-compartmental Petri plate. Chopped agar containing AMF propagules is spread on the young parts of the hairy roots. After a few days, new hyphae growing from spores colonize the *A. tinctoria* hairy root, producing new spores and extensive mycelium after several months.

All these three techniques should be conducted under a laminar flow hood with sterilized laboratory materials.

Hairy root cultures (HRCs) - Hairy root cultures are cultures raised after the infection of explants/cultures by the gram-negative soil bacterium *Agrobacterium rhizogenes*² (Tepfer and Casse-Delbart, 1987). This bacterium leads to the neoplastic growth of roots which are characterized by high growth rates in hormone-free media and genetic stability (Pistelli et al., 2010). Ri T-DNA transformed hairy roots grow faster than adventitious roots, or even conventional plant cultures in which plants are growing in soil or substrate (Paek et al., 2009; Yu et al., 2005). For instance, *Panax ginseng*, a valuable medicinal plant originating from Asia, has been used as a healing drug and health tonic since ancient times (Tang and Eisenbrand, 1992) due to its production of triterpene saponins, collectively called ginsenosides (Dewick, 1997; Huang, 1998). However, it generally takes 5 to 7 years in the field to attain maturity and to reach the harvesting stage for extraction of

² now called *Rhizobium rhizogenes*.

STATE OF THE ART

bioactive compounds (Murthy et al., 2014). To solve this problem, many different techniques have been explored, such as culture of callus tissues, suspended cells, adventitious roots, and HRCs (Furuya et al., 1973; Furuya et al., 1983; Paek et al., 2009; Shi et al., 2021). It has been reported that HRCs grow more rapidly and produce a higher level of ginsenosides than the suspended cells and adventitious root cultures (Inomata et al., 1993; Yoshikawa and Furuya, 1987). Moreover, HRCs produce the same phytochemical patterns as the wild type root organs and accumulate higher levels of certain valuable compounds compared with adventitious roots and native-grown plant roots (Kai et al., 2011; Hao et al., 2020; Miao et al., 2017). For instance, the total tanshinone content reached 15.4 mg/g dry weight (DW) in transgenic *Salvia miltiorrhiza* hairy roots, while only 1.7–9.7 mg/g DW tanshinone was produced in roots of field-grown plants (Kai et al., 2011; Hao et al., 2020). High stability and productivity, high biomass production and efficient biosynthetic capacity make HRCs valuable biotechnological tools for the production of plant secondary metabolites (Pistelli et al., 2010; Gutierrez-Valdes et al., 2020). Some examples of metabolites produced using HRCs are tropane alkaloids, such as scopolamine and hyoscyamine (Jouhikainen et al., 1999; Häkkinen et al., 2016; Guo et al., 2018; Khezerluo et al., 2018), catharanthine (Hanafy et al., 2016), ginsenosides (Woo et al., 2004; Ha et al., 2016), solanoside (Putalun et al., 2004), and anthraquinones (Perassolo et al., 2017). The studies mentioned here, however, did not involve inoculation with AMF.

In order to upscale production and commercialize secondary metabolites, various conventional bioreactors, broadly classified as liquid phase, gas phase or hybrid reactors, have been employed for the mass production of HRCs, which permit the growth of interconnected tissues normally unevenly distributed throughout the vessel. For instance, increased production of terpenoid indole alkaloid and artemisinin were obtained with HRCs of *Rauwolfia serpentina* and *Artemisia annua* grown in different reactors (no AMF involved) (Mehrotra et al., 2015; Patra et al., 2014). Interestingly, the *in vitro* large-scale production of AMF is mostly based on HRCs (Declerck et al., 1996; 2006) which involve the association of AMF propagules with transformed hairy roots on synthetic mineral media. Arbuscular mycorrhizal fungi species grown in HRCs produce viable and contaminant-free spores (Fortin et al., 2005) (**Figure 11e**). From these studies, we can suggest HRCs of medicinal plants associated with AMF in bioreactors for the commercial production of secondary metabolites.

STATE OF THE ART

Table 3: Detailed summary of studies on the relationship between AMF and medicinal plants.

Plant Family	Plant Species	AMF ^a	Secondary metabolites group and active ingredients	Medicinal Value of the plant	Plant Organ studied	Change in secondary metabolites production	Change in plant growth/biomass	Reference
Alliaceae	<i>Allium sativum</i>	<i>Rhizophagus fasciculatus</i>	alliin	antibacterial, antifungal, antiviral, antiprotozoa, antioxidative and anticancerogenic properties; against arteriosclerosis and high blood pressure	leaves, flowers, and cloves	significant increase	increase in plant height, total biomass and bulb diameters, bulb weight, and yield.	Borde et al., 2009
Amaranthaceae	<i>Achyranthes aspera</i>	<i>Rhizophagus fasciculatus</i>	flavonoids	treatment of cough, bronchitis, rheumatism, malarial	_____ ^b	increased the contents of active principles	positive effect on plant growth parameters	Tejavathi and Jayashree, 2011

^a the column “AMF” shows the current names not the one at the time of publication.

^b - means there is no studies or available data found online.

STATE OF THE ART

				fever, dysentery, asthma, hypertension and diabetes (Bhosale et al., 2012)				
Anacardiaceae	<i>Myracrodruon urundeuva</i>	<i>Acaulospora longula</i>	total phenols and flavonoids	anti- inflammator y, anti-ulcer, astringent, anti-allergic, and antidiarrheal activities (Teixeira et al., 2020)	leaves	81.03% increased	promote plant growth	Oliveira et al., 2013
Apiaceae	<i>Angelica dahurica</i>	<i>Glomus spp.</i>	Imperatorin and total coumarins	treatment for colds, headache, dizziness, toothache, supraorbital pain, nasal congestion, acne, ulcer, carbuncle, and rheumatism (Lee et al., 2015)	root, seed, and fruit	significant increase	plant growth and biomass promoted	Zhao and He, 2011

STATE OF THE ART

	<i>Angelica archangelica</i>	<i>Funneliformis mosseae</i>	monoterpenoid and coumarin	dyspeptic complaints such as mild gastrointestinal spasms, sluggish digestion, flatulence and feeling of fullness, loss of appetite, anorexia and bronchitis	rhizome and roots	a marked increase in yield	biomass increased	Zitterl-Eglseer et al., 2015
	<i>Anethum graveolens</i>	<i>Glomus macrocarpum</i> , <i>Rhizophagus fasciculatus</i>	anethole	treatment for abdominal discomfort and colic and also for promoting digestion (Jana and Shekhawat, 2010)	seed	90% increased	improved the growth	Kapoor et al., 2002
	<i>Bupleurum scorzonerifolium</i>	_____	flavonoids	treating cold, fever, chest pain, irregular menstruation, uterine fall off and rectocele	_____	_____	_____	Teng and He, 2005

STATE OF THE ART

	<i>Coriandrum sativum</i>	<i>Glomus hoi</i>	β -caryophyllene p-cymene, geraniol	antioxidant, antidiabetic, hepatoprotective, antibacterial, and antifungal activities (Asgarpanah and Kazemivash, 2012)	seeds or leaves	significant improvement	————	Rydlová et al., 2016
	<i>Foeniculum vulgare</i>	<i>Glomus macrocarpum</i> and <i>Rhizophagus fasciculatus</i>	essential oil concentration	used for digestive, endocrine, reproductive, and respiratory systems (Badgujar et al., 2014)	seeds	significantly increased	improved plant growth	Kapoor et al., 2004
	<i>Trachyspermum ammi</i>	<i>Rhizophagus fasciculatus</i>	thymol	antifungal, antioxidant, antimicrobial properties and used for antinociceptive, hypolipidemic, antihypertensive,	fruits	72% increased	————	Kapoor et al., 2002

STATE OF THE ART

				antispasmodic, broncho-dilating actions, antilithiasis and diuretic (Bairwa et al., 2012)				
Apocynaceae	<i>Catharanthus roseus</i>	<i>Glomus spp.</i>	vinblastine and vincristine rutin, quercetin, and kaempferol	treatment of diuretic, hemorrhagic, wound healing, coughs, sore throats, lung infections and diabetes (Gupta et al., 2017)	aerial part	significant increase	————	Andrade et al., 2013
	<i>Gymnema sylvestre</i>	<i>Rhizophagus fasciculatus</i> and <i>Funneliformis mosseae</i>	gymnemic acid	control diabetes mellitus	shoots and leaves	positive increased	higher shoot and root length and fresh and dry weight	Zimare et al., 2013
Araliaceae	<i>Panax ginseng</i>	————	————	reinforcing vital energy and restoring physiological weakness and possess antioxidant	————	————	plant seedlings biomass significantly increased	Cho et al., 2009

STATE OF THE ART

				, anti-inflammatory, antiallergic, antidiabetic, and anticancer properties (Kim, 2018)				
		<i>Rhizophagus intraradices</i>	ginsenosides		roots	increased total content	————	Tian et al., 2019
	<i>Panax notoginseng</i>	————	————	used for stanch bleeding, invigorating, and supplementing blood (Yang et al., 2014)	————	————	only AMF community study from plant	Ren et al., 2007
Araceae	<i>Pinellia ternate</i>	<i>Rhizophagus intraradices</i> , <i>Funneliformis mosseae</i>	L-ephedrine and guanosine	treating cough and vomiting	tubers	significant increase	increasing fresh weight and dry weight	Guo et al., 2010
	<i>Acorus calamus</i>	<i>Funneliformis mosseae</i> and <i>Acaulospora laevis</i>	————	anti-spasmodic and anti-anthelmintic properties and also used for treatment	——	————	significantly increase in plant height, plant spread, number of leaves per plant and	Yadav et al., 2011

STATE OF THE ART

				of epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and tumors			leaf area	
Asteraceae	<i>Atractylodes macrocephala</i>	<i>Funneliformis mosseae</i>	atractylol	strengthenin g the spleen, benefiting vital energy, eliminating dampness, hidroschesis, and soothing fetuses (Gu et al., 2019)	rhizome	significant increase	————	Lu and He, 2005
	<i>Atractylodes lancea</i>	<i>Funneliformis mosseae</i>	————	used to treat rheumatic diseases, digestive disorders, night blindness, and influenza and also exert anti-	————	no effect on essential oil contents	improved plant growth	Guo et al., 2006

STATE OF THE ART

				cancer, anti-obesity and anti-inflammatory effects (Jun et al., 2018)				
		<i>Claroideoglomus etunicatum</i> <i>Glomus tortuosum</i> and <i>Funneliformis mosseae</i>	essential oils hinesol, β -eudesmol and atractylodin		—	increased	increased the survival rate of seedlings, plant height, root length, and leaf number significantly increased	Liang et al., 2018
	<i>Artemisia annua</i>	<i>Rhizophagus irregularis</i>	artemisinin content	treat fever, inflammation, malaria, cough, stomach and intestinal upset	leaves	17% increased	significant increase in fresh and dry plant biomass	Domokos et al., 2018
	<i>Arnica montana</i>	<i>Funneliformis geosporum</i> , <i>Funneliformis constrictum</i>	sesquiterpene lactones	stimulate blood flow, promote healing, and soothe arthritic pains	fresh or dried flower	significant Increase	—	Jurkiewicz et al., 2010
		several <i>Glomus</i>	phenolic acids		roots	increased concentration	—	Jurkiewicz et al., 2010

STATE OF THE ART

		strains				n		
	<i>Artemisia umbelliformis</i>	<i>Planticonsortium tenue</i> , <i>Rhizophagus intraradices</i> , <i>Claroideoglomus claroideum/e</i> <i>tunicatum</i> and a new <i>Acaulospora</i> <i>species</i>	essential oil E- β -ocimene	against coughs	shoots	significantly increased	increase of P concentration in shoots	Binet et al., 2011
	<i>Baccharis trimera</i>	<i>Rhizophagus clarus</i>	phenolics	antioxidant, anti- microbial, anti-fungal, anti-parasitic and anti- inflammatory properties and used for gastric and hepatic- protector (Rabelo and Costa 2018)	—	marked increases	dry weight of the aerial part and height of plants increased	Freitas et al., 2004
	<i>Cynara cardunculus</i>	<i>Rhizophagus intraradices</i> and <i>Funneliformis mosseae</i>	phenolics	prevent carcinogenes is and atherosclerosis	leaves and flowers	marked increases	—	Ceccarelli et al., 2010

STATE OF THE ART

			total phenolic content		—	no impact	significantly increased plant yield	Colonna et al., 2016
	<i>Echinacea purpurea</i>	<i>Rhizophagus intraradices</i>	phenolics and cichoric acid	treatment of toothache, bowel pain, snake bite, skin disorders, seizure, chronic arthritis, and cancer (Grimm and Muller 1999)	root and aerial parts	significant increase	plant growth increased	Araim et al., 2009
	<i>Eclipta alba</i>	<i>Glomus aggregatum</i> , <i>Funneliformis mosseae</i> and <i>Rhizophagus fasciculatus</i> .	flavonoids	treatment of gastrointestinal disorders, respiratory tract disorders (including asthma), fever, hair loss and graying of hair, liver disorders (including jaundice), skin disorders,	—	increased	positive effect on plant growth	Tejavathi and Jayashree, 2011

STATE OF THE ART

				spleen enlargement, and cuts and wounds (Jahan et al., 2014)				
	<i>Eclipta prostrata</i>	<i>Rhizophagus irregularis</i> , <i>Funneliformis mosseae</i> , <i>Claroideoglossus etunicatum</i> , <i>Claroideoglossus claroideum</i> , <i>Rhizoglossus microaggregatum</i> and <i>Funneliformis geosporum</i>	scopolamine	treatment of diabetes type II, dizziness, haemoptysis, and liver diseases	leaves	0.34% increased	————	Vo et al., 2019
			quercetin		whole plant	0.87% increased	————	Vo et al., 2019
	<i>Inula ensifolia</i>	<i>Rhizophagus clarus</i>	thymol derivatives	possess antiproliferative activity against human cancer	roots	increased	————	Zubek et al., 2010
	<i>Stevia rebaudiana</i>	<i>Rhizophagus fasciculatus</i>	stevioside, rebaudioside -A	used as a substance strengthening the heart, the	leaves	significant increased	————	Mandal et al., 2013

STATE OF THE ART

				circulatory system and regulating blood pressure (Marcinek and Krejpcio, 2016)				
		<i>Rhizophagus irregularis</i>	————		————	positive increased	leaf dry biomass increased	Tavarini et al., 2018
	<i>Spilanthes acmella</i>	<i>Funneliformis mosseae</i> and <i>Acaulospora laevis</i>	————	antiseptic, antibacterial, antifungal, and antimalarial properties and used as remedy for toothache, flu, cough, rabies diseases, and tuberculosis	————	————	improved the survival rate, plant growth and biomass yield of micropropagated plantlets	Yadav et al., 2012
	<i>Tagetes erecta</i>	————	————	used as antiseptic and in kidney troubles, muscular pain, piles	————	————	positively improved plant growth, and flower quality under drought stress	Asrar and Elhindi, 2010

STATE OF THE ART

				and applied to boils and carbuncles (Singh et al., 2020)				
	<i>Wedelia chinensis</i>	<i>Rhizophagus fasciculatus</i>	total phenols, ortho dihydroxy phenols, flavonoids, alkaloids, tannins and saponins	treatment of bites, stings, fever, infection, kidney dysfunction, cold, wounds and amenorrhea problems (Rehana and Nagarajan 2018)	seedlings	increased	————	Nisha and Kumar, 2010
Burseraceae	<i>Commiphora leptophloeos</i>	<i>Gigaspora albida</i> and <i>Claroideoglomus etunicatum</i> (native)	total phenols and tannins	treatment of bronchitis, cough, renal problems, general inflammation and stomachache	seedling leaves	significantly increased	————	Lima et al., 2017
Caprifoliaceae	<i>Valeriana Jatamansi</i>	<i>Rhizophagus intraradices</i>	gallic acid, chlorogenic acid, catechin, hydroxyl	possess sedative, neurotoxic, cytotoxic, antidepressant	rhizome and root	significant increase	significantly increased in above ground fresh and dry	Jugran et al., 2015

STATE OF THE ART

			benzoic acid	nt, antioxidant, and antimicrobial activities (Jugran et al., 2019)			weight, and below ground fresh and dry weight	
	<i>Valeriana officinalis</i>	<i>Rhizophagus intraradices</i>	valerenic acid	possess sedative and antispasmodi c and sleep- inducing effects (Mungali et al., 2021)	roots	relative increasing	biomass of rhizomes and roots negatively effected	Nell et al., 2010
Colchicaceae	<i>Gloriosa superba</i>	<i>Funneliformi s mossae, Rhizophagus fasciculatus, Gigaspora margarita and Gigaspora gilmorei</i>	colchicine content	treatment of gout, rheumatic arthritis, diseases of the skin and liver	tubers	increased	improved plant growth	Pandey et al., 2014
Dioscoreacea e	<i>Dioscorea spp. yam</i>	<i>Rhizophagus clarus, Claroideoglo musetunicatu m, Rhizophagus fasciculatus,</i>	polyphenols, flavonoids, and anthocyanin	anti- oxidative property to inhibit lipid peroxidation, resist the attack of free	bulbils	significantly increased	tube weights significantly increased	Lu et al., 2015

STATE OF THE ART

		<i>Gigaspora</i> <i>sp.</i> , <i>Funneliformi</i> <i>s mosseae</i> , and <i>Acaulospora</i> <i>sp.</i>		radicals, diminish low-density lipoproteins (LDLs), and reduce the occurrence of cardiovascul ar diseases				
Euphorbiace ae	<i>Euphorbia</i> <i>hirta</i>	<i>Funneliformi</i> <i>s mosseae</i>	phenols, flavonoids alkaloids and terpenoids	treatment for respiratory ailments (cou gh, coryza, bronchitis, and asthma), worm infestations in children, dysentery, jaundice, pimples, gonorrhea, digestive problems, and tumors (Kumar et al., 2010)	————	increased	positive effect on plant growth parameters	Tejavathi and Jayashree, 2011
Fabaceae	<i>Astragalus</i> <i>membranace</i> <i>us</i>	————	————	increasing telomerase activity and possing	————	————	AMF community study	Liu and He, 2008

STATE OF THE ART

				antioxidant, anti-inflammatory, immunoregulatory, anticancer, hypolipidemic, antihyperglycemic, hepatoprotective, expectorant, and diuretic effects (Liu et al., 2017)				
	<i>Anadenanthera colubrina</i>	<i>Acaulospora longula</i> and <i>Gigaspora albida</i>	catechin	treat for respiratory problems and inflammations (Monteiro et al., 2006)	bark and leaves	significant increase	proteins and carbohydrates were significantly increased	Pedone-Bonfim et al., 2013
	<i>Castanospermum australe</i>	<i>Rhizophagus intraradices</i> and <i>Gigaspora margarita</i>	castanospermine	possess anti-cancer and anti-inflammatory properties and as HIV	seeds	significant increasing with <i>R. intraradices</i>	increased the growth and P contents	Abu-Zeyad et al., 1999

STATE OF THE ART

				inhibitors and treatment of AIDS				
	<i>Glycyrrhiza inflata</i>	_____	_____	clearing away toxic materials, eliminating phlegm and relieving cough	_____	_____	study under water stress	Liu and He, 2009
	<i>Glycine max</i>	<i>Funneliformis mosseas</i>	isoflavonoids	reduction of different types of cancer, cardiovascular diseases, postmenopausal problems, diabetes, and some neurodegenerative disorders (Ahmad et al., 2014)	roots, seeds, leaves and flowers	significant increase	_____	Morandi and Bailey, 1984
	<i>Glycyrrhiza glabra</i>	<i>Glomus hoi</i> , <i>Claroideoglossum etunicatum</i> , <i>Claroideoglossum musclaroide</i>	glycyrrhizic acid	antiviral effects and act as a multifunctional drug carrier	roots	increased	_____	Johny et al., 2021

STATE OF THE ART

		<i>um,</i> <i>Rhizophagus</i> <i>irregularis,</i> and <i>Acaulospora</i> <i>delicata</i>						
	<i>Glycyrrhiza</i> <i>uralensis</i>	<i>Funneliformis</i> <i>mosseae</i>	contents of glycyrrhizic acid, liquiritin, and isoliquiritigenin	having immune- modulating and anti- tumor potential (Ayeka et al., 2016)	roots	significantly enhanced	significantly increased the shoot and root biomass	Chen et al., 2017
	<i>Libidibia</i> <i>ferrea</i>	<i>Claroideoglossum</i> <i>etunicatum</i>	total flavonoids	possessing antiulcerogenic, antiinflammatory, anticarcinogenic, antihistaminic, antimicrobial, anticoagulant and cicatrizizing properties	leaves	increased	improving the production of seedlings, a larger stem diameter, higher chlorophyll a leaf content	Silvia et al., 2014
		<i>Claroideoglossum</i> <i>etunicatum</i> and <i>Acaulospora</i>	flavonoids		stem's bark and leaves	significantly increased	————	Dos Santos et al., 2017

STATE OF THE ART

		<i>longula</i>						
		<i>Acaulospora longula</i>	tannins		_____	significantly increased	_____	Dos Santos et al., 2017
	<i>Medicago sativa</i>	<i>Rhizophagus intraradices</i>	formononetin	antioxidant, anti-inflammatory, immunomodulatory, and anticancer properties (Zagórska-Dziok et al., 2020)	roots	significant increase	_____	Volpin et al., 1994
	<i>Prosopis laevigata</i>	<i>Gigaspora rosea</i>	trigonelline	cardioprotection potential and treatment of heart diseases, throat infections, dysentery and eye inflammations (Matta et al., 2017)	roots and leaves	1.8-fold increase in roots	_____	Rojas-Andrade et al., 2003

STATE OF THE ART

Ginkgoaceae	<i>Ginkgo biloba</i>	<i>Funneliformis mosseae</i> <i>Rhizophagus intraradices</i> and <i>Diversispora epigaea</i>	_____	regulating cerebral blood flow, protection against free radicals, and delaying the progress of dementia and diabetes (Isah, 2015)	_____	_____	plant seedling growth significantly increased	Qi et al., 2002; 2003
Hypericaceae	<i>Hypericum perforatum</i>	<i>Rhizophagus intraradices</i> alone or mixture of <i>Funneliformis constrictum</i> <i>Funneliformis geosporum</i> <i>Funneliformis mosseae</i> , and <i>Rhizophagus intraradices</i> .	naphthodianthrone-es, hypericin and pseudohypericin	possess sedative and astringent properties and utilized for excitability, neuralgia, anxiety and depression	shoots	higher concentration	no impact on shoot biomass	Zubek et al., 2012
Hypoxidaceae	<i>Curculigo orchioides</i>	crude consortium of AMF spores isolated from rhizosphere	_____	anticancerous properties	_____	_____	increase biomass production, number of leaves and roots per	Sharma et al., 2008

STATE OF THE ART

		soil of <i>C. orchioides</i>					plant and higher concentrations of photosynthetic pigments as well as minerals	
Lamiaceae	<i>Coleus forskohlii</i>	<i>Glomus bagyarajii</i> and <i>Scutellospora calospora</i>	forskolin	treatment of eczema, asthma, psoriasis, cardiovascular disorders and hypertension (Kavitha et al., 2010)	roots	increased	positive effect on plant growth	Sailo and Bagyaraj, 2005
	<i>Leucas aspera</i>	<i>Funneliformis mosseae</i>	alkaloids	carminative, antihistaminic, antipyretic, and antiseptic properties to treat jaundice, anorexia, dyspepsia, fever, helminthic manifestatio	————	increased	enhanced growth and total biomass	Tejavathi and ayashree, 2011

STATE OF THE ART

				n, respiratory and skin diseases (Nirmala and Kanchana, 2018)				
	<i>Mentha arvensis</i>	<i>Rhizophagus fasciculatus</i>	terpenes content	used for stomach problems, allergy, liver and spleen disease, asthma and jaundice (Thawkar et al., 2016)	aerial parts	significantly increased	significantly increasing plant height, fresh herbage and dry matter yield	Gupta et al., 2002
	<i>Mentha spicata</i>	commercial AMF consortium 'Rhizagold'	————	antiseptic, restorative, carminative and antispasmodic properties	————	————	significantly positive effect of increasing various plant growth parameters	Birje and Golatka, 2016
	<i>Melissa officinalis</i>	<i>Claroideoglomus etunicatum</i> , <i>Claoideoglomus claroideum</i> and <i>Rhizophagus intraradices</i>	citronellal and neral	to treat nervous disturbances (anxiety, insomnia and stress) and gastrointestinal disorders and possess	leaves	increased	no impact	Engel et al., 2016

STATE OF THE ART

				sedative, spasmodic, antimicrobial, antioxidant and antitumoral actions				
	<i>Ocimum basilicum</i>	<i>Gigartaria margarita</i> and <i>Gigartaria rosea</i>	linalool and geraniol	treat for headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions (Joshi, 2014)	seeds	significant Increase	plant growth parameters and yield increased	Rasouli-Sadaghiani et al., 2010
		<i>Funneliformis caledonium</i>	rosmarinic and caffeic acids		shoots	increased	—	Toussaint et al., 2007
	<i>Origanum onites</i>	<i>Claroideoglossum musetunicatum</i>	total essential oil production	treatment of indigestion, coughs, toothache and to stimulate menstruation	leaves	increased	significantly higher shoot and root dry weight	Karagiannidis et al., 2012

STATE OF THE ART

	<i>Origanum vulgare</i>	<i>Claroideoglo musetunicatum</i>	essential oil composition of p-Cymene, and γ -Terpinene	treatment for indigestion, coughs, toothache and to stimulate menstruation	leaves	increased	significantly higher shoot and root dry weight	Karagiannidis et al., 2012
	<i>Plectranthus amboinicus</i>	<i>Rhizophagus clarus</i>	carvacrol, trans-caryophyllene, α -bergamotene and α -humulene	possess digestive, expectorant, antispasmodic, healing and antiseptic actions	shoots	significant improvement	improved shoot dry matter, root dry matter and total dry matter	Merlin et al., 2020
	<i>Pogostemon cablin</i>	<i>Claroideoglo musetunicatum</i>	essential oils	used to treat nausea, diarrhea, colds and headaches	—	increased essential oil content	greater plant height, number of branches and spread, biomass	Arpana et al., 2008
		<i>Acaulospora laevis</i> , <i>Funneliformis mosseae</i> and <i>Scutellospora calaspora</i>	patchoulol		leaves	significant improvement	—	Singh et al., 2012
	<i>Satureja macrostema</i>	<i>Rhizophagus irregularis</i>	β -linalool, menthone, pulegone, and verbenol	antimicrobials	aerial parts	significantly increased	significantly increased biomass, shoot and	Carreón-Abud et al., 2015

STATE OF THE ART

			acetate				root length	
	<i>Salvia officinalis</i>	<i>Rhizophagus clarus</i>	essential oil camphor, α -humulene, viridiflorol, manool, α -thujone and β -thujone	treatment of different kinds of disorders including seizure, ulcers, gout, rheumatism, inflammation , dizziness, tremor, paralysis, diarrhea, and hyperglycemia (Ghorbani et al., 2017)	shoots	increased	plant biomass increased	Sete da Cruz et al., 2019
	<i>Salvia miltiorrhiza</i>	<i>Funneliformis geosporum</i> or <i>Acaulospora laevis</i>	total phenolic acids	treatment of menstrual disorders, cardiovascular, and cerebrovascular disease	roots	significant increase	roots biomass, fresh and dry weight of the plant effectively increased	Wu et al., 2021
	<i>Scutellaria integrifolia</i>	————	————	a strong emmenagogue and as a female medicinal herb	————	————	positive effects on micropropagated plantlets growth, particularly root	Joshee et al., 2007

STATE OF THE ART

							development	
	<i>Schizonepeta tenuifolia</i>	_____	essential oil	used for headaches, colds, allergies, and eczem (Jeon et al., 2019)	_____	increased	_____	Wei and Wang, 1991
	<i>Thymus daenensis</i>	<i>Funneliformis mosseae</i> and <i>Rhizophagus intraradices</i>	essnetial ois	possess digestive, carminative, antitussive, antispasmodic, and expectorant attributes (Elahian et al., 2021)	_____	improve essential oil under drought stress	_____	Arpanahi et al., 2020
	<i>Thymus vulgaris</i>	<i>Funneliformis mosseae</i>	thymol, p-cymene and γ -terpinene	possess antiseptic, antibacterial, antifungal, antispasmodic, antitussive, expectorant and analgesic properties		increased	improved yield under drought condition	Machiani et al., 2021
Leguminosae	<i>Puerraria lobata</i>	_____	_____	to relieve body heat, eye soring, dry mouth,	_____	_____	AMF community study	Wang et al., 2006a

STATE OF THE ART

				headache associated with high blood pressure, and stiff neck problems (Liu et al., 2019)				
Oleaceae	<i>Forsythia suspense</i>	<i>Rhizophagus fasciculatus</i> and <i>Funneliformis constrictum</i>	————	anti-inflammatory, antioxidant, antibacterial, anti-cancer, anti-virus, anti-allergy and neuroprotective effects (Wang et al., 2018a)	————	————	strengthen the anti-drought of the seeding	Zhao et al., 2007
Poaceae	<i>Cymbopogon citratus</i>	<i>Funneliformis mosseae</i>	essential oils geraniol, nerol, and β -pinene	to treat cough, cold, rheumatism, digestive problems, bladder issues, toothache and swollen gums	aerial parts	enhanced	————	Mirzaie et al., 2020

STATE OF THE ART

	<i>Coix lachrymal- jobi</i>	————	————	diuretic, anti- rheumatic, antispasmodi c, anti- inflammator y, antidiarrheal, anthelmintic, antipyretic, antispasmodi c, diuretic, hypoglycaem ic, anti- cancer and tonic properties (Patel et al., 2017)	————	————	plant growth study	Li, 2003
Passifloracea e	<i>Passiflora alata</i>	<i>Claroideoglo mus etunicatum, Rhizophagus intraradices</i>	total phenols content	treatment of several diseases, such as insomnia, anxiety and hysteria (Simao et al., 2018)	shoots	————	dry mass of shoot and leaf number were greater	Riter et al., 2014
		<i>Rhizophagus clarus</i> and <i>Glomus spurcum</i>	————			significant increase	higher plant height	Riter et al., 2014

STATE OF THE ART

Rutaceae	<i>Phellodendron amurense</i>	<i>Funneliformis mosseae</i> , <i>Claroideoglossum musetunicatum</i> , <i>Diversispora epigaea</i> , and <i>Glomus diaphanum</i>	berberine, jatrorrhizine, palmatine	treatment of jaundice, dysentery, hypertension, inflammation , and liver-related diseases (Kwete, 2014)	barks	significant increase	_____	Fan et al., 2006
	<i>Phellodendron chinense</i>	_____	berberine	treating dysentery, detoxifying and curing furuncles	_____	_____	_____	Zhou and Fan, 2007
	<i>Citrus aurantium</i>	_____	_____	possess antiseptic, antioxidant, antispasmodic, aromatic, astringent, carminative, digestive, sedative, stimulant, stomachic and tonic properties treatment of gastrointestinal disorders, insomnia,		_____	plant growth and root antioxidative enzymes study	Wu et al., 2010

STATE OF THE ART

				head aches, cardiovascular diseases and cancer (Suryawanshi, 2011)				
Solanaceae	<i>Datura stramonium</i>	<i>Funneliformis mosseae</i> and <i>Glomus epigaeum</i>	hyoscine and hyoscyamine	treatment of stomach and intestinal pain from worm infestation, toothache, and fever from inflammation (Soni et al., 2012)	seeds and fruits	significant increase	————	Wei and Wang, 1989
	<i>Solanum viarum</i>	<i>Glomus aggregatum</i> and bacteria <i>Bacillus coagulans</i> and <i>Trichoderma harzianum</i>	flavonoids	used for cancer, patients with addison' s disease and rheumatic arthritis treatment	seedlings	increased	————	Hemashenpagam and Selvaraj, 2011
	<i>Withania somnifera</i>	<i>Rhizophagus irregularis</i>	withaferin-A	treatment of cancer	root	significantly increased	————	Johny et al., 2021
Taxaceae	<i>Taxus chinensis</i>	————	————	anticancer effect (Jian et al., 2016)	————	————	AMF infection and colonization study	Ren et al., 2008

STATE OF THE ART

Violaceae	<i>Viola tricolor</i>	<i>Rhizophagus irregularis</i>	caffeic acid concentration	treatment of various skin disorders and upper respiratory problems	aerial part	significant increase	no impact on root mass and negative impact on shoot biomass	Zubek et al., 2015
Zingiberaceae	<i>Curcuma longa</i>	<i>Glomus, Gigaspora</i> and <i>Acaulospora sp</i>	curcumin	a natural antioxidant with antitumor activity, an inhibitor of arachidonic acid metabolism and a good antiinflammatory agent	rhizomes	increased	————	Dutta and Neog, 2016
		<i>Gigaspora margarita</i>	curcumin		————	no impact on curcumin content (field)	no impact on plant growth parameters, biomass production, nutrient uptake	Yamawaki et al., 2013
		<i>Gigaspora margarita</i>	curcumin		————	concentration of curcumin increased (greenhouse)	higher biomass production and nutrient uptake	Yamawaki et al., 2013

III. Endophytic fungi

Endophytic fungi belong to mitosporic and meiosporic ascomycetes that *colonize internal living plant tissues including fruits, seeds, leaves, shoots, stems and roots without causing any visible manifestation of disease and live in mutualistic association with plants for at least a part of their life cycle* (Bacon and white, 2000; Jia et al., 2016). They often form sporadically hyphae in the intercellular fluids and wall spaces of their host plants and sporulate upon senescence of plant or host tissues (Bacon and white, 2000).

The number of endophytic fungi in nature is estimated at more than one million species (Faeth and Fagan, 2002). They have been isolated from nearly 300,000 plant species in hot deserts, arctic tundra, mangroves, temperate and tropical forests, grasslands and savannas, and croplands (Arnold, 2008; Khiralla et al., 2017). The community structure of endophytes in plants depends on various factors, such as host, developmental stage, inoculum density and environmental factors (e.g., temperature, humidity, light intensity, geographic location, and vegetation) (Suryanarayanan et al., 2005; Song et al., 2007; Dudeja and Giri, 2014). In some cases, an endophyte can survive as a latent pathogen, without causing infection for a long time, and with symptoms appearing only when physiological or ecological conditions favor virulence (Firáková et al. 2007). An endophyte in one plant can be a pathogen in another plant depending on the balance between pathogenicity and endophytism of the microorganism in different hosts (Saikkonen et al., 2004).

III.1. Phylogeny of endophytic fungi

Endophytic fungi have been first divided into two major groups based on taxonomy, host range, colonization patterns, tissue specificity and ecological function (Schaechter, 2011). Group one contains the Clavicipitaceous (Ascomycetes of the family Clavicipitaceae) endophytes (C-endophytes) which infect some grasses. Many of its members produce alkaloids which are toxic to animals and humans. Group two contains the Nonclavicipitaceous endophytes (NC-endophytes) which include hyperdiverse endophytic fungi. They are associated with leaves of tropical trees (Lodge et al., 1996; Fröhlich and Hyde, 1999; Arnold et al., 2000;

STATE OF THE ART

Gamboa and Bayman, 2001), as well as diverse above-ground tissues of non-vascular plants, seedless vascular plants, conifers, and woody and herbaceous angiosperms in biomes ranging from tropical forests to boreal and Arctic/Antarctic ecosystems (Carroll and Carroll, 1978; Petrini, 1986; Stone, 1988).

Rodriguez et al. (2009) later classified NC-endophytes into three distinct functional groups based on host colonization, horizontal or vertical transmission, plant diversity and fitness conferred to hosts. They pointed out that one group consists of endophytes capable of colonising the aerial part and roots, and that they are transmitted horizontally and vertically, and another group consists of endophytes commonly associated with the leaves of tropical trees, a very diverse group with only horizontal transmission. Finally, the last group includes the so-called dark septate endophytes fungi which have melanin in their septa and occur exclusively in the roots of their hosts and show only horizontal transmission.

Morphological identification of endophytic fungi –

Traditionally, endophytic fungi have been identified by two basic methods, i.e., direct observation and culture-dependent methods (Sun and Guo, 2012).

Endophytic fungi with spore-producing structures and sexual or asexual spores can be detected under a light and electron microscope within the plant tissues. This is particularly important for biotrophic endophytic fungi that cannot be cultured on standard growth media (Deckert et al., 2001; Lucero et al., 2011). However, most endophytic fungi within plant tissues only produce hyphae, which cannot be used for identification (Sun and Guo, 2012).

Unlike direct observation, culture-dependent methods are routinely used in studies of endophytes diversity (e.g. Petrini et al., 1982; Rodrigues and Samuels, 1990; Guo et al., 2000; Sun et al., 2011; Vieira et al., 2011). Their isolation on appropriate media allows detailed studies on population dynamics and species diversity, or for the production of inoculum to improve plant growth and health, or finally for the screening of new biologically active SMs (Ding et al., 2009; Wang et al., 2010; Li et al., 2011; Tejesvi et al., 2011).

With culture-dependent methods, the isolated endophytic fungi that

STATE OF THE ART

produce spores can be observed under a light and electron microscope and identified to the genus level by observation of the presence of conidial structures, spore mass color, distinctive reverse colony color, production of diffusible pigments, and spore morphology (Barnett and Hunter, 1998). However, a consistent number of isolates do not produce spores or conidia in Petri plates and thus cannot be identified to any taxonomic category using morphological characteristics. Therefore, various methods such as the use of different media and inclusion of sterile host tissue in cultures media, have been assayed to stimulate spore production (Guo et al., 1998; 2000; Taylor et al., 1999). Even under these conditions, a high number of isolates (up to 54% of the total) do not sporulate in cultures and are called *mycelia sterilia* (e.g., Petrini et al., 1982; Fisher et al., 1993; Guo et al., 2000; 2008; Photita et al., 2001; Cannon and Simmons, 2002; Kumaresan and Suryanarayanan, 2002; Wang and Guo, 2007; Sun et al., 2008; 2011). Also, some fungi may be missed due to the inability to culture them or due to their slow growth in artificial media which are then easily outcompeted by fast growing species (Sun and Guo, 2012). Therefore, molecular methods are needed to address potential technical limitations of traditional endophyte identification.

Molecular method to identify endophytic fungi – The application of molecular methods brings a new perspective to studies of endophyte diversity, circumventing the obstacles caused by traditional culture-dependent methods.

Molecular techniques can be used to identify non-sporulating isolates that cannot be identified via morphological characters. Also, they can be employed for the detection of endophytic fungi directly within the host tissues allowing to identify the endophytic fungi that cannot grow or grow slowly in artificial media. Recently, high-throughput sequencing (pyrosequencing, Illumina sequencing...) has enabled metagenomic and metagenetic analyses, providing a powerful tool for the study of fungal communities in clinical (Gharizadeh et al., 2004), phyllosphere (Jumpponen and Jones, 2009), forest (Ovaskainen et al., 2010), soil (Jumpponen et al., 2010), freshwater (Monchy et al., 2011) environments. Diversity of mycorrhizal fungi has also been investigated via these methods (Tedersoo et al., 2010; Dumbrell et al., 2011).

The internal transcribed spacer (ITS) region is the most widely sequenced

STATE OF THE ART

genetic marker for fungi and has also been formally proposed as a standard fungal barcode marker (Nilsson et al., 2015; Schoch et al., 2012). Thus, depending on the objectives of the study, some authors only used the ITS region to identify endophytic fungi (Promputtha et al., 2005; Doilom et al., 2017). However, multi-gene analyses are more accurate for identifying endophytic fungal species and are nowadays largely used (Ko et al., 2011; Tibpromma et al., 2018).

III.2. General benefits of endophytic fungi

Endophytic fungi confer several benefits to their host plants through their proliferation in tissues. First, they promote the fitness and growth of their host plants. For example, the root endophyte, *Serendipita Indica* (formerly called *Piriformospora indica*) inoculated to plant roots, has been shown to improve biomass of *Zea mays*, *Nicotiana tabacum*, *Petroselinum crispum*, *Artemisia annua* and *Bacopa monnieri* (Varma et al., 1999). Some endophytic fungi can increase the fitness and growth of host plants by increasing the concentration of hormones, such as indole-3-acetic acid, indole-3-acetonitrile, and cytokinins (Jia et al., 2016) or by mobilizing mineral elements such as nitrogen and phosphorus that are useful for plants (Zhang et al., 2006; Hartley and Gange, 2009). For example, *Mycena dendrobii* has been reported to promote seed germination and growth of the host plant *Gastrodia elata* by secreting indoleacetic acid (Guo and Wang, 2001). In another study, *Metarhizium robertsii* was shown to translocate nitrogen directly from insects to *Phaseolus vulgaris* and *Panicum virgatum* (Behie et al., 2012). Several studies have also reported that certain endophytic fungi can activate the expression of certain enzymes and genes in their host plants, thereby promoting plant growth and fitness (Chen et al., 2005). For example, *S. indica* increased root growth of *N. abacum* by stimulating expression of nitrate reductase and starch-degrading enzyme (glucan-water dikinase) (Sherameti et al., 2005).

In addition, endophytic fungi have been shown to increase the resistance of plants to biotic (pathogens) and abiotic (salt, drought, heat) stresses (Ren and Dai, 2012; Liu et al., 2011; Wang et al., 2009; Khan et al., 2013). Most endophytes have been reported to produce SMs and some of them exhibit antibacterial and antifungal properties (antibiotics) that help inhibiting the growth of plant pathogenic microorganisms (Gunatilaka, 2006). For example, *Phomopsis cassia* isolated from *Cassia spectabilis*, has been

shown to synthesize five substances similar to 3,11,12-trihydroxycadalene and cadinane sesquiterpenes in which one of the five derivatives produce an antifungal metabolite active against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* (Silva et al., 2006). Similarly, two strains of *Trichoderma harzianum* were shown to significantly control the disease of rice brown spot and one strain of *T. atroviride* to increase the seedling growth of rice (*Oryza sativa* L.) plants (Khalili et al., 2012). In addition, *S. indica* has been reported to induce drought tolerance (Boorboori et al., 2022), salt tolerance and disease resistance in various plants such as *H. vulgare*, *Oryza sativa* and *Triticum aestivum* (Ghabooli et al., 2013; Jogawat et al., 2013; Rabiey et al., 2015). Song et al. (2011) also reported that *C. globosum* can increase the salt-tolerant of *Chrysanthemum morifolium* seedlings by increasing peroxidase (POD) activity and soluble protein content.

III.3. Endophytic fungi and production of secondary metabolites in medicinal plants

Endophytic fungi have been extensively studied in medicinal plants (Nisa et al., 2015; Jia et al., 2016) because they can produce analogues of plant molecules (Jia et al., 2016) which have a large potential for application in the medical, pharmaceutical, industrial, agricultural and environmental fields (Kaul et al., 2012). For example, taxol, a bioactive compound in cancer drugs was originally produced from the bark of the yew tree (*Taxus brevifolia*) (Souza and Santos, 2017). Later, Stierle et al. (1993) isolated the paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *T. brevifolia*. Some other samples of bioactive compounds produced by endophytic fungi are pestacin, camptothecin, ergoflavin, podophyllo toxin, benzopyran, isopestacin, phloroglucinol, tetrahydroxy-1-methylxanthone, salidroside, borneol, dibenzofurane, methyl peniphenone, lipopeptide, peniphenone (Adeleke and Babalola, 2021).

Recent studies have shown that endophytic fungi not only produce SMs that their host plants originally produce, but also play a very important role in promoting the accumulation of bioactive compounds in medicinal plants, thus affecting the quality and quantity of crude drugs (Jia et al., 2016).

Several studies have shown that the symbiosis between endophytic fungi and medicinal plants can promote the accumulation of different SMs,

STATE OF THE ART

grouped according to their chemical structures into several classes (Hussein and El-Anssary, 2018).

Alkaloids – *Catharanthus roseus* is one of the most studied medicinal plants. It is used as a model species for the study of plant secondary metabolism and plant-microbe interactions (Shukla et al., 2013; Torres et al., 2013; Ferreira Filho et al., 2012; Lacava et al., 2007; Monteiro et al., 2001) because it is the only source of antitumor bisindole alkaloids [belonging to the class terpenoids indole alkaloids (TIAs)] vinblastine and vincristine, which are widely used in cancer chemotherapy (Pandey et al., 2016). Many studies have been conducted with this plant inoculated with endophytes. For example, *Curvularia sp.* (CATDLF5) and *Choanephora infundibulifera* (CATDLF6) isolated from the alkaloid-rich genotype *C. roseus* cultivar Dhawal increased serpentine content by 211.7–337.6%, while *Aspergillus japonicus* (CATDRF2) and *Pseudomonas sp.* (CATDS5) increased the content of ajmalicine by 123.4–203.8% in the low alkaloid yielding cultivar Prabal, respectively. Interestingly, upregulated expression of key genes, geraniol 10- hydroxylase, tryptophan decarboxylase and strictosidine synthase involved in terpenoid indole alkaloid biosynthetic pathway was observed in *Catharanthus roseus* plant inoculated with endophytes *Curvularia sp.* CATDLF5 and *Choanephora infundibulifera* CATDLF6, individually (Pandey et al., 2016). It has also been reported that *Curvularia sp.* (CATDLF5) and *C. infundibulifera* (CATDLF6) increased the content of vindoline by 229-403% in leaves of *C. roseus* cultivar Prabal (Pandey et al., 2016).

Terpenoids – The endophytic fungi *Macrophomina pseudophaseolina* and *Fusarium redolens* isolated from stem and root of the medicinal plant *Coleus forskohlii* have been reported to significantly improve the andrographolide content in *Andrographis paniculata* under field conditions (Mastan et al., 2019). In another study, *Colletotrichum gloesporioides* isolated from *Artemisia annua* has been shown to induce the production of artemisinin in hairy-root cultures of this plant (Wang et al., 2006b). In the study by Yong et al. (2009), endophytic fungi (*Fusarium sp.* I and II) isolated from *Euphorbia pekinensis* increased the contents of isoeuphpekinensin and euphol in tissue cultured in bottles and pots. In addition, polysaccharides from the root endophyte *Trichoderma atroviride* D16 stimulated the biosynthesis of tanshinone in hairy roots of *Salvia*

STATE OF THE ART

miltiorrhiza by upregulating transcriptional activity of the gene involved in tanshinones biosynthetic pathway for the synthesis of tanshinones (Ming et al., 2013).

Phenolics – *Aspergillus sp.* isolated from the medicinal plant *Rumex gmelina* Turcz has been reported to significantly enhance the bioactive content of chrysophaein, resveratrol (polyphenols), chrysophanol, emodin and physcion (natural anthraquinone) in the seedlings of this plant grown *in vitro* (Ding et al., 2018). Co-cultivation of *Curcuma longa* with the endophyte *S. indica* resulted in increased curcumin production under farmers' field conditions (Bajaj et al., 2014).

Other chemical compounds – *Fusarium redolens* has been shown to significantly increase the content and yield of davanone (which belongs to the class of organic compounds known as tetrahydrofurans) and ethyl cinnamate (the ester of cinnamic acid and ethanol) in the medicinal plants *Pelargonium graveolens* and *Artemisia pallens* (Mastan et al., 2019). Cell-free extracts of *Aspergillus niger* and *Saccharomyces cerevisiae* were used as elicitors to increase gymnemic acid production in cell suspension cultures of *Gymnema sylvestre* (Chodisetti et al., 2013).

III.3.1 Cultivation techniques for secondary metabolites production in endophytic fungi-associated medicinal plants

As discussed in section II.4.4, there are several disadvantages associated with extracting bioactive compounds from plants grown in the wild. These are the generally low yield, heterogeneity in production levels due to unpredictable environmental conditions and limited supply leading to overexploitation. Therefore, cultivation techniques associating endophytic fungi with medicinal plants are required for securing SMs production.

III.3.1.1. Production of secondary metabolites by endophytic fungi under controlled condition

Greenhouse cultivation

Inoculation of two endophytic fungi (*Serendipita indica* and *Sebacina vermifera*) was reported to significantly increase the oil yield of micropropagated *Thymus vulgare* plants grown in pot cultures compared to uninoculated control plants (Dolatabadi et al., 2011). *Alternaria sp.* A13

STATE OF THE ART

isolated from *Salvia miltiorrhiza* increased the root dry weight and SMs accumulation of micropropagated *S. miltiorrhiza* under greenhouse condition (Zhou et al., 2018). *Mycena* sp. F-23 isolated from the roots of *Dendrobium officinale* also stimulated the kinsenoside and flavonoid contents of micropropagated *Anoectochilus formosanus* Hayata (a rare and threatened medicinal Orchidaceae plant) grown in pot cultures (Zhang et al., 2013b).

III.3.1.2. Production of secondary metabolites associated with endophytic fungi under *in vitro* condition

Compared to growing whole plants in the wild or on farms, plant tissue culture under *in vitro* conditions has the advantages of well-controlled and reproducible conditions without the limitations of natural factors such as geographical location and climatic variations (Kieran et al., 1997; Zhou and Wu, 2006). However, plant cells, tissues or organs usually produce low amounts of desired SMs when compared with intact plants. Therefore, great efforts have been made to research strategies to improve SMs production in plant tissue cultures, such as cultivation of specific plant organs, selection of high productivity cell lines, optimization of medium and culture conditions, application of genetic engineering and biotransformation, use of immobilization and permeabilization of cell cultures, and improvement of SMs production using elicitors (Dicosmo and Misawa, 1995).

The most effective method to increase the productivity of SMs in plant tissue culture is the use of fungal elicitors (Takeuchi et al., 2013) which induces the expression of specific plant genes and activates secondary metabolic pathways thus increasing SMs (Han et al., 2007) and plant phytoalexins involved in plant disease resistance (Zhang et al., 2013c). *Fusarium*, *Pythium*, *Aspergillus*, *Penicillium*, *Trichoderma* species, etc. have been frequently used as fungal elicitors (fungal cell wall fragments) to induce SMs in medical plants (Namdeo et al., 2002; Liang et al., 2018).

Whole plant culture system

The *in vitro* co-cultivation of *Centella asiatica* plants with the endophytic fungus *Colletotrichum gloeosporioides* (isolated from *in vivo* grown plants of *C. asiatica*) increased plant growth and production of asiaticoside (a

STATE OF THE ART

major active terpenoid compound with several biological activities) (Gupta and Chaturvedi, 2019). Similarly, the co-cultivation of *Dracaena cochinchinensis* with an endophytic *Fusarium* sp. led to the formation and accumulation of red resin (Jiang et al., 2003) which is well known for the treatment of traumatic and visceral haemorrhages in China (Hussain et al., 2021).

In addition, elicitors extracted from fungal endophytes have also been used with whole plants grown *in vitro* to modulate the biosynthesis of bioactive compounds. For example, the total content of some volatile oils (e.g., atractylone) was increased in *Atractylodes lancea* plants treated with elicitors of the endophytic fungus *Gilmaniella* sp. (Wang et al., 2012).

Plant cells culture system

Plant cells have also been co-cultured with endophytic fungi to enhance the production of bioactive compounds. For example, a co-culture of the suspension cells of *Taxus chinensis* var. *mairei* and its endophytic fungus *Fusarium mairei* increased the production of paclitaxel by 38 (Li et al., 2009). This compound was also increased in cell cultures of *Taxus chinensis* in presence of the endophytic fungus *Aspergillus niger* (Wang et al., 2001a). Cell cultures of *Catharanthus roseus* with its endophytic fungus *Fusarium oxysporum* F9, increased the production of total content of alkaloids, with 48% compared to the control plants (Tang et al., 2011). Similarly, cambial meristematic cells of *Catharanthus roseus* grown in presence of elicitors extracted from *Aspergillus flavus* showed an increase in the production of terpenoid indole alkaloids (TIAs): vindoline, catharanthine, and ajmaline (Liang et al., 2018).

Hairy roots culture system

Hairy roots can produce metabolites levels similar to those seen in the whole plant, with the benefit of rapid growth rates (in hormone-free media), genetic and biochemical stability. Thanks to these characteristics, the use of hairy roots has been widely explored in plant science, having the advantage of a practical, stable, and efficient plant tissue culture system for the production of valuable metabolites present in roots of the wild-type plant (Srivastava and Srivastava, 2007).

The extracts of mycelium and the polysaccharide fraction of *Trichoderma atroviride* D16 isolated from *Salvia miltiorrhiza* was shown to promote the

STATE OF THE ART

hairy root growth and biosynthesis of tanshinones in root organs of *S. miltiorrhiza* (Ming et al., 2013). In another study conducted by Savitha et al. (2006) the hairy roots of *Beta vulgaris* treated with *Penicillium notatum* extracts showed a significantly higher productivity of betalain in shake-flasks and bioreactors. Similarly, artemisinin content in hairy roots of *Artemisia annua* was increased from 0.8 to 1 mg g⁻¹ dry weight by using mycelial extracts from the endophytic fungus *Colletotrichum* sp isolated from *A. annua* (Wang et al., 2001b).

Fermentation culture of endophytic fungi with elicitors

The discovery of endophytic fungi capable of mimicking their host's SM repertoire in most plants of medicinal or industrial value has given impetus to the possibility of using microbial fermentation to meet the ever-increasing demand for several life-saving drugs (Venugopalan and Srivastava, 2015). In addition, endophytes are known to develop new mechanisms of resistance to toxic metabolites produced by them and the host as a co-evolutionary adaptation (Kusari and Spiteller, 2011), which simplifies bioprocessing of these organisms without risk of growth inhibition due to the self-toxicity of the product.

Endophytic fungi can directly produce bioactive SMs by fermentation through the optimisation of fermentation medium, time, pH, temperature, and agitation rate. However, separation from their hosts always resulted in the loss of this ability. For example, it has been reported that the endophytic fungus *Trichoderma atroviride* D16 isolated from the root of *Salvia miltiorrhiza* was capable of producing tanshinone I (T-I) and tanshinone IIA (T-IIA) under shake-flask conditions. However, a substantial decrease in the production of tanshinones occurred in the *in vitro* cultured endophyte following repeated subculturing (Ming et al., 2013). Therefore, many studies have used different kinds of elicitors to maintain or promote fungal production of bioactive components (Wang et al., 2012; Ding et al., 2018). For example, water-extracted polysaccharide from *Dioscorea zingiberensis* was found to be the most effective elicitor to enhance diepoxin zeta production of the endophytic fungus *Berkleasmium* sp. Dzf12 in liquid culture (Li et al., 2012). Methyl jasmonate was proven to be an optimum elicitor in the production of Camptothecin (the third largest anticancer drug) by *Trichoderma atroviride* LY357 isolated from its host plant *Camptotheca acuminata* (Pu et al., 2013).

RESEARCH QUESTIONS

RESEARCH QUESTIONS

Arbuscular mycorrhizal fungi and endophytic fungi form symbiotic associations with their host plants, providing several benefits such as improved plant growth and resistance to abiotic and biotic stress. More importantly, these beneficial microorganisms have been repeatedly shown to modulate the production of secondary metabolites (SMs) in their host plants which is extremely interesting for medicinal plants.

To the best of our knowledge, no study has ever reported the effect of these microorganisms on growth and secondary metabolites (especially alkannin/shikonin and their derivatives – A/Sd) production of plant species belonging to the Boraginaceae family (in particular *Alkanna tinctoria* and *Echium vulgare*). In the present thesis four main research questions were addressed:

1. Does the root microbiome of *A. tinctoria* differ with developmental stage of the plant and how does it influence the production of A/Sd?
2. Are native AMFs (*R. irregularis* and *S. viscosum*) isolated from *A. tinctoria* in the wild more efficient than non-native AMF (*R. irregularis* MUCL 41833) in stimulating growth and A/Sd production in *A. tinctoria*? Do these AMFs modulate different relative gene expression involved in A/Sd biosynthesis?
3. Is the native AMF (*R. irregularis*) more efficient than the non-native AMF (*R. irregularis* MUCL 41833) to increase A/Sd production in hairy roots of *A. tinctoria*?
4. Does endophytic fungi isolated from *A. tinctoria* and non-native AMFs (from GINCO collection) impact growth of *Echium vulgare* and A/Sd production in roots?

RESEARCH QUESTIONS

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Biological Materials

I.1. Arbuscular mycorrhizal fungi

I.1.1 Arbuscular mycorrhizal fungi provided by the Glomeromycota *in vitro* collection

These five AMF strains were tested in **Chapter IV** for their effects on plant growth and the production of secondary metabolites (A/Sd) of *E. vulgare* grown under greenhouse conditions.

These five AMF strains were provided by the Glomeromycota *in vitro* collection (GINCO – <http://www.mycorrhiza.be/ginco-bel>): *Rhizophagus irregularis* (Błaszk, Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833, *Rhizophagus clarus* (TH Nicolson and NC Schenck) C. Walker and A. Schüßler MUCL 46238, *Rhizophagus intraradices* (NC Schenck and GS Sm) C. Walker and A. Schüßler MUCL 49410, *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker MUCL 49408, *Glomus hoi* (Berch and Trappe) MUCL 45686 (**Table 4**). The strains were provided as cultures associated with Ri T-DNA transformed roots of carrot (*Daucus carota*) on the Modified Strullu–Romand (MSR) medium (Declerck et al., 1998)

MATERIALS AND METHODS

Table 4: Geographic origin and host plant/habitat of AMF strains (from the GINCO)

Scientific name	Accession number	State, Country	Habitat, Climate
<i>R. irregularis</i>	MUCL 41833	Canary Islands, Spain	Arid, hot
<i>R. clarus</i>	MUCL 46238	Cuba	Tropical, savannah
<i>R. intraradices</i>	MUCL 49410	Florida, USA	Temperate, without dry season, hot summer
<i>R. aggregatus</i>	MUCL 49408	Florida, USA	Humid, subtropical
<i>G. hoi</i>	MUCL 45686	Laukaa, Vihtavuón, Finland	Temperate, meadow vegetation

Moreover, *R. irregularis* MUCL 41833 was also used in **Chapter II** to assess its effects on the growth and production of secondary metabolites (A/Sd) of *A. tinctoria* under greenhouse condition compared to native strains (see section Isolation of AMF, below). This AMF was also used in **Chapter III** to assess its effects on growth and production of secondary metabolites (A/Sd) of hairy roots of *A. tinctoria* compared to native strains (see section *In vitro* AMF culture establishment, below). *Rhizophagus irregularis* was selected because it is widely distributed all over the world and is known as a generalist symbiont (IJdo et al., 2010; Chagnon et al., 2013). In addition, this AMF, particularly stain MUCL41833, is easily cultivable *in vitro* (IJdo et al., 2011) and produces an extensive hyphal network bearing thousands of spores (e.g. Voets et al., 2009). Thus, it is a model fungus widely used for *in vivo* (Rufyikiri et al., 2000; Anene and Declerck, 2016; Buysens et al., 2017) as well as *in vitro* (Koffi et al., 2013; Buysens et al., 2015; Loján et al., 2017) studies.

I.1.2. Arbuscular mycorrhizal fungi isolated from *A. tinctoria* in the wild

Rhizophagus irregularis and *Septoglomus viscosum* isolated from *A. tinctoria* in the wild in Greece were used in **Chapter II** to evaluate their

MATERIALS AND METHODS

effects on the growth and production of secondary metabolites (A/Sd) of *A. tinctoria* under greenhouse conditions compared to the standard strain *Rhizophagus irregularis* MUCL 41833. However, prior to their use, the AMF were isolated from the field, identified and mass-produced.

Two sources of material (spores and root fragments) from the field are often used. However, spores collected directly from a field soil suffer from many problems: (a) they can appear healthy but are not viable (some persisting as dead husks for years or possibly decades); (b) they can lose or change appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture, and microbial activity; and (C) they represent only those colonizing AMF with enough activity and biomass to trigger sporulation. Root fragments can also serve as an alternative source of inoculum (Biermann and Linderman 1983). However, it should be noted that roots from field inevitably host numerous AMF species. Trapping is necessary to obtain many healthy spores of colonizing fungi for identification and as inoculum to establish monospecific cultures (<https://invam.wvu.edu/methods/culture-methods/trap-culture>).

-In vivo AMF culture establishment

Generally speaking, the isolation of AMF involves sampling of inoculum, trapping culture of AMF, monosporal culture establishment. The detailed procedures can be seen in ANNEX II (Establishment of AMF trap cultures and Establishment of AMF mono-species pot cultures).

-In vitro AMF culture establishment

The establishment of root organ cultures of AMF can be started with spores or AMF-colonized root fragments.

For spores, active and healthy-looking individuals were collected as described above and disinfected in a vacuum filtration apparatus under a laminar hood (**Figure 12**). Briefly, spores were first cleaned with tap water and then transferred to the filtration apparatus connected to a vacuum outlet. They were rinsed 3 times with sterile water and gently shaken with a Pasteur pipette. The spores were then treated with chloramine T 2% solution (with 2 drops of Tween 20) with continuing manual agitation with the Pasteur pipette for 10 min. The spores were again rinsed three times with sterile water and then treated with an antibiotic solution (a mixture of 0.02% streptomycin and 0.01% gentamycin) for 10 min following the same

MATERIALS AND METHODS

process as described above. At the end of the procedure, the membrane supporting the disinfected spores were transferred into a plastic Petri plate containing 20 ml of filter-sterilized antibiotics solution. Healthy-looking spores were selected under a binocular microscope and placed in 90 mm Petri plates containing the MSR medium with five spores per plate. The Petri plates were sealed with plastic wrap and incubated at 27°C in the dark until germination.

MATERIALS AND METHODS

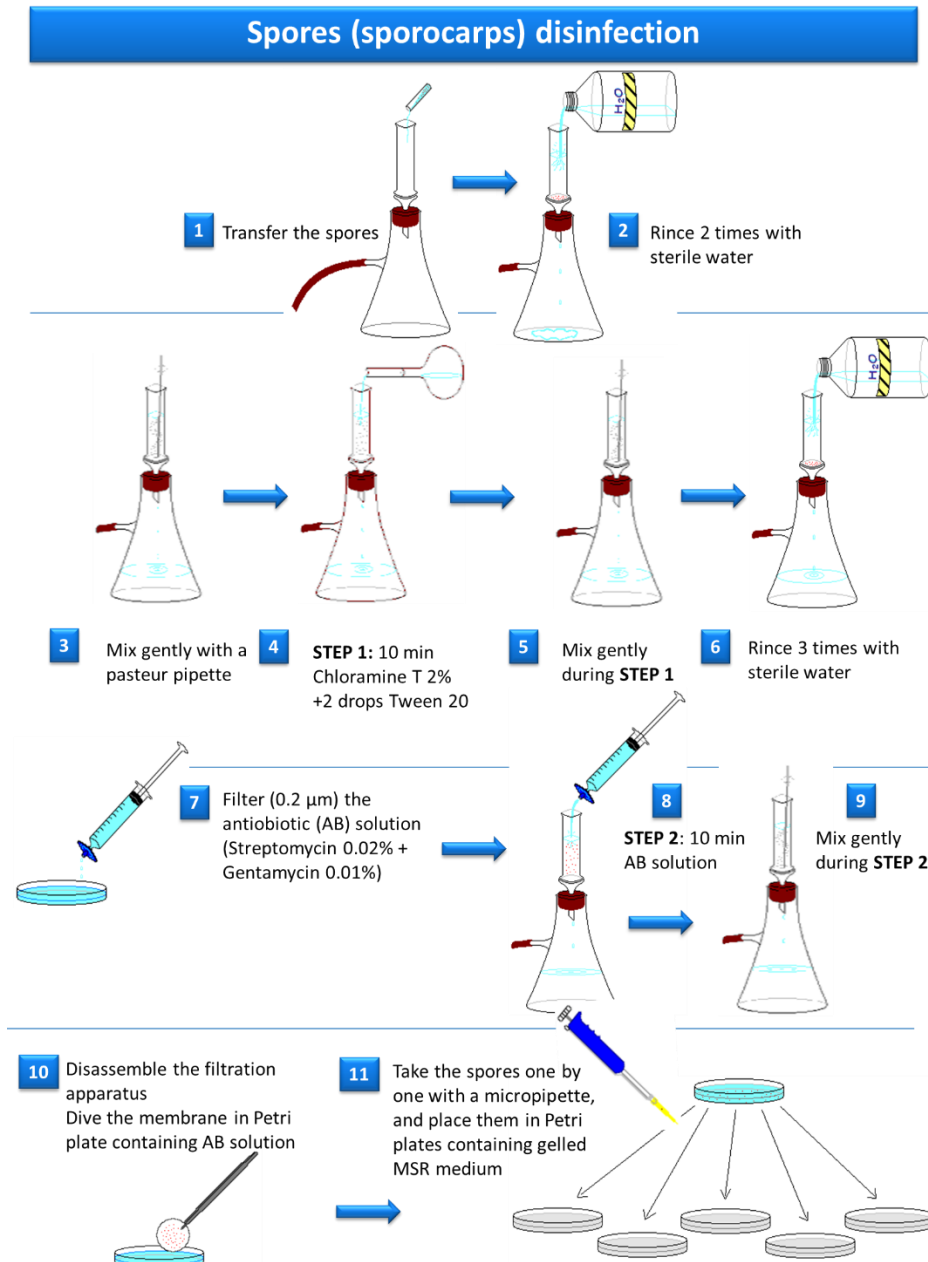


Figure 12: The spore disinfection procedure used from GINCO and adapted from Cranenbrouck et al. (2005)

MATERIALS AND METHODS

For colonized root fragments, pieces of 5 cm long were treated in an ultrasonic apparatus. Briefly, root fragments were washed with tap water and surface disinfected with 98% ethanol for 10 s, Ca Hypochlorite for 2 min, Chloramine T for 10 min, and a solution of antibiotics (a mixture of 0.02% streptomycin and 0.01% gentamycin) for 10 min in a sterilized Falcon tube. Between each treatment, the roots were rinsed at least 3 times with sterilized water. The sterilized roots were then maintained in a fresh antibiotic solution. Around 5 pieces of 1 cm long root fragments were placed on the MSR medium in 90 mm Petri plates. The Petri plate was wrapped with plastic film and maintained in the incubator at 27°C in the dark until germination.

After hyphal re-growth from spores or root fragment (**Figure 13**), a slice of agar supporting the germinated propagule was cut with a cork borer (the size is depending on the edge of the active growing hyphal) and transferred into another new Petri plate (90 mm) containing MSR medium in which a slice of agar was already removed with the same size cork borer so that the propagule on the agar slice could be inserted in the hole. Ri T-DNA transformed actively growing carrot (*D. carota* L. clone 1E) roots were placed in the near vicinity of the propagule. The associated AMF cultures plates were closed with plastic wrap and were maintained at 27°C in the dark in an inverted position (**Figure 13**).

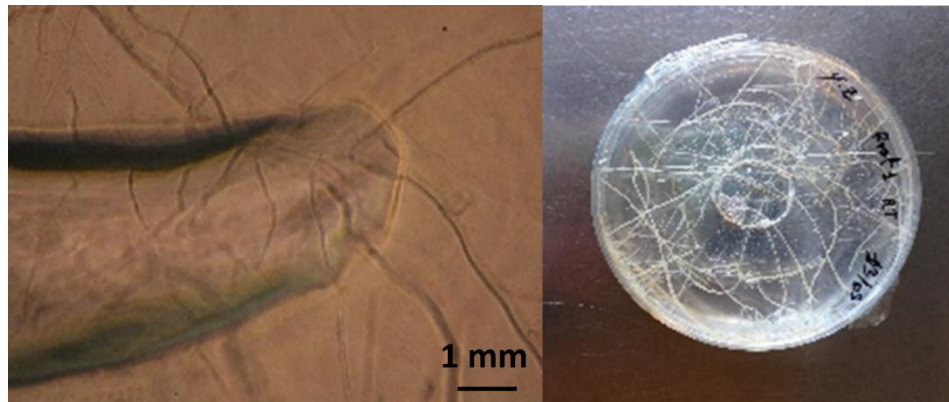


Figure 13: left: AMF germinated from root fragment; right: Association of germinated AMF inside root fragment with transformed carrot root (*Daucus carota* L. clone 1E)

MATERIALS AND METHODS

I.1.3. Identification of AMF

In order to clearly distinguish morphologically more similar spores and species level diversity, DNA based molecular techniques are used.

The DNA was first extracted from AMF spores. A 1.5 kb fragment, covering a part of the SSU rRNA gene, the complete ITS region (including the 5.8S rRNA gene) and approx. 800 bp of the LSU rRNA gene, named SSU-ITS-LSU rDNA region for AMF as an extended DNA barcode providing species-level resolution, was amplified by polymerase chain reaction (PCR). It is used to identify AMF from trap cultures (i.e., single spores or mycorrhizal roots) and also in field studies (Krüger et al., 2009; Stockinger et al., 2010; Senés Guerrero et al., 2014). The amplification of the SSU-ITS-LSU region was made according to Krüger et al. (2009) [see ANNEX II (Molecular identification of isolated AMF)].

Cloning

It is well known that AMF spores contain heterogeneous nuclei. Therefore, there is a huge genetic diversity within one AMF species. Cloning the PCR products help to identify the possible different patterns present in a multi-spores trap culture or in environmental samples (i.e. colonized roots or soil).

Cloning, selection and sequencing were conducted as described in Krüger et al. (2009). In brief, PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol, with only one-third of the specified volume of all components used. The ligation reaction between the PCR product and the PCR vector lasted one hour at room temperature. Cells of *Escherichia coli* genetically modified (DH5 α / chemically competent) were transformed with the ligation product by thermal shock (42°C/ 1 min) and immediately stored in ice to finish the process. Super Optimal broth with Catabolite repression (SOC, Thermo Fisher Scientific, USA) medium was used for initial bacterial growth after transformation. After 2 hours, 100 μ L of the bacterial inoculum was spread in the Lysogeny Broth (LB) (Fluka Sigma-Aldrich, India) medium with kanamycin antibiotic (Alfa Aesa, Germany) (50 μ g mL⁻¹) and incubated in the dark at 37°C for 24 hours. Five clones from each sample were analyzed for correct length of plasmid inserts and clone selection (RFLP). Colony-PCR was performed with 1x GoTaq DNA Polymerase (Promega, USA) and M13F and M13R primers. The colony-

MATERIALS AND METHODS

PCR products were loaded on 1.0 % agarose gels with 1× TAE buffer and GelRed™ Nucleic Acid Gel Stain, 10,000× in water (Biotium, USA). Migration parameters were 100 V, 200 mA for 30 minutes and visualized in camera UV light. After confirmation, a PCR using M13 primers was performed and sequenced at Macrogen Inc. (Korea) with Sanger sequencing.

Sanger sequencing was used for AMF species identification. This method was developed by Frederick Sanger and colleagues in the 1970s and is based on selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication (Sanger and Nicklen 1977). It is used for sequencing of single gene (a single DNA fragment) at a time which can produce DNA sequence reads of more than 500 nucleotides. It is still considered the gold standard of sequencing technology today since it provides a high degree (99.99%) accuracy (Shendure and Ji 2008), long-read capabilities (compared with short-read sequencing technologies such as Illumina), and the flexibility to support a diverse range of applications in many research areas.

A phylogenetic tree is an estimate of the relationships among taxa (or sequences) and their hypothetical common ancestors (Nei and Kumar 2000; Felsenstein 2004; Hall 2011). In order to differentiate the newly isolated AMF strains, phylogenetic tree was made.

The detailed procedures for identification of AMF is reported in **Annex II (Molecular identification of isolated AMF)**.

I.2. Endophytic fungi isolated from *A. tinctoria* in the wild

Nineteen selected endophytic fungi isolated from *A. tinctoria* in the wild in Greece were used in **Chapter IV** to evaluate their effects on growth and production of secondary metabolites (A/Sd) of *E. vulgare* under greenhouse condition.

I.2.1. Isolation of endophytic fungi

The isolation procedure includes several steps (1) washing of the plant tissues (i.e. root fragments in the present work), under tap water to remove adhering soil particles, debris and major epiphytes, (2) disinfection of the root surface to kill any unwanted microorganisms, (3) isolation of the endophytic fungi present in the internal tissues of the roots, (4) purification

MATERIALS AND METHODS

and sporulation of endophytic isolates, and (5) identification of the endophytic fungi based on morphological characteristics in cultures and further to be identified with molecular method.

To be effective, the isolation procedure must be adapted to the respective plant species, tissues and fungi. In practice, isolated strains can be assumed to be endophytic if complete surface sterilisation is confirmed, i.e. no fungal growth is observed from the surface-sterilised plant tissues or from the last water rinsing. The sterilised plant tissue is cut into small segments (ca. 5 mm diam.), macerated or ground, then transferred onto nutrient agar medium for regrowth of fast and slow-growing fungi.

The procedure to obtain endophytic fungi from *A. tinctoria* plants include: 1) root surface disinfection of *A. tinctoria* plants; 2) growing sterilized and small sized root on Malt Extract Agar (Duchefa) supplemented with 200 mg L⁻¹ chloramphenicol (MEAC) or Potato Dextrose Agar (Oxoid) supplemented with 200 mg L⁻¹ chloramphenicol (PDAC) in Petri plates (90 mm diam); 3) establishment of monosporic culture; 4) sporulation of endophytic fungi. The detailed procedures is reported in **Chapter IV (material and methods)**.

I.2.2. Identification of endophytic fungi

-Primers

Based on the morphological characteristics of spores or conidia of endophytic fungi, different primer pairs were chosen for molecular identification. For *Fusarium* sp. elongation-factor gene was used and for *Penicillium* sp. béta-tubuline gene was used by using primers EF1T/ EF2T and Bt2a/ Bt2b, respectively. For the non-sporulating endophytic fungi, internal transcribed spacer (ITS) region and 28S rDNA region using primers ITS4/ITS5 and LROR/LR6 (Raja et al., 2017), respectively were amplified by PCR (**Table 5**).

MATERIALS AND METHODS

Table 5: Forward and reverse primers used to amplify genes of different endophytic fungi.

Region	Primer	Nucleotide sequence (5'-3')	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC	
28S	LROR	ACCCGCTGAACTTAAGC	Raja et al., 2017
	LR6	CGCCAGTTCTGCTTACC	
Béta-tubuline	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Isshiki et al., 2014
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Elongation-Factor	EF1T	ATGGGTAAGGA(A/G)GACAAGAC	O'Donnell et al., 2000
	EF2T	GGA(G/A)GTACCAGT(G/C)ATCATGTT	

-PCR conditions

The PCR conditions used to amplify sequence from endophytic fungi were summarized below in **Table 6**.

MATERIALS AND METHODS

Table 6: Thermal cycler conditions for amplifying genes of endophytic fungi

Amplified region	Procedure	Temperatures (°C)	Duration (minutes)	Number of cycles
ITS/28S/ Béta- Tubuline	initial denaturation	94	3	30X
	denaturation	94	1	
	annealing	56	1.30	
	extension	72	2	
	final extension	72	10	
		4	INFINI	
Elongation- Factor	initial denaturation	92	5	40X
	denaturation	92	30s	
	annealing	52	30s	
	extension	72	1.30	
		4	INFINI	

I.3. Plant species

Different plants species were used in the form of hairy roots or seedlings (Table 7).

MATERIALS AND METHODS

Table 7: Plant species and growth conditions

Plant type	Plant species	Growth condition	Chapter and Use
Hairy roots	<i>Daucus carota</i> L. clone 1E	<i>In vitro</i>	Used as root host to maintain the AMF GINCO strains <i>in vitro</i> . Used in Chapter III to establish <i>in vitro</i> cultures of AMF isolated in the wild from <i>A. tinctoria</i>
	<i>Alkanna tinctoria</i> (L.) Tausch	<i>In vitro</i>	Used in Chapter III
	<i>Alkanna tinctoria</i> (L.) Tausch	In pot	Used in Chapter I and II
Seedlings	<i>Echium vulgare</i> L.	In pot	Used in Chapter IV
	<i>Plantago lanceolata</i> L.	In pot	Used in Chapter II as host plant to trap AMF isolated in the wild from <i>A. tinctoria</i>
	<i>Medicago truncatula</i> Gaertn	In pot	
	<i>Zea Mays</i> L. cv. ES Ballade	In pot	Used in Chapter II as host plant for mass production of AMF

***Daucus carota* L.** clone 1E was supplied as Ri T-DNA transformed roots by the Glomeromycota *in vitro* collection (GINCO – <http://www.mycorrhiza.be/ginco-bel>). It was grown on MSR medium and incubated in an inverted position in the dark at 27°C. The roots were sub-cultured every month on the same growth medium.

***Alkanna tinctoria* (L.)** Tausch was provided as: (1) transformed hairy roots, provided by Angelique Rat (PhD at the University of Ghent, Belgium) and (2) micropropagated plants, provided by the Hellenic Agricultural Organization (HAO, Thessaloniki, Greece) under accession number GR-1-BBGK-17,5975 maintained at the International Plant Exchange Network - IPEN).

The transformed roots were grown on solid Murashige and Skoog (MS,

MATERIALS AND METHODS

Duchefa) medium supplemented with 3% sucrose (Sigma) and 0.8% phytoagar (Duchefa, Haarlem in Netherlands) and incubated in the dark at 27°C. The roots were sub-cultured every 3-4 weeks on the same growth medium. The plant seedlings were provided as rooted acclimatized individuals, originally collected and identified from natural populations before micropropagation. They were proliferated on MS basal medium enriched with plant growth regulators (PGRs) and rooted on Root Culture 1 (RC1) modified medium free of ammonium nitrate (Cartabia et al., 2021).

Echium vulgare L. was provided as commercial seeds by Templiner Kräutergarten (Germany). The seeds were cleaned with 70% ethanol and germinated on filter papers in plastic box (0.3L). Twenty-day old seedlings were used for the greenhouse experiment.

Plantago lanceolata L., *Medicago truncatula* Gaertn. and *Zea Mays* L. cv. ES Ballade were provided as seeds by Ecosem (Belgium), SARDI (Australia) and Euralis (France), respectively. The seeds were first surface-disinfected by soaking in 70% ethanol for 1 min. and in sodium hypochlorite (8% active chloride) for 5 min., and rinsed 3 times with demineralized water. The cleaned seeds of *P. lanceolata* and *M. truncatula* were then germinated in 1.3 L pot (15×11.4 cm) filled with sterilized (121 °C for 15 min) substrate (calcinated clay (DCM, Belgium), quartz (0.4-0.8 mm), quartz (1-2 mm)) in a volume ratio 2: 2: 1. The cleaned seeds of *Z. Mays* were germinated in 4 L pot filled with sterilized (121°C for 15 min) lava (DCM, Belgium). Pots were kept under greenhouse condition set at 20°C, 50% RH, photoperiod of 16 h day⁻¹ and PPFD of 96 µmol m⁻²s⁻¹. Two-week old seedlings of *P. Lanceolata* and *M. truncatula* were used as host plants to trap AMF isolated in the wild (Greece) from *A. tinctoria*. Ten-day-old seedlings of *Z. Mays* were used as host plants for mass-production of AMF in the greenhouse under the same condition as described above.

II. Endophytic fungal community study

In **Chapter I**, we used the Illumina sequencing technology to explore the microbiome (especially bacterial and fungal communities) associated with *A. tinctoria* roots in three different field soils under greenhouse condition.

Illumina MiSeq technique has provided a powerful tool for the analysis of

MATERIALS AND METHODS

fungus and bacterial communities in different substrates and plant tissues, by the ability to sequence hundreds to thousands of genes or gene regions simultaneously per run. It allows to detect culturable and non-culturable microorganisms (Schmidt et al., 2013). Generally, a typical sequencing workflow (see **Figure 14**) comprises four steps: 1) sample/library preparation 2) cluster amplification 3) DNA sequencing and 4) alignment and data analysis.

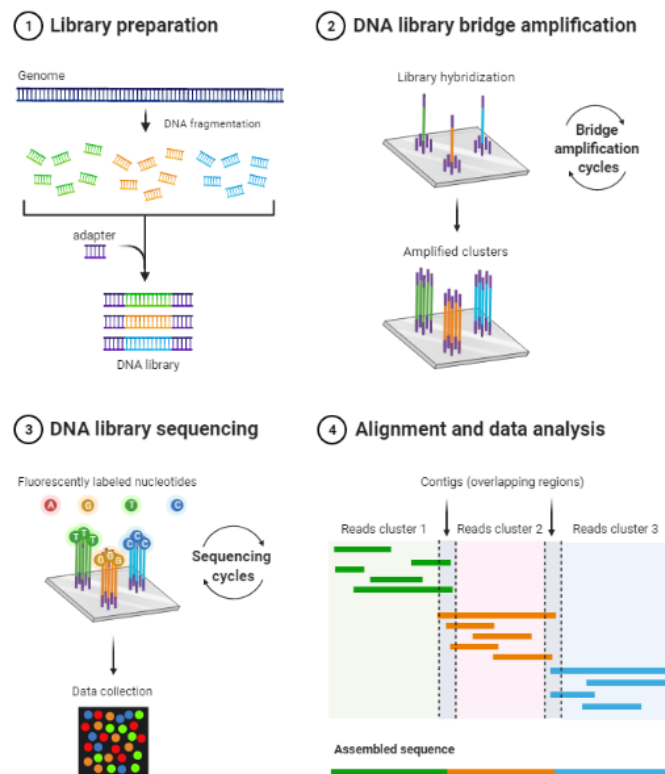


Figure 14: Illumina sequencing workflow (<https://app.biorender.com/biorender-templates/t-5ef134a11c72b100ad8d13ac-next-generation-sequencing-illumina>)

The detailed procedures for the sample/library preparation of this work were listed below:

MATERIALS AND METHODS

-Genomic DNA extraction

Before using glass beads (Sigma-Aldrich), they were soaked for 16 h in concentrated HCl and then rinsed thoroughly in distilled water. Later, they were baked for 16 h at 150°C and finally were kept at 4°C or on ice before use. The solutions used to extract DNA were first sterilized by autoclaving.

The DNA extraction method was adapted from a modified cetyltrimethylammonium bromide (CTAB) protocol combined with a classical phenol-chloroform-isoamyl-alcohol extraction protocol (Wakelin et al., 2007).

The procedure to extract DNA from the roots for the bacterial and fungal community study includes 1) the sterilization of each root sample in clean 50 ml Falcon tube with 50 ml sterile distilled water followed by bathing in 15 ml 2.4% NaClO for 5 minutes, respectively, with a horizontal shaker; 2) the washing off the NaClO with 50 ml sterile water. Hundred mg root-material was placed in safe-lock tubes pre-chilled with liquid nitrogen; 3) the homogenization of the root material by ballmixer at 30Hz for 2 minutes with 3-4 pieces of 3.5 mm UFO beads (Mixer Mill MM400, Retsch®, Haan, Germany) per tube; 4) the removing of the steel balls with magnet and addition of 310 µL of STE buffer [0,35 M D-Sorbitol, 0,1 M Tris-HCl (pH 8,0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8,0)], 440 µL lysis buffer [0,2 M Tris-HCl, 1 M Tris-HCl (pH 8,0), 0,05 M EDTA (pH 8,0) 2 M NaCl, 2% CTAB)] and 150 µL sarkosyl; 5) the addition of 200 µL 1mm glass beads, vortexing for 10 seconds and bead beating in Retsch mill (Retsch) for 30 seconds at 30 Hz; 6) the addition of 1 µL mercaptoethanol for each sample and incubation for 2 hours in 65°C water bath with occasional mixing; 7) the chilling for 5 minutes on ice, then addition of 950 µL phenol/chloroform/isoamylalcohol (25:24:1), inverting the tubes a few times and centrifuging at 4°C at 14000 rcf for 10 minutes; 8) the transfer of the aqueous phase (~900 µL) to 2 ml yellow phase lock tubes and addition of the same amount of chloroform/isoamylalcohol (24:1), inverting the tubes a few times, centrifuging at 15°C at 14000 rcf for 10 minutes; 9) the repetition of the above step and transfer of the aqueous phase (~800 µL) to clean 2 ml Eppendorf tubes, add an equal amount of ice-cold isopropanol + 1/10 volume of 4M ammonium acetate, invert the tubes several times and store at -20°C overnight; 10) the centrifugation at 4°C at 13600 rcf for 30 minutes and discarding of the

MATERIALS AND METHODS

supernatant; 11) the washing of the pellet with 1mL ice-cold 70% ethanol and centrifuging at 4°C at 13600 rcf for 30 minutes; 12) the discarding of the supernatant, air drying of the pellet and dissolving of the DNA in 100 µL sterile water.

To get rid of additional PCR inhibiting substances still inside the extracted solution, an additional DNA purification step with DNeasy® PowerClean® CleanUp Kit (Qiagen) was applied on all samples.

-Primers

For general fungal community analysis an improved primer pair (Taylor et al., 2016) amplifying partial 5.8S ribosomal subunit gene 5.8S-Fun (5'-AACTTTYRRCAAYGGATCWCT-3') and whole internal transcribed spacer 2 ITS4-Fun (5'-AGCCTCCGCTTA TTGATATGCTTAART-3') and index primers were used (see **Table 8**).

MATERIALS AND METHODS

Table 8: Illumina PCR primers (a) and barcoding primer (b) sequences used in this study

(a)

Primer name	Sequence	Primer name	Sequence
5.8S-Fun	GGAACCTTTYRRCAAYGG ATCWCT	ITS4-Fun	AAAGCCTCCGCTTATTGAT ATGCTTAART
Forward	TCGTCGGCAGCGTCAGA	Reverse	GTCTCGTGGGCTCGGAGAT
Illumina Tag	TGTGTATAAGAGACAG	Illumina Tag	GTGTATAAGAGACAG

(b)

Primer name	Index Sequence	Primer name	Index Sequence
Amplicon_IDX- S502_A	CTCTCTAT	Amplicon_IDX- N701_1	TCGCCTTA
Amplicon_IDX- S503_B	TATCCTCT	Amplicon_IDX- N702_2	CTAGTACG
Amplicon_IDX- S505_C	GTAAGGAG	Amplicon_IDX- N703_3	TTCTGCCT
Amplicon_IDX- S506_D	ACTGCATA	Amplicon_IDX- N704_4	GCTCAGGA

MATERIALS AND METHODS

Amplicon_IDX-S507_E	AAGGAGTA	Amplicon_IDX-N705_5	AGGAGTCC
Amplicon_IDX-S508_F	CTAAGCCT	Amplicon_IDX-N706_6	CATGCCTA
Amplicon_IDX-S510_G	CGTCTAAT	Amplicon_IDX-N707_7	GTAGAGAG
		Amplicon_IDX-N710_8	CAGCCTCG
		Amplicon_IDX-N711_9	TGCCTCTT
		Amplicon_IDX-N712_10	TCCTCTAC
		Amplicon_IDX-N714_11	TCATGAGC
		Amplicon_IDX-N715_12	CCTGAGAT

MATERIALS AND METHODS

-PCR conditions

PCRs were conducted in a 25 µl reaction mixtures with 2 µl of template DNA with KAPA HiFi HotStart PCR Kit system in which 0.75 µL 10 µM forward Primer, 0.75 µL 10 µM reverse Primer, 0.75 µL 10 mM KAPA dNTP Mix, 0.5 µL HiFi polymerase, 5.0 µL 5X KAPA HiFi Buffer and 15.25 µL of MiliQ H₂O were used. For amplification the thermocycling conditions were initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C for 20 s, 56°C for 15 s, 72°C for 20 s, and a final extension at 72°C for 5 min. Three replicates of each sample were mixed and 1µl of amplicon DNA was used for index PCR with initial denaturing at 95°C for 3 min, followed by 8 cycles of denaturation at 98°C for 20 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min.

After indexing PCR, the resulting amplicons were pooled in equimolar amounts. Paired-end sequencing (2×300 bp) was performed with a MiSeq Reagent Kit v3 at a final loading concentration of 6 pM in a MiSeq System (Illumina, San Diego, CA, United States) in Austrian Institute of Technology (AIT, Austria).

III. qPCR to study gene expression regulating A/Sd biosynthesis

In **Chapter II**, we used qPCR method to study gene expression regulating A/Sd biosynthesis in *A. tictoria* plants.

Specific metabolites often show a restricted presence in only a few organs, tissues or cell types (Li et al., 2016), and can be extensively regulated by environmental factors (e.g., pathogen attack, UV-B light) (Li et al., 2015; Tohge et al., 2016). Intuitively, the biosynthetic enzymes and their mRNAs should only be present in the cells where the metabolite is made. This assumption can be exploited to identify the biosynthetic genes by correlating gene expression and metabolite levels (Delli-Ponti et al., 2021).

Quantitative real-time polymerase reaction (qPCR) is well-known as the most effective method to analyse modulations in gene expression due to its efficiency of detection and precise quantification of target genes, even at low expression levels (Holland, 2002).

MATERIALS AND METHODS

A detailed procedures in the gene expression regulating A/Sd production in *A. tinctoria* inoculated with AMF is presented below:

III.1. Selection of genes for Real-Time quantitative PCR (RT-qPCR) analysis

-Reference gene

The prerequisite for a suitable housekeeping gene is that it must be adequately expressed in the tissue of interest, but most importantly, it shows minimal variability in expression between samples and under the experimental conditions used (Dheda et al., 2004). For example, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a commonly used genes which encodes for an enzyme involved in glycolysis and glyconeogenesis (Barber et al., 2005). This gene was used in the present work to make the normalization of genes expression obtained in the RT-qPCR analysis.

-Target gene

Three target genes involved in the shikonin (A/Sd) biosynthesis pathway were selected:

Geranylhydroquinone 3''-hydroxylase (*GHQH*): a CYP enzyme hydroxylation of C-3'' of the isoprenoid side chain of GHQ and provides the key intermediate 3''-hydroxy-geranylhydroquinone (GHQ-3''-OH) (Wang et al., 2019).

4-hydroxybenzoate-m-geranyltransferases (*PGT1* and *PGT2*): coding enzyme in catalysing the coupling of p-hydroxybenzoic acid and geranyl diphosphate to produce m-geranyl-p-hydroxybenzoic acid (Yazaki et al., 2002).

Sequences of primers of reference and target genes were listed in **Table 9**.

MATERIALS AND METHODS

Table 9: Primers used for gene expression study

Gene	Primer sequence 5'-3'	Accession number or reference used for primer designing
<i>GAPDH</i>	ACCGTCCACTCCA TTACCG ATGAGGCAGCCCT TCCACC	Wu et al., 2009
<i>GHQH/ G10H</i>	ATTGCTGGGACTG ATACAAC CGTGCAACATCGG ATTCTTC	MH077962 (Wang et al., 2019) MN056184 (Song et al., 2020)
<i>LePGT1</i>	CTCTTAGGCTCCT CTGCT CGTCGTCCACCTT ATCTT	Wu et al., 2008
<i>LePGT2</i>	AGAAAGGCAAGC AACCATC CCCACCATCCAAA TATTGCC	KT991522 (Liu et al., 2016) AB055079.1 (Yazaki et al., 2002)

III.2. Total RNA extraction

The frozen *A. tinctoria* roots were first ground in liquid nitrogen (-196°C) with pestle and mortar. Total RNA extraction was done on 0.2 g starting root material and adapting the protocol from Xu et al. (2010) with a slight change. Briefly, the process including 1) the extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid (EDTA), 1.4 M sodium chloride (NaCl), 2% cetyltrimethylammonium bromide (CTAB) (w/v), 2% polyvinylpyrrolidone (PVP) (w/v)] was prewarmed at 65 °C for 10 min; 2) Quickly transfer frozen powder (around 200 mg) to the prewarmed extraction buffer (0.6 mL) and vortex well. Add 12 µL of β-mercaptoethanol and incubate at 65 °C for 15 min, during incubation vertexing several times; 3) Add 0.5 µL of chloroform, vortex well and centrifuge at 13, 000 rpm for 10 min at 4°C; 4) Transfer the viscous supernatant to a new tube and add 0.1mL of 5 M NaCl and 0.3 mL of

MATERIALS AND METHODS

chloroform. Vortex well and centrifuge at 13,000 rpm for 10 min at 4°C; 5) Transfer the upper phase to a new tube and repeat the above procedure; 6) Carefully and slowly collect the supernatant into a new tube and add a half volume of isopropanol and a half volume of high salt solution (0.38 M trisodium citrate dihydrate + 1.2 M NaCl) and incubate at room temperature for 15 min; 7) Centrifuge at 13,000 rpm for 10 min at 4°C; 8) Completely discard the viscous supernatant and wash the pellet with 70% ethanol to remove the remaining mucilage, and air dry it till there was no ethanol; 9) Dissolve the RNA in 55 µL diethyl pyrocarbonate (DEPC)-treated water.

Then the total RNA was treated with TURBO DNA-free™ Kit (Thermo Fisher Scientific, United States), according to the manufacturer protocol. The RNA of all the samples was loaded on 1.5% agarose gel, electrophoresed to separate RNA, stained with GelRed® (Biotium, USA), and visualised under UV light to assess the integrity of ribosomal bands. In addition, the concentration of each RNA sample was measured using NanoDrop® -ND 1000 UV-vis Spectrophotometer (NanoDrop Technologies, United States) and RNA purity estimated from the A260/A280 and A260/A230 absorbance ratios. Finally, a 1 µg aliquot of total RNA was used for the first-strand cDNA synthesis according to the protocol of the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Montreal, QC, Canada). For each RNA sample, a reaction without Transcriptor High Fidelity Reverse Transcriptase (Hifi RT) enzyme was performed as a control for contamination by genomic DNA.

III.3. Relative quantification in RT-qPCR analysis

Since no published genomic resources are available for *A. tinctoria*, a previously published primer for amplification of *LePGT1* in *Onosma paniculatum* Bur. et Franch was used, while for *PGT2* and *GHQH* new primer pairs were designed. Sequences of each gene (*PGT2* or *GHQH*) from two phylogenetically distant Boraginaceae species [*L. erythrorhizon* and *Arnebia euchroma* (Royle) Johnston] were obtained from NCBI, aligned, and primers were generated from conserved regions. Once reference and targets primer genes were designed, their efficiency and specificity were analyzed by RT-qPCR. Briefly, to calculate the primers' efficiency, the cDNA from the different treatments (i.e., control, mycorrhized plants, from different time point) were mixed to serve in the

MATERIALS AND METHODS

standard curve. Six levels of dilution were used, from pure mix to a dilution of 1/1000. To increase the stability of the standard curve, chain dilution (1/5, 1/10, 1/50, 1/100, 1/500) was made. For all primer genes tested, three technical replicates of each level of standard curve were made (**Figure 15**, blue). Additionally, three replicates of negative control (i.e., water instead of DNA template, **Figure 15**, red) were used. Efficacy of the reaction was assessed for each gene, normally around efficacy (E) = 2 with curve slope of -3,5.

Selected primers were then used on all tested treatments. One plate was used by gene for each treatment (**Figure 15b**): biological replicate tested (**Figure 15b**, green), negative control (**Figure 15b**, red), std/positive control (**Figure 15b**, blue).

MATERIALS AND METHODS

a

*	1	2	3	4	5	6	7	8	9	10	11	12
A	S	S	S	S	S	S						
	d11 LePQT1 [1.000E+6]	d11 LePQT1 [1.000E+6]	d11 LePQT1 [1.000E+6]	d11 GHQH	d11 GHQH	d11 GHQH						
B	S	S	S	S	S	S						
	d5 LePQT1 [1.000E+6]	d5 LePQT1 [1.000E+6]	d5 LePQT1 [1.000E+6]	d5 GHQH	d5 GHQH	d5 GHQH						
C	S	S	S	S	S	S						
	d10 LePQT1 [1.000E+7]	d10 LePQT1 [1.000E+7]	d10 LePQT1 [1.000E+7]	d10 GHQH	d10 GHQH	d10 GHQH						
D	S	S	S	S	S	S						
	d50 LePQT1 [1.000E+6]	d50 LePQT1 [1.000E+6]	d50 LePQT1 [1.000E+6]	d50 GHQH	d50 GHQH	d50 GHQH						
E	S	S	S	S	S	S						
	d100 LePQT1 [1.000E+5]	d100 LePQT1 [1.000E+5]	d100 LePQT1 [1.000E+5]	d100 GHQH	d100 GHQH	d100 GHQH						
F	S	S	S	S	S	S						
	d500 LePQT1 [1.000E+4]	d500 LePQT1 [1.000E+4]	d500 LePQT1 [1.000E+4]	d500 GHQH	d500 GHQH	d500 GHQH						
G	S	S	S	S	S	S						
	d1000 LePQT1	d1000 LePQT1	d1000 LePQT1	d1000 GHQH	d1000 GHQH	d1000 GHQH						
H	-	-	-	-	-	-						
	LePQT1	LePQT1	LePQT1	GHQH	GHQH	GHQH						

b

*	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	S	S	S
	1c LePQT1	1c LePQT1	1c LePQT1	3M LePQT1	3M LePQT1	3M LePQT1	R5 LePQT1	R5 LePQT1	R5 LePQT1	d1500 LePQT1	d1500 LePQT1	d1500 LePQT1
B	+	+	+	+	+	+	+	+	+	S	S	S
	2c LePQT1	2c LePQT1	2c LePQT1	4M LePQT1	4M LePQT1	4M LePQT1	R6 LePQT1	R6 LePQT1	R6 LePQT1	d1000 LePQT1	d1000 LePQT1	d1000 LePQT1
C	+	+	+	+	+	+	+	+	+	-	-	-
	3c LePQT1	3c LePQT1	3c LePQT1	5M LePQT1	5M LePQT1	5M LePQT1	S2 LePQT1	S2 LePQT1	S2 LePQT1	LePQT1	LePQT1	LePQT1
D	+	+	+	+	+	+	+	+	+			
	5c LePQT1	5c LePQT1	5c LePQT1	6M LePQT1	6M LePQT1	6M LePQT1	S3 LePQT1	S3 LePQT1	S3 LePQT1			
E	+	+	+	+	+	+	+	+	+			
	6c LePQT1	6c LePQT1	6c LePQT1	R1 LePQT1	R1 LePQT1	R1 LePQT1	S4 LePQT1	S4 LePQT1	S4 LePQT1			
F	+	+	+	+	+	+	+	+	+			
	7c LePQT1	7c LePQT1	7c LePQT1	R2 LePQT1	R2 LePQT1	R2 LePQT1	S5 LePQT1	S5 LePQT1	S5 LePQT1			
G	+	+	+	+	+	+	+	+	+			
	1M LePQT1	1M LePQT1	1M LePQT1	R3 LePQT1	R3 LePQT1	R3 LePQT1	S6 LePQT1	S6 LePQT1	S6 LePQT1			
H	+	+	+	+	+	+	S	S	S			
	2M LePQT1	2M LePQT1	2M LePQT1	R4 LePQT1	R4 LePQT1	R4 LePQT1	d10 LePQT1	d10 LePQT1	d10 LePQT1			

Figure 15: Plate organization: (a) standard curve and (b) gene analyses with a square for each technical replicate, in blue standard level, in green positive control and in red negative control.

Normalization was achieved for each experiment separately using the reference gene (i.e., *GAPDH*) following the “Pfaffl” method (Pfaffl, 2001).

MATERIALS AND METHODS

Relative quantification analysis results were expressed as a ratio: the relative amount (fold change) of a target nucleic acid (test) compared to a control sample (calibrator) following “Pfaffl” method (Pfaffl, 2001) that accounted for differences in primer efficiencies. The advantage of using a reference gene is that this method prevents the need for accurate quantification and loading, of the starting material. The drawback is that this method requires the availability of a known reference gene or genes, with constant expression in all samples tested and whose expression is not changed by the treatment under study.

Average cycle threshold (“Average Ct”) for each gene in each sample analyzed is calculated. Control treatment (i.e., non-mycorrhized plants) is selected and the Ct average of all control samples calculated (“control average”). Then, ΔCt is calculated separately for each gene in each sample (i.e., subtract the “Control average” value from the “Average Ct” of each sample including all of the control samples). The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ with the standard curve (Rasmussen 2001). Finally, these values are used to calculate the gene expression ratio (fold change compared to a calibrator) applying this formula (Pfaffl, 2001):

$$\text{Gene expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct, \text{target}}}{(E_{\text{ref}})^{\Delta Ct, \text{ref}}}$$

IV. Evaluation of root colonization by AMF

In **Chapter II, III and IV**, we measured the root colonization ration by AMF following the methods described below.

The AMF structures (i.e. hyphae, arbuscules and vesicles) within the roots are usually hidden by the natural pigments (i.e. tannins, polyphenols) and contents of root cells. To evaluate root colonization by AMF it is thus necessary first to clear the roots using KOH (10%) for 30 min at 70°C. This process soften the cells wall, empty the cortical cells from their cytoplasm, removes tannins and improve the penetration of ink within the cells. If the roots are pigmented such as is the case of *A. tinctoria*, roots should be first incubated in H₂O₂ (3.5%) for 5 min at 70°C. The roots are then neutralized in HCl (1%) for 1 min at room temperature to neutralize the pH and

MATERIALS AND METHODS

increase the affinity with the ink solution. They are finally stained in 2 % Parker Super Quink Permanent Ink (Parker, blue ink) in 1% HCl for 30 min at 70°C. Stained roots are maintained in lactoglycerol (v:v-v-1:1:1-lactic acid: glycerol: H₂O).

In our study, we used the method of McGonigle et al. (1990) to evaluate root colonization. Briefly this method is based on the observation of a representative length of randomly selected roots fragment, focusing on the presence of arbuscules, vesicles and hyphae. The presence of these structures is then used to infer the global rate of root system colonization. For each sample, 150 intersections were observed under a compound microscope at 40 X magnification (Olympus BH2, Olympus Optical, GmbH, Germany) and the percentage of total root colonization (%RC), percentage of arbuscules (%A) and percentage of spores/vesicle (%V) were estimated. Around 300 intersections were observed per plant. At each intersection the presence or absence of the fungus was noted (**Figure 16**).

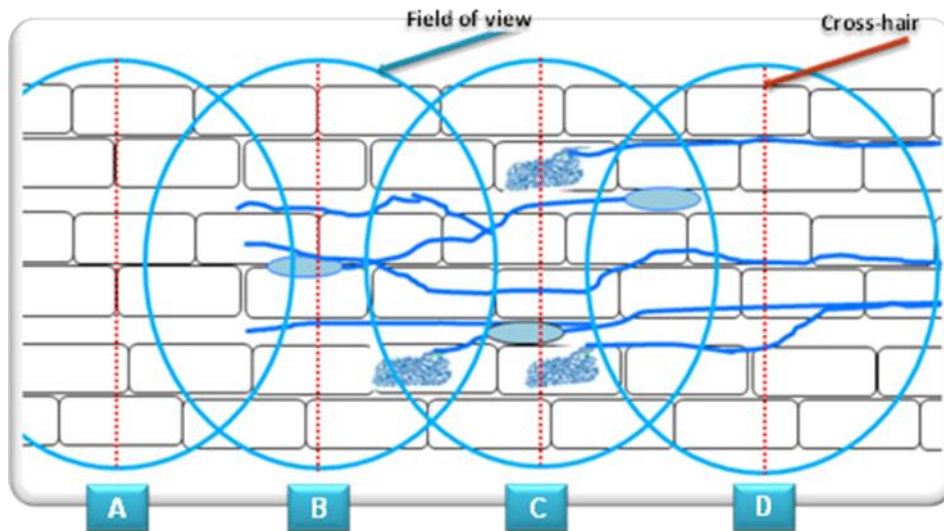


Figure 16: Evaluation of the AMF root colonization using the method of McGonigle et al. (1990).

During observation of stained roots fragments, different observations are possible (**Figure 16**):

- In case of absence of AMF at cross-hair: note “negative-intersect”

MATERIALS AND METHODS

(Figure 16A)

- In case of presence of spores/vesicles or hyphae: note “presence” of the observed structure (spores/vesicles, hyphae) (**Figure 16B**).
- When at the same time spores/vesicles, arbuscules and hyphae are observed in the intersection (**Figure 16C**): note “presence” of spores/vesicles and “presence” of arbuscules.
- When only hyphae are observed at the intersection (**Figure 16D**): note “presence” of hyphae.
- Percentage of hyphae colonization (%H), percentage of arbuscules colonization (%A) and percentage of spores/vesicles colonization (%V) are then calculated by dividing the number of intersections with the presence of hyphae or arbuscules or spores/vesicles, respectively by the total number of intersections examined. Total colonization (%RC) is calculated as the proportion of non-negative intersections.

MATERIALS AND METHODS

RESEARCH RESULTS

RESEARCH RESULTS

RESEARCH RESULTS

RESEARCH RESULTS-PART I

PART I *ALKANNA TINCTORIA* STUDIES

RESEARCH RESULTS-PART I

Preface

In nature, plants are associated with diverse microbial communities, which confer many advantages to the host plant, including growth promotion, resistance to biotic and abiotic stresses as well as modulation of the secondary metabolites (SMs) production. *Alkanna tinctoria* is a medicinal plant, producing alkannin/shikonin and derivatives (A/Sd). Yet, no study has reported the relationship between the root microbiome of *A. tinctoria* and the production of A/Sd at different developmental stages.

In **Chapter I**, *A. tinctoria* plants were grown in three distinct soils (two from Greece and one from Austria), and thus microbiomes. The objectives were (i) to investigate the link between microbiome structure and A/Sd production and (ii) to link plant growth stage with A/Sd production. The plants were grown in pots in the greenhouse and harvested at four defined plant developmental stages (vegetative growth, blooming, fruiting and decay stages). At each stage, an in-depth assessment of the bacteria and fungi associated to the roots was assessed by next generation sequencing. The primary and secondary metabolites content (target and non-target) of roots were also analyzed by ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) and HPLC with UV/Vis detection. Finally, microbiome-metabolome correlations and co-occurrence network analyses were made.

**Chapter I Metabolite production in *Alkanna tinctoria* links
plant development with the recruitment of individual
members of microbiome thriving at the root soil interface**

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Abstract

Plants are naturally associated with diverse microbial communities, which play significant roles in plant performance, such as growth promotion or fending off pathogens. The roots of *Alkanna tinctoria* L. are rich in naphthoquinones, particularly the medicinally used enantiomers alkannin, shikonin and their derivatives. Previous studies have shown that individual, isolated microorganisms may influence secondary metabolism of plants and induce or stimulate the production of medicinally relevant secondary metabolism. To further investigate the potential interaction between *A. tinctoria* and associated microorganisms we performed a greenhouse experiment, in which *A. tinctoria* plants were grown in the presence of three distinct soil microbiomes. At four defined plant developmental stages we made an in-depth assessment of bacterial and fungal root-associated microbiomes as well as all extracted primary and secondary metabolite content of root material.

Our results showed that the plant developmental stage was the most important driver influencing the plant metabolite content, revealing peak contents of alkannin/shikonin derivatives at the fruiting stage. Plant root microbial diversity was both influenced by bulk soil origin and to a small extent by the developmental stage. Metabolomes of plant roots of the same developmental stage across different soils were highly similar, pinpointing to the plant maturity as the primary driver of SM production. The performed correlation analyses and co-occurrence networks on the measured metabolite content and the abundance of individual bacterial and fungal taxa suggested a dynamic, at times positive or negative relationship between root-associated microorganisms and root metabolism. In particular, the bacterial genera *Labrys* and *Allorhizobium*-*Neorhizobium*-*Pararhizobium*-*Rhizobium*, as well as four species of the fungal genus *Penicillium* were found to be positively correlated with higher content of alkannins.

Keywords: Plant, Microbiome, Alkannin, Alkanet, Metabolomics, Metabolome, Medicinal Plant

Introduction

Medicinal plants represent a valuable source of new drugs due to the production of bioactive secondary metabolites (SMs) (Nalawade et al.,

RESEARCH RESULTS-PART I-CHAPTER I

2003; Chacko et al., 2010; Hamilton, 2004; Chen et al., 2016). Among them, members of the *Boraginaceae* family, which comprise more than 150 genera and approximately 2,700 species worldwide, represent a valuable source of bioactive SMs, primarily naphthoquinones, alkaloids, flavonoids, polyphenols, phytosterols, and terpenoids (Dresler et al., 2017). In particular, the naphthoquinone alkannin, its enantiomer shikonin, and their derivatives (A/Sd) are commercially interesting due to their broad spectrum of biological activities, such as wound healing (Kheiri et al., 2017; Papageorgiou et al., 2009), anti-inflammatory (Kourounakis et al., 2002; Papageorgiou et al., 1999), and anticancer (Andujar et al., 2013) activities.

Environmental factors, developmental stage, light intensity, soil composition, or various abiotic stress factors have been described to qualitatively and quantitatively influence primary and secondary metabolism of plants (Chaparro et al., 2013; Li et al., 2020). For example, the root and rhizome of 2-year-old *Echinacea purpurea* M. was reported to produce larger amounts of the phenylpropanoid cichoric acid in the fruiting stage (Xu et al., 2014), while the main flavonoids in the roots of *Scutellaria baicalensis* G. accumulated rapidly before the full-bloom stage (Xu et al., 2018).

Plants are associated with highly diverse microbial communities composed primarily of bacteria and fungi and termed as the plant microbiome. The microbiome plays a key role for plant health, development, and productivity by mobilizing plant nutrients, increasing nutrient uptake, or antagonizing plant pathogens and by enhancing plant resilience to abiotic stresses (Mendes et al., 2011; Castrillo et al., 2017; de Vries et al., 2020). Some microorganisms release phytohormones, small molecules, or volatile compounds that may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis (Ortiz-Castro et al., 2009). A number of studies also reported plant-associated microorganisms modulating plant metabolite synthesis (Brader et al., 2014; Company et al., 2021) and influencing the quantity and quality of the produced SMs (Chen et al., 2018). For example, inoculation of *Chamomilla recutita* L. with a *Bacillus subtilis* and a *Paenibacillus polymyxa* strain (Company et al., 2018) and coinoculation of *Papaver somniferum* L. with *Acinetobacter* sp. and *Marmoricola* sp. (Ray et al., 2019) resulted in the enhancement of specific bioactive SMs (apigenin-7-O-glucoside and morphine,

RESEARCH RESULTS-PART I-CHAPTER I

respectively). Furthermore, endophytic bacteria isolated from *Echinacea purpurea* L. have been reported to stimulate changes in the expression of plant valine decarboxylase (VDC) genes for SM alkamide biosynthesis (Xu et al., 2014). Also, fungi have been reported to enhance SM production in plants. Yams inoculated with different arbuscular mycorrhizal fungal (AMF) species (Lu et al., 2015) and *Rumex gmelini* T. seedlings cocultured with *Aspergillus* sp. isolated from the roots of tissue culture seedlings (Ding et al., 2018) showed significantly enhanced production of bioactive SMs in *R. gmelini*.

Alkanna tinctoria (L.) Tausch, commonly known as alkanet, is a European perennial flowering plant of the *Boraginaceae* family and an important producer of A/S. The derivatives of alkannin include mainly acetylalkannin, propionylalkannin, isobutylalkannin, angelylalkannin, isovalerylalkannin, α -methyl-*n*-butylalkannin, β -hydroxy-isovalerylalkannin, and β,β -dimethylacrylalkannin as well as others (Assimopoulou et al., 2006). *A. tinctoria* was an ideal naphthoquinone-producing candidate for our study as it has a high A/S content (Papageorgiou et al., 1999; Tappeiner et al., 2014), it is endemic in Europe, and good quality clonal material is available. Recently, it was shown that A/S production can be enhanced by the application of microorganisms. *A. tinctoria* hairy roots inoculated with four bacterial strains belonging to *Chitinophaga* sp., *Allorhizobium* sp., *Duganella* sp., and *Micromonospora* sp., all isolated from wild *A. tinctoria* roots, showed a significantly higher production of A/S than the uninoculated controls (Rat et al., 2021). However, the mechanisms leading to this enhanced SM production are unknown. Similarly, the ecology of plant-associated microbiomes of *A. tinctoria* or their link with plant SM production have so far not been investigated. Furthermore, although protocols for untargeted plant metabolomics analysis by using ultrahigh performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) have been published (De Vos et al., 2007), holistic studies of *Boraginaceae* metabolomes are still missing. Here, we present a comprehensive metabolomics study on a *Boraginaceae* species in which we utilize UHPLC-HRMS for analyzing the metabolome of *A. tinctoria* roots.

Although secondary metabolite production is mostly driven by the plant

RESEARCH RESULTS-PART I-CHAPTER I

and its physiology, it is our hypothesis that the plant microbiome (besides other environmental parameters) may modulate secondary metabolism. Root-associated microorganisms may influence the production of A/S and other SMs of *A. tinctoria* roots, and the plant metabolome is also likely to affect the microbiome structure. Consequently, we expected that the root microbiome or specific taxa would correlate with the overall metabolome or specific plant SMs. Furthermore, our hypothesis was that different A/S concentrations are produced in different plant growth stages, in line with a growth stage-dependent colonization of plant microbiota. To test our hypotheses, we performed next-generation sequencing of the root-associated bacterial and fungal microbiome of *A. tinctoria* grown for 5 months in the greenhouse in the presence of three different soil microbiomes and correlated the presence of individual bacterial and fungal taxa with the metabolic content results of HPLC-UV/Vis and UHPLC-HRMS performed on the root extracts (**Figure 17**). Apart from addressing A/Sd and naphthoquinone compounds, we also performed a comprehensive, untargeted metabolome analysis of *A. tinctoria*, which was used in microbiome-metabolome correlations and cooccurrence network analyses.

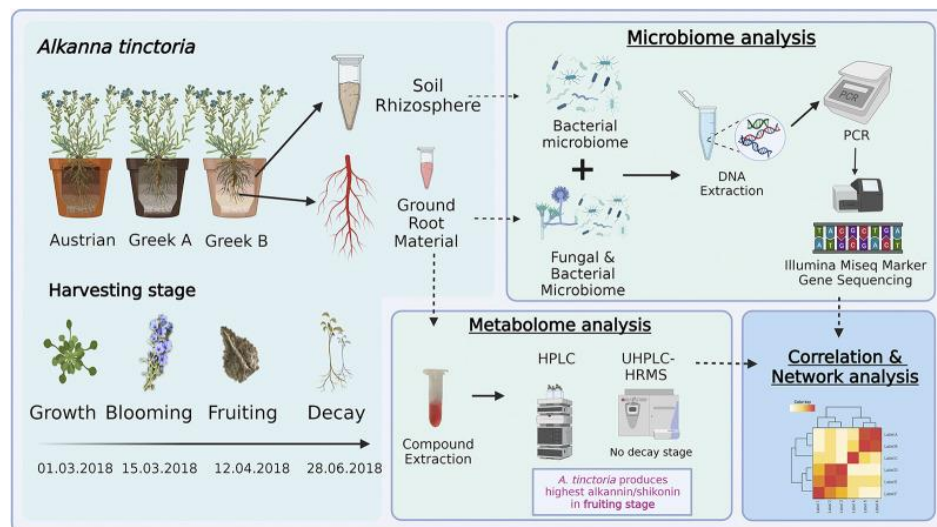


Figure 17. Graphical overview of the experiment. Created with Biorender.com

Materials and Methods

Greenhouse experiment

Alkanna tinctoria plants were provided as rooted acclimatized individuals that were originally collected and identified from natural populations and then produced by micropropagation from several mother plants by the Hellenic Agricultural Organization (HAO, Thessaloniki, Greece) in January 2018. The plants were transferred to 5-L pots containing 4.5 L of sterilized (121°C for 15 min) peat moss and perlite (volume ratio 2:1) mixed with 200 g of field soil collected either in Austria or in Greece. Thus, all plants were grown in a substrate with highly similar chemical and physical characteristics but that hosted those microbial communities prevailing in these three distinct soils. In nature, all three soils hosted various plant species related to *A. tinctoria* and belonging to *Boraginaceae*. The Austrian soil showed vegetation with *Echium vulgare*, whereas both Greek soils had *A. tinctoria* populations. Conductivity, pH, Ca, Fe, K, Mg, Mn, and P content of these soils were measured (**Table S1** in the Annex I) by extracting available soil nutrients in a chemical solution (0.5 M ammonium acetate/0.5 M acetic acid/0.02 M EDTA).

Plants were grown in the greenhouse with a 16-h light/8-h dark photoperiod at 25°C with 50% relative humidity (RH) and a photosynthetic photon flux density (PPFD) of 96 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered twice per week with deionized water and moved randomly once per week.

Plants were harvested at four different defined developmental stages; the first stage (“vegetative growth”) was defined when more than 50% of the individuals started to produce new leaves, “blooming” was the stage when more than 50% of the individuals had flowers, and “fruiting” was the stage when more than 50% of the individual plants began to produce fruits. The final stage was “decay,” when more than 50% of the plants had withered inflorescences. At each sampling stage of each soil, six individuals were harvested. Roots were cut at the root-stem transition and gently washed using tap water to eliminate the adhering debris. Rhizosphere samples were collected by washing roots in 45 mL of phosphate-buffered saline Tween 20 (PBST) solution and placed in a sonicator (Branson ultrasonic cleaner) for 10 min. The tubes were then centrifuged at 4°C at 3,500 rpm for 20 min after taking roots out, and pellets were collected by removing the

RESEARCH RESULTS-PART I-CHAPTER I

supernatant (White et al., 2015). Corresponding bulk soil samples were also collected from each pot. The root system was divided into two equal parts; one part was freeze-dried and shipped to Greece for SM analysis, while the other part together with the rhizosphere and bulk soil samples were kept at -80°C until performing DNA extraction. Fresh weights of roots and shoots were measured at each harvest point.

Microbiome analysis

DNA analysis and microbial community sequencing

For extracting whole community DNA of roots, 100 mg of root material was freeze-ground by using 3.5-mm stainless steel UFO beads in a ball mill (Mixer Mill MM400, Retsch, Haan, Germany). The powdered material was then used for DNA extraction by using a modified cetyltrimethylammonium bromide (CTAB) protocol combined with a classical phenol-chloroform-isoamyl-alcohol extraction protocol (Wakelin et al., 2007). To get rid of additional PCR-inhibiting substances still inside the extracted solution, an additional DNA purification step with a DNeasy PowerClean cleanup kit (Qiagen) was applied on all samples. The rhizosphere and soil samples were processed with the FastDNA spin kit for soil and the FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA) by following the instructions in the provided manual. For bacterial community analysis, we used the primer pair 799F-1175R (5'-AACMGGATTAGATACCCCKG-3' and 5'-ACGTCRTCCCCDCCTTCCTC-3', respectively) (Hanshew et al., 2013) to target the V5-V7 region of the 16S rRNA gene. For fungal community analysis, an improved primer pair (Taylor et al., 2016) amplifying the partial 5.8S ribosomal subunit gene 5.8S-Fun (5'-AACTTTYRRCAYGGATCWCT-3') and the whole internal transcribed spacer (ITS) 2 ITS4-Fun (5'-AGCCTCCGCTTATTGATATGCTTAART-3') was used.

Paired-end sequencing (2×300 bp) was performed with a MiSeq reagent kit with v3 chemistry at a final loading concentration of 6 pM. The fungal ITS library was sequenced on a MiSeq system (Illumina, San Diego, CA, United States), whereas the bacterial 16S rRNA gene library was sequenced on the same Illumina platform by LGC Genomics (Berlin, Germany).

Microbial data and cooccurrence network analysis

RESEARCH RESULTS-PART I-CHAPTER I

Raw reads were filtered with Bowtie2 v.2.3.4.3 (Langmead and Salzberg, 2012) to avoid the presence of Illumina's PhiX contamination, and quality was preliminarily checked with FastQC v.0.11.8. Primers were stripped using Cutadapt v.1.18 (Martin, 2011). Sequences were quality filtered, trimmed, and denoised, and amplicon sequence variants (ASVs) were generated with DADA2 v1.14 (Callahan et al., 2016). Denoised forward and reverse ASV sequences were merged, and chimeras were removed. Filtered ASVs were checked using Metaxa2 v2.2.1 (Bengtsson-Palme et al., 2016) and ITSx v1.1.2 (Bengtsson-Palme et al., 2013) for targeting the presence of V5-V7 16S rRNA and ITS2 regions in archaeal and bacterial sequences and fungal sequences, respectively. Taxonomic assignment of the 16S rRNA gene ASVs and ITS-based ASVs was performed using the RDP classifier (Wang et al., 2007) against the SILVA v138 (Quast et al., 2013) database and UNITE 8.2 (Nilsson et al., 2019) database, respectively. BIOM objects with bacterial and fungal counts, respectively, were built and imported into the R-4.0.3 statistical environment for further analyses. Contaminating sequences were identified and eliminated by using the package decontam (Davis et al., 2018) by setting the threshold to 0.1 together with batch correlation to this particular experiment. Rare ASV filtering was applied with a 0.1% threshold in the RAM package filter.OTU function. The resulting tables were normalized and analyzed by using the vegan (Dixon, 2003) rtk, emmeans, multcomp, dplyr, DeSeq2 (Love et al., 2014), phyloseq (McMurdie and Holmes, 2013), and mvabund packages. The 2,261 bacterial ASVs in root samples were assigned to 20 phyla, 43 classes, and 267 genera. In the rhizosphere, we identified 2,457 bacterial ASVs belonging to 19 phyla, 45 classes, and 298 genera. In bulk soil samples, we assigned 2,232 bacterial ASVs to 22 phyla, 46 classes, and 209 genera. Alpha-diversity values were calculated by adopting a multiple rarefaction method in which richness (observed ASVs) and diversity values (Simpson's diversity index [SDI]) were generated by averaging the results inferred after 999 rarefactions, starting from a minimum number of 1,422 and 2,632 for 16S rRNA gene and ITS data, respectively. ANOVA based on a linear model was calculated on the alpha-diversity values between groups, and significances were confirmed and investigated with a *post hoc* analysis (estimated marginal means). Beta-diversity Bray-Curtis distance matrices were created based on DeSeq2 count-normalized tables. Bray-Curtis values were analyzed in a pairwise PERMANOVA by using

RESEARCH RESULTS-PART I-CHAPTER I

999 permutations. A canonical analysis of principal coordinates (CAP) ordination plot based on the Bray-Curtis dissimilarity matrix was used to distinguish between the groups. Reproducibly occurring ASVs were calculated by first normalizing our data with DeSeq2's median of ratios method and creating a hybrid artificial factor by combining developmental stage and soil origin with the package dplyr and then using the core.OTU function from RAM with a 4/6 threshold, which calculated the ASVs present in 4 of 6 replicates in each artificial factor. Afterwards, to calculate the significantly differentially abundant rASVs, manyglm analysis (multivariate glm in mvabund package) was used considering both factors, followed by an ANOVA based on manyglm with 999 permutations.

We identified core microbiomes for each sample type (i.e., rhizosphere, root, and bulk soil), considering rASVs shared between soils or between developmental stage. In addition, we calculated the stable core microbiome according to the definition by Pfeiffer et al. (2017) comprising rASVs across soils and developmental stages. For visualizing the core through Venn diagrams, venny 2.1.0 was used. The HPLC and liquid chromatography-mass spectrometry (LC-MS) metabolome data before correlation and network analysis were centered and scaled as a normalization method. Spearman correlations were done in the psych package, with false-discovery rate (FDR)-corrected *P* values and correlation plots visualized with the ggcorrplot package. To create the cooccurrence networks, we used the DeSeq-normalized microbial and center-scaled metabolome data and inspected the data sets with t-distributed stochastic neighbor embedding (t-SNE) with the Rtsne package. R packages parallel, doParallel, caret, and rfPermute were used for performing random forest analysis on all the fungal, bacterial, and UHPLC-HRMS data. The significant features from the random forest analysis were submitted to Spearman correlation analysis in the psych package, and the correlation results were used to build undirected cooccurrence networks with the packages igraph, tidygraph, and ggraph. For creating box plots, bar plots, and ordinations, the ggplot2, ggvegan, reshape, and ggpubr packages were used.

Metabolome analysis

Chemicals and reagents

RESEARCH RESULTS-PART I-CHAPTER I

Several standard compounds were used for metabolite identification and quantitation as previously described (Tappeiner et al., 2014; Assimopoulou and Papageorgiou, 2004a). The following (purified by column chromatography) standards were used: alkannin (Ikeda, Japan), shikonin (Ichimaru, Japan), acetylshikonin (ABCR GmbH, Germany), propionylshikonin (synthesized by Elias Kouladouros, Agricultural University of Athens, Greece), deoxyshikonin (TCI, Belgium), β,β -dimethylacrylshikonin (ABCR GmbH, Germany), and isovalerylshikonin (TCI, Belgium). For metabolite extraction and UHPLC-HRMS analysis, methanol and formic acid (LC-MS reagent-grade, Honeywell Fluka, USA) were used. To perform HPLC analysis, acetonitrile (HPLC-grade, Honeywell Riedel de Haën, USA), ultrapure water (Merck Millipore, Germany), and formic acid (HPLC-grade, Merck KGaA, Germany) were utilized. For hydrolyzing the alkannin/shikonin esters from root extracts to determine the alkannin:shikonin enantiomeric ratio, the following reagents were used: sodium hydroxide (Merck KGaA, Germany), hydrochloric acid (37%, Carlo Erba Reagents, France), and chloroform (ChemLab, Belgium). Hexane (HPLC-grade, Honeywell Riedel de Haën, USA) and 2-propanol (for liquid chromatography; Merck Millipore, USA) were used for chiral HPLC-UV/Vis.

Sample preparation for metabolite analysis

Samples of *A. tinctoria* roots were stored at -80°C before being ground to a fine powder using a ball mill (Fritsch Pulverisette 0, Germany). Each powdered sample was weighed (70 mg) into microcentrifuge tubes, followed by extraction with 3 mL of methanol by ultrasound at 10% power for 3 h (Bandelin Sonorex Digital 10P, Berlin, Germany) and centrifugation for 10 min at 12,500 rpm (Hermle Z 216 MK, Wehingen, Germany). The supernatants were collected and subjected to UHPLC-HRMS and HPLC-UV/Vis analyses after filtering with 0.22- μm syringe filters. To determine A/S enantiomeric ratio by chiral HPLC, the sample preparation procedure described by Tappeiner et al. (2014) was followed.

HPLC-UV/Vis method for the quantitation of A/S and determination of the alkannin:shikonin enantiomeric ratio

To quantify the contents of alkannins/shikonins (each naphthoquinone derivative separately and total A/S content) in the methanolic root extracts

RESEARCH RESULTS-PART I-CHAPTER I

prepared as described above, HPLC with UV-Vis detection set at 520 nm was used. All samples, regardless of growth stage or soil type, were analyzed. Analyses were performed on an Agilent 1200 HPLC instrument (Santa Clara, CA, USA) utilizing a VDSpher PUR C18-M-E 5- μ m, 150 $\times$ 4.6 mm column (Berlin, Germany). The mobile phase was composed of ultrapure water (A) and acetonitrile (B). Each run lasted for 13 min with a flow rate of 1 mL/min, and samples were run in a randomized sequence to avoid bias. Elution was performed using the following solvent gradient: 0 min 30A/70B in 8 min to 100B and kept at that composition for 5 min. Before the next injection, the column was equilibrated for 5 min with the initial solvent composition. Column temperature was kept at 35°C. Quantitation of each A/S derivative using HPLC was performed by using calibration curves of A/S standards (shikonin, acetylshikonin, propionylshikonin, deoxyshikonin, β,β -dimethylacrylshikonin, and isovalerylshikonin). Data acquisition and processing of raw HPLC data were accomplished via the Agilent ChemStation software. Statistical analyses on the data set were performed using R v4.0.3 software.

The hydrolyzed root extract was analyzed by an adaptation of the normal phase HPLC-UV/Vis method (Tappeiner et al., 2014), with the detection wavelength set to 520 nm with the aid of a Chiralcel OD-H chiral column (Daicel, Osaka, Japan). The isocratic elution program consisted of *n*-hexane and 2-propanol in a 65:35 ratio, and elution time was 15 min with the mobile phase flow rate set to 0.8 mL/min. The retention time of each enantiomer was determined with A/S standards. The peak areas of alkannin and shikonin in hydrolyzed root extracts were estimated by integration through the HPLC software, and percent A:S enantiomeric ratio was estimated in each hydrolyzed extract. One sample of each soil type within each growth stage was randomly selected for hydrolysis and normal phase chiral HPLC analysis.

UHPLC-HRMS method

The UHPLC-HRMS data of the *A. tinctoria* root extracts were recorded on an LTQ Orbitrap Discovery (Thermo Scientific, Waltham, MA, USA) instrument. All samples from the vegetative growth, blooming, and fruiting stages were analyzed. The column used was an Acquity UPLC HSS C18 SB 1.8- μ m, 2.1 $\times$ 100 mm column (Waters, Milford, MA, USA) kept at 50°C, with the mobile phase flow rate set to 0.3 mL/min. The solvents used

RESEARCH RESULTS-PART I-CHAPTER I

were ultrapure water (A) and methanol (B), both with 0.1% formic acid added. The following gradient elution program was used: 0 min 95A/5B, 1 min 50A/50B, 8 min 0A/100B, 13 min 0A/100B, 13.01 min 95A/5B, and 16 min 95A/5B. Data were recorded in both positive and negative ESI modes, with the capillary temperature set to 300°C using the MS/MS feature of the instrument. The MS/MS data were obtained for the six most intense m/z peaks in each full scan, with the normalized collision energy set to 35 eV. The acquisition and initial processing of the data were performed using Xcalibur (Thermo Scientific, USA) software. Data alignment and feature extraction were performed utilizing the XCMS online platform (The Scripps Research Institute, USA). Parameters used involved a maximum tolerated m/z deviation of 2.5 ppm in consecutive scans and a signal-to-noise threshold of 10. Retention time alignment was done with Obiwrap, while the maximum allowed shift in retention time was set to 5 s. The extracted data were passed through a filter, which removed features with a relative standard deviation in quality control (QC) samples of over 30% to avoid mistaking unreliable measurements with biological variation. The data were then normalized by median and underwent log transformation, resulting in normal distribution of the detected features. The identified/annotated features were subjected to correlation based on the Pearson coefficient. A hierarchical clustering heatmap of the normalized data set containing identified/annotated features was created using the Ward clustering method based on Euclidean distance. One-way ANOVA on the obtained data matrix containing all detected features was performed to estimate the number of statistically significant features present in the data set. The listed statistical analyses as well as the related data visualization were done with the help of MetaboAnalyst 5.0 (Pang et al., 2021) and R v4.0.3 software. Compound identification was performed by comparing m/z values, MS/MS fragmentation spectra, and retention times to those of commercially available A/S standards, while compound annotation was largely done by matching m/z values and MS/MS fragmentation spectra to the mzCloud database by using Compound Discoverer 3.2 (Thermo Fisher Scientific, Waltham, MA, USA) while also taking into account retention times. For maximum hit confidence, matching was done by comparing our MS/MS spectra to database spectra obtained on reference compounds with Orbitrap systems by using the same collision-induced dissociation energy. Additional publicly available online

RESEARCH RESULTS-PART I-CHAPTER I

databases, such as MassBank and PubChem, as well as available literature on studied compounds containing information on MS/MS fragmentation were used for metabolite annotation.

Results

Root bacterial and fungal microbial diversity and taxonomic composition

We focused on the analysis of the root-associated bacterial and fungal microbiome; in addition we report on the bacterial microbiome of rhizosphere and bulk soil samples. Analysis of variance (ANOVA) analyses on alpha-diversity estimators (Simpson's diversity and richness) in root samples (comprising endophytic and rhizoplane microorganisms) showed that the soil microbiome was the only significant factor among the analyzed components (**Table 10** and **Figure S1** in Annex I). As we inoculated 200 g of nonsterile soil in 5 kg of sterilized soil substrate, we consider any observed differences between soils mostly due to the different microbiomes present in these soils. In the rhizosphere, both soil (microbiome) and developmental stage significantly affected the alpha-diversity values (bacterial diversity and richness; **Table 10**), whereas in bulk soil samples, only the plant developmental stage influenced alpha-diversity. According to pairwise permutational multivariate analysis of variance (PERMANOVA) calculations on the Bray-Curtis dissimilarity values (**Table S2** in Annex I), the bacterial beta-diversity of root samples in the different soil microbiome subsets was significantly different only between the fruiting and decay stages. There was only one significant difference in beta-diversity values between developmental stages in the root fungal microbiome, which was in the Greek B soil between blooming and fruiting stages (**Table S2** in Annex I). However, the different soil microbiomes had significant effects on the root bacterial and fungal beta-diversity values after reaching the blooming stage (**Table S2** in Annex I and **Figure 18**). In the rhizosphere and bulk soil samples, the measured beta-diversity values were significantly influenced by both developmental stage and soil microbiome (**Figure 18**), although the difference between plant developmental stages varied in the different soil subsets (**Table S2**). Both rhizosphere and bulk soil bacterial communities exhibited significant differences in their composition between Austrian and Greek soils in all stages, except in the fruiting stage in which Austrian and Greek A groups

RESEARCH RESULTS-PART I-CHAPTER I

were similar (**Table S2** in Annex I). The most abundant bacterial phyla assigned from 2,261 amplicon sequence variants (ASVs) in all root samples were *Proteobacteria* (~67% relative abundance), *Bacteroidetes* (~16% relative abundance), and *Actinobacteria* (~11% relative abundance). The bacterial classes with the highest abundance in all root samples were *Gammaproteobacteria* (~44% relative abundance), *Alphaproteobacteria* (~23% relative abundance), *Bacteroidia* (~16% relative abundance), and *Actinobacteria* (~10% relative abundance). The bacterial genus groups with the highest relative abundances in all stages and soils were *Burkholderia-Caballeronia-Paraburkholderia* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (**Figure 19A**). Root fungal ASVs mostly belonged to the phylum *Ascomycota* (97% relative abundance) and to the class *Sordariomycetes* (84% relative abundance). The fungal genus *Thielaviopsis* dominated the vegetative growth (61% relative abundance) and blooming (73% relative abundance) stages of roots of plants grown in the presence of the Austrian soil amendment (**Figure 19B**). The fungal genus composition of *A. tinctoria* roots grown in Greek A soil microbiome had a dynamic turnover throughout plant development, although the genus *Penicillium* was the most abundant in the fruiting stage in all soils (**Figure 19B**).

Table 10: Alpha-diversity statistics

Sample type	Bacteria Factor	Richness ^a		Diversity ^a	
		<i>F value</i>	<i>P value</i> ^b	<i>F value</i>	<i>P value</i> ^b
Root	Soil ^c	2.76	0.071	5.46	0.007**
	Stage	2.74	0.051	0.51	0.678
	Soil:Stage	2.04	0.075	0.52	0.794
Rhizosphere	Soil ^c	5.92	0.004**	47.09	0.000***
	Stage	23.48	0.000***	17.31	0.000***

RESEARCH RESULTS-PART I-CHAPTER I

	Soil:Stage	2.95	0.014*	15.52	0.000***
Soil	Soil ^c	1.55	0.221	2.40	0.099
	Stage	6.99	0.000***	12.33	0.000***
	Soil:Stage	2.71	0.021*	1.26	0.289

Sample type	Fungi factor	Richness ^a		Diversity ^a	
		<i>F value</i>	<i>P value</i> ^b	<i>F value</i>	<i>P value</i> ^b
Root	Soil ^c	5.39	0.007**	4.47	0.015*
	Stage	1.77	0.161	2.07	0.113
	Soil:Stage	1.25	0.294	0.53	0.781

Bacteria ^d	Richness values ^d	Diversity values ^d	Fungi	Richness values	Diversity values
Growth stage			Growth stage		
Austrain	157	0.974	Austrain	31.9	0.677
Greek A	165	0.979	Greek A	39.8	0.789
Greek B	141	0.959	Greek B	43.7	0.782
Blooming			Blooming		
Austrain	134	0.966	Austrain	28.6	0.642

RESEARCH RESULTS-PART I-CHAPTER I

Greek A	142	0.971	Greek A	36.6	0.763
Greek B	118	0.952	Greek B	40.4	0.747
Fruiting			Fruiting		
Austrain	127	0.967	Austrain	38.3	0.764
Greek A	135	0.973	Greek A	46.2	0.885
Greek B	111	0.953	Greek B	50.1	0.869
Decay			Decay		
Austrain	150	0.966	Austrain	32.8	0.703
Greek A	158	0.971	Greek A	40.8	0.885
Greek B	134	0.952	Greek B	44.7	0.808

^aANOVA (Analysis of variance) based on linear model fitted to richness (observed number of ASVs) and Simpson's diversity values (SDI).

^bStatistically significant P values presented in bold letters, *, P < 0.05; **, P < 0.01; ***, P < 0.001.

^cSoil refers to soil communities of different origin.

^dFor root-associated bacteria and fungi also the estimated mean values without statistics per group are provided.

RESEARCH RESULTS-PART I-CHAPTER I

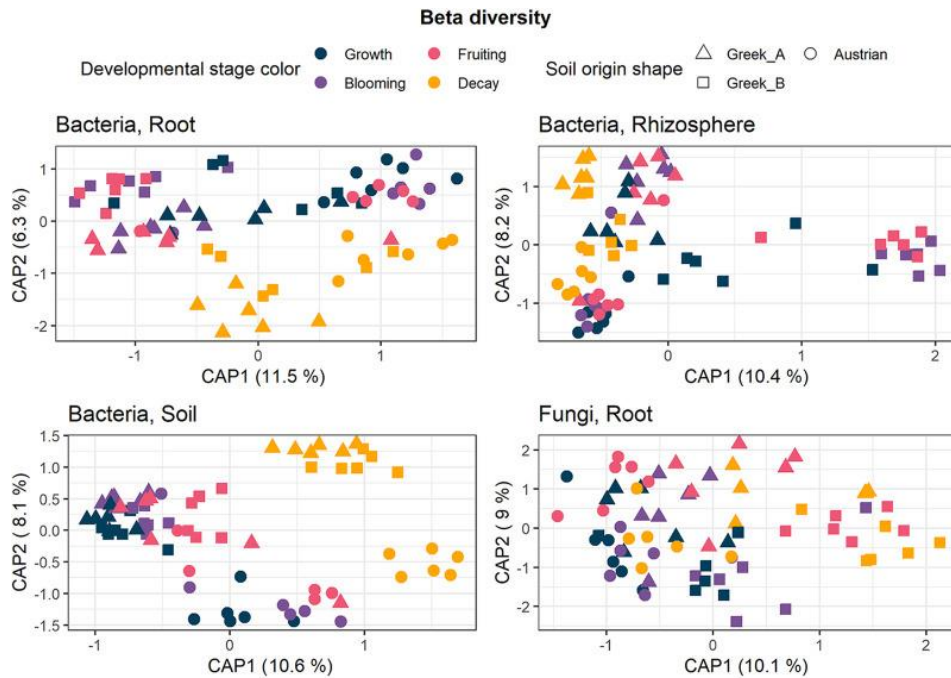


Figure 18. CAP beta-diversity plots based on Bray-Curtis dissimilarities showing the differences in the microbial community structures ($n = 6$ constrained by the soil origin and by the different developmental stages in root, rhizosphere, and soil bacterial samples as well as in root fungal samples).

RESEARCH RESULTS-PART I-CHAPTER I

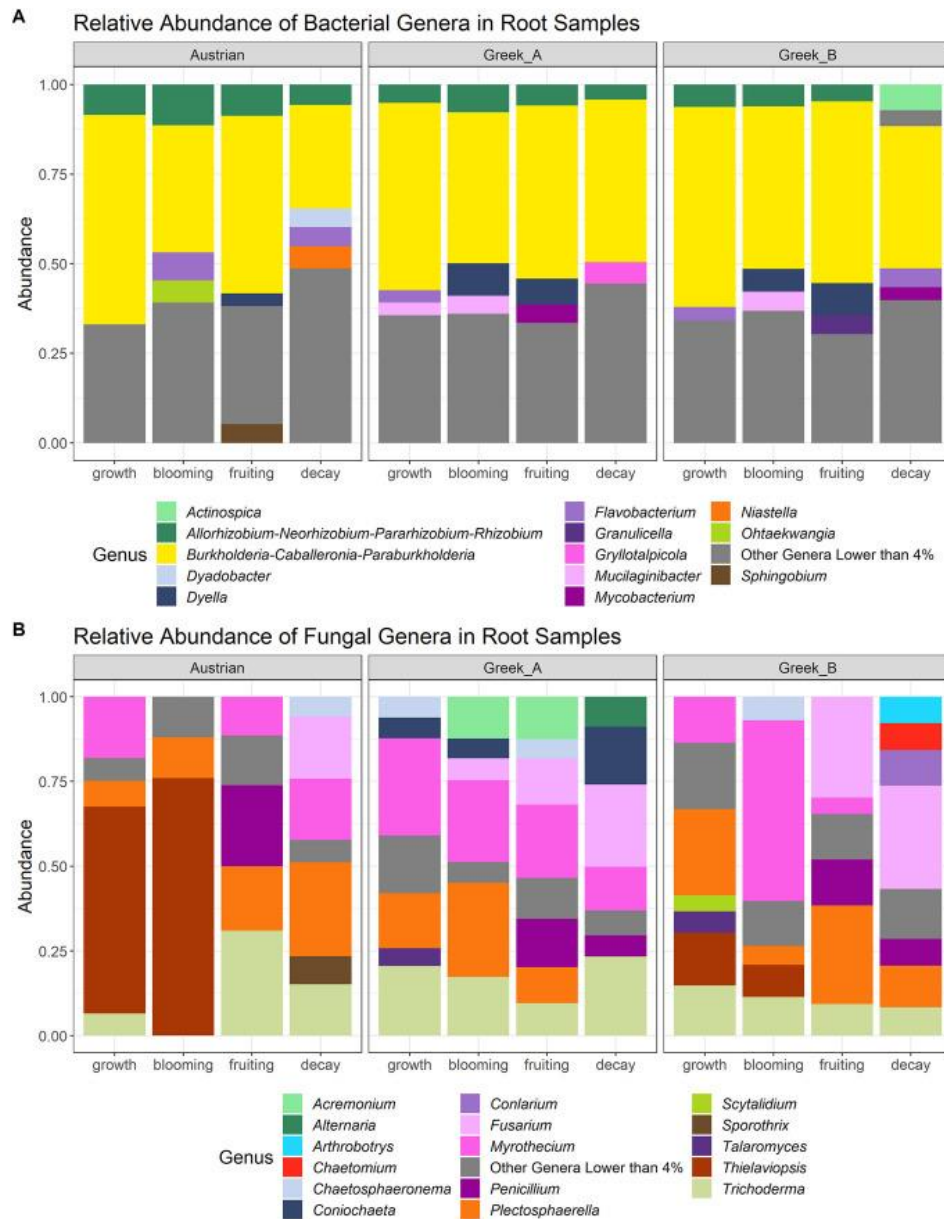


Figure 19. Changes in relative abundance of genera in root samples during plant development. (A and B) Bacteria (A) and fungi (B) in the root samples of *A. tinctoria* faceted by the three soil microbial communities. Genera lower than 4% were merged.

RESEARCH RESULTS-PART I-CHAPTER I

In the rhizosphere, the genera *Burkholderia-Caballeronia-Paraburkholderia* were most abundant in the fruiting stage (34%), followed by *Mucilaginibacter* (only in the growth and decay stage; 6% relative abundance) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (5% relative abundance). In bulk soil samples, the genera *Burkholderia-Caballeronia-Paraburkholderia* were most abundant (22% relative abundance), followed by *Mucilaginibacter* (11% relative abundance), *Nocardioides* (9% relative abundance), *Dyella* (6% relative abundance), and *Gryllotalpicola* (5% relative abundance).

For each sample type, we identified core microbiomes composed of shared reproducibly occurring ASVs (rASVs) across all soils or developmental stages as well as stable core microbiomes of shared rASVs across soils and developmental stages. Among root-associated bacteria, 204 core rASVs were found in all root samples irrespective of the soil, whereas 193 rASVs were shared between all plant developmental stages. We found 174 stable core rASVs shared among all samples (**Figure S2A** in Annex I) comprising 9 phyla, 11 classes, and 57 genera and dominated by *Burkholderia-Caballeronia-Paraburkholderia* (36% relative abundance among core genera; **Figure S2B** in Annex I). In the root-associated fungal core microbiome, 59 rASVs were found in all root samples irrespective of the soil and 61 rASVs in all developmental stages, and we identified 56 stable core rASVs. The latter could be assigned to 2 phyla, 7 classes, and 22 genera and was dominated by *Trichoderma* (19% relative abundance), *Plectosphaerella* (19% relative abundance), *Fusarium* (16% relative abundance), and *Myrothecium* (15% relative abundance; **Figure S3** in Annex I).

The majority of the core bacterial rASVs in rhizosphere samples belonged to *Burkholderia-Caballeronia-Paraburkholderia* (69% relative abundance among all rASVs) and *Asticcacaulis* (10% relative abundance). The bulk soil core rASVs consisted mainly of *Nocardioides* (21% relative abundance), *Burkholderia-Caballeronia-Paraburkholderia* (19% relative abundance), and *Dyella* (13% relative abundance). Overall, 114 bacterial reproducibly occurring core rASVs were shared between root, rhizosphere, and bulk soil samples (**Figure S4** in Annex I) and belonged mainly to *Burkholderia-Caballeronia-Paraburkholderia*.

A. tinctoria roots produce highest A/S amounts in the fruiting stage

RESEARCH RESULTS-PART I-CHAPTER I

To determine the *A. tinctoria* developmental stage in which the roots exhibit the highest A/S content, a high-throughput gradient-elution HPLC-DAD? UV_Vis? method was applied. External calibration with commercially available and purified standards allowed the quantitation of individual A/S compounds as well as their sum, that is, “Total A/S.”

The measured root A/S concentrations were highest in the fruiting stage, followed by the vegetative growth and blooming stages (**Table S3** in Annex I). In the decay stage, roots contained the lowest A/S amounts, whereas in the fruiting stage, A/S total content of roots represented up to 1.7% of the total root weight based on methanolic extractions. The A/S content variability was high across all sample groups (**Figure 20A**). Changes in total A/S content were primarily driven by the developmental stages (ANOVA, $P < 0.01$), while the variance due to different soil types was found to be statistically insignificant ($P > 0.05$; **Table 11**). This trend was displayed also with concentrations of individual A/S compounds, except for alkannin and acetylalkannin. The latter compound showed to be influenced by the soil ($P = 0.02$). In contrast, the alkannin data set consisted mainly of nondetectable and nonquantifiable values. This is a consequence of the total A/S content being dominated by two compounds: β,β -dimethylacrylalkannin and isovalerylalkannin. Other A/S were mostly present in small amounts, often eluding reliable HPLC detection/quantitation. The list of annotated compounds identified in the greenhouse *A. tinctoria* root samples by using UHPLC-HRMS is shown in **Table S4** in Annex I.

RESEARCH RESULTS-PART I-CHAPTER I

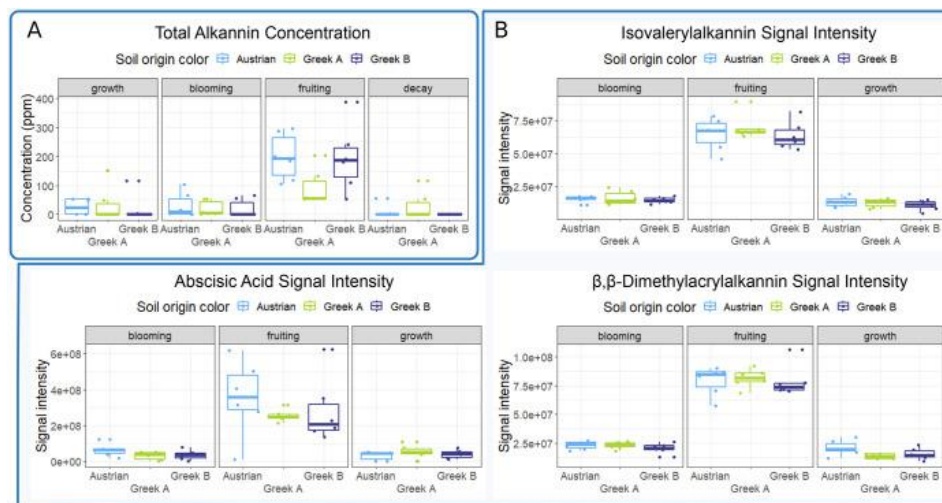


Figure 20. (A) Total A/S concentration (mg/L) measured by HPLC in the methanolic extracts of *A. tinctoria* roots across four different growth stages and three different soil microbiomes. (B) UHPLC-HRMS signal intensities of important metabolites in the extracts of *A. tinctoria* roots across three different growth stages and three different soil microbial communities. Created with [BioRender.com](https://www.biorender.com).

Table 11. Statistics on A/S concentrations obtained from HPLC-UV/Vis data

ANOVA ^a	Alkannins/shikonins		Concn
Compound	Factor	F value	P value ^b
Total A/S	Soil	1.24	0.296
	Stage	28.26	0.000***
	Soil:stage	2.12	0.063
Alkannin	Soil	3.91	0.026*
	Stage	0.00	1
	Soil:stage	0.00	1
Acetylalkannin	Soil	3.94	0.024*
	Stage	16.07	0.000***
	Soil:stage	2.29	0.047*
Deoxyalkannin	Soil	0.24	0.784
	Stage	9.56	0.000***

RESEARCH RESULTS-PART I-CHAPTER I

	Soil:stage	0.19	0.977
	Soil	0.39	0.679
Propionylalkannin	Stage	14.69	0.000***
	Soil:stage	0.50	0.804
	Soil	1.18	0.314
β , β -Dimethylacrylalkannin + isovalerylalkannin	Stage	26.63	0.000***
	Soil:stage	2.11	0.065
PERMANOVA ^a	Factor	F value	P value ^b
UHPLC-HRMS metabolome	Soil	1.33	0.133
	Stage	11.97	0.000***

a ANOVA based on a linear model. PERMANOVA on the obtained UHPLC-HRMS data.

b *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Chiral HPLC measurements were used for determining the enantiomeric ratio of A/S in selected root samples, which was shown to remain stable regardless of the developmental stage. The type of soil microbiome used in our study did not influence the enantiomeric ratio. Analyzed *A. tinctoria* roots contained, on average, ~95% alkannin and ~5% shikonin.

Untargeted UHPLC-HRMS-based metabolomics revealed developmental stage as the dominant driver of metabolome changes

A high-throughput UHPLC-HRMS method was used to analyze samples from the first three developmental stages. The untargeted UHPLC-HRMS metabolomics data set was used for metabolite identification and annotation.

We identified 4 A/S compounds and annotated 27 metabolites from the UHPLC-HRMS data set. Among these were compounds that belong to the alkannin biosynthesis pathway (Yazaki, 2017; Singh et al., 2010), which was the main focus of our study. Plant hormones (abscisic acid, methyl jasmonate, and 3-methylsalicylic acid) were also detected in the data set. Other highlighted annotated metabolites included sinapinic acid, caffeic acid 2-amino-1,3,4-octadecanetriol or phytosphingosine, 6-

RESEARCH RESULTS-PART I-CHAPTER I

methoxyflavanone, ostruthol, and compounds belonging to the anthraquinone class (a complete list of identified features is shown in **Table S5** in Annex I). Moreover, we were able to detect citrinin, a mycotoxin from *Penicillium*.

In UHPLC-HRMS, isovalerylalkannin and β,β -dimethyacrylalkannin could be separated, and these two most abundant A/Sd in *A. tinctoria* roots showed the highest signal intensities across different sample groups, with obvious maxima in the fruiting stage. Absciscic acid showed similar trends in signal intensities as the two A/S, reaching maximum concentrations in the same developmental stage, albeit with higher variability between individual samples (**Figure 20B**). In a correlation analysis, absciscic acid was found to cluster with 6-methoxyflavanone, ostruthol, maltotetraose, and A/S (all metabolites present in high relative concentration in the fruiting stage). Another distinct cluster contained caffeic acid, rosmarinic acid, sinapinic acid, methyl jasmonate, and 4-(3,4-dihydroxyphenyl)-6,7-dihydroxy-2-naphthoic acid, metabolites that are present in high relative concentrations at the growth and blooming stages (**Figure 21A**).

We combined ANOVA, PERMANOVA, and t-distributed stochastic neighbor embedding (t-SNE; **Figure 21B**) to analyze the large untargeted metabolomics data sets obtained via UHPLC-HRMS. These indicated no significant variations in the metabolome of *A. tinctoria* roots in the three different soil substrates. PERMANOVA confirmed the t-SNE patterns on our data, and only the developmental stage was found to be a significant factor. When ANOVA was used to compare samples from different soil substrates (differing in their microbiomes) within the same developmental stage, only a single feature (that unfortunately eluded annotation) was statistically significant. ANOVA highlighted hundreds of statistically significant features in the UHPLC-HRMS data set when samples from the same soil microbiome type were compared across different developmental stages. Due to the lack of confident tandem mass spectrometry (MS/MS) spectral matches, the majority of these features did not result in compound annotation or identification. The t-SNE dimensionality reduction (**Figure 21B**) also discriminated between the three different developmental stages.

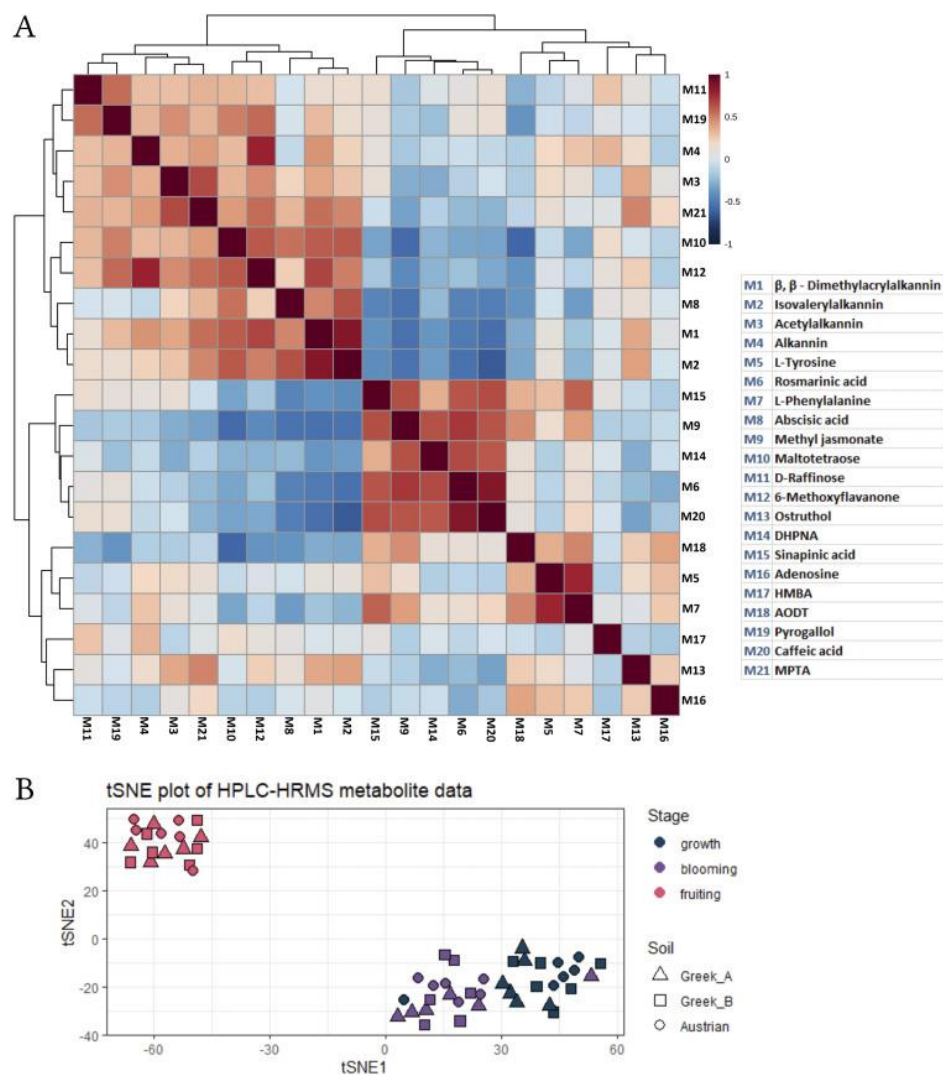


Figure 21. (A) Compound correlation heatmap from the UHPLC-HRMS data set. DHPNA, 4-(3,4-dihydroxyphenyl)-6,7-dihydroxy-2-naphthoic acid; HMBA, 4-hydroxy-3-(3-methylbut-2-en-1-yl)benzoic acid; AODT, 2-amino-1,3,4-octadecanetriol; MPTA, methyl (7-hydroxy-11-methyl-2,9-dioxo-12,15-dioxatetracyclo[8.4.1.0^{1,10}.0^{3,8}]pentadeca-3,5,7-trien-13-yl)acetate. (B) t-SNE dimensionality reduction plot on centered and scaled total UHPLC-HRMS data. Created with [BioRender.com](https://www.biorender.com).

Individual bacterial and fungal taxa associated with roots positively

RESEARCH RESULTS-PART I-CHAPTER I

correlated with higher levels of alkannins

For correlating alkannin concentrations with root-associated microorganisms in our data set, we calculated significantly differentially abundant rASVs (sign. rASV) from *A. tinctoria* root samples. The identified 31 bacterial and 10 fungal sign. rASVs were present in at least 4 of 6 replicates in their individual group but showed significantly different abundances between the groups (one group for each soil/developmental stage combination). We used these root sign. rASVs for the correlation analysis with the targeted HPLC-UV/Vis root metabolite analysis results to identify possible links between A/S production and the presence of individual bacterial or fungal taxa. In a Spearman correlation between the root bacterial sign. rASVs and the concentrations of the targeted HPLC metabolites, rASV 223 had the strongest positive correlation ($R = 0.43$) with alkannin (**Figure 22A**). This rASV was assigned to the genus group *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, which was present in all stages in roots but only when plants were cultivated in one of the Greek soils (**Table S4** in Annex I). Root bacterial sign. rASV 194, from the same genus group, was positively correlated with acetylalkannin ($R = 0.33$) and was only present in samples grown in substrate amended with the Austrian soil. Bacterial sign. rASV 194 was also found both in soil and rhizosphere samples in the fruiting stage of all three soils and in all stages when plants were grown in the Austrian soil (**Table S4** in Annex I). Additionally, bacterial sign. rASV 1231 of the genus group *Burkholderia-Caballeronia-Paraburkholderia* positively correlated with alkannin ($R = 0.3$).

The root fungal sign. rASV 41, assigned to *Penicillium jensenii*, positively correlated with β,β -dimethylacryl-isovalerylalkannin ($R = 0.30$; **Figure 22B**). This sign. rASV was found in the fruiting stage in all root samples, with the highest abundance in the Greek B samples at the fruiting stage (**Table S5** in Annex I).

RESEARCH RESULTS-PART I-CHAPTER I

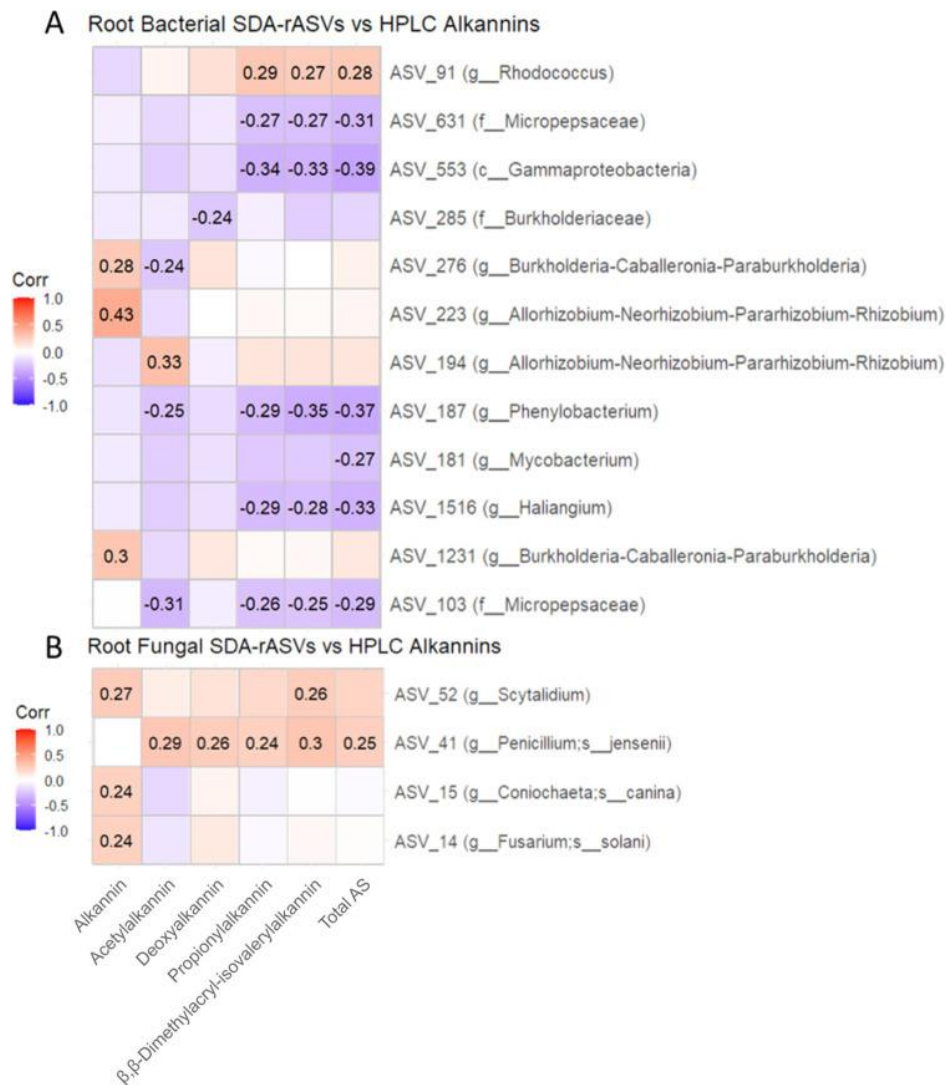


Figure 22. (A and B) Correlation plots of Spearman correlation values calculated from the abundance of alkannin concentrations from HPLC analysis and significantly differentially abundant root bacterial rASVs (A) and significantly differentially abundant root fungal taxa (B). *R* values are written in the boxes on the plots, and positive or negative attribution of the correlations are indicated by the color legend. Nonsignificant correlations ($P > 0.05$) are without *R* values on the plot.

We built cooccurrence networks from our root microbiome and UHPLC-

RESEARCH RESULTS-PART I-CHAPTER I

HRMS data for association discovery. We created two networks from all multiomics data via performing two random forest analyses based on development stages or soil types on all data separately and then calculated Spearman correlation matrices based on the significant ($P < 0.05$) features. We used a higher threshold of the correlation coefficients ($R > 0.5$) in the creation of the adjacency matrices to have transparent networks.

The network based on the soil types (**Figure 23A**) resulted in 700 total significant edges and 122 nodes. Key nodes in this network were bacterial ASV 54 (*Dyella* sp.) and bacterial ASV 194 (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*). In this network, the largest cliques consisted only of bacterial and fungal ASVs, which highlights the importance of microbial nodes and their connection in this scenario.

The developmental stage network (**Figure 23B**) contained 25,541 total significant edges and 362 nodes. The key nodes in this network were metabolites “M352T3” and “M284T3_1,” while the largest cliques consisted exclusively of metabolite nodes. M352T3 is likely senecivernine N-oxide, while the latter metabolite unfortunately eluded annotation.

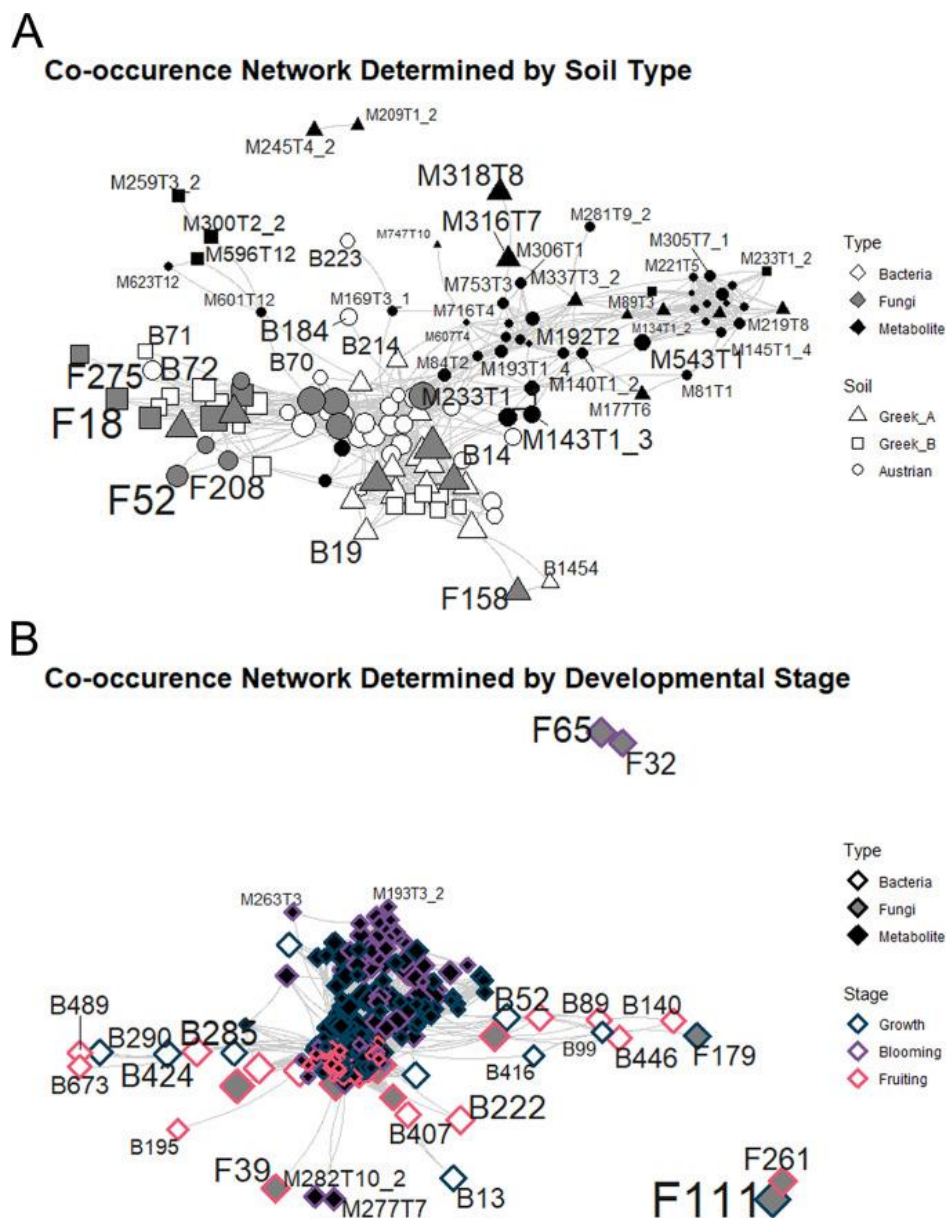


Figure 23. Cooccurrence network of metabolites and microbial ASVs. (A) Cooccurrence network based on soil type differentiation; the key nodes of the network were B54 and B194. (B) Cooccurrence network based on developmental stages; the key nodes of the network were M352T3 and M284T3_1. Nodes

RESEARCH RESULTS-PART I-CHAPTER I

starting with M are metabolites, B nodes are the bacterial rASVs, and F nodes are the fungal rASVs. Key nodes and labels are not visible on either network due to high node density in the central hubs.

Additionally, we created a filtered subnetwork based on the developmental stage random forest analysis with only the identified/annotated UHPLC-HRMS compounds (**Figure S5** in Annex I). We correlated those against the random forest-filtered root microbiome and used a threshold of $R > 0.3$ for building a more detailed, but still positively correlating, network. This subnetwork contained only 6 identified or annotated metabolites and in total contained 306 significant edges and 50 nodes. The key nodes/vertices of this network were isovalerylalkannin, which was also the node with the maximum betweenness, and bacterial ASV 294, an alphaproteobacterium from the genus *Labrys* with the highest eigenvector centrality in the network. Both of these important nodes were associated with the fruiting stage. The direct neighbors of isovalerylalkannin included positive correlations with alkannin, β,β -dimethylacryalkannin, abscisic acid, maltotetraose, and 6-methoxyflavanone. Direct fungal connections to isovalerylalkannin were ASV 39 (*Penicillium simplicissimum*, $R = 0.38$), ASV 41 (*Penicillium jensenii*, $R = 0.46$), ASV 56 (*Penicillium* sp., $R = 0.38$), ASV 85 (*Penicillium citreonigrum*, $R = 0.46$), and ASV 111 (*Saitozyma podzolica*, $R = -0.33$). The direct bacterial neighbors to isovalerylalkannin were ASV 18 (*Nocardioides* sp., $R = -0.35$), ASV 77 (*Conexibacter woesei*, $R = 0.38$), ASV 80 (*Micropepsaceae* sp., $R = -0.34$), ASV 156 (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, $R = -0.36$), ASV 259 (*Asticacaulis* sp., $R = -0.41$), ASV 271 (*Sphingomonas* sp., $R = 0.34$), ASV 285 (*Burkholderiaceae* sp., $R = -0.31$), ASV 290 (*Chryseolinea* sp., $R = -0.35$), ASV 294 (*Labrys* sp., $R = 0.54$), ASV 407 (*Novosphingobium resinovorum*, $R = -0.40$), ASV 416 (*Rhodopseudomonas* sp., $R = 0.30$), ASV 424 (*Cellvibrio* sp., $R = -0.35$), and ASV 673 (*Methylophilaceae* sp., $R = -0.31$).

Discussion

Several studies have focused on the microbiome and metabolome of medicinal plants with prominent SM content, although multidisciplinary approaches combining molecular microbiological and advanced chemical analytical methods are still rare. The focus of former studies in the topic has been restricted mainly to testing microbial isolates regarding their

RESEARCH RESULTS-PART I-CHAPTER I

effect on plant secondary metabolism (Ray et al., 2019; Rat et al., 2021; Schmidt et al., 2014). Here, we combined next-generation sequencing of root-associated microbiomes of *A. tinctoria* with a multilayered metabolome analysis.

We identified the plant developmental stage as the most important driver of the root metabolome change, whereas the microbiome was, to a major extent, influenced by the soil with its specific microbiome. The influence of other soil parameters can be mostly ruled out, as plants were grown in a sterilized soil substrate, which was amended with a comparably small amount of nonsterile soils obtained from different geographic regions. In this study, the soils had very similar pH values; however, soil pH may strongly influence plant microbiomes and metabolism. In this study, metabolomes of plant roots of the same developmental stage across different soils were highly similar, pinpointing to plant maturity as the primary driver of SM production. However, we found correlations and cooccurrence network connections between bacterial or fungal taxa with plant metabolites. This indicates the possibility of microbial induction of specific pathways leading to the synthesis of specific metabolites. Alternatively, these results could also pinpoint to the selection of specific microorganisms enriched by a specific metabolite. There are reports of distinct plant metabolic contents in different developmental stages. Chaparro et al. (2013) reported plant developmental stage-dependent root exudation in *Arabidopsis thaliana*, which positively correlated with metabolism-specific rhizosphere microbial functions. Furthermore, soil and root microbial shifts were directly connected to metabolome-level changes in *Populus tremula* × *alba* (Mangeot-Peter et al., 2020). Here, we also found compounds in the roots known to be produced exclusively by microorganisms, like the mycotoxin citrinin (Silva et al., 2020).

In line with a recent publication characterizing bacteria isolated from *A. tinctoria* roots (Rat et al., 2021), we found some overlap between the culturable and unculturable *A. tinctoria* root microbiome. Rat et al. (2021) isolated *Gammaproteobacteria* and *Alphaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* as the most abundantly occurring root endophytes, and inoculation of a *Allorhizobium* strain led to significantly enhanced A/S concentrations in *A. tinctoria* hairy root cultures. This is in accordance with our results, as we identified bacterial rASVs (ASV 223 and ASV 194) of

RESEARCH RESULTS-PART I-CHAPTER I

the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group, which positively correlated with higher concentrations of acetylalkannin. The rASV 194 was also found in rhizosphere and bulk soil samples, suggesting that bacteria belonging to this ASV derive from the soil microbiome.

We found rASVs assigned to the genus group *Burkholderia-Caballeronia-Paraburkholderia* in positive correlation with alkannin (HPLC, **Figure 22**) or in a negative correlation toward isovalerylalkannin (UHPLC-HRMS cooccurrence network, Fig. S5 in the supplemental material) of *A. tinctoria*. The genus *Burkholderia* has been regularly described as a highly diverse and environmentally adaptable plant-associated group (Company et al., 2008) prevalent in the rhizosphere or plant endosphere and was reported to cause metabolic changes in plants (Mitter et al., 2013), for example, through their 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Onofre-Lemus et al., 2009). Herpell et al. (2020) isolated a *Paraburkholderia* sp. strain from the sweet potato phyllosphere that encoded several genes for plant hormone regulation, detoxification of xenobiotics, and aromatic compound degradation. Similarly, *Paraburkholderia phytofirmans* strain PsJN is a well-known plant colonizer that shows hormone and ACC deaminase production and is known to elicit plant responses (Esmaeel et al., 2018; Sessitsch et al., 2005). Fungal elicitors, which enhance A/S production, have been previously described for the *Boraginaceae* species *Arnebia euchroma* (Arghavani et al., 2015; Fu and Lu, 1999). In our study we found a moderate positive correlation between the root fungal rASV41 (*Penicillium jensenii*) and higher A/S levels (HPLC, **Figure 22**).

We found one of the key metabolite nodes in the developmental stage network to likely be senecivernine N-oxide, a pyrrolizidine alkaloid that was recently reported in *L. erythrorhizon*, a *Boraginaceae* species (Ko et al., 2021). Pyrrolizidine alkaloids and their N-oxides serve as plants' defense against herbivores and have been extensively studied (Avula et al., 2015; Hungerford et al., 2019) due to their toxicity and potential for contamination of foods such as honey.

In the developmental stage subnetwork with the identified/annotated metabolites, the key metabolite of the network isovalerylalkannin had direct connections of positive correlations with four *Penicillium* species.

RESEARCH RESULTS-PART I-CHAPTER I

Several species of this genus were already shown to affect plant growth and metabolism (Wakelin et al., 2007). In a study with citrus fruit, *Penicillium digitatum* infection induced the secondary and amino acid metabolism of the fruit (González-Candelas et al., 2010). The strongest positive correlation ($R=0.54$) to a direct neighbor of isovalerylalkannin in the network is a bacterial rASV from the genus *Labrys*. Plant rhizosphere isolates of this genus were previously described with indole acetic acid production and phosphate-solubilizing capabilities (Inui et al., 2012) and urea-decomposing and tryptophan-producing genes (Kim et al., 2021), which makes them potential plant growth-promoting strains. *Labrys* species were isolated several times from root nodules (Chou et al., 2007; Gerding et al., 2017; Bautista et al., 2017) but were not associated with nodulation and at present have unknown roles in the root nodule microbial community.

In accordance with previous publications (Assimopoulou and Papageorgiou, 2004a), A/S derivatives in *A. tinctoria* at different developmental stages and grown in different soils consisted of >95% of the alkannin enantiomer.

Despite the increasing literature behind the metabolic content of *A. tinctoria* roots and the biological activities of A/S (Papageorgiou et al., 2008; Guo et al., 2019; Jaradat et al., 2018), not many interactions between specific chemical constituents of the plant have previously been discovered. In our study, we performed correlations between identified/annotated metabolites and found that two of the most abundant A/S in *A. tinctoria* roots, β,β -dimethylacrylalkannin (M1) and isovalerylalkannin (M2), exhibit moderate or weak correlations with abscisic acid ($R=0.61$ and $R=0.48$, respectively). Because the two mentioned A/S derivatives represented the majority of total A/S derivatives in *A. tinctoria* roots, their connection to abscisic acid is noteworthy. The potential role of abscisic acid in enhancing A/S yield in a production setting should be analyzed in more detail in a further study. Abscisic acid was shown to inhibit the growth of cultured cells of the *Boraginaceae* species *Onosma paniculatum* B. F., while it simultaneously reduced shikonin biosynthesis (Sun et al., 2007). However, our metabolomics data suggest this is not replicated in *A. tinctoria* roots; on the contrary, an increased abscisic acid concentration correlated with increased content of the most abundant A/S

RESEARCH RESULTS-PART I-CHAPTER I

derivatives in the root system. With UHPLC-HRMS, we also annotated methyl jasmonate, which may also be produced by plant-associated microorganisms and which is known to induce metabolic changes in plants (Carlson et al., 2020; Hammerbacher et al., 2019).

Previous experiments have suggested the importance of developmental stages in identifying an ideal time point for harvesting plants for higher yield of the metabolites of interest (Li et al., 2020; Xu et al., 2014; Xu et al., 2018). This knowledge was not available in the *in vivo* A/S production of *A. tinctoria*. We discovered that the fruiting developmental stage of *A. tinctoria* was the time point with the highest total A/S content under *in vivo* greenhouse conditions. Harvesting *A. tinctoria* roots at the fruiting stage in a production setting may represent an opportunity to make use of some of the other compounds with concentration peaks at the same stage, for example, abscisic acid, maltotetraose, 6-methoxyflavanone, ostruthol, and pyrogallol (**Figure 20A**). Flavonoids, compounds which can possess anticancer, antiviral, and anti-inflammatory effects, among others (Wang et al., 2018b), are represented in the analyzed roots by 6-methoxyflavanone, a metabolite reported as anxiolytic (Akbar et al., 2017), a gamma-aminobutyric acid (GABA) allosteric modulator (Hall et al., 2014), and a bitter taste receptor inhibitor (Roland et al., 2014). Ostruthol, an anti-inflammatory coumarin described in *Peucedanum ostruthium* roots (Zwirschmayr et al., 2020), is a potent acetylcholinesterase inhibitor with potential applications in treating Alzheimer's disease (Urbain et al., 2005). Pyrogallol is a metabolite that was found to have antimicrobial properties (Lima et al., 2016). The biosynthesis of the above-mentioned metabolites would, due to their positive correlation with A/S, likely be positively affected by using certain microbes herein identified as beneficial toward A/S biosynthesis. The transition to the fruiting developmental stage also caused microbiome shifts, such as the abundance of rASVs in the root core microbiome, specifically higher abundances of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Penicillium*, and *Trichoderma* associated with plants grown in the presence of the Austrian soil microbiome, the appearance of high abundances of *Dyella*, *Myrothecium*, and *Chaetosphaeronema* in Greek A samples, and a sudden dominance of *Fusarium* species in the core of Greek B samples. The core microbiome is known as an important reservoir of microorganisms potentially influencing plant metabolism (Chen et al.,

RESEARCH RESULTS-PART I-CHAPTER I

2018b). Here, we analyzed core microbiome shifts through plant development together with metabolome shifts.

Our main hypothesis was that plant microbiota may influence plant metabolism and can enhance A/S production of *A. tinctoria* and/or that specific plant metabolites may specifically select or enrich certain microorganisms. Our findings support this hypothesis, as we successfully identified potential individual bacterial and fungal ASVs that correlated with higher A/S production. We observed core microbiome shifts, minor diversity changes in root, rhizosphere, and soil samples, and several significant correlations and network connections regarding metabolome-microbiome patterns in different stages of plant development. Such shifts have been identified to be connected to the peak of A/S biosynthesis, which is reached in the fruiting stage. This matches our hypothesis that different A/S concentration levels occur in different developmental stages, in line with a developmental stage-dependent colonization of plant microbiota.

Furthermore, we described previously unreported secondary metabolism constituents present in *A. tinctoria* roots and how their relative abundances change with plant development and also assessed how the identified and annotated compounds correlate to one another. We tested three different soils containing different microbiota for the cultivation of *A. tinctoria* resulting also in distinct root and rhizosphere microbiomes, however, not in significant changes in plant metabolite production. A comprehensive, untargeted metabolome analysis of *A. tinctoria* root extracts together with an untargeted microbiome analysis and Spearman correlations revealed microbial taxa correlating with the abundance of relevant A/S targeted.

Our findings can serve as a good basis for later multiomics studies and field experiments on *Boraginaceae* and other naphthoquinone-producing species. These taxa may be further explored for the involved mechanisms, both at the plant and microbial sides and for the development of microbe-based approaches to enhance A/S content in *A. tinctoria*. Such an enhancement would not only lead to increased production yields of A/S but also open the door toward using *A. tinctoria* roots as a starting material toward the isolation and production of metabolites positively correlated with A/S, calling for more studies on the feasibility of using the plant as a source of these compounds.

RESEARCH RESULTS-PART I-CHAPTER I

Acknowledgements

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Preface

In **Chapter I**, a positive relationship between root-associated microorganisms (bacterial and fungal taxa) and root metabolites in *A. tinctoria* was observed. In particular, the bacteria genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Labrys* as well as fungal species belonging to the genus *Penicillium* were positively correlated with the content of alkannins. This indicates the possibility of microbial induction of specific pathways leading to the synthesis of specific metabolites. These results also pinpoint the selection of specific microorganisms enriched by specific metabolites.

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts, belonging to the Glomeromycota Phylum. They provide their hosts with nutrients (especially N and P) and have been shown to modulate the synthesis of plant secondary metabolites (SMs). However, no study so far has investigated the effects of AMF strains on the growth and production of A/Sd in this plant.

In **Chapter II**, two native AMFs isolated from *A. tinctoria* plants in the wild in Greece (i.e., *R. irregularis* and *S. viscosum*) and *R. irregularis* MUCL 41833 from the GINCO were associated to *A. tinctoria* and grown for 85 days in pots under greenhouse conditions. The objectives were to determine whether (i) a standard strain of AMF can increase the production of A/Sd and whether this effect is more/less significant compared to AMF isolated from *A. tinctoria* grown in the wild and (ii) AMF can modulate the expression of genes involved in the A/Sd biosynthetic pathway. Quantification and identification of A/Sd were performed by HPLC-PDA and by HPLC-HRMS/MS, respectively. *LePGT1*, *LePGT2* and *GHQH* genes involved in the A/Sd biosynthesis were analysed through RT-qPCR.

Chapter II Arbuscular mycorrhizal fungi impact the production of alkannin/shikonin and their derivatives in *Alkanna tinctoria* Tausch. grown in semi-hydroponic and pot cultivation systems

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Abstract

Alkanna tinctoria is a medicinal plant, producing alkannin/shikonin and derivatives (A/Sd). It associates with arbuscular mycorrhizal fungi (AMF), which are known to modulate the plant secondary metabolites (SMs) biosynthesis. However, no study has compared the effects of AMF strains from international collections (i.e., GINCO) versus strains isolated from wild-growing *A. tinctoria*, on the growth and production of A/Sd in this plant. Here, three experiments were conducted. In Experiment 1, plants were associated with the GINCO strain *R. irregularis* MUCL 41833 and in Experiment 2, with two strains of GINCO (*R. irregularis* MUCL 41833 or *R. aggregatus* MUCL 49408) and two native strains (*R. irregularis* and *S. viscosum*) in a semi-hydroponic (S-H) cultivation system. Plants were harvested after 9 and 37 days in Experiment 1 and 9 days in Experiment 2. In Experiment 3, plants were associated with the two native AMF strains and with *R. irregularis* MUCL 41833 and were grown for 85 days in pots under greenhouse conditions. Quantification and identification of A/Sd were performed by HPLC-PDA and by HPLC-HRMS/MS, respectively. *LePGT1*, *LePGT2* and *GHQH* genes involved in the A/Sd biosynthesis were analysed through RT-qPCR. In Experiment 1, no significant differences were noticed in the production of A/Sd, while in Experiment 2 and 3, plants associated with native AMF *R. irregularis* had the highest content of total A/Sd expressed as shikonin equivalent. In Experiment 1, a significantly higher relative expression of both *LePGT1* and *LePGT2* was observed in plants inoculated with *R. irregularis* MUCL 41833 compared with control plants after 37 days in the S-H system. Similarly, a significantly higher relative expression of *LePGT2* in plants inoculated with *R. irregularis* MUCL 41833 was noticed after 9 days versus 37 days in the S-H cultivation system. In Experiment 2, a significant lower relative expression of *LePGT2* was observed in native AMF *R. irregularis* inoculated plants compared to the control. Overall, our study showed that the native *R. irregularis* strain increased A/Sd production in *A. tinctoria* regardless of the system used, further suggesting that the inoculation of native/best performing AMF is a promising method to improve the production of important SMs.

Keywords

Arbuscular mycorrhizal fungi, *Alkanna tinctoria*, semi-hydroponic

RESEARCH RESULTS-PART I-CHAPTER II

cultivation system, *Rhizophagus irregularis*, Alkannin/Shikonin derivatives, gene expression

Introduction

The hydroxynaphthoquinones (HNQs) of natural origin, and in particular the isohexenylnaphthazarins (IHNs), such as the chiral pair alkannin and shikonin (A/S), are lipophilic red pigments characterized by a wide spectrum of wound healing, antibacterial, anti-inflammatory, anticancer, and antithrombotic activities (Papageorgiou et al., 1999; Tappeiner et al., 2014). Monomeric A/S derivatives, mainly esters of the side chain hydroxyl group, have been found in the root periderm of several plants of the Boraginaceae family, including *Alkanna tinctoria* Tausch. This important medicinal plant, commonly known as alkanet or dyers' bugloss/alkanet, is found across southern Europe, northern Africa, and southwestern Asia, with a central distribution in the Mediterranean region (Valdés, 2011). Preparations made with *A. tinctoria* roots are commonly used in traditional medicine to treat wounds, burns, and ulcers, while extracted A/S are employed as active ingredients in different marketed pharmaceutical formulations (e.g., Histoplastin Red[®], HELIXDERM[®]) as strong wound healing medicines. Root extracts are also used as cosmetics, food additives or natural dyes for staining silk (Papageorgiou et al., 2008; Malik et al., 2016).

Today, nature remains the main source of commercial A/S and their derivatives (A/Sd), despite significant efforts to synthesize these compounds over the years (Papageorgiou et al., 2008). The only successful example of shikonin scaling up from cell cultures was with *L. erythrorhizon* Siebold & Zucc. (Boraginaceae) by Mitsui Petrochemical Industries Ltd. (now Mitsui Chemicals Inc., Tokyo, Japan) in 1984 (Yazaki, 2017). Few more attempts have been made with cell suspension cultures of *Arnebia* spp. (Boraginaceae) in stirred-tank (New Brunswick Scientific Company Inc., Edison, NJ) and in air-lift bioreactors, but no further commercial successes have been reported (Gupta et al., 2014; Malik et al., 2016). For *A. tinctoria*, cell tissues and root cultures have been used, but the level of A/S production remained insufficient for commercial use (Urbanek et al., 1996; Gerardi et al., 1998). Yet, these important compounds remain mostly extracted from plants grown in the wild, with a risk of extinction as already documented for other Boraginaceae spp. (e.g.,

RESEARCH RESULTS-PART I-CHAPTER II

L. erythrorhizon in Japan, *Alkanna sieheana* Rech. Fil. and *Alkanna orientalis* L. Boiss. in Turkey). Indeed, *A. tinctoria* is considered as very rare in some European countries, such as Slovakia, Bulgaria, and Hungary (Yazaki, 2017; Yaman et al., 2019; Ahmad et al., 2021). Moreover, this plant is characterized by a very low seed germination rate and its cultivation under conventional agriculture practices is hampered by several constraints (e.g., time needed for A/S production, high costs of harvesting, exposure to biotic/abiotic stresses) (Malik et al., 2016). For these reasons, *in vitro* and *ex vitro* cultivation protocols have been developed for the propagation and preservation of this plant (Cartabia et al., 2022). In addition, hydroponic cultivation of medicinal plants has attracted the attention of the scientific community and industrial sector, as it can effectively meet the nutrients needs of the plants under controlled/stable environmental conditions (Gontier et al., 2002; Sgherri et al., 2010). Interestingly, these cultivation systems are adapted to the growth of microorganisms such as arbuscular mycorrhizal fungi (AMF) (Ijdo et al., 2011; Garcés-Ruiz et al., 2017). These obligate plant symbionts, belonging to the Glomeromycota Phylum, provide their hosts with nutrients (especially N and P) in exchange for carbon and lipids (Smith and Read, 2008; Chen et al., 2018). A number of studies have reported their impact on the production of secondary metabolites (SMs) in leaves, roots or fruits/tubers of different crops used as food or for medicinal purposes (Zeng et al., 2013; Pandey et al., 2018; Avio et al., 2018; Kaur and Suseela, 2020; Zhao et al., 2022). For instance, artemisinin in leaves of *Artemisia annua* L. (Chaudhary et al., 2008), caffeic acid and rosmarinic acid in shoots of *Ocimum basilicum* L. (Toussaint et al., 2007), and triterpenes and phenolics in *Dioscorea* spp. (Lu et al., 2015) were increased in AMF-colonized plants. In addition, the AMF *Rhizophagus irregularis* MUCL 41833 was shown to modulate the primary and secondary metabolites (PMs and SMs, respectively) production of *Anchusa officinalis* L. (i.e., shoot and root tissues as well as exudates in the nutrient solution), another important Boraginaceae plant, under a semi-hydroponic (S-H) cultivation system (Cartabia et al., 2021). A similar study, conducted with *A. officinalis* associated with different AMF species (*R. irregularis* MUCL 41833, *R. intraradices* MUCL 49410, *R. clarus* MUCL 46238, *R. aggregatus* MUCL 49408), demonstrated that specific symbiotic associations can affect the production of bioactive compounds differently in the same host (Tsiokanos

RESEARCH RESULTS-PART I-CHAPTER II

et al., 2022).

Biosynthesis of alkannin/shikonin and their derivatives involves two pathways, the phenylpropanoid and mevalonate pathways (Song et al., 2020). Several genes, responsible for encoding enzymes involved in the biosynthesis of shikonin, have been identified in Boraginaceae plant species (Wang et al., 2014; Wu et al., 2017; Takanashi et al., 2019). The *p*-hydroxybenzoate geranyltransferase (*PGT*) gene plays a key role in coding the enzyme catalysing the coupling of *p*-hydroxybenzoic acid and geranyl diphosphate to produce *m*-geranyl-*p*-hydroxybenzoic acid, which is the first step in the formation of the basic carbon skeleton leading to A/Sd, and it has been cloned and characterized in *L. erythrorhizon* and *Arnebia euchroma* (Royle) Johnston cell cultures (Yazaki et al., 2002; Singh et al., 2010). In addition, geranylhydroquinone 3"-hydroxylase (*GHQH*), an enzyme hydroxylating the isoprenoid side chain of geranylhydroquinone (GHQ), a well-known precursor of shikonin, has also been identified in *L. erythrorhizon* cell suspension cultures (Yamamoto et al, 2000). However, to our knowledge, no study has investigated the gene regulation of the biosynthesis of A/Sd in *A. tinctoria*, in particular in the presence of AMF.

In this study, the main objectives were to determine whether (1) standard strains of AMF could enhance the production of A/Sd and whether this effect is more/less significant compared to AMF isolated from *A. tinctoria* grown in the wild; (2) AMF can modulate the expression of genes involved in the A/Sd biosynthetic pathway; (3) the production of A/Sd is affected similarly under semi-hydroponic and pot cultivation systems. To address these objectives, the quantification of A/Sd was performed through High-Performance Liquid Chromatography coupled with Photodiode Array detection (HPLC-PDA) and the related genes expression through Real-Time Quantitative Reverse Transcription PCR (RT-qPCR). Moreover, identification of the main A/Sd was performed using HPLC coupled with High-Resolution Mass Spectrometry detection (HPLC-HRMS/MS).

Material and Methods

Chemicals

All used organic solvents (i.e., n-hexane 97%, methanol, trifluoroacetic acid (TFA), and acetonitrile (ACN) (VWR INTERNATIONAL, Leuven, Belgium) were HPLC/LC-MS grade. Water was purified and

RESEARCH RESULTS-PART I-CHAPTER II

demineralized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA). Shikonin (purity > 98%) was purchased from Cayman Chemical Company (Biomol GmbH, Hamburg, Germany) and used as internal standard. Discovery® DSC-C18 Supelco Solid-Phase Extraction (SPE) cartridges of 500 mg bedweight and 3-mL tubes were purchased from Sigma-Aldrich (Taufkirchen, Germany).

For gene expression analysis (i.e., RNA extraction) all non-disposable materials (e.g., glass materials, mortars, pestles) were first treated with RNase AWAY™ Surface Decontaminant (ThermoFisher Scientific™, Belgium) and 0.1% diethylpyrocarbonate (DEPC)-treated sterile water. All the reagents were prepared with sterilized (121°C for 15 min) DEPC-treated water. Reagents used in RNA extraction are as follows: extraction buffer [100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB (w/v) and 2% PVP (w/v)], 2% β-mercaptoethanol (v/v), 5 M NaCl, 1.2 M NaCl, 0.38 M trisodium citrate dihydrate, chloroform, isopropanol and 70% ethanol (stored at -20°C).

Biological Materials

Rhizophagus irregularis (Błaszk, Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare']) MUCL 41833 and *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker MUCL 49408 were supplied by the *Glomeromycota in vitro* collection (GINCO)³. Both AMF were mass-produced on *Zea mays* L. cv. ES Ballade (Euralis, Lescar, France) in 10 L plastic boxes containing sterilized (121°C for 15 min) lava (DCM, Grobbendonk, Belgium). Two other AMF (*R. irregularis* and *Septoglomus viscosum*) were isolated from *A. tinctoria* growing in a suburban pine forest, altitude 50 m of Northern Greece (Evaggelistria, Seih Sou, Thessaloniki, special collection permit obtained by the Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization Demeter - IPBGR, HAO Demeter). They were first trapped on *Plantago lanceolata* L. (Ecosem, Belgium) and *Medicago truncatula* Gaertn. (SARDI, Australia) to stimulate the production of numerous spores and then grown as monospores on *P. lanceolata* at the GINCO premises to obtain single species cultures (see Isolation and purification of AMF in Annex II, **Figure S6-9** and **Table S6**). Two identified AMF species (*R.*

³ <http://www.mycorrhiza.be/ginco-bel/>

RESEARCH RESULTS-PART I-CHAPTER II

irregularis and *S. viscosum*) were finally mass-produced on *Zea mays* L. in 5 L plastic boxes containing sterilized lava substrate as above.

The four AMF strains were grown under greenhouse conditions at 25°C/18°C (day/night), a relative humidity (RH) of 38%, a photoperiod of 16 h day⁻¹ and a photosynthetic photon flux (PPF) of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Alkanna tinctoria unrooted *in vitro* explants (International Plant Exchange Network -IPEN- accession number GR-1-BBGK-17,5975) were provided by IPBGR, HAO Demeter (Thessaloniki, Greece). The plants were proliferated on Murashige-Skoog (MS) basal medium enriched with plant growth regulators (PGRs) and rooted on Root Culture 1 (RC1) modified medium free of ammonium nitrate (Cartabia et al., 2022). The rooted *A. tinctoria* plants were further acclimatized following different steps resulting in an optimal plant survival rate (see *Alkanna tinctoria* acclimatization protocol in Annex II, **Figure S10**). The acclimatization protocol was applied for all the experiments in order to have plants of the same age and established under identical growth conditions (first acclimatization from 2nd November 2020 to 7th December 2020 and second from 1st February 2021 to 10th March 2021).

Colonization of *A. tinctoria*

After the acclimatization phase, the plants were carefully removed from the substrate, gently washed under a stream of demineralized water, and transplanted into 1 L pots (11x11x12 cm) containing a sterilized (121°C for 15 min) substrate mixture (2 peatmoss/2 compost/1 perlite/1 quartz 0.4-0.8 mm/1 quartz 1-2 mm) (see Annex II **Figure S10**). Total fresh weight (TFW) of the plants was evaluated before AMF inoculation.

In each experiment, plants were associated [i.e., the mycorrhizal (M) treatments] or not [i.e., the non-mycorrhizal (NM) treatments] with AMF. For the M plants, the substrate was half mixed with the AMF-inoculum substrate above, whereas for the NM treatments, the substrate was half mixed with the same AMF-inoculum substrate above but sterilized (121°C for 15 min). The plants were grown in the substrates for two months under greenhouse conditions set at 24°C/22°C (day/night), RH of 50%, photoperiod of 16 h day⁻¹ and LED light (lumigrow) intensity of about 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

RESEARCH RESULTS-PART I-CHAPTER II

Experiment 1: A/Sd production and genes expression of *A. tinctoria* associated with *R. irregularis* before and after 9 and 37 days in an S-H cultivation system

In Experiment 1, the objective was to evaluate the production of A/Sd and the expression of genes involved in the biosynthetic pathways of A/Sd in *A. tinctoria* roots colonized (later abbreviated for fluency as M^{irr}) or not (NM) with *R. irregularis* MUCL 41833, before (T0) and after 9 (T1) and 37 days (T2) of growth in the S-H cultivation system.

Two-month-old M^{irr} and NM plants were gently removed from the pots above and their roots cleaned with demineralized water to eliminate substrate debris (see Annex II **Figure S10**). Six plants belonging to the M^{irr} and NM treatments were randomly harvested, and AMF root colonization assessed before (T0) transfer to the S-H cultivation system (paragraph 2.7). Fourteen additional plants from the M^{irr} and NM treatments were transferred to the S-H cultivation system as detailed in Cartabia et al. (2021). The plants were placed in 500 mL plastic bottles (VWR INTERNATIONAL, Leuven, Belgium), cut at the base and covered with a 100 µm size pore nylon mesh (Prosep B.V.B.A., Zaventem, Belgium) glued on the top. The bottles (called containers thereafter) were used bottom-up, filled with 32 g of perlite (KNAUF GMBH, Dortmund, Germany), covered with a superficial layer of black lava rock (1-3 mm) and wrapped in aluminium foil to avoid algae development and inhibition of shikonin production (as shikonin is sensitive to light) (Yazaki, 2017). The containers were transferred randomly in holes made in flex foam supports and were maintained in the greenhouse set at the same conditions as described above. A 90% P-impoverished modified Hoagland solution (see Garcés-Ruiz et al., 2017) lacking ammonium nitrate (NH₄NO₃) [as NH₄⁺ is an inhibitor of shikonin production (Yazaki, 2017)] was used at two different concentrations as detailed in Cartabia et al. (2021). After 7 days of acclimatization, the circulatory system was started. Each container was connected to a 1 L glass bottle covered with aluminium foil containing Hoagland^{dil100X} solution. A 4.6 mm diameter black supply pipe (GARDENA®, Micro-Drip System, Ulm, Germany) connected a dropper cap fixed on the bottom of the plant container with the glass bottle, and another black pipe of the same diameter connected the glass bottle to the upper part of the plant container via a multichannel peristaltic pump

RESEARCH RESULTS-PART I-CHAPTER II

(Gilson's Minipuls Evolution, France). Once the circulation started, the nutrient solution was pumped from the glass bottle into the plant container and the liquid flowed back by gravity into the bottle. Before starting with the circulation, initial flushing was performed as described in Cartabia et al. (2021). Then, four successive circulations were performed at a velocity of 7.5 mL min^{-1} for different durations: 42 h at day 9 (T1), 8 h at day 17, 24 and 31, and finally 42 h at day 37 (T2).

Experiment 2: A/Sd production and genes expression of *A. tinctoria* associated with different AMF strains after 9 days in an S-H cultivation system

In Experiment 2, the objective was to evaluate the production of A/Sd and the expression of genes involved in the biosynthetic pathways of A/Sd in *A. tinctoria* roots colonized (later abbreviated for fluency as M^{irr} , M^{aggreg} , M^{Rhiz} , M^{Sept}) or not (NM) with *R. irregularis* MUCL 41833, *R. aggregatus* MUCL 49408, the native *R. irregularis* and *S. viscosum* and grown for 9 days in the S-H cultivation system. The same procedure as in Experiment 1 was applied. Briefly, after cleaning the roots system, two-month-old plants (6 per treatment) were transferred in the S-H cultivation system, and the containers were randomly placed in the holes made in the flex foam supports. Then, after the acclimatization period of 7 days, a regular circulation was initiated and maintained at 7.5 mL min^{-1} for 42 h.

Experiment 3: A/Sd production and genes expression of *A. tinctoria* associated with different AMF strains in an 85 days pot-experiment in the greenhouse

In Experiment 3, the objective was to evaluate the production of A/Sd and the expression of genes involved in the biosynthetic pathways of A/Sd in *A. tinctoria* roots colonized (M^{irr} , M^{Rhiz} , and M^{Sept}) or not (NM) with *R. irregularis* MUCL 41833, the native *R. irregularis* and *S. viscosum* and growing for 85 days in pots under greenhouse conditions. In this Experiment, *R. aggregatus* MUCL 49408 was not considered due to inoculum limitation. The plants were gently removed from the 1 L pots above and transplanted in 3 L pots (3x18.5x17 cm) containing a sterilized (2x 121°C for 15 min) substrate mixture (2 peatmoss/2 compost/1 perlite/1 quartz 0.4-0.8 mm/1 quartz 1-2 mm). They received 200 mL Hoagland^{dil200X} solution and were randomly moved every week until

RESEARCH RESULTS-PART I-CHAPTER II

harvest, 45 days later. The plants were grown under the same conditions as for the S-H cultivation system in Experiments 1 and 2.

Plants biomass and AMF root colonization

For the three experiments, plants were harvested and biomass as well as AMF root colonization evaluated. In Experiment 1, plants were harvested before transfer to the S-H cultivation system (T0) and after 9 (T1) and 37 days (T2) corresponding to 57 and 94 days after AMF inoculation of the plants, respectively. In Experiment 2, they were harvested after 9 days (i.e., 79 days after AMF inoculation of the plants), and in Experiment 3, 85 days after AMF inoculation of the plants.

Whatever the experiment, shoots were separated from roots (cleaned under demineralized water and gently dried). Shoot fresh weight (SFW) and roots fresh weight (RFW) were then measured. Root colonization was further evaluated by McGonigle et al. (1990). Roots (an approximate of 2 g fresh material) were cut into circa 1 cm length pieces and placed in 50-mL Falcon tubes. Twenty-five mL of KOH 10% was added to the roots before incubation at 70°C for 40 min. The KOH solution was then removed and replaced with H₂O₂ 3.5% before incubation at 70°C for 5 min. The roots were then rinsed with demineralized water and HCl 1% was added for 1 min at room temperature. After discarding the solution, the roots were stained with ink 2% (Parker Blue Ink, United States) in HCl 1% (Walker, 2005) by placing the tubes at 70°C for 30 min. The roots were finally rinsed with demineralized water and kept in lactoglycerol (lactic acid/glycerol/distilled water, 1:1:1, v/v) until observation. For colonization assessment, the root fragments were placed on microscope slides and covered with 40×22 mm coverslips before observation under a bright field light microscope (Olympus BH2-RFCA, Japan) at ×10 magnification. Around 200 root intersections were observed for each plant in Experiment 1 and 2 and around 300 intersections for the plants in Experiment 3. The total colonization percentage (TC%) of roots (e.g., hyphae, arbuscules, and vesicles/spores), and arbuscules colonization percentage (AC%) was further calculated (McGonigle et al., 1990).

The remaining root system of each replicate was divided in two parts of around 5 g each for metabolites and gene expression analysis. Regarding the analysis of metabolites, roots were freeze-dried during 72 h and kept at

RESEARCH RESULTS-PART I-CHAPTER II

-80°C before proceeding with A/Sd extraction. For analysis of gene expression, roots were transferred within 5-10 min after sampling into liquid nitrogen and kept at -80°C before proceeding with the RNA extraction.

Quantitative and qualitative analysis of A/Sd in *A. tinctoria* roots

Standards stock solutions, calibration curves and validation parameters

The standard stock solution of shikonin was serially diluted in HPLC-grade methanol to obtain a range of concentrations from 0.05 to 0.8 mg mL⁻¹ (i.e., stock solution of 1 mg mL⁻¹ of shikonin was prepared and diluted in HPLC-grade methanol to obtain the calibration solutions of 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mg mL⁻¹). The calibration curve was made by plotting the average peak areas of three independent experiments vs the concentration of each analyte. The method was validated with three independent series of experiments based on total error and tolerance intervals (Hubert et al., 2003). Specificity was evaluated by analysis of an extract sample by HPLC-HRMS and comparison of MS¹ signals of blank, shikonin standard, and *A. tinctoria* extract at 0.8 µg mL⁻¹, respectively. Linear regression equation, response function, linearity (R²), precision, trueness, accuracy, limit of detection (LOD), limit of quantification (LOQ), and stability were provided (see HPLC quantification: methodology validation, **Figures S11-12** and **Table S7** in Annex II).

Samples Preparation

For the three experiments, primary and secondary roots of *A. tinctoria* plants were freeze-dried separately and reduced into powder using liquid nitrogen, mortar and pestle. Fifty mg of each root type was then mixed in glass test tube (labbox 10 mL neutral glass, TU04-160-100) closed with a plastic cap. Then, the total 100 mg of root material was subjected to a 20-min ultrasound-assisted extraction (two cycles), using *n*-hexane 97% (2 mL) at room temperature. The samples were finally centrifuged at 1500 rpm for 10 min at 4°C and the supernatants of each cycle were combined and evaporated at room temperature. The extraction protocol was adapted from Bossard et al. (2022).

Prior to analysis, the plant extracts and nutrient solution residues were weighed and solubilised in methanol and were filtered through a 45-µm PTFE membrane (WhatmanTM, Maidstone, UK). Each sample was

RESEARCH RESULTS-PART I-CHAPTER II

adjusted to the final concentration of 2 mg mL⁻¹, using methanol LCMS-grade.

HPLC-PDA and HPLC-HRMS/MS analysis

For the three experiments, the quantification of HNQ enantiomers (A/Sd) in their free form (not linked to sugars) was performed following the protocol of Bossard et al. (2022). For HPLC-PDA analysis, the system consists in an Accela HPLC system (Thermo Fischer ScientificTM, Bremen, Germany) coupled with a photodiode array (PDA) detector, an autosampler equipped with a conventional sample tray compartment with its cooler (set at 4°C), an injection system with a sample loop of 20 µL, and a quaternary pump, all piloted by ChromQuest software. The column used was an Alltech ALLTIME C8 250x4.6 mm, packed with 5 µm particles. Twenty µL of sample was injected in full loop injection mode by the autosampler. The column was eluted at constant flow rate of 1 mL min⁻¹ using a binary solvent system: solvent A, MilliQ water 0.1% TFA and solvent B, ACN, isocratic mode (25% A: 75% B). Quantification analyses were conducted at a wavelength of 510 nm corresponding to the maximum absorption of A/Sd and their content was calculated according to the shikonin standard curve. The total shikonin equivalent production was calculated as the sum of peaks corresponding to A/Sd free forms (i.e., by integration of the different peaks identified as A/Sd-type compounds by their UV spectra) contained in the root powder, reported as mg g⁻¹ root powder using the same shikonin standard curve (so not corresponding to an absolute quantification of each compound). Peaks were identified by comparing their retention times (RT) and UV spectra with the standard chromatogram of shikonin. The chromatographic profiles are in agreement with the chemical profile reported by Bossard et al. (2022), for A/Sd obtained from *A. tinctoria* roots. In total, 18 M and 18 NM root samples (6 NM and 6 M^{irr} plants for each time harvest-T0, T1 and T2) were considered in Experiment 1, 30 root samples (6 M^{irr}, 6 M^{agg}, 6 M^{Rhiz}, 6 M^{Sept}, and 6 NM) in Experiment 2, and 24 root samples (6 M^{irr}, 6 M^{Rhiz}, 6 M^{Sept}, and 6 NM) in Experiment 3. All samples were analysed in duplicates.

The HPLC-HRMS/MS analysis was performed at the end of Experiments 2 and 3 on an HPLC-PDA-HRMS system consisting of an Accela pump and PDA detector (ThermoFisher ScientificTM, Bremen, Germany) connected with a LTQ orbitrap XL mass spectrometer (ThermoFisher

RESEARCH RESULTS-PART I-CHAPTER II

ScientificTM, Bremen, Germany). The instrument was controlled using a ThermoFisher ScientificTM Xcalibur X software. The LC separation was done as reported above except for the solvent A: MilliQ water 0.1% formic acid (FA). The chromatograms were recorded between 200 and 600 nm. HRMS analyses were realised in ESI positive and negative modes with the following inlet conditions for the positive mode: capillary temperature 250°C; sheath gas flow 10 a.u.; auxiliary gas flow 5 a.u. and sweep gas flow 5 a.u.; ionization spray voltage 3 kV; capillary voltage of 15 V; tube lens voltage of 90 V. For negative mode, the only differences from the positive mode were the capillary voltage (-10 V) and tube lens voltage (-125 V). The data-dependent MS/MS events were performed on the three most intense ions detected in full scans MS. Peaks were tentatively identified by comparing their HRMS and MS/MS spectra with the literature data of A/Sd.

MS data treatment, organization and dereplication

All HRMS run data (.RAW) files were treated using MZmine software suite version 2.5.3 (Pluskal et al., 2010). For mass detection at MS¹ level, the noise level was set to 1.5×10^4 for positive mode and to 8.5×10^3 for negative mode. For MS² detection, the noise level was set to 1. The ADAP chromatogram builder was used and set to a minimum group size of scans of 4, a minimum group intensity of 1.0×10^4 , a minimum highest intensity of 1.0×10^4 , and m/z tolerance of 8 ppm. The ADAP algorithm (wavelets) was used for chromatogram deconvolution. The intensity window signal to noise (S/N) was used as a S/N estimator with S/N ratio set at 10, a minimum feature height of 1.0×10^4 , a coefficient area threshold of 25, a peak duration ranging from 0.02 to 0.8 min, and a RT wavelet range from 0.02 to 0.2 min. Isotopes were detected using the isotope peak grouper with a m/z tolerance of 8 ppm, a RT tolerance of 0.02 min (absolute), a maximum charge set at 1, and the representative isotope used was the most intense. Then, the aligned list peak was gap-filled with RT range of 0.05 min and m/z tolerance of 8 ppm. The resulting list was filtered using the peak list rows filter option to remove all the duplicates and all the features without MS² spectrum associated.

A molecular network was constructed from the .mgf file exported from MZmine, using the online workflow on the GNPS website (Wang et al., 2016). The precursor ion mass tolerance was set to 0.02 Da with a MS/MS

RESEARCH RESULTS-PART I-CHAPTER II

fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine of 0.7 and more than 3 matched peaks. The spectra in the network were then searched against GNPS's spectral libraries filtered under the same conditions as before. Putative identification was carried out comparing available MS/MS fragmentation patterns from the literature. Data visualization was achieved using Cytoscape 3.8.0 (Shannon et al., 2003). Peak area data from the .csv file obtained from MZmine was added to the network. Size nodes were set proportionally to the total area of each peak detected in both analysed extracts.

Analysis of A/Sd target genes expression in *A. tinctoria* roots

Total RNA extraction

Frozen roots of each replicate were ground in liquid nitrogen (-196°C) with a pestle and mortar. Total RNA extraction was done on 0.2 g root material using the protocol from Xu et al. (2010) slightly modified. Main differences were in the extraction buffer composition and in the last steps of the protocol, where the pellet was washed with 70% ethanol and the total RNA was dissolved in 55 µL DEPC-treated water. Further, the total RNA was treated with TURBO DNA-free™ Kit (Thermo Fisher Scientific™, Belgium), according to the manufacturer protocol. The RNA of all samples was loaded on 1.5% agarose gel, electrophoresed to separate RNA, stained with GelRed® (Biotium, United States), and visualised under UV light to assess the integrity of ribosomal bands. Moreover, concentration of each RNA sample was measured using NanoDrop® -ND 1000 UV-vis Spectrophotometer (NanoDrop Technologies, United States) and RNA purity estimated from the A260/A280 and A260/A230 absorbance ratios. Finally, a 1 µg aliquot of total RNA was used for the first-strand cDNA synthesis according to the protocol of the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Montreal, QC, Canada). For each RNA sample, a reaction without Transcriptor High Fidelity Reverse Transcriptase (Hifi RT) enzyme was performed as a control for contamination by genomic DNA.

Five biological replicates (i.e., means of relative genes expression) for M and NM treatments at each time harvest (T0, T1, and T2) were considered in Experiment 1, six biological replicates for treatments M^{irr}, M^{aggreg}, M^{Rhiz}, M^{Sept}, and NM in Experiment 2, and six biological replicates for treatments M^{irr}, M^{Rhiz}, and NM and five for M^{Sept} in Experiment 3.

RESEARCH RESULTS-PART I-CHAPTER II

For all the experiments, tissue samples for A/Sd and molecular determinations were simultaneously collected at the same period of the day, between 9:00 and 11:00 a.m.

Real-Time quantitative PCR (RT-qPCR)

For RT-qPCR, the expression of three target genes: *LePGT1*, *LePGT2* and *GHQH* involved in the shikonin (A/Sd) biosynthesis was analysed (see Analysis of A/Sd target genes expression in *A. tinctoria* roots, Supplementary materials, Table S8). Since no published genomic resources are available for *A. tinctoria*, a previously published primer for amplification of *LePGT1* in *Onosma paniculatum* Bur. et Franch was used, while for *PGT2* and *GHQH* new primer pairs were designed. Sequences of each gene (*LePGT2* or *GHQH*) from two phylogenetically distant Boraginaceae species (*L. erythrorhizon* and *A. euchroma*) were obtained from NCBI, aligned, and primers generated from conserved regions (see Alignments used to design primers, Supplementary materials). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as internal reference control. RT-qPCR was performed using a LightCycler® FastStart Essential DNA Green Master (Roche) in 10 µL volume of reaction formed as follows: 5 µL Master mix (or Mix SYBR 2x), 0.5 µL of each primer from the pair (10 µM), and 4 µL cDNA (dil 5x). The reaction was carried out in a Roche LightCycler® 96 System using the following parameters: 10 min at 95°C, followed by 40 (for housekeeping gene) and 50 (for target genes) cycles of denaturation (95°C, 10 s)/annealing (60°C housekeeping gene/56°C target genes, 15 s)/extension (72°C, 10 s), and finalized by a standard melting curve analysis (95°C). Reactions were performed in three replicates. Normalization was achieved for each experiment separately using the reference gene (i.e., *GAPDH*) and the “Pfaffl” method (Pfaffl, 2001).

Statistical Analysis

For all the experiments, a one-way ANOVA followed by HSD Tukey post-hoc test ($p < 0.05$) was applied to discriminate between means for plant growth parameters (i.e., SFW, RFW, and TFW), AMF colonization (i.e., TC% and AC%), A/Sd content, and relative genes expression at different time points (Experiment 1) or different AMF treatments (Experiments 2 and 3). Moreover, in Experiment 1, differences between M and NM treatments were highlighted by pairwise comparison with Bonferroni

RESEARCH RESULTS-PART I-CHAPTER II

correction ($p < 0.05$) at each harvesting time (T0, T1, and T2). For all parameters, normal distribution of residuals variance and normality was checked before analyses. Non-normal data were normalized by log10 transformation before analysis. Data analyses were performed by IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA).

Results

Plants biomass and root colonization by AMF

In Experiment 1, no significant differences between harvesting times (before plants transfer to the containers-T0, and after 9-T1 and 37-T2 days of growth in the S-H cultivation system) were noticed for SFW, RFW, and TFW of plants in the Mirr treatment and for SFW of plants in the NM treatment. Conversely, a significantly greater RFW was noticed at T1 and T2 as compared to T0, and greater TFW at T2 compared to T0 for plants in the NM treatment. Irrespective of the harvest time, no significant difference was reported between the SFW of plants in the Mirr and NM treatments, while at T2, the RFW and TFW of the plants in the NM treatment was significantly greater than that in the Mirr treatment. In the Mirr treatment, a significant decrease in TC% was observed at T2 compared to T0 and T1 and in AC% at T1 and T2 compared to T0 (**Table 12**). No root colonization was observed in plants of the NM treatment.

RESEARCH RESULTS-PART I-CHAPTER II

Table 12. Shoot, root and total fresh weights (SFW, RFW, and TFW, respectively), total colonization percentage (TC%), arbuscules colonization percentage (AC%), and total A/Sd expressed as shikonin equivalent content of *A. tinctoria* plants inoculated (M^{irr}) or not (NM) with *R. irregularis* MUCL 41833 before (T0) and after 9 (T1) and 37 (T2) days in the S-H cultivation system (Experiment 1), and of *A. tinctoria* plants inoculated (M^{irr} , M^{aggreg} , M^{Rhiz} , M^{Sept}) or not (NM) with different AMF strains (two from GINCO - *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408, and two isolated from wild *A. tinctoria* – *R. irregularis* and *S. viscosum*) after 9 days in the S-H cultivation system (Experiment 2).

Experiment 1

Treatments	Time	SFW (g)	RFW (g)	TFW (g)	TC%	AC%	Total shikonin equivalent mg g ⁻¹ root powder
M^{irr}	T0	14 ± 2.3	10 ± 1.6	24 ± 2.4	61 ± 7 a	34 ± 4 a	3.3 ± 1.5
	T1	13 ± 2.7	10.8 ± 2.2	23.7 ± 3.6	53 ± 7 a	17 ± 5 b	1.9 ± 1.5
	T2	13 ± 1.2	12.4 ± 1.1*	25.7 ± 2*	29 ± 4 b	12 ± 3 b	2.1 ± 1
NM	T0	13.5 ± 2	7.9 ± 2.3 b	21.4 ± 3.8 b	-	-	2.5 ± 1
	T1	14.2 ± 2	11 ± 2.4 a	25.2 ± 3.7 ab	-	-	2.6 ± 1.1
	T2	13.6 ± 0.6	14 ± 1 a*	28 ± 1 a*	-	-	2.3 ± 0.7

RESEARCH RESULTS-PART I-CHAPTER II

Experiment 2

Treatments	SFW (g)	RFW (g)	TFW (g)	TC%	AC%	Total shikonin equivalent mg g ⁻¹ root powder
M ^{Rhiz}	9.8 ± 1	11.3 ± 1	21 ± 1.8	44 ± 7	21 ± 5 a	8.7 ± 1.3 a
M ^{Sept}	11.1 ± 2	13.1 ± 2.6	24.2 ± 1	39 ± 9	17 ± 7 ab	6.8 ± 2.2 ab
M ^{irr}	11.2 ± 1	12.8 ± 1.7	24.2 ± 2.4	42 ± 6	16 ± 2 ab	4.3 ± 2.1 b
M ^{aggreg}	10.3 ± 5	11 ± 3.3	20 ± 5	43 ± 9	12 ± 4 b	5 ± 0.6 b
NM	10.3 ± 2	11 ± 2.1	21.5 ± 3.3	-	-	4.9 ± 1.9 b

The parameters measured are expressed as mean ± standard deviation (SD) of five replicates per treatment (M^{irr} and NM) and harvesting time (T0, T1, and T2) in Experiment 1, and 6 replicates per treatment (M^{irr}, M^{aggreg}, M^{Rhiz}, M^{Sept} and NM) in Experiment 2. Means followed by different lowercase letters within the same column are significantly different according to HSD Tukey post-hoc test ($p < 0.05$) (Experiment 1 and 2). Means followed by * within the same column are significantly different according to pairwise comparison with Bonferroni correction ($p < 0.05$) (Experiment 1).

RESEARCH RESULTS-PART I-CHAPTER II

In Experiment 2, no significant differences were observed in SFW, RFW and TFW and in TC% between the different treatments (Mirr, Maggreg, MRhiz, MSept, and NM) after 9 days of growth of the plants in the S-H cultivation system. Conversely, a significant higher AC% was observed for the plants in the MRhiz treatment compared to those in the Maggreg treatment, while intermediate values were noticed in the plants of the MSept and Mirr treatments (**Table 12**). No root colonization was observed in plants of the NM treatment.

In Experiment 3, no significant differences were observed in SFW and RFW of plants between the different treatments (Mirr, MRhiz, MSept, and NM) after 85 days of growth in pots. Similarly, no significant differences were observed for TC% between the different treatments, while a significantly higher AC% was observed for the plants in the MSept treatment compared to those of the Mirr treatment. Plants in the MRhiz treatment had intermediate value (**Table 13**). No root colonization was observed in plants of the NM treatment.

RESEARCH RESULTS-PART I-CHAPTER II

Table 13. Shoot and root fresh weights (SFW, RFW, respectively), total colonization percentage (TC%), arbuscules colonization percentage (AC%), shikonin and total A/Sd expressed as shikonin equivalent of *A. tinctoria* inoculated (M^{irr} , M^{Rhiz} , M^{Sept}) or not (NM) with different AMF strains (one from GINCO - *R. irregularis* MUCL 41833, and two isolated from wild *A. tinctoria* – *R. irregularis* and *S. viscosum*) and growing for 85 days in pots under greenhouse conditions.

Treatments	SFW (g)	RFW (g)	TC%	AC%	mg shikonin g ⁻¹ root powder	Total shikonin equivalent mg g ⁻¹ root powder
M^{irr}	15.8 ± 2	13.3 ± 2.6	25 ± 6.6	4 ± 2 b	0.04 ± 0.01 b	7.2 ± 1 b
M^{Rhiz}	17.7 ± 0.6	14 ± 2.1	23 ± 16	8 ± 10 ab	0.12 ± 0.07 a	9.6 ± 0.7 a
M^{Sept}	16.3 ± 2.3	12.8 ± 1.7	25 ± 13	22 ± 11 a	0.06 ± 0.02 b	7.8 ± 1 b
NM	16.1 ± 1.3	14.4 ± 0.6	-	-	0.04 ± 0.01 b	8 ± 1 b

The parameters measured are expressed as mean ± standard deviation (SD) of 6 replicates per treatment (M^{irr} , M^{Rhiz} , M^{Sept} and NM). Means followed by different lowercase letters within the same column are significantly different according to HSD Tukey post-hoc test ($p < 0.05$).

RESEARCH RESULTS-PART I-CHAPTER II

Alkannin/shikonin and their derivatives content

In Experiment 1, whatever the harvesting time (T0, T1, or T2) or treatment (Mirr or NM) no significant differences in content of A/Sd were noticed (**Table 12**).

In Experiment 2, a significant higher content of total A/Sd expressed as shikonin equivalent was observed in the plants of the MRhiz treatment compared to those in the Mirr, Magreeg, and NM treatments, while an intermediate value was observed for the plants in the MSept treatment (**Table 12**).

In Experiment 3, a significantly higher content of shikonin, whose values were nevertheless below the LOQ (Supplementary Table 2), and in total A/Sd expressed as shikonin equivalent, was reported in the roots of the plants in the MRhiz treatment compared to those in the Mirr and NM treatments, while an intermediate value was observed for the plants in the MSept treatment (**Table 13**).

Alkannin/shikonin and their derivatives target genes expression

In Experiment 1, whatever the time of evaluation (T0, T1, or T2) or treatment (Mirr or NM) no significant differences were noticed in gene expression of *GHQH*. Similarly, no significant differences between harvesting times were observed in expression of *LePGT1* gene of the plants in the Mirr treatment and expression of *LePGT1* and *LePGT2* genes of the plants in the NM treatment. Conversely, a significantly higher expression of *LePGT2* gene was observed at T2 compared to T1 for the plants in the Mirr treatment, while an intermediate gene expression was noticed at T0. A significant higher relative expression of both *LePGT1* and *LePGT2* genes was observed in the plants of the Mirr treatment compared to those in the NM treatment at T2 (**Figure 24A**).

RESEARCH RESULTS-PART I-CHAPTER II

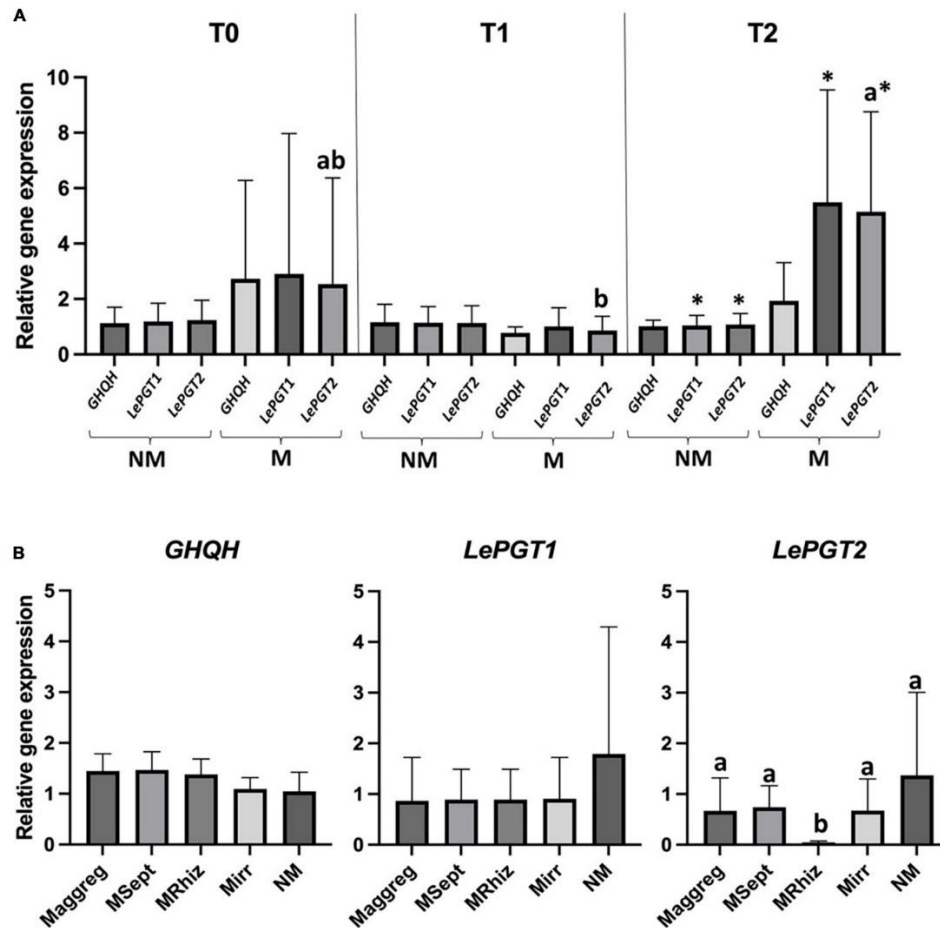


Figure 24. (A) Graphical representation of RT-qPCR relative genes expression analysis of *GHQH*, *LePGT1*, and *LePGT2* in *A. tinctoria* roots inoculated (M^{irr}) or not (NM) with *R. irregularis* MUCL 41833 before (T0) and after 9 (T1) and 37 (T2) days in the S-H cultivation system (Experiment 1); (B) graphical representation of RT-qPCR relative genes expression analysis of *GHQH*, *LePGT1*, and *LePGT2* in *A. tinctoria* roots inoculated (M^{irr} , M^{aggreg} , M^{Rhiz} , and M^{Sept}) or not (NM) with different AMF strains (two from GINCO – *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408, and two isolated from wild *A. tinctoria* – *R. irregularis* and *S. viscosum*) after 9 days in the S-H cultivation system (Experiment 2). Means followed by different lowercase letters within the same column are significantly different according to HSD Tukey *post-hoc* test ($p < 0.05$). Means followed by asterisk within the same column are significantly

RESEARCH RESULTS-PART I-CHAPTER II

different according to pairwise comparison with Bonferroni correction ($p < 0.05$).

In Experiment 2, whatever the treatment (Mirr, Maggreg, MRhiz, MSept, and NM), no significant differences were observed in expression of *GHQH* and *LePGT1* genes. A significant lower relative expression of *LePGT2* was observed in the plants of the MRhiz treatment compared with the other treatments that did not differ among them (**Figure 24B**).

In Experiment 3, *LePGT2* target gene was not considered due to contamination by the primer dimer. Whatever the treatment (Mirr, MRhiz, MSept, and NM), no significant differences were observed in expression of *GHQH* and *LePGT1* genes (data not presented).

HPLC-HRMS/MS analysis

To better recognize the metabolites produced in *A. tinctoria* roots, the main chemical compounds were tentatively identified in Experiments 2 and 3 by performing a dereplication strategy based on HPLC-HRMS/MS analysis (**Figure 25** and **Tables 14, 15**) and molecular network representation (**Figures 26–28**). These analyses were not performed in Experiment 1, since no significant differences were reported in the production of A/Sd.

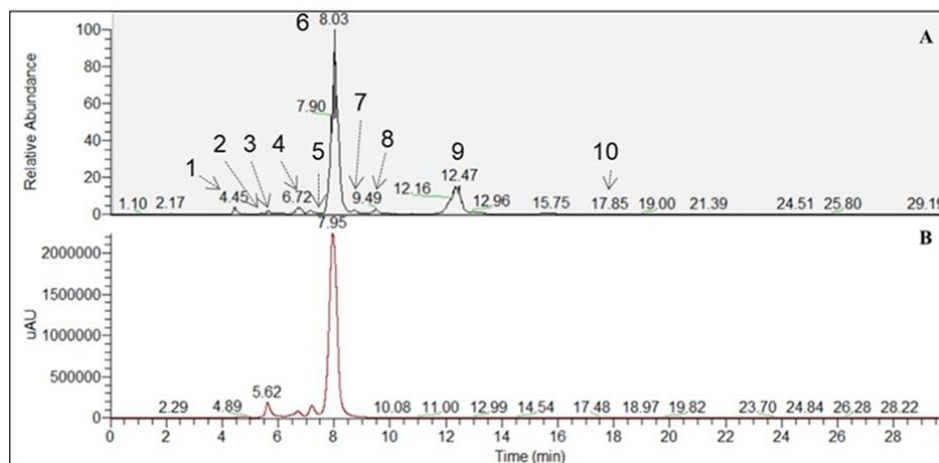


Figure 25. Chromatographic profile of *A. tinctoria* root samples associated with *Septoglomus viscosum* detected under (A) HPLC-MS (BP+) and (B) HPLC-PDA (510 nm). Each peak defined by a number referred to a tentative identified compound, which has been described in Table 14.

RESEARCH RESULTS-PART I-CHAPTER II

Table 14. Putative identification of major chemical constituents in *A. tinctoria* extracts from Experiment 2 (positive mode ESI).

	Code	Retention time [min]	UV	[m/z]	MS Major Ion(s)	Molecular Formula	Δ ppm	Δ mDa	MS/MS Fragments [m/z]	Putative Identification
Cluster A	1	4.60	513, 272	303.1224	[M+H] ⁺	C ₁₇ H ₁₈ O ₅	-2.80	-0.85	285.1119 243.1013 233.0442	methylshikonin isomer I
	2	5.41	n.d.	220.1119	[M+H] ⁺	C ₁₆ H ₁₃ N	-3.29	-0.72	205.0883 142.0648 128.0618	N-phenyl-naphthylamine
	3	5.66	515, 272, 489	287.1267	[M+H] ⁺	C ₁₇ H ₁₈ O ₄	-5.69	-1.63	219.0649 269.1168 231.0648 245.0804	O-methyl-1'-deoxyshikonin
	4	6.74	415, 258	285.1125 303.1228	[M+H-H ₂ O] ⁺ [M+H] ⁺	C ₁₇ H ₁₈ O ₅	-0.65	-0.18	267.1012 243.0649 233.0443	methylshikonin isomer II
	5	7.16	n.d.	403.2317	[M+H] ⁺	C ₂₀ H ₃₄ O ₈	-3.70	-1.49	361.2221 329.1594 273.0968 213.0757	Not identified

RESEARCH RESULTS-PART I-CHAPTER II

	6	8.00	517, 484, 281	271.0955 541.1843	[M+H] ⁺ [2M+H] ⁺	C ₁₆ H ₁₄ O ₄	-5.66	-1.53	229.0493 253.0855 165.0181 191.0338 179.0338 243.1014	anhydroalkannin
Cluster B	7	8.76	n.d.	254.2475	[M+H] ⁺	C ₁₆ H ₃₁ NO	-3.50	-0.89	237.2209 219.2104	palmitoleamide
	8	9.52	n.d.	280.2627 559.5198	[M+H] ⁺ [2M+H] ⁺	C ₁₈ H ₃₃ NO	-4.78	-1.34	263.2365 245.2260	linoleamide
	9	12.33	n.d.	282.2793 563.2793	[M+H] ⁺ [2M+H] ⁺	C ₁₈ H ₃₅ NO	-1.38	-0.39	265.2522 247.2417	oleamide
	10	17.87	n.d.	284.2943	[M+H] ⁺	C ₁₈ H ₃₇ NO	-3.66	-1.04	267.2267	stearamide

Δm = mass errors; [M-H]⁺ = m/z of the protonated molecular ion in positive ionization mode; m/z = mass to charge ratio.

Table 15. Putative identification of major chemical constituents in *A. tinctoria* extracts (Experiment 3, negative mode, ESI).

Code	Retention time [min]	UV	[m/z]	MS Major Ion(s)	Molecular Formula	Δ ppm	Δ mDa	MS/MS Fragments [m/z]	Putative Identification
1	4.60	513, 272.	301.1086	[M-H] ⁻	C ₁₇ H ₁₈ O ₅	3.30	1.00	286.0847 232.0375	methylshikonin isomer I

RESEARCH RESULTS-PART I-CHAPTER II

11	4.78	515, 274, 490	287.0934	[M-H] ⁻	C ₁₆ H ₁₆ O ₅	5.06	1.45	218.0227 190.0273	shikonin
12	5.26	n.d.	797.2762	[M-H] ⁻	C ₃₃ H ₅₀ O ₂₂	5.83	4.65	527.1894	Not identified
13	5.85	516, 274, 490	329.1030	[M-H] ⁻	C ₁₈ H ₁₈ O ₆	1.48	0.49	269.0829 251.0709 241.0865	acetylshikonin
14	7.62	n.d.	1111.3696	[M-H] ⁻	C ₄₇ H ₆₈ O ₃₀	-1.90	-2.12	841.2741 571.1847 1023.3120	Not identified
15	7.98	n.d.	1147.3676	[M-H] ⁻	C ₅₀ H ₆₈ O ₃₀	-3.59	-4.12	877.2743 607.1854 1047.3101	Not identified
6	8.00	517, 484, 281	269.0829	[M-H] ⁻	C ₁₆ H ₁₄ O ₄	5.63	1.52	251.0712 241.0868	anhydroalkannin

Δm = mass errors; [M-H]⁻ = m/z of the deprotonated molecular in negative ionization mode; m/z = mass to charge ratio.

RESEARCH RESULTS-PART I-CHAPTER II

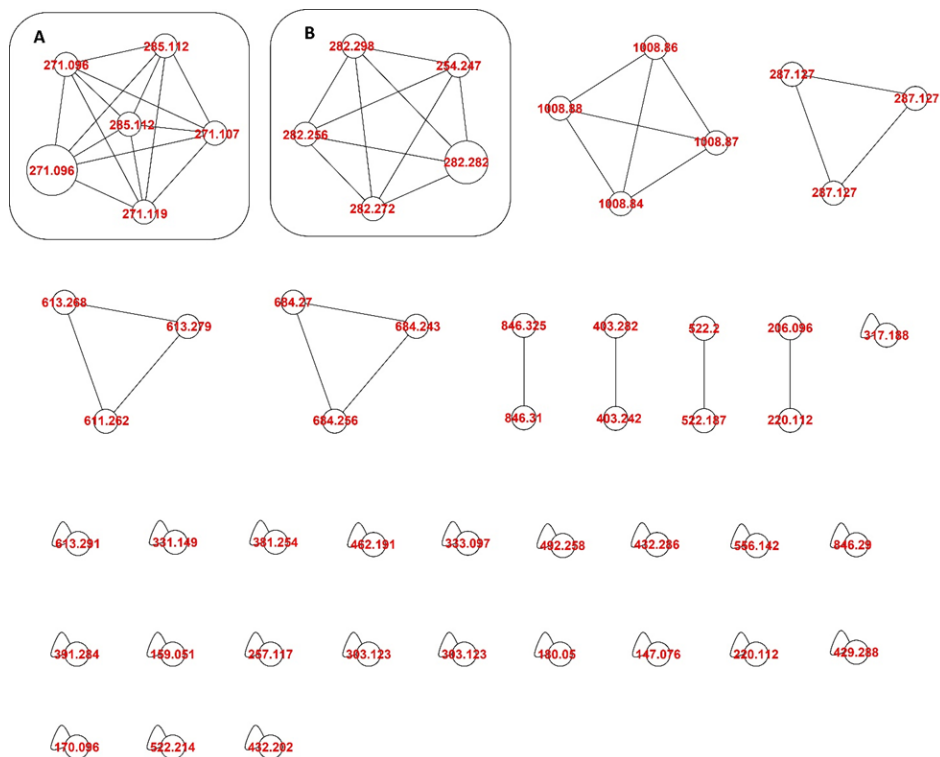


Figure 26. Molecular network of *A. tinctoria* root extracts obtained in Experiment 2 in positive mode. (A) HNQ naphthoquinone's enantiomers (A/Sd); (B) lipid amides. Clusters were built with a cosine of 0.7 with a minimum of 3 common ions. Size nodes are proportional to corresponding peak area.

RESEARCH RESULTS-PART I-CHAPTER II

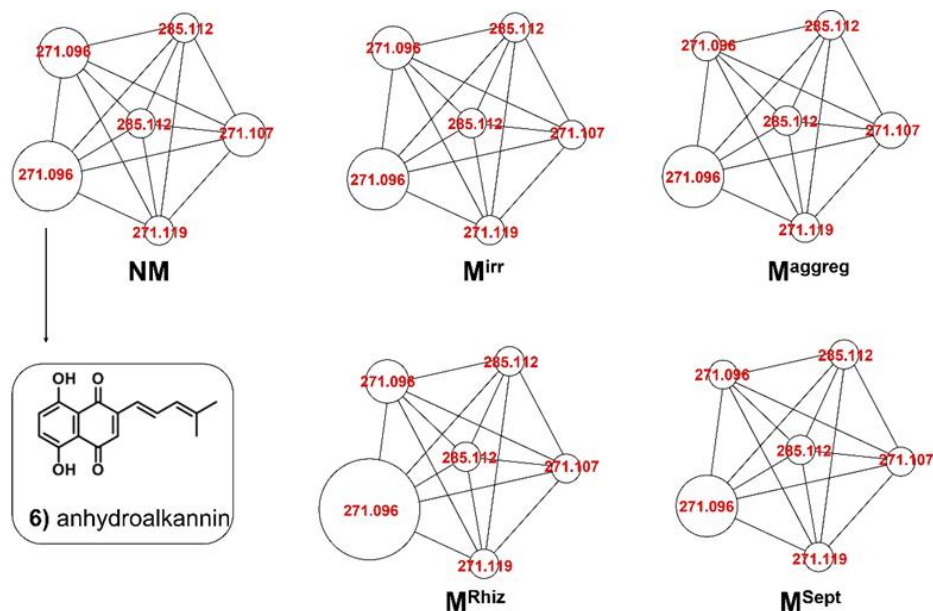


Figure 27. Comparative molecular networking of shikonin derivatives cluster (different nodes) between *A. tinctoria* roots inoculated or not (control) with different AMF strains (two from GINCO - *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408, and two isolated from wild *A. tinctoria* – *R. irregularis* and *S. viscosum*) and growing for 9 days in the S-H cultivation system (Experiment 2).

RESEARCH RESULTS-PART I-CHAPTER II

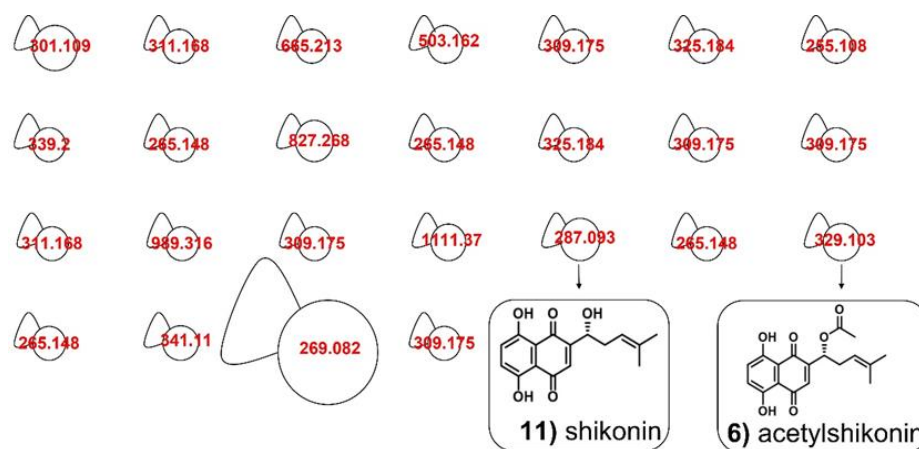


Figure 28. Molecular network in *A. tinctoria* root extracts obtained in Experiment 3 in negative mode. Size nodes are proportional to corresponding peak area.

In Experiment 2, the HPLC-HRMS/MS analysis was performed in positive and negative modes. However, shikonin was not detected in any root sample analyzed. Dereplication analyses of shikonin derivatives were done on positive mode to compare our results with published data available in the literature (Bossard et al., 2022). The major detected compounds were organized in two distinctive clusters: A/Sd naphthoquinones (cluster A, **Figures 26A** and **Table 14**) and lipid amides derivatives (cluster B, **Figure 26B** and **Table 14**). In cluster A, the first eluted derivative was putatively identified as a methylshikonin isomer (1) which gave a protonated molecular ion $[M + H]^+$ at m/z 303 and fragmented to m/z 285, 243, and 233, corresponding to the loss of a H_2O molecule, a $C_2H_4O_2$ and a C_5H_{10} fragment from the alkyl chain. Similar fragmentation pattern was observed for compound 4 with a protonated dehydrated molecular ion $[M + H - H_2O]^+$, putatively identified as a second methylshikonin derivative. Similar loss was observed for compounds 3 and 6, putatively corresponding to methyl-1'-deoxyshikonin and anhydroalkannin at m/z of 287 and 271, respectively. Data also indicated that the major shikonin derivative produced by the plants under these conditions was anhydroalkannin (6) (Kyogoku et al., 1973; Bai and Jin, 1994). Moreover, a group of lipid amides were also putatively detected from the *A. tinctoria* roots samples and represented in cluster B. These amides derivatives were tentatively identified as palmitoleamide (7), linoleamide (8), oleamide (9), and

RESEARCH RESULTS-PART I-CHAPTER II

stearamide (10) with protonated molecular ions $[M + H]^+$ at m/z 254, 280, 282, and 284, presenting a loss of a fragment of m/z 17, corresponding to the loss of an ammoniac molecule (Divito et al., 2012). Other isomeric compounds were also detected by HPLC-HRMS/MS, but in the absence of their fragmentation patterns, putative identities were not proposed. No significant differences were noticed in the metabolites' production profile of *A. tinctoria* roots associated or not with different AMF strains (**Figure 27**).

In Experiment 3, shikonin was detected only in negative mode and this result was corroborated by co-injection with shikonin standard solution (**Table 15**). Due to the important differences in the fragmentation spectra in positive mode of A/Sd, clusters of these compounds were not formed (**Figure 28**). In addition, the abovementioned shikonin derivatives isomers (1 and 6) were also observed as deprotonated molecular ion $[M - H]^-$ at m/z 301 with fragmentation signals at m/z 286 and 232 corresponding to the loss of a CH_3 and C_5H_9 groups, for 1, and at m/z 269 with fragment loss at m/z 251 and 241, corresponding to the loss of a H_2O and CO molecules, for 6. The deprotonated molecular ion $[M - H]^-$ of shikonin (11) was observed at m/z 287 with fragmentation signals at m/z 218 and 190, corresponding to the loss of C_5H_9 and the subsequent loss of a CO group (Liao et al., 2015). Moreover, an additional shikonin derivative was observed with a deprotonated molecular ion $[M - H]^-$ at m/z 329, putatively identified as acetylshikonin (13), with fragmentation loss at m/z 269, 251, and 241 corresponding to a McLafferty rearrangement (i.e., neutral loss of acetic acid), and the subsequent loss of a H_2O and CO molecules (Liao et al., 2015). Three others unidentified compounds (12, 14, and 15) were detected under negative ionization mode with deprotonated molecular ion $[M-H]^-$ at m/z 797, 1111, and 1147. However, no clusters were observed between compounds 1, 6, 11, and 13 (shikonin derivatives) and for 12, 14, and 15 (unknown compounds) because they show a low degree of similarities in their fragmentation spectra (in positive ionization mode).

Slight differences in metabolites production were observed between Experiments 2 and 3, when samples were analyzed in the same ionization mode. Nevertheless, the most important difference between the two experiments was the detection of shikonin only in Experiment 3. Analyses

RESEARCH RESULTS-PART I-CHAPTER II

performed in negative mode for root samples of Experiment 2 did not show the presence of shikonin. Similarly, in Experiment 3, no significant differences were observed in the metabolites' production profile of *A. tinctoria* roots associated or not with different AMF strains, thus producing similar chromatographic profiles.

Discussion

In the present study, two native AMF strains isolated from *A. tinctoria* roots and two strains from GINCO were tested and compared on the production of A/Sd and on the expression of genes involved in A/Sd biosynthesis in *A. tinctoria* plants grown either in a semi-hydroponic or in a pot cultivation system. In Experiment 1, conducted only with the GINCO strain *R. irregularis* MUCL 41833, no effect was observed on the production of A/Sd, while a significantly higher relative expression of *LePGT2* was observed after 37 days compared to 9 days of growth in the S-H cultivation system. Furthermore, the relative expression of *LePGT1* and *LePGT2* genes were significantly higher in the plants colonized with this AMF compared to the non-mycorrhized ones. In Experiment 2, four AMF strains (two from GINCO: *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408 and two isolated from *A. tinctoria*: *R. irregularis* and *S. viscosum*) were associated to *A. tinctoria* in the S-H cultivation system. The native strain *R. irregularis* significantly increased the production of A/Sd in the roots of *A. tinctoria*, although this was not accompanied by an increased expression of genes involved in the A/Sd biosynthesis pathway. In Experiment 3, the two native strains (*R. irregularis* and *S. viscosum*) and the GINCO one (*R. irregularis* MUCL 41833) were associated to *A. tinctoria* in pots. The native strain *R. irregularis* was confirmed to significantly enhance the content of shikonin and total A/Sd in *A. tinctoria* roots. However, no significant increase in relative genes expression was observed.

The S-H and pot cultivation systems are adequate for growth and AMF colonization of *A. tinctoria*

Whatever the growth system (S-H or pot), an increase in biomass of *A. tinctoria* was observed in the presence as well as absence of AMF. No significant difference was observed in SFW between the plants in the M and NM treatments after 9 days in the S-H cultivation system (Experiments

RESEARCH RESULTS-PART I-CHAPTER II

1 and 2) or after 85 days in the pots cultivation system (Experiment 3). However, a significantly lower RFW and TFW was reported for the plants in the M treatment compared to those in the NM treatment after 37 days in the S-H cultivation system (Experiment 1), unlike the results obtained with *A. officinalis* colonized by *R. irregularis* MUCL 41833 in the same S-H cultivation system (Cartabia et al., 2021). These results are not surprising as it has often been reported that plant growth response to AMF inoculation can vary among AMF species and that the direction (e.g., increase or decrease in plant biomass) and magnitude of the response strongly depends on the combination of plant and AMF taxa (Klironomos, 2003).

Root colonization was observed in each AMF-inoculated plant. However, colonization measured at transfer and after 9 and 37 days in the S-H cultivation system (Experiment 1), decreased steadily, probably because of damages caused to the extraradical mycelium at transfer to the S-H cultivation system with no full recovery and development within the roots throughout the experiment (Cartabia et al., 2021). It is not excluded that this is also a reason for the lower plant biomass reported at the end of Experiment 1 compared to the control, due to an imbalance between the carbon resources transported from plant to the fungus in exchange for nutrients transported from fungus to plant. Interestingly, in both Experiments 2 and 3, the total root colonization was almost similar among the AMF strains, while the percentages of arbuscules were higher in presence of the native *R. irregularis* and *S. viscosum* strains, suggesting that they were better adapted to *A. tinctoria* than the GINCO strains. This has often been reported by comparing commercial strains with local strains; the latter being more prone to rapid and effective colonization (Chenchouni et al., 2020; Wu et al., 2021). Unfortunately, this was not translated into higher plant biomass, probably because of the same justification explained above.

AMF isolated from wild *A. tinctoria* impact the production of A/Sd

The production of A/Sd was not impacted by the GINCO strain *R. irregularis* MUCL 41833 (Experiment 1), whereas a significant increase in production was observed with the native *R. irregularis* strain (Experiments 2 and 3) and an intermediate production between this strain and the GINCO strains with the other native strain *S. viscosum* (Experiment 2). Similarly, no significant effect was observed with the GINCO strains *R. irregularis*

RESEARCH RESULTS-PART I-CHAPTER II

MUCL 41833 and *R. aggregatus* MUCL 49408 (Experiments 2 and 3). It cannot be ruled out that these results are related to the fungal genotypes, with the native ones being more prone to stimulate the production of A/Sd than the GINCO ones. The difference between the two native strains further suggests some degree of functional specialization. This is supported by recent studies revealing that various species/strains of AMF can induce different changes in the production of metabolites in the same plant species (Kapoor et al., 2002; Copetta et al., 2006; Khaosaad et al., 2006; Toussaint et al., 2007; Zubek et al., 2010). For example, Toussaint et al. (2007) showed that *Funneliformis caledonium* increased rosmarinic acid and caffeic acid production in *O. basilicum*, whereas *Funneliformis mosseae* only increased caffeic acid production. In another study, Jurkiewicz et al. (2010) showed that total phenolic acids accumulation was significantly higher in the roots of *Arnica montana* L. associated with AMF collected from particular plant's natural stands (in Kurpie or in Karkonosze), while intermediate increase was observed with AMF isolated from other regions (i.e., *R. intraradices* UNIJAG PL24-1 and *R. intraradices* BEG 140 or a mixture composed of the above and of *Funneliformis geosporum* UNIJAG PL 12-2, *Septoglomus constrictum* 265-5 Walker and *F. mosseae* BEG 12). Finally, Frew (2020) reported that the inoculation with a mixture of four commercial species of AMF (*Claroideoglossum etunicatum*, *Funneliformis coronatum*, *F. mosseae*, and *R. irregularis*) had a greater effect on phenolic concentrations in *Hordeum vulgare* L. cv. "Hindmarsh" as compared to a single commercial inoculant (*R. irregularis*). Interestingly, the author also demonstrated that the effects of the commercial mixture was not different from the results obtained with a native multi-species AMF inoculant extracted from field soil. This suggested that a commercial AMF mixture provided little to no additional benefits (Frew, 2020). Conversely, several specialized compounds produced by plants may be synthesized as a chemical defense against the presence of AMF in the roots (Copetta et al., 2006). However, this defense mechanism would be likely to occur with AMF strains that have never been associated to the plant. Our native strains isolated from the wild *A. tinctoria* in their natural habitat are more adapted to the plant and are likely to have evolved as good associates. Natives *R. irregularis* and *S. viscosum* could be better adapted to the presence of A/Sd, which are characterized by different biological activities and by various effects on soil microorganisms (e.g., antifungal activities), and thus better

RESEARCH RESULTS-PART I-CHAPTER II

interact with their host plants to regulate and enhance the production of these important therapeutic metabolites (Yan et al., 2019). As reported above, a significant higher AC% was observed with MRhiz and MSept treatments in Experiments 2 and 3, respectively. An increase in nutrients uptake via the arbuscules can lead to an enhanced production of precursor compounds, such as NADPH, ATP, acetyl-CoA (mevalonic acid pathway), and pyruvate glyceraldehyde and phosphate (methylerythritol-4-phosphate pathway) that are required for the biosynthesis of various SMs (e.g., terpenoids, phenolic, and alkaloids) (Kapoor et al., 2017). In our study, a higher AC% could have led to an increase in the precursor compounds essential to produce A/Sd. However, this was not confirmed in Experiment 3, where the higher AC% in MSept treatment was not accompanied by a higher production of A/Sd. If we parallel this to plant biomass, it is well known that the response of plants to inoculation with an AMF may vary depending on the AMF species as previously reported (Klironomos, 2003). It is not excluded that similar effect is observed on metabolites production, although this needs to be demonstrated.

AMF modulate the expression of genes involved in the A/Sd biosynthetic pathway in *A. tinctoria* roots

At the end of Experiment 1, a significantly higher relative expression of *LePGT1* and *LePGT2* genes was observed in *A. tinctoria* associated with *R. irregularis* MUCL 41833 compared to the NM treatment. In addition, a significantly higher relative expression of *LePGT2* was observed in the AMF-colonized plants at day 37 compared to day 9. However, these increases were not accompanied by an enhanced production of A/Sd. Conversely, in Experiment 2, a significant lower relative expression of *LePGT2* was observed in plants associated with the native *R. irregularis* strain, compared with the other AMF and NM treatments, whereas a significantly higher production of A/Sd was observed with this strain. In both Experiments, no differences were reported for *GHQH*. In Experiment 3, no significant differences between the treatments were noted for all the genes tested (data not presented).

Although these results may seem surprising, similar outcomes were reported in the literature. For example, Wang et al. (2014) demonstrated that the application of methyl jasmonate (MeJA) on shikonin-deficient *A. euchroma* cell lines rapidly led to the overexpression of several genes

RESEARCH RESULTS-PART I-CHAPTER II

involved in the biosynthesis of A/Sd, including *PGT*, but was not accompanied by a significant higher production of A/Sd. In another study, Ahmad et al. (2022b) showed a significantly higher expression of *LePGT1*, *LePGT2*, and *LeGHQH1* in *Lithospermum officinale* L. roots treated with MeJA, but no higher accumulation of total A/Sd, after eight weeks. Conversely, the same authors (Ahmad et al., 2022a) demonstrated that the bacterium *Chitinophaga* sp. strain R-73072 significantly upregulated *LePGT1* and *LePGT2*, and a cytochrome P450-*LeCYP76B101* genes, resulting in a significant enhanced production of total A/Sd in *L. erythrorhizon* roots, after 2-3 weeks.

In our study, a few hypotheses can be advanced to explain why upregulation of *PGTs* was not followed by higher production of A/Sd and, conversely, why downregulation was followed by higher production of A/Sd. Firstly, it is not excluded that the duration of Experiment 1 (37 days) was too short to result in a significant increased production of A/Sd, or that the timing of plant harvesting in our experiments was not optimal (i.e., gene expression behavioral oscillations due to the plant circadian cycle) (Morrow et al., 2005). The *PGTs* regulate the first biosynthetic steps forming the basic carbon skeleton that leads to A/Sd, and therefore, it is possible that the resulting modulation of A/Sd production took longer after the upregulation of the relative expression of target *PGT* genes, as supported by Ahmad et al. (2022b), or that the genes oscillations were not timely targeted during our harvest times, as suggested by Wang et al. (2014) (**Figure 29**). Secondly, in a study by Andrade et al. (2013), a mismatch between alkaloid levels, another important SM group, and gene expression was reported in different *Catharanthus roseus* (L.) G. Don tissues. In this study, the AMF *C. etunicatum* had a greater influence (i.e., alkaloids production) in roots than in shoots, and a higher gene expression was reported in the older leaves of M plants as well as in the youngest leaves of NM plants (leaves were harvested at the same time) (Andrade et al., 2013). These results suggest a very precise phenological and spatial regulation process during alkaloid biosynthesis (Mahroug et al., 2007). Moreover, the reported influence of AMF on idioblast and laticifer density in *C. roseus* plants might have enhanced the expression of enzymes specifically located in these cells (St-Pierre et al., 1999). An increase in glandular trichome density upon mycorrhization was also linked with an enhanced concentration of another group of SMs, the terpenoids (Zhao et

al., 2022). This is an interesting aspect that needs to be further verified. In fact, A/Sd compounds are sequestered as granules in the phospholipid layer and are accumulated in the apoplastic spaces, and they can be found in the cork layer of mature roots (Brigham et al., 1999; Singh et al., 2010; Tatsumi et al., 2016). A difference in A/Sd accumulation might have occurred (i.e., higher content in the primary roots as compared to the secondary roots) upon AMF colonization and might have not been fully displayed across *A. tinctoria* root samples used for our RT-qPCR analysis (i.e., no homogenous samples analyzed with mostly secondary roots utilized). Thirdly, the regulation and accumulation of SMs in plants is usually controlled by a complex network characterized by transcription factors (TFs), which promote or inhibit the expression of multiple genes involved in one or more biosynthetic pathways (Yang et al., 2012; Wu et al., 2021). Transcription factors can act alone or in combination with other TFs to modulate the expression of target genes, and also one TF can regulate the expression of multiple genes participating in one or more biosynthetic pathways (Pinson et al., 2009; Goossens et al., 2017; Hassani et al., 2020). In our study, different TFs might have regulated the target genes (*PGTs* and *GHQH*), yet the full landscape of the A/Sd biosynthesis pathway is not entirely understood, with co-expressed genes still to be identified (Suttiyut et al., 2022). Finally, even after a gene has been transcribed, its expression could still be regulated at various stages. Post transcriptional modifications changes might occur to a newly transcribed primary RNA transcript after transcription has occurred and prior to its translation into a protein.

RESEARCH RESULTS-PART I-CHAPTER II

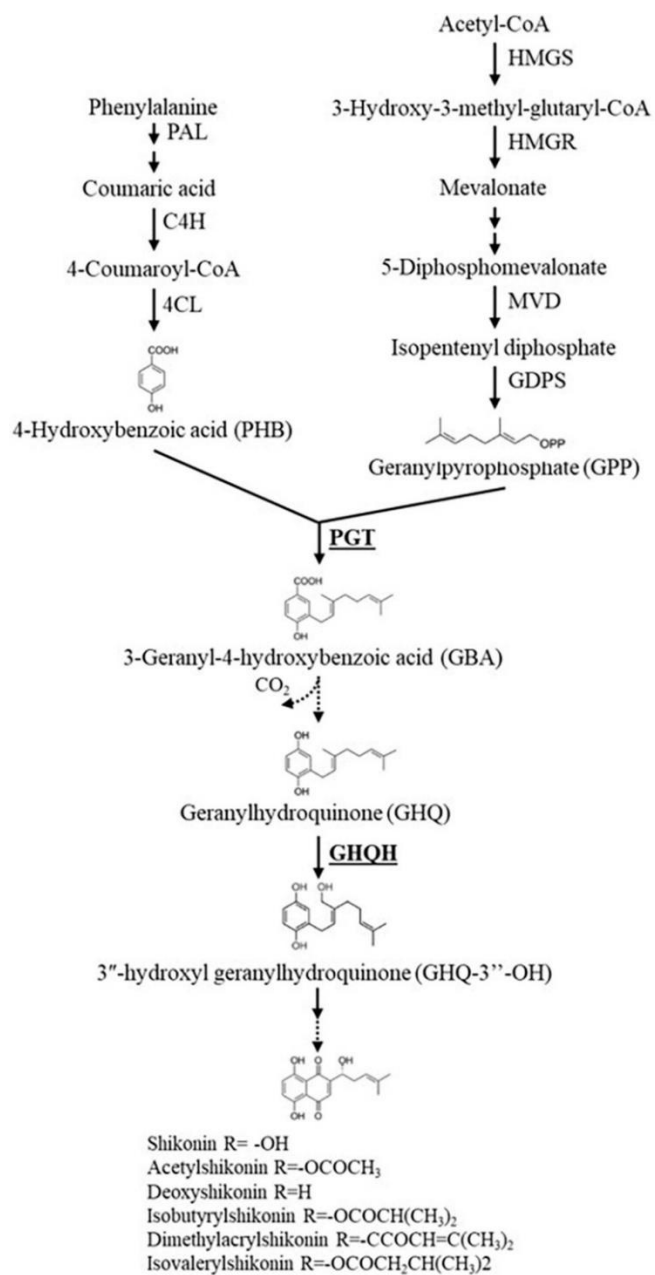


Figure 29. An abridged scheme of the shikonin derivatives biosynthesis pathway. The genes that were investigated in this study are reported in bold font. Single

RESEARCH RESULTS-PART I-CHAPTER II

arrows represent one step reaction, while double arrows represent multiple step reactions. Dashed arrows signify undefined steps or the enzymes have not been verified yet. HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVD, mevalonate diphosphate decarboxylase; GDPS, geranyl diphosphate synthase; PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; PGT, p-hydroxybenzoate geranyltransferase; GHQH, geranylhydroquinone 3''-hydroxylase.

Chemical profile of *A. tinctoria* does not markedly differ between semi-hydroponic and pot cultivation systems and in presence of different AMF

A similar chemical composition was reported in *A. tinctoria* roots associated with the different AMF and non-colonized control plants in Experiments 2 (S-H cultivation system) and 3 (pot cultivation system) (**Figures 27, 28**) except for the detection of shikonin in Experiment 3 (**Tables 14, 15**).

The major compound detected in both experiments was putatively identified as anhydroalkannin (6). This compound is also a main product of shikonin biotransformation by several human intestinal bacteria in aerobic conditions and it was found to be less cytotoxic against a series of human tumor cell lines in vitro, in comparison with shikonin (Meselhy et al., 1994; Min et al., 2000). Methyl A/Sd isomers, as detected in our samples, were also identified from *Lithospermum*, *Alkanna*, and *Onosma* species. 1'-methyl shikonin was isolated and characterized from roots of *L. erythrorhizon*, exerting moderate antioxidant activity (Han et al., 2008). To the best of our knowledge, 5- or 8- O-methoxyshikonin derivatives have not yet been identified from natural sources. To better clarify the chemical composition of our samples, putative methylshikonin derivatives (1 and 4) must be purified and the methoxyl position elucidated in the structure of these isomers. In addition, in both experiments, four main lipid amides were tentatively identified as palmitoleamide (7), linoleamide (8), oleamide (9), and stearamide (10). Fatty acids amides are a group of nitrogen-containing, lipid-soluble fatty acid derivatives, which act against a variety of diseases such as cancer, bacterial infections, parasitic infection, inflammations, diabetes, and obesity (Kim et al., 2010; Tanvir et al., 2018). Further, it has been reported that fatty acid amides as oleamide from plant root exudates can participate in strong plant-microbe interactions,

RESEARCH RESULTS-PART I-CHAPTER II

stimulating nitrogen metabolism in rhizospheric bacteria (Sun et al., 2016).

The data of chemical composition obtained from *A. tinctoria* growing in our cultivation systems cannot be accurately compared with data from literature (i.e., from nature, commercial samples, and/or from cell plant suspension). Indeed, the proportion and quantity of A/Sd varies depending on the level of stress and microorganisms present in the rhizosphere (Brigham et al., 1999). The influence of the cultivation systems on the production of these methoxy and anhydro A/Sd must be deeply studied to better understand their significance. Secondary metabolites' production and compositional changes have a strong correlation and association with the environment, and thus synthesized only under specific growth conditions (Peñuelas and Llusà, 1997). Variations in an environmental factor, such as light, temperature, soil water, soil fertility, and salinity, may alter the plant metabolites content (Yang et al., 2018). Indeed, many chemical and physical factors have been found to inhibit shikonin production, such as NH_4^+ , 2,4-dichlorophenoxyacetic acid (a synthetic auxin), low pH, temperature higher than 28°C, and light, especially blue light (Tatsumi et al., 2016). Skoneczny et al. (2017), using a metabolomic approach profiling hydroxynaphthoquinones (HNQs) and pyrrolizidine alkaloids (PAs), demonstrated the influence of high temperatures and water withholding on the accumulation of A/Sd in *Echium plantagineum* L. Abundance of HNQs, especially deoxyshikonin, shikonin, and dimethylacrylshikonin, rapidly increased in roots exposed to elevated temperatures. Water withholding initially increased NQ abundance, but prolonged drought resulted in reduced total PAs and HNQs (Skoneczny et al., 2017). In our study, *A. tinctoria* growing in the S-H cultivation system were kept in perlite, while a mixture of peatmoss, perlite, and quartz was used in the pots experiment. Perlite is a growing medium frequently used in hydroponic cultivation system since it has a high-water retention and provide the plants' roots with strong anchor points for stability and strength. Moreover, in this system, plants received minerals by the circulating nutrient solution flowing directly through the plants' container, while in the pots it was added at constant intervals and left to be completely absorbed by the plants. Therefore, it is possible that the temperature and the water/nutrient solution retention was higher in the conventional pots than in the plant containers of the S-H cultivation system. Moreover, shikonin and A/Sd might have better accumulated in the pots, while

RESEARCH RESULTS-PART I-CHAPTER II

leached out in the S-H system. Finally, plant developmental stage qualitatively and quantitatively influences primary and secondary metabolism. A recent study by Csorba et al. (2022) reported this aspect as the most important driver influencing *A. tinctoria* metabolites content, revealing a peak content of A/Sd at the fruiting stage. In our study, plants were harvested during the vegetative growth (around five months old), and thus, might have played a role in the chemical profile reported.

Conclusion

For the first time, to the best of our knowledge, AMF isolated from wild-growing *A. tinctoria* were identified and applied under the cultivation systems described in this study. Native *R. irregularis* significantly increased A/Sd production in *A. tinctoria* roots, whatever the system used (S-H or pots), thus opening new perspectives towards the application of AMF in the production of these valuable therapeutic compounds in medicinal plants. A better adaptation of this indigenous strain towards its host was demonstrated, with higher arbuscules formation and production of A/Sd reported in *A. tinctoria* roots. This result suggests that the selection of the most effective AMF species (native or not; single or combinations of different AMF strains) remains a key point in studying the modulation/increase of SMs. However, the mechanisms behind AM symbiosis and their impact on the A/Sd biosynthetic pathways still need to be further clarified. Additionally, the condition characterizing the conventional pots system seemed to be the optimal one in term of shikonin detection. Therefore, the application of the best growing conditions should be further investigated as well. Since shikonin was reported only in the pots system, the recovery of SMs can yet be conducted only in a destructive way (i.e., using the roots of *A. tinctoria*). For this reason, further studies applying S-H system/innovative systems are required, especially for testing non-destructive ways of trapping the metabolites exudates by the roots in the circulating nutrient solution.

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RESEARCH RESULTS-PART I-CHAPTER II

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Preface

In **Chapter II**, the effects of two native (i.e., *R. irregularis* and *S. viscosum*) and one non-native (i.e., *R. irregularis* MUCL 41833 from the GINCO) AMF strains were tested on the production of A/Sd by *A. tinctoria* plants grown under semi-hydroponic and pot culture conditions. The native *R. irregularis* strain increased the total A/Sd production under both conditions and it also increased shikonin production in pot cultures. In both systems whole plants of *A. tinctoria* were considered. However, the germination rate of seeds is very low, the time needed for alkannin/shikonin and their derivatives (A/Sd) production is quite long, and harvest may be at high costs. This is particularly the case in the field, where plants can, in addition, be exposed to numerous biotic and abiotic stresses, making secondary metabolites (SMs) production uncertain. Therefore, hairy root cultures (HRCs) have been proposed as an alternative way to produce SMs under highly controlled and reproducible conditions. Indeed, HRCs have become popular in the two last decades for producing SMs. These organs grow fast, are easy to cultivate and are genetically stable. Interestingly, it has been reported that applying abiotic and/or biotic elicitors in the HRCs of medicinal plants can increase the biosynthesis of certain SMs.

Therefore, in **Chapter III**, it was the objective to grow HRCs of *A. tinctoria* and to compare the effects of a native strain of *R. irregularis* (the one from Chapter II) with the standard strain *R. irregularis* MUCL 41833, on the production of A/S and their derivatives (A/Sd). It is well known that A/Sd compounds have antifungal properties. Thus, the antifungal effect of HRCs of *A. tinctoria* was further assessed on three *R. irregularis* strains (native, MUCL 41833 and MUCL 43194) by evaluating the germination rate of the spores incubated in the vicinity of these roots.

**Chapter III Impact of arbuscular mycorrhiza fungi on the
production of alkannin/shikonin and their derivatives in
the hairy roots of *Alkanna tinctoria***

This research work has not been published

Abstract

Hairy root cultures (HRCs) of medicinal plants have become popular over the past two decades to produce secondary metabolites (SMs). They could be a potential way to produce alkannin/shikonin and their derivatives (A/Sd) under highly controlled and reproducible conditions. In addition, it has been reported that applying abiotic and/or biotic elicitors in the HRCs of medicinal plants can enhance the biosynthesis of certain SMs. In nature, *A. tinctoria* interacts with various microorganisms including arbuscular mycorrhizal fungi (AMF). However, it is unknown whether the application of AMF to HRCs of this plant can increase the production of A/Sd.

In the present study, a strain of *Rhizophagus irregularis* isolated from nature on *A. tinctoria* and *Rhizophagus irregularis* MUCL 41833 from the Glomeromycota *in vitro* collection (GINCO), were compared for their potential to increase the production of A/Sd in HRCs of *A. tinctoria*. Identification and quantification of A/Sd in roots and growth medium were done using HPLC-HRMS/MS. In parallel, the effects of HRCs of *A. tinctoria* (potentially exuding A/Sd, which are known to have antimicrobial activities) were tested on spore germination of *R. irregularis* (one native and two from GINCO: MUCL 41833 and MUCL 43194). After 17 months of association, no significant effect of AMFs (native and MUCL 41833 strains) was noticed on A/Sd production. Moreover, regardless of the AMF strain, no significant difference in germination rate was observed between spores incubated close to the HRCs of *A. tinctoria* and carrot after 5 weeks incubation, suggesting that there was no effect of A/Sd on AMF spore germination. However, whatever the HRCs, the spore germination of *R. irregularis* MUCL 41833 was significantly lower as compared to the two other strains. The absence of colonization and lower germination rate of MUCL 41833 versus the native strains is thus possibly related to the state of the culture of this strain at the time of the experiment. It is thus advisable to carry out further research in a shorter time frame and with other AMFs isolated from nature on *A. tinctoria* and from culture collections. This should help to understand whether an AMF strain is able to impact the production of SMs in HRCs of *A. tinctoria* and whether this plant can affect the germination rate of AMF spores.

Key words: Hairy root cultures (HRCs), *Alkanna tinctoria*, alkannin/shikonin and their derivatives (A/Sd), arbuscular mycorrhizal

fungi (AMF), spore germination

Introduction

Secondary metabolites (SMs) of medicinal plants are economically important as drugs, flavours and fragrances, dye and pigments, pesticides, and food additives (Hussain et al., 2012). Among them are the hydroxynaphthoquinones (HNQs) and in particular the isohexenylnaphthazarins (IHNs), such as the chiral pair alkannin and shikonin (A/S) and their derivatives (A/Sd). They are lipophilic red pigments characterized by a wide spectrum of wound healing, antibacterial, anti-inflammatory, anticancer and antithrombotic activities (Papageorgiou et al., 1999; Tappeiner et al., 2014). These naphthoquinones compounds are mainly produced in plants from the Boraginaceae family.

Alkanna tinctoria is the main producer of A/S (Rat et al., 2021). However, it is considered as very rare in some European countries, such as Slovakia, Bulgaria, and Hungary (Yazaki, 2017; Yaman et al., 2019; Ahmad et al., 2021). Moreover, it has a very low seed germination rate and its cultivation under conventional agriculture practices is hampered by several constraints (e.g., time needed for A/S production, high costs of harvesting, exposure to biotic/abiotic stresses) (Malik et al., 2016). Furthermore, the only successful example of shikonin scaling up from cell cultures was with *L. erythrorhizon* Siebold & Zucc. (Boraginaceae family) by Mitsui Petrochemical Industries Ltd. (now Mitsui Chemicals Inc., Tokyo, Japan) in 1984 (Yazaki, 2017). Few more attempts have been made with cell suspension cultures of *Arnebia* spp. (Boraginaceae family) in stirred-tank (New Brunswick Scientific Company Inc., Edison, NJ) and in air-lift bioreactors, but no further commercial successes have been reported (Gupta et al., 2014; Malik et al., 2016). The industrial production of A/Sd is thus mostly from field-grown plants, increasing the pressure on this species.

Hairy root cultures (HRCs), which are differentiated cultures of transformed roots generated by the infection of wounded higher plants with *Rhizobium rhizogenes* (Pistelli et al., 2010), has become popular in the two last decades for producing SMs (Srivastava and Srivastava, 2007). These HRCs are unique in their genetic and biosynthetic stability, grow fast and are easy to cultivate. Moreover, they often accumulate phytochemicals at a

RESEARCH RESULTS-PART I-CHAPTER III

higher level than cell/callus cultures that contain undifferentiated cells (Mehrotra et al., 2015). It has allowed the production of a wide range of chemical compounds such as scopolamine, hyoscyamine, catharanthine, ginsenosides, and anthraquinones (Häkkinen et al., 2016; Guo et al., 2018; Hanafy et al., 2016; Palazón et al., 2003; Perassolo et al., 2017). Interestingly, some studies have reported that applying abiotic and/or biotic elicitors in the HRCs of medicinal plants can increase growth and biosynthesis of certain bioactive compounds (Sivanandhan et al., 2014; Halder et al., 2019). For example, in the study conducted by Zhao et al. (2014), the application of yeast polysaccharides in the growth medium effectively increased flavonoids (i.e., rutin and quercetin) production in HRCs of *Fagopyrum tataricum*. Similarly, two bacterial strains (*Bacillus cereus* and *Staphylococcus aureus*) used as biotic elicitors have been shown to induce scopolamine production in HRCs of *Datura metel*, which was not observed in the absence of these bacteria (Shakeran et al., 2017).

Arbuscular mycorrhiza fungi (AMF), a ubiquitous group of soil microorganisms belonging to the Glomeromycota Phylum, are well-known to provide their hosts with nutrients (especially N and P) (Smith and Read, 2008; Chen et al., 2018). Interestingly, they have also been reported to affect both quantitatively and qualitatively the production of SMs produced by their hosts (Salam et al., 2017; Kaur and Suseela 2020). For instance, *Rhizophagus irregularis* MUCL 41833 has been shown to modulate the SMs production of *Anchusa officinalis* L., a *Boraginaceae* species, under semi-hydroponic cultivation system (Cartabia et al., 2021). In another study Tsiokanos et al. (2022), demonstrated that *A. officinalis* accumulated more primary and secondary metabolites when associated to *R. irregularis* and *Rhizophagus intraradices* compared to *Rhizophagus clarus* and *Rhizophagus aggregatus*.

Therefore, in the present study, the main objectives were to determine whether (1) a native AMF isolated from nature on *A. tinctoria* plants can increase the production of A/Sd in hairy roots of *A. tinctoria* and if this effect is significantly higher than with a standard strain of AMF and (2) exudates of *A. tinctoria* have an antifungal effect on spore germination of AMF. To address these objectives, *A. tinctoria* hairy roots colonized with the native *R. irregularis* strain or with a strain from the Glomeromycota *in vitro* collection (GINCO) (i.e., *R. irregularis* MUCL 41833) were grown

RESEARCH RESULTS-PART I-CHAPTER III

in bi-compartmental Petri plate on the modified Strullu-Romand (MSR) medium (Declerck et al., 1998) for 17 months in the dark at 27°C. Identification and quantification of the A/Sd from roots and MSR medium was performed using High-Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry detection (HPLC-HRMS/MS). Meanwhile, the content of A/Sd in the root compartment (RC) and hyphal compartment (HC) via a potential transport by hyphae from RC to HC was measured by HPLC-HRMS/MS. Finally, the antifungal effect of A/Sd on AMF was assessed by evaluating the germination of spores belonging to 3 different *R. irregularis* strains placed in the vicinity of *A. tinctoria* hairy roots or carrot roots (as control).

Materials and Methods

Biological materials

Arbuscular mycorrhizal fungi (AMF)

Three AMF strains were considered: *Rhizophagus irregularis* isolated from *A. tinctoria* plants in nature (in process of deposit in MUCL) and two strains (*Rhizophagus irregularis* MUCL 41833 and *R. irregularis* MUCL 43194) purchased from the GINCO (<http://www.mycorrhiza.be/ginco-bel>). The strains were grown *in vitro* in association with Ri T-DNA transformed roots of carrot (*Daucus carota* L. clone 1E).

Transformed hairy roots of A. tinctoria

Ri T-DNA transformed hairy roots of *A. tinctoria* were provided by Rat et al. (2021). Roots were first grown on the Murashige-Skoog (MS) (Duchefa, Netherlands) medium containing 1% sucrose, 0.8% phytoagar (Duchefa, Netherlands), and 500 mg L⁻¹ of cefotaxime (Duchefa, Netherlands). The roots (approximately 1 cm in length) were sub-cultured weekly on this medium during 3–4 weeks. The roots were then propagated in liquid MS medium supplemented with 3% sucrose (Brigham et al., 1999; Fukui et al., 1999; Kim et al., 2009) for mass production.

Experiment 1: Effects of AMF strains on the concentration of A/Sd in hairy roots of A. tinctoria

Bi-compartmented Petri plates were set up associating a 2 cm long *A. tinctoria* hairy root containing healthy apexes, with approx. 200 spores of

RESEARCH RESULTS-PART I-CHAPTER III

the native *R. irregularis* or *R. irregularis* MUCL 41833 strain. They were associated in the root compartment (RC) of the bi-compartmented Petri plate on the MSR medium as described by Cranenbrouck et al. (2005). Non-inoculated *A. tinctoria* hairy roots were used as control. Six replicates were considered per treatment. The Petri plates were maintained at 27°C in the dark (**Figure 30**). To avoid development of hairy roots in the hyphal compartment (HC), roots were trimmed at regular intervals. After three months, the hyphae started to cross the plastic barrier separating the RC from the HC, developing in the HC.

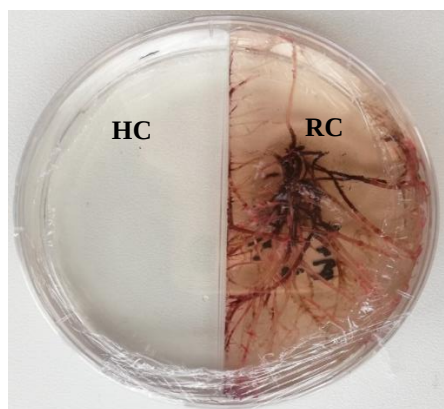


Figure 30: Bi-compartmented Petri plate (90 mm diam.) with Ri T-DNA transformed hairy roots of *A. tinctoria* associated with an AMF in the root compartment (RC). Only the AMF was allowed to cross the barrier separating the RC from the HC to proliferate in the hyphal compartment (HC).

After 17 months of culture, the hairy roots and agar from the RC and HC were harvested. A fraction of hairy roots from each replicate was used to assess the AMF roots colonization. All the remaining hairy roots as well as the agar samples in both compartments were collected and subjected to freeze-drying for 72 h and then reduced in powder using liquid nitrogen and mortar with pestle. The powders obtained were stored at -20 °C for LC/MS analysis.

AMF colonization of *A. tinctoria* of hairy roots

Root colonization was evaluated by the method of McGonigle et al. (1990). Firstly, roots (an approximate of 2 g fresh material) were cut into circa 1 cm length pieces and placed in 15 mL Falcon tubes. Ten mL of a solution

RESEARCH RESULTS-PART I-CHAPTER III

of KOH 10% was added to the roots before incubation at 70°C for 30 min. The KOH solution was then removed and replaced with a solution of H₂O₂ 3.5% before incubation at 70°C for 5 min. The roots were subsequently rinsed with demineralized water and a solution of HCl 1% was added for 1 min at room temperature. After discarding the solution, the roots were stained with ink 2% (Parker Blue Ink, United States) in HCl 1% (Walker, 2005) by placing the tubes at 70°C for 30 min. The roots were finally rinsed with demineralized water and kept in lactoglycerol (lactic acid/glycerol/distilled water, 1:1:1, v/v) before observation. For colonization assessment, the root fragments were placed on microscope slides and covered with 40×22 mm coverslips before observation under a bright field light microscope (Olympus BH2-RFCA, Japan) at ×10 magnification. For each sample, around 200 root intersections were observed per root system. The percentage of total colonization (%TC) of roots (i.e., presence of hyphae, arbuscules, or vesicles/spores), percentage of arbuscules (%AC) and percentage of vesicles/spores (%VC) were calculated using the method of McGonigle et al. (1990).

Experiment 2: Effects of hairy roots of *A. tinctoria* on spore germination of AMF

Petri plates (90 mm diam.) were filled with MSR medium. A sterilized 40 µm mesh nylon membrane (VWR R Cell Strainers) containing a hairy root of *A. tinctoria* was incorporated into the Petri plate. Only the root exudates were able to pass through the membrane, diffusing in the MSR medium. Hairy roots of carrot similarly enclosed in a nylon membrane were used as control. In total, three *A. tinctoria* and carrot (as a control) hairy roots fragments of about 2 cm each, containing healthy apexes from one-week old culture were transferred into the membrane. After one week, spores of three AMF strains (*R. irregularis* MUCL 41833, *R. irregularis* MUCL 43194 and the native *R. irregularis*) were transferred randomly on the MSR medium 1cm away from the membranes. A control that consists of spores inoculated 1cm far from the membrane without any hairy root was also included. Forty-eight spores (16 for each *R. irregularis* strain) were considered per treatment. The systems were maintained at 27°C in the dark. Spore germination was checked twice a week during one-month as well as hyphal growth length through a gridline attached to the stage plate of the stereomicroscope.

Quantitative and qualitative analysis of A/Sd in hairy roots of *A. tinctoria*

Chemicals and reagents

The following (purified by column chromatography) standards were used: alkannin (Ikeda, Japan), shikonin (Ichimaru, Japan), acetylshikonin (ABCR GmbH, Germany), propionylshikonin (synthesized by Prof. Elias Kouladouros, Agricultural University of Athens, Greece), deoxyshikonin (TCI, Belgium), β,β -dimethylacrylshikonin (ABCR GmbH, Germany) and isovalerylshikonin (TCI, Belgium). All used organic solvents (i.e., n-hexane 97%, methanol, trifluoroacetic acid (TFA) and acetonitrile (ACN) (VWR INTERNATIONAL, Leuven, Belgium) were HPLC/LC-MS grade. Water was purified and demineralized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA).

Samples preparation

For the experiment associating the native *R. irregularis* strain and *R. irregularis* MUCL 41833 with hairy roots of *A. tinctoria*, root samples and MSR medium from RC and HC of the bi-compartmented Petri plates were freeze-dried separately and reduced into powder using liquid nitrogen, mortar and pestle. For each plant/replicate, 50 mg of each root material was subjected to a 20-min ultrasound-assisted extraction (two cycles), using n-hexane 97% (1 mL) at room temperature (25-30°C). The samples were finally centrifuged at 1500 rpm for 10 min at 4°C and the supernatants of each cycle were combined and evaporated at room temperature. The extraction protocol was adapted from Bossard et al. (2022). Prior to analysis, the root extracts were weighed and solubilised in methanol and were filtered through a 20- μ m PTFE membrane (WhatmanTM). Each sample was adjusted to the final concentration of 2 mg mL⁻¹, using methanol LCMS-grade.

For the MSR medium from RC and HC of each treatment, 50 mg of the powder was subjected to a 20-min ultrasound-assisted extraction using 60% acetonitrile (1.5 mL) at room temperature (25-30°C). The samples were finally centrifuged at 1500 rpm for 10 min at 4°C and the supernatants were collected and evaporated in speedvac. The remaining 100 μ L solution was centrifuged and collected for the LC-MS analysis.

RESEARCH RESULTS-PART I-CHAPTER III

LC/MS analysis

Alkanin/shikonin and their derivatives (deoxyshikonin, isovalerylshikonin, dimethyl-acryl shikonin and acetyl-shikonin) were identified and quantified by UPLC-MS. Peak areas of extracted ion chromatograms of the different molecules were compared with calibration curves previously made with 10 μL of each commercial standard (as described above) injected at concentration ranging from 1 μM to 40 μM . The analyses were performed using a Agilent 1290 Infinity II HPLC system coupled to mass detector (Jet Stream ESI-qTOF 6530, Agilent) in negative mode with MS parameters set up as follows : capillary voltage: 3.5 kV; nebulizer pressure: 35 psi; drying gas: 9 l min^{-1} ; drying gas temperature: 250°C; flow rate of sheath gas: 11 mL min^{-1} ; sheath gas temperature: 350°C; fragmentor voltage: 135 V; skimmer voltage: 65 V; octopole RF: 750 V. Accurate mass spectra was recorded in the range of m/z =50–1200. Separation was performed using a C18 Acquity UPLC BEH column (2.1 $\times$ 50 mm $\times$ 1.7 μm ; Waters) and 0.1% formic acid (solvent A) acetonitrile acidified with 0.1% formic acid (solvent B) as mobile phase with constant flow rate at 0.4 mL min^{-1} and column temperature set at 40°C. First, gradient raising from 60% to 65% B was applied during 1.38 min before increasing to 85% B in 2.12 min. Then, 100% solvent B was applied for 4 min before going back to initial conditions and kept as such for 4 min. Masshunter Qualitative Analysis software (Agilent) was used for data analysis.

Statistical analysis

Data were analysed using JMP 16.0 and IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA) software. Levene and Shapiro-Wilk tests were run prior to the analysis to confirm homogeneity of variance and normality of distribution. To fulfill the assumptions of the method, data were log10 or root transformed, prior to the analysis. If data remained non-normal after transformation, they were analyzed by Kruskal-Wallis. Dunn test, a non-parametrical test for pairwise or with a control comparison, was used to identify significant differences ($p \leq 0.05$) between treatments.

Data for root colonization percentages were analyzed using a one-way ANOVA followed by comparison using a Students'-test ($p \leq 0.05$). Data for A/Sd concentration in AMF colonized and non-colonized root samples

RESEARCH RESULTS-PART I-CHAPTER III

was analyzed using Kruskal–Wallis non-parametrical test followed by a pairwise comparison Dunn test ($p \leq 0.05$). Data for AMF spore germination rate for each strain and each plant hairy root were analyzed using a two-ways contingency. Fisher's exact test was used to test the independence of the variable.

Results

Exp1: AMF root colonization

After 17 months of association, the %TC, %AC and %VC of the hairy roots of *A. tinctoria* associated with the native *R. irregularis* strain was 15.6 ± 5.7 , 1.2 ± 1.5 and 6.5 ± 2.9 %, respectively. Conversely, no root colonization was observed with *R. irregularis* MUCL 41833, although spores germination was observed. Curiously, some spores of the two strains used as inoculum had a reddish color 3 weeks after their association with the hairy roots of *A. tinctoria* (**Figure 31**).

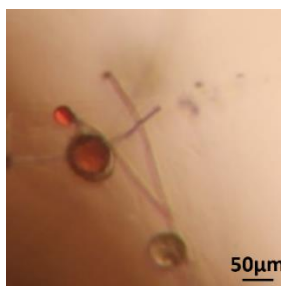


Figure 31: Reddish color of spores of the native *R. irregularis* strain associated with *A. tinctoria* hairy roots *in vitro*.

Effect of AMF on A/Sd concentration in *A. tinctoria* hairy roots

After 17-month of association, the A/Sd concentration of the hairy roots of *A. tinctoria* associated with the two strains of AMF was assessed (**Table 16**). Four A/Sd compounds (i.e., Dimethylacrylshikonin, Acetylshikonin, shikonin and Isovalerylshikonin) were detected in the hairy roots. None of the two strains tested affected significantly the concentration of these compounds as compared to the control treatment. Moreover, no A/Sd compounds were detected in the MSR medium of the RC and HC.

RESEARCH RESULTS-PART I-CHAPTER III

Table 16: A/Sd concentration ($\mu\text{g/g}$) in hairy roots of *A. tinctoria* after 17 months of association with the native strain of *R. irregularis* or the strain *R. irregularis* MUCL 41833.

Treatment	Dimethylacrylshikonin	Acetylshikonin	Shikonin	Isovaleryshikonin
Control	5.71157E-05 $\pm$	3.6333E-06 $\pm$	3.45948E-07 $\pm$	4.34787E-05 $\pm$
	2.37797E-05	1.78362E-06	1.72974E-07	1.64355E-05
MUCL 41833	4.96336E-05 $\pm$	3.6333E-06 $\pm$	3.45948E-07 $\pm$	3.77165E-05 $\pm$
<i>R. irregularis</i>	2.88171E-05	1.3212E-06	1.15316E-07	1.9513E-05
Native	4.02254E-05 $\pm$	3.03876E-06 $\pm$	2.8829E-07 $\pm$	3.04482E-05 $\pm$
	<i>R. irregularis</i> 6.74128E-06	1.91574E-06	1.15316E-07	5.50032E-06
Wilcoxon / Kruskal-Wallis test				
P-value	0.595	0.761	0.863	0.486

The parameters measured are expressed as mean $\pm$ standard deviation (SD) of 6 replicates per treatment. P-values higher than 0.05 showed no significant impact between treatments (Wilcoxon/Kruskal-Wallis test).

RESEARCH RESULTS-PART I-CHAPTER III

Exp2: Effect of *A. tinctoria* hairy roots on AMF spore germination

Spores of the AMF strains were placed close to the hairy roots of *A. tinctoria* and carrot (as control) and germination rate evaluated after 5 weeks. The germination rate of the native strain *R. irregularis* and the strain *R. irregularis* MUCL 43194 was not impacted in presence of *A. tinctoria* or carrot hairy roots (**Table 2**). No significant differences were further observed between the two stains, whatever the hairy root. Conversely, the germination rates of the strain *R. irregularis* MUCL 41833 were significantly lower compared to the other strains in presence as well as absence of hairy roots (**Table 17**). Moreover, the germination rates were significantly higher in the presence of the hairy roots as compared to the control without any root.

Table 17: Germination rate of spores of three strains of *R. irregularis* cultured in the presence of hairy roots of *A. tinctoria* or carrot or in absence of roots, after 5 weeks under *in vitro* culture conditions.

Treatment	<i>A. tinctoria</i> (%)	Carrot (%)	No root (%)
Native <i>R. irregularis</i>	100	100	100
<i>R. irregularis</i> MUCL 41833	80*,b	71*,b	8* ^a
<i>R. irregularis</i> MUCL 43194	96	96	96
Fisher's exact test			
P-value	<,0005	<,0001	<,0001

P-value lower than 0.05 showed a significant impact on spore germination between the AMF strains according to Fisher's exact test of independence. Values in the same column followed by * indicate significant difference ($p \leq 0,05$). Values in the same lines followed by lower-case letter indicate significant difference ($p \leq 0,05$).

Discussion

In the present study, the objectives were to evaluate (1) whether AMF could increase the concentration of A/Sd in hairy roots of *A. tinctoria* and (2) if exudates of *A. tinctoria* could have an impact on spore germination of AMF. Three *Rhizophagus irregularis* strains were considered and compared: a

RESEARCH RESULTS-PART I-CHAPTER III

native strain isolated from the roots of *A. tinctoria* plants grown in nature and two strains (MUCL 41833 and MUCL 43194) from the GINCO. After 17 months of association, no significant difference in A/Sd concentration was observed in the hairy roots of *A. tinctoria* associated or not with the native or MUCL 41833 strains. Interestingly, some reddish spores were observed in both strains after three weeks of association with *A. tinctoria* hairy roots. It is not excluded that A/Sd accumulated in spores, thus impacting their viability. Moreover, this accumulation may have hindered the potential role of hyphae to transport A/Sd from RC to HC. Indeed, no A/Sd were detected in the MSR medium of the RC and HC. No impact of the hairy roots was observed on the germination rate of the native *R. irregularis* strain and MUCL 43194, while it was significantly lower for MUCL 41833. Curiously, spore germination rate of this strain was significantly higher in presence of *A. tinctoria* and carrot root compared to the control (i.e., in the absence of roots).

Impact of hairy roots of *A. tinctoria* on AMF root colonization

Root colonization of *A. tinctoria* was observed with the native strain of *R. irregularis*, while it was absent with the strain MUCL 41833. This suggested a specificity between the native strain and its host plant. This was consistent with the study by Zhao et al. (2023) where the same native *R. irregularis* associated with *A. tinctoria* plants under pot or semi-hydroponic condition showed a higher AMF colonization compared with AMF from the GINCO. Few spores of MUCL 41833 germinated, suggesting an impact of *A. tinctoria* roots on this AMF and thus reducing considerably the possibility of colonization. However, it is not excluded that MUCL 41833 failed to germinate because of the state of the culture at the time of the experiment, as sometimes observed. It would have been nice to have a control, consisting of hairy roots of carrot on which this fungus is associated routinely in GINCO, to explore this hypothesis. Compared with pots or semi-hydroponic cultivation systems (Zhao et al., 2023), the association with root organs resulted in drastically lower root colonization, as noticed with the native *R. irregularis* strain. It is not unusual to have low root colonization *in vitro*. However, the extreme poor colonization in hairy roots of *A. tinctoria* may be related to the production of A/Sd during the first weeks (with viable growing roots) impacting germination and colonization. After several weeks, it could have been related to the aging

RESEARCH RESULTS-PART I-CHAPTER III

(and lignification) of the roots, not allowing further colonization. Curiously, some spores of both strains showed reddish color when associated with *A. tinctoria* hairy roots. We speculated that A/Sd may have accumulated in the spores impacting their capacity to germinate and colonize the roots. This corroborates the study of Brigham et al. (1999), showing that *Rhizophagus intraradices* was able to accumulate shikonins in their spores. This accumulation was observed in less than 5% of the spores, suggesting a sequestration mechanism to protect the other spores produced in the cultures (Brigham et al., 1999). Indeed, it has been reported that these naphthoquinone derivatives in the shikonin family have antimicrobial activity against many soil microorganisms (Brigham et al., 1999; Mone et al., 2021; Yadav et al., 2022; Pang et al., 2023). For instance, *in vitro* antifungal activity of shikonin against *Candida albicans* has been reported through induction of cellular apoptosis and necrosis (Pang et al., 2023). In our study, the development of mycelium in the RC and HC was very low for the native strain and even inexistent for MUCL 41833. This suggested A/Sd accumulated in the AMF spores could be toxic to the AMF colonization capacity, limiting the growth and development of the AMF in the RC and HC. The slight growth and colonization of the native strain, further support the hypothesis that this fungus is more adapted to *A. tinctoria* than the GINCO strain, possibly related to a co-evolution in the field.

Impact of AMF on A/Sd concentration in *A. tinctoria* hairy roots

A number of studies have reported the impact of AMF on the production of SMs in medicinal plants grown in pots or field (Zhao et al., 2022), but none considered hairy roots.

In the present study, neither the native strain *R. irregularis* nor the strain *R. irregularis* MUCL 41833 increased the concentration of A/Sd in hairy roots of *A. tinctoria* after 17-month association. The most probable hypothesis is that the hairy roots died after such a long period of time, the MSR medium being exhausted in carbon and minerals, resulting in the degradation of the A/Sd in the roots and medium. It is well-known that A/Sd compounds are susceptible to light exposure (Cho et al., 1999; Cheng et al., 1995), to heat degradation (Cho et al., 1999) and have the tendency to polymerize in alkaline media, polar solvents and under high temperature (Assimopoulou and Papageorgiou 2004b). It has also been reported that

RESEARCH RESULTS-PART I-CHAPTER III

ammonium or benzoic acid could inhibit the production of these compounds (Brigham et al., 1999; Pal et al., 2019). During the summer season 2023, there was a higher temperature (around 30°C) outside and inside the incubator room. This high temperature may have degraded the A/Sd compounds. However, Skoneczny et al. (2019) noticed the reverse. They reported that the roots of *Echium plantagineum* exposed to a temperature regime of 30/25°C had an increased accumulation of deoxyshikonin over time in contrast to roots produced at 20/15°C in pot culture (Skoneczny et al., 2019). These contradictory results may be related to the conditions under which both experiments were conducted. In the study of Skoneczny et al. (2019), the plants were grown in pots for a short period (35, 49, 56 and 63 days respectively), while in our study, the plants were grown *in vitro* for a long period (i.e., 17 months). Skoneczny et al. (2019) further observed that *Echium plantagineum* plants grown under water stress had elevated levels of deoxyshikonin, suggesting the impact of water withholding on the accumulation of A/Sd. So, we speculated that the duration of the present experiment was too long, resulting in the degradation of A/Sd compounds under high humidity in the Petri plates (the MSR medium is made of almost 99% of water).

There was no A/Sd compounds detected in the RC and HC of the MSR medium. This was probably due to the degradation of the A/Sd in the MSR medium after such a long period of time as discussed before. Another reason could be due to the fact that the A/Sd were sequestered in the AMF spores as discussed above and were not leached in the MSR medium leading to the absence of detection of these compounds. Finally, it is not excluded that the amount of A/Sd in the growth medium was under the limit of detection with the LC-MS.

Impact of *A. tinctoria* hairy roots on AMF spore germination

Spores of AMF are able to germinate in the absence of signals from a suitable host plant (Kowalczyk and Hryniewicz, 2018). This was also shown in the present study. Indeed, for the three AMF strains tested, germination of spores was observed in the absence of roots. However, a significantly lower germination rate was observed for *R. irregularis* MUCL 41833 compared with the two other strains in presence as well as absence of roots. Moreover, with this AMF, spore germination was dramatically lower in absence of roots compared to the treatments with *A. tinctoria* or

RESEARCH RESULTS-PART I-CHAPTER III

carrot root. This suggest that the hairy roots may stimulate the germination of this fungus. This supports the literature showing that some metabolites released by roots [e.g. strigolactones (Akiyama and Hayashi, 2006; Lidoy et al., 2023)] may stimulate germination of spores and hyphal branching. However, this was not observed with the two other AMF. Therefore, the reason for a lower germination of *R. irregularis* MUCL 41833 can not exclude another factor related with the physiological state of this specific culture, which led to almost no association with *A. tinctoria* hairy root in the first *in vitro* experiment. Finally, whatever the AMF, no significant difference in germination rate was noticed when associated with *A. tinctoria* and carrot. This seems to support the hypothesis that the host root had no or limited impact on spore germination. And no effect of hairy root on AMF spore germination can be concluded from this study. However, more diverse of AMF species should be included to investigate the impact of *A. tinctoria* hairy root on AMF spore germination.

Conclusion

For the first time, this study investigated the impact of two AMF strains of *R. irregularis* (native and MUCL 41833) on the concentration of A/Sd in transformed hairy roots of *A. tinctoria* and impact of these root organs on spore germination of three *R. irregularis* strains (native, MUCL 41833 and MUCL 43194 *R. irregularis*). No significant impact of AMFs (native and MUCL 41833 strains) was observed on A/Sd concentration in roots which may be attribute to the age of the roots and their probable death and subsequent degradation of the A/Sd in the roots and medium after such a long period of time. A significantly higher AMF colonization with the native *R. irregularis* strain compared with *R. irregularis* MUCL 41833 suggest that the native strain was probably better adapted to *A. tinctoria*, which is consistent with previous studies carried out under pot and semi-hydroponic cultivation systems. The hairy roots (and potential exudates) had no impact on spore germination of the AMF (native, MUCL 41833 and MUCL 43194). The lower germination rate of MUCL 41833 versus the two other strains was thus probably attributed to the physiological state of this specific culture. Since A/Sd are light and temperature sensitive, it is advised to conduct further research within a shorter time frame to evaluate the effect of AMF on these secondary metabolites (i.e., A/Sd) production in *A. tinctoria* hairy roots.

RESEARCH RESULTS-PART II

PART II *ECHIU* *VULGARE* STUDY

RESEARCH RESULTS-PART II

Preface

Part I of the thesis was focused on *A. tinctoria*. However, this plant is characterized by a very low seed germination rate and its cultivation under conventional agriculture practices is hampered by several constraints. Therefore, **Part II** of the thesis was focused on *E. vulgare*.

Endophytic fungi colonizing internal living plant tissues without causing any visible manifestation of disease build a relationship with their host plants ranging from mutualistic or symbiotic to antagonistic or mildly pathogenic. Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota, are another important group of root inhabitants. All plant species, including medicinal plants, form associations with one or the other or both types of fungi. Many studies have shown that these microorganisms can improve the production of secondary metabolites in plants.

In **chapter IV**, endophytic fungi isolated from *A. tinctoria* plants collected from the wild in Greece were identified by molecular methods and mass-produced. Ten endophytic fungi were selected and together with 5 AMF strains from the GINCO were inoculated on *E. vulgare* plants in pots under greenhouse conditions. The objective was to investigate their effects on (i) plant growth and (ii) A/Sd production. Plant growth parameters were assessed and quantification of A/Sd and their identification was performed by HPLC-PDA.

Chapter IV Effects of fungal endophytes and arbuscular mycorrhizal fungi on growth of *Echium vulgare* and alkannin/shikonin and their derivatives production in roots

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Abstract

Endophytic fungi as well as arbuscular mycorrhizal fungi (AMF) are known to stimulate plant growth and production of secondary metabolites in medicinal plants. Here, 10 endophytic fungi isolated from roots of wild *Alkanna tinctoria* plants and 5 AMF purchased from the Glomeromycota *in vitro* collection were evaluated, during two successive three-month greenhouse experiments, on the growth of *Echium vulgare* and alkannin/shikonin and their derivatives (A/Sd) production in the roots. Some of the endophytic fungi tested significantly increased plant growth parameters as compared to the control: *Cladosporium allicinum*, *Cadophora* sp., *Clonostachys* sp., *Trichoderma hispanicum* and *Leptosphaeria ladina* increased root volume, *Plectosphaerella* sp. and *T. hispanicum* root fresh weight and root water retention and *T. hispanicum* plant water retention. However, none of these fungi impacted A/Sd production. Conversely, none of the AMF strains tested impacted plant growth parameters, but those inoculated with *R. intraradices* MUCL 49410 had a significantly higher concentration of alkannin/shikonin (A/S), acetyl-A/S, β,β -dimethylacryl-A/S, isovaleryl-A/S and total A/Sd, compared to the control plants. Further studies are needed to investigate the mechanisms involved in the production of A/Sd in plants associated to specific endophytic fungi /AMF and on the cultivation conditions required for optimal production of these compounds.

Keywords

Endophytic fungi, Arbuscular mycorrhizal fungi, *Alkanna tinctoria*, *Echium vulgare*, Alkannin/Shikonin and their derivatives

Introduction

Plants host diverse communities of microorganisms which provide them with several benefits, including growth promotion, nutrients uptake, resistance/tolerance to abiotic and biotic stresses (Noman et al., 2021). Among these microorganisms are the endophytic microfungi (mainly members of the Ascomycota) that colonize internal living plant tissues such as roots, stems and/or leaves without causing any visible manifestation of disease (Wilson 1995). They build a relationship with their host plants ranging from mutualistic or symbiotic to antagonistic or mildly pathogenic

RESEARCH RESULTS-PART II-CHAPTER IV

(Arnold 2007). Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota (Schüßler et al., 2001), are another important group of root inhabitants, forming symbiosis with above 72% of vascular plants (Brundrett and Tedersoo 2018). All plant species, including medicinal plants, form associations with one or both types of fungi.

Medicinal plants are a valuable source of therapeutic agents to treat many ailments thanks to their different types of active secondary metabolites (SMs). In the last decade, several studies have focused their attention on the relationships between endophytic fungi or AMF and medicinal plants. It has gradually been recognized that fungal endophytes not only produce SMs that their host plants originally produce, but also play a very important role in influencing the production of metabolites in plants affecting the quality and quantity of the crude drugs produced (Jia et al., 2016). For instance, endophytic fungi have been reported to promote the accumulation of bioactive compounds in *Wollemia nobilis* (Strobel et al., 1997), *Phellodendron amurense* (Duan 2009), *Cinchona ledgeriana* (Maehara et al., 2012) or *Piper nigrum* (Chithra et al., 2014). Besides, it has been shown that endophytic fungi (i.e., *Catharanthus roseus*, *Coleus forskohlii* and *Macleaya cordata*) isolated from the medicinal plant *Salvia sp.*, were able to synthesize pharmacologically active SMs identical to those produced by their host (Jia et al., 2016). Similarly, growing evidence suggest that AMF can directly or indirectly improve the production and accumulation of important active ingredients (e.g., terpenes, phenols, alkaloids) in medicinal plants and ultimately improve the quality of herbal drugs (Araim et al., 2009; Yaghoub and Weria 2013; Zeng et al., 2013).

Alkanna tinctoria belongs to the Boraginaceae family and is native to Europe, the Mediterranean basin and Western Asia (Salimikia et al., 2015). It produces more alkannin/shikonin (A/S) and A/S derivatives (A/Sd) in its roots compared to other plants of the family Boraginaceae (Rat et al., 2021). These SMs possess multiple biological activities (e.g. wound healing, anti-microbial, anti-tumor) (Papageorgiou et al., 1999) and are therefore of commercial interest. Many studies have focused their attention on the production of shikonin in cell cultures of *L. erythrorhizon*, and other plants from the Boraginaceae. Several factors (e.g. light, methyl jasmonate, nitric oxide, ethylene and fungal elicitors) have been identified as crucial regulators of shikonin biosynthesis (Yazaki et al., 1999, 1997b; Dong et al.,

RESEARCH RESULTS-PART II-CHAPTER IV

1990). Interestingly, Mollaei et al. (2019) isolated endophytic fungi from roots of *Lithospermum officinale* L. (another plant species from the boraginaceae family) and found that shikonin could also be produced by *Fusarium tricinctum*.

So far, there is no study focusing on the isolation of endophytic fungi from *A. tinctoria* and the relationship between endophytic fungi and this plant. *Echium vulgare* L., native to most of Europe as well as western and central Asia (Kapusterynska et al., 2020) also belongs to the family Boraginaceae and produces A/Sd in its roots. Both plants have many medicinal properties but the production of *E. vulgare* is much easier than *A. tinctoria*. This plant has therefore been considered in the present study aimed to investigate the effects of (1) endophytic fungi isolated from *A. tinctoria* in Greece (Rat et al., 2021) and (2) AMF from the Glomeromycota *In vitro* Collection (GINCO – <http://www.mycorrhiza.be/ginco-bel>), on plant growth and A/Sd production in roots of *E. vulgare*. The study was conducted under greenhouse conditions in two successive experiments, firstly with the endophytic fungi and secondly with the AMF. To address this objective, the quantification of A/Sd was performed through High-Performance Liquid Chromatography coupled with Diode Array detection (HPLC-DAD).

Materials and Methods

Endophytic fungi

Isolation of endophytic fungi from *Alkanna tinctoria* roots – In October 2017, twelve plants of *A. tinctoria* were collected from nature in Thessaloniki (Greece), 600 m northwest of the Theatre of the Earth (Theatro Gis) at an altitude of 130 m, latitude of 40°63' and longitude of 22°97'. The climate is Mediterranean with an average annual rainfall and temperature of 445 mm and 15.9°C, respectively. These plants were used for the first-round isolation of endophytic fungi. A second-round of isolation was conducted in April 2018. Six *A. tinctoria* plants were collected in Southern Greece (at the University of Athens, Campus of Zografou, Athens), at latitude 37° 59' and longitude 23° 43'. The mean annual rainfall and temperature is 376.2 mm and 18.8°C, respectively.

Root surface sterilization – Roots of *A. tinctoria* plants were carefully selected and surface-cleaned with tap water. For thick roots, bark was

RESEARCH RESULTS-PART II-CHAPTER IV

removed before surface disinfection. The roots of each plant were placed in 50 mL Falcon tubes and surface-disinfected by bathing in 70% ethanol for 30 s, 1%NaClO for 3 min, 95% ethanol for 5 min and finally in sterile (121°C for 15 min) water for three successive rinses. During each step, the Falcon tubes were shaken to remove debris attached to the roots. Finally, the roots were gently dried with sterilized paper (121°C for 15 min) and cut into 2-3 mm long pieces. Five pieces from each root segment were placed in Petri dishes (90 mm diameter) on Malt Extract Agar (MEA) or Potato Dextrose Agar (PDA) supplemented with chloramphenicol (50 µg mL⁻¹) to prevent bacterial growth. To check the efficacy of the surface sterilization, 100 µL of the last rinsing sterilized water was spread on the isolation agar. The Petri dishes were incubated at 25°C in the dark and observed daily for mycelium growth. Hyphal tips growing out of the root segments were cut with a sterile scalpel and transferred to fresh growth medium (MEA or PDA) for fungal isolation.

Single- conidium culture establishment – Single-conidium cultures were obtained for each isolate by using a sterilized loop, scraping and dipping the fungal mycelium in peptone water, then spreading the solution on a thin layer of PDA. The Petri dishes were incubated at 25°C until conidia production. Conidia were then germinated and single colony was gently picked one by one and transferred on a fresh PDA medium to establish a pure culture.

For the non-sporulating isolates, one plug of the growth medium containing the fungal hyphae was transferred on oatmeal agar (OA) or V8 juice agar (V8) media to induce sporulation. Cultures that repetitively failed to sporulate on different media were not considered in the study.

Molecular identification of endophytic fungi – Genomic DNA of endophytic fungi was extracted from innuPREP Plant DNA kit (IST Innuscreen GmbH) according to the manufacturer's instructions, to yield a total volume of 70 µL purified DNA. Internal transcribed spacer (ITS) region using universal primers ITS4 and ITS5 were amplified by PCR for the sporulating endophytic fungi (White et al., 1990). For the non-sporulating endophytic fungi, ITS and 28S rDNA region using primers ITS4/ITS5 and LROR/LR6 (Raja et al., 2017), respectively were amplified by PCR. In both amplifications, the reaction mix contained Phusion High Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific,

RESEARCH RESULTS-PART II-CHAPTER IV

Lithuania) with 0.5 μM concentration of each primer (Sigma, Germany) and 0.2 $\mu\text{g mL}^{-1}$ BSA (Albumin Bovine, AMRESCO, United States). For each PCR reaction mix, 5 μL of template DNA (as above), 10 μL of Phusion High-Fidelity PCR Master Mix, 1 μL of ITS4/ITS5 and LROR/LR6 primer, and 3 μL of ultra clean water (Sigma) were used in 20 μL of final reaction. Thermal cycling was performed in an Eppendorf Master-cycler Gradient (Eppendorf Nexus X2, Germany) using the following parameters: initial denaturation of 3 min at 94°C, followed by 30 cycles of denaturation (1 min at 94°C)/ annealing (1.30 min at 56°C)/ elongation (2 min at 72°C), and a final elongation of 10 min at 72°C. PCR products for endophytic fungi were visualized on 1.0 % agarose gel with 1 $\times$ TAE buffer and GelRedTM Nucleic Acid Gel Stain, 10,000 $\times$ in water (Biotium, USA) with the following migration parameters: 100 V, 200 mA for 30 minutes and visualization under camera UV light. After confirmation, amplicons were sequenced with the same primers bi-directionally a single time in Macrogen Inc. (Korea), and the resulting sequences were aligned and edited in Sequencher 5.4.6 version (Gene Codes Corporation, USA). The sequences were confronted with those in GeneBank database (<http://www.ncbi.nlm.nih.gov>), using BLASTN 2.2.28.

Inoculum preparation of endophytic fungi – Only the fungal endophytes producing conidia on OA and V8 were considered. After three weeks, conidia were scraped from the medium and suspended in sterile tap water. A Thoma hemocytometer was used to count the conidia concentration through the formula:

$$\frac{\text{Number of conidia}}{\text{Counted area (mm}^2\text{)} \times \text{chamber depth (mm)} \times \text{dilution}} = \text{conidia per } \mu\text{L of suspension}$$

Finally, approximately 10^6 conidia of each fungal strain was prepared and suspended in 5 mL of sterilized tap water and stored at +4 °C one day before being used to inoculate plant roots.

Arbuscular mycorrhizal fungal strains

Rhizophagus clarus (TH Nicolson and NC Schenck) C. Walker and A. Schüßler MUCCL 46238, *Rhizophagus irregularis* (Błaszk, Rbet, Renker

RESEARCH RESULTS-PART II-CHAPTER IV

and Buscot) C. Walker and A. Schüßler as ['irregulare']) MUCL 41833, *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker MUCL 49408, *Rhizophagus intraradices* (NC Schenck and GS Sm) C. Walker and A. Schüßler MUCL 49410 and *Glomus hoi* (Berch and Trappe) MUCL 45686, were provided by the Glomeromycota *in vitro* collection (GINCO, Belgium). They were provided in bi-compartmented Petri plates on root organ cultures (ROCs) of carrot (*Daucus carota* L.) clone DC2. They were kept in a growth chamber in the dark at 27 °C until thousands of spores were produced.

Echium vulgare

Commercial seeds of *Echium vulgare* were purchased from Templiner Kräutergarten (Germany). The seeds were surface-disinfected with 70% ethanol, rinsed with sterilized (121 °C for 15 min) water and germinated on filter papers in a plastic box (0.3 L). After 20 days, each seedling was transferred in a 2 L pot containing a mixture of calcinated clay (Park et al., 2010) and quartz (0.4-0.8mm) at a volume ratio 1:1.

The plants were grown in the greenhouse set at 16h light/8h dark photoperiod, 20°C with 50 % relative humidity (RH) and a photosynthetic photon flux density (PPFD) of 96 $\mu\text{mol m}^{-2}\text{s}^{-1}$. After one week of growth, they were inoculated with the endophytic fungi or AMF (see below). The plants were placed at random and moved twice a week from week 1 to 8 and once a week from week 9 to 12. They were watered 2 or 3 times a week with 150 mL deionized water. After 20 days, 150 mL of Hoagland solution (Hoagland and Arnon 1950) modified with low P and without ammonium nitrate, at 200X dilution was added weekly.

Experiment 1: Impact of fungal endophytes on the growth of *E. vulgare* and A/Sd production in roots

From the endophytic fungi isolated, only those that were able to produce conidia in OA and V8 medium were selected to evaluate their effect on *E. vulgare* plant growth and A/Sd production in roots. In total, 10 endophytic fungal strains were selected (**Table 18**). For each fungal strain, two-week old *E. vulgare* plantlets were inoculated with 5 mL conidia suspension (10^6 conidia mL^{-1} water) of three weeks old fungal cultures prepared as described above. Conidia suspension was gently pipetted into the substrate

RESEARCH RESULTS-PART II-CHAPTER IV

in the vicinity of the plant roots. Non-inoculated plants were used as control. Six replicates were considered per treatment.

RESEARCH RESULTS-PART II-CHAPTER IV

Table 18: Endophytic fungi considered to assess *Echium vulgare* plant growth and A/Sd production in roots.

Phylum /class	Isolate number	Closest match with online data (NCBI)	Primers Used
Ascomycota	10	<i>Plectosphaerella sp.</i>	ITS, ITS4/ITS5
	11	<i>Cladosporium allicinum</i>	ITS, ITS4/ITS5
	13	<i>Cadophora sp.</i>	ITS, ITS4/ITS5
	14	<i>Clonostachys sp.</i>	ITS, ITS4/ITS5
	16	<i>Trichoderma hispanicum</i>	ITS, ITS4/ITS5
	17	<i>Cadophora luteo-olivacea</i>	ITS, ITS4/ITS5
	94	<i>Phialophora sp.</i>	ITS, ITS4/ITS5
	259	<i>Scytalidium sp.</i>	28S, LROR/LR6
	265	<i>Fusarium acuminatum</i>	ITS, ITS4/ITS5
	476	<i>Leptosphaeria ladina</i>	28S, LROR/LR6

After inoculation, the plants were maintained under the same greenhouse conditions and watered as described above. The plants were harvested after 3 months. Shoot and roots were separated and shoot fresh weight (SFW) measured. Five g of fresh shoot was then placed in a plastic bag and oven-dried (at 60°C for 2 days) to extrapolate to total shoot dry weight (SDW). The remaining shoot material was placed in a 250 mL tube and lyophilized. Roots were carefully cleaned from substrate in 3 L demineralized water. Root volume (RV) was estimated by putting the root system in a cylinder of 500 mL containing 200 mL demineralized water. Each root system was surface-dried with paper and root fresh weight (RFW) measured. Five g of each root system was homogenously collected in a plastic bag and dried in the oven (at 60°C for 2 days) to extrapolate to total root dry weight (RDW). The remaining root material was kept in the 250 mL tube and stored at -

RESEARCH RESULTS-PART II-CHAPTER IV

20°C for lyophilization. In addition, root water retention (RWR) and plant water retention (PWR) were measured.

Experiment 2: Impact of AMF on *E. vulgare* plant growth and A/Sd production in roots

Each AMF strain was maintained *in vitro* in bi-compartmented Petri dishes (90 mm diameter) (Declerck et al., 1996) in association with Ri T-DNA transformed carrot (*Daucus carota* L. clone DC2) or chicory (*Cichorium intybus* L.) roots on the modified Strullu-Romand (MSR) medium (Declerck et al., 1996).

For each strain, the MSR medium from the root-free compartment of the bi-compartmented Petri dishes, was collected, cut with a scalpel into small pieces and mixed homogenously to recover the spores and hyphae. Around 1000 spores were then inoculated in the planting hole of two-week old seedlings of *E. vulgare*. The controls consisted of plants inoculated with MSR medium free of AMF. Nine replicates were considered per treatment. Plants were watered and maintained as described above.

The plants were harvested after 3 months following the same procedures as in experiment 1. The same plant growth parameters as in experiment 1 were measured. Roots were also used to evaluate root colonization (see below).

AMF root colonization – Root colonization by AMF was evaluated as follows: each root sample was stained using the protocols of Vierheilig et al. (1998) and Walker (2005). In brief, roots were first cleaned with water, then cleared in 10% KOH for 30 min at 70°C, bleached in 3.5 % H₂O₂ for 5 min at 70°C, neutralized in 1% HCl for 1 min at room temperature and finally stained in 2 % Parker Super Quink Permanent Ink in 1% HCl for 30 min at 70°C. Stained roots were maintained in lactoglycerol (1:1:1-lactic acid: glycerol: H₂O, v:v:v). Forty root fragments of 1 cm length were then used to evaluate root colonization. The percentages of total colonization (%TC), arbuscules (%AC) and spores/vesicles (%VC) colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10x magnification according to McGonigle et al. (1990). Around 300 intersections were observed per plant. At each intersection the presence or absence of AMF was recorded.

Quantitative and qualitative analysis of A/Sd in *E. vulgare* roots

RESEARCH RESULTS-PART II-CHAPTER IV

following inoculation of endophytic fungi or AMF

Chemicals and reagents – The following standards were used for the identification and quantification of alkannins/shikonins: shikonin (Ichimaru, Japan), acetylshikonin (ABCR GmbH, Germany), propionylshikonin (synthesized by Prof. Elias Kouladouros, Agricultural University of Athens, Greece), deoxyshikonin (TCI, Belgium), β,β -dimethylacrylshikonin (ABCR GmbH, Germany) and isovalerylshikonin (TCI, Belgium). LC-MS grade methanol (Honeywell Riedel de Haën, USA) was used as the extraction solvent. Acetonitrile (HPLC-grade, Honeywell Riedel de Haën, USA), ultrapure water (Merck Millipore, Germany) and formic acid (HPLC-grade, Merck KGaA, Germany) were used as mobile phase components in the HPLC-DAD analysis.

Sample preparation – Samples of *E. vulgare* roots were freeze-dried and stored at -20°C prior to being ground to a fine powder using a ball mill (Fritsch Pulverisette 0, Germany). Leachates were freeze-dried into powder and stored at -20°C . Thirty-five mg of each powdered sample was placed in a microcentrifuge tube. Extraction was performed in 1.5 mL of methanol by ultrasound at 10% power for 3 h (Bandelin Sonorex Digital 10P, Germany) followed by centrifugation for 10 minutes at 12500 rpm (Hermle Z 216 MK, Germany). The supernatants were collected and subjected to HPLC-DAD analyses after filtering with $0.22\ \mu\text{m}$ syringe filters.

HPLC-DAD method for the quantitation of A/Sd – The method used to quantify A/Sd in the root samples was previously described in Varela Alonso et al. (2022). In short, the detection wavelength of 520 nm was chosen to quantify the concentrations of alkannins/shikonins (each naphthoquinone derivative separately, and total A/Sd concentration) in the methanolic root extracts. An ECOM HPLC-DAD instrument (model ECS05, Czech Republic) was used for the analyses, utilizing a Fortis SpeedCore C18 column (United Kingdom). Ultrapure water (A) and acetonitrile (B) made up the mobile phase, with 0.1% formic acid added. Elution was performed using the following solvent gradient: 0 min 30A/70B, 8 min 100B, 13 min 100B with $1\ \text{mL min}^{-1}$ flow rate. Prior to each injection, the column was equilibrated for 5 min with the initial solvent composition. Column temperature was kept at 35°C . Quantification of each A/Sd was performed by using calibration curves of A/Sd standards (shikonin, acetylshikonin, propionylshikonin, deoxyshikonin, β,β -

RESEARCH RESULTS-PART II-CHAPTER IV

dimethylacrylshikonin and isovalerylshikonin). Data acquisition and processing of raw HPLC data were accomplished through the Clarity software (DataApex, Czech Republic). The concentration of each A/S derivative and total A/Sd was calculated in ppm units, referring to the mass concentrations of compounds in the methanolic extracts prepared as described above. Additional calibration and method validation data is available in Annex III (**Table S9** and **Figure S13**).

Statistical analyses

Statistical analyzes were performed with JMP 16.0 and SAS 9.4 statistical software (SAS Institute Inc., Cary, NC, United States). Levene and Shapiro-Wilk tests were run prior to the analysis to confirm homogeneity of variance and normality of distribution. To fulfill the assumptions of the method, data were log10 or root transformed, prior to the analysis. If data remained non-normal after transformation, they were analyzed by Kruskal-Wallis test.

Plant growth parameters (i.e., SFW, RV, RFW and RDW), AMF colonization (i.e., %TC, %AC and %VC) and A/Sd concentration in roots, were analyzed using a one-way ANOVA followed by a pairwise comparison post-hoc Tukey HSD test ($p \leq 0.05$) to discriminate between means. A/Sd content in roots were analyzed using Kruskal-Wallis test.

Results

Experiment 1: Impact of fungal endophytes on the growth of *E. vulgare* and A/Sd production in roots

Impact of endophytic fungi on growth of *E. vulgare* – Three months after inoculation of the fungal endophytes, plant growth parameters were evaluated (**Table 19**). With the exception of SFW and SDW, a significant impact of the treatments was observed for all parameters. The plants inoculated with *C. allicinum*, *Cadophora* sp., *Clonostachys* sp., *T. hispanicum* and *L. ladina* had a significantly higher RV compared to the plants in the control treatment. The plants inoculated with *Plectosphaerella* sp. and *T. hispanicum* had a significantly higher RFW and RWR compared to the plants in the control treatment. *T. hispanicum* also significantly increased PWR compared to the control plants. Whatever the fungal inoculant, no detrimental effect was noticed compared to the plants in the

RESEARCH RESULTS-PART II-CHAPTER IV

control treatment.

RESEARCH RESULTS-PART II-CHAPTER IV

Table 19: Impact of fungal endophytes on root volume (RV), shoot fresh and dry weight (SFW and SDW, respectively), root fresh and dry weight (RFW and RDW, respectively), root water retention (RWR), plant water retention (PWR) of *Echium vulgare* after three months of growth in pots under greenhouse conditions.

Treatment	RV (mL)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	RWR (g)	PWR (g)
Control	20.17± 3.43 b	15.46± 1.70	3.00± 0.35	12.90± 1.62 b	2.22± 0.28	10.68± 1.62 b	23.14± 1.68 b
<i>Plectosphaerella</i> sp.	28.50±4.28 ab	14.97± 2.07	2.79± 0.47	19.04± 3.00 a	2.87± 0.56	16.16± 2.63 a	28.35± 2.76 ab
<i>Cladosporium allicinum</i>	30.67±4.23 a	14.56± 1.34	2.87± 0.19	18.12± 2.50 ab	3.76± 0.83	14.36± 2.13 ab	26.06± 2.95 ab
<i>Cadophora</i> sp. REF040	30.33± 6.44 a	15.31± 1.05	2.95± 0.35	18.52± 3.75 ab	3.61± 0.84	14.91± 3.27 ab	27.27± 3.73 ab
<i>Clonostachys</i> sp. G5182	29.33± 3.27 a	14.84± 1.11	2.77± 0.21	14.97± 4.82 ab	3.11± 1.31	11.87± 4.22 ab	23.93± 4.06 ab
<i>Trichoderma hispanicum</i>	33.50± 5.05 a	16.31± 2.26	3.00± 0.31	19.71± 3.65 a	3.01± 0.88	16.70± 2.84 a	30.00± 4.19 a
<i>Cadophora</i> sp.	27.83± 2.40 ab	16.06± 3.22	3.07± 0.52	17.73± 2.07 ab	3.25± 0.50	14.48± 1.94 ab	27.47± 3.72 ab
<i>Scytalidium</i> sp.	26.00± 4.47 ab	15.15± 1.34	2.95± 0.26	17.76± 1.21 ab	3.59± 0.56	14.17± 1.18 ab	26.36± 1.14 ab
<i>Fusarium acuminatum</i>	28.17± 4.22 ab	15.85± 1.24	3.09± 0.17	16.25± 1.75 ab	2.48± 0.41	13.77± 1.72 ab	26.53± 2.27 ab
<i>Leptosphaeria ladina</i>	32.67± 5.35 a	16.40± 1.21	3.07± 0.19	17.90± 3.24 ab	2.71± 0.54	15.19± 2.88 ab	28.52± 2.90 ab
<i>Phialophora</i> sp.	28.17± 4.45 ab	15.81± 1.08	2.87± 0.17	16.14± 2.65 ab	3.29± 1.54	12.86± 2.10 ab	25.80± 2.76 ab
Analysis of variance							
Prob. > F	0,0005*	0,6689	0,6474	0,0091*	0,0464*	0,0056*	0,0142*

The parameters measured are expressed as mean ± standard deviation (SD) of 6 replicates per treatment. The asterisks show the significant effect of the treatments according to one-way ANOVA ($p < 0.05$). Values in the same column, followed by different lowercase letters differ significantly according to the HSD Tukey post-hoc test ($p < 0.05$).

Impact of endophytic fungi on A/Sd production in roots - The impact of the fungal endophytes on the A/Sd concentration in plant roots are presented in **Table 20** and on A/Sd content in **Table S10** in Annex III. Overall, a significant effect of treatment was observed on the concentration of deoxy-A/S and isovaleryl-A/S. However pairwise comparisons did not show any differences between treatments. For the other A/S derivatives, the endophytic fungal strains did not impact their concentration in plant roots compared to the control plants (**Table 20**).

RESEARCH RESULTS-PART II-CHAPTER IV

Table 20: A/S, Acetyl-A/S, Deoxy-A/S, Isovaleryl-A/S, Propionyl-A/S, β,β -Dimethylacryl-A/S and total A/Sd concentrations (mg g^{-1}) in roots of *E. vulgare* inoculated with 10 fungal endophytes, after three months of growth in pots under greenhouse condition.

Treatment	A/S	Acetyl-A/S	Deoxy-A/S	Isovaleryl-A/S	Propionyl-A/S	β,β -Dimethylacryl-A/S	Total A/Sd
Control	0.42±0.60	1.69±1.91	ND	0.29±0.15	ND	ND	2.41±2.64
<i>Plectosphaerella</i> sp.	0.09±0.08	1.39±0.62	ND	0.26±0.11	ND	0.04±0.06	1.77±0.71
<i>Cladosporium allicinum</i>	0.18±0.24	0.80±0.56	ND	0.10±0.12	ND	0.01±0.03	1.10±0.81
<i>Cadophora</i> sp. REF040	0.08±0.06	0.77±0.71	ND	0.11±0.11	ND	ND	0.97±0.83
<i>Clonostachys</i> sp. G5182	0.08±0.05	0.60±0.27	ND	0.07±0.07	ND	ND	0.75±0.26
<i>Trichoderma hispanicum</i>	0.07±0.06	1.43±1.19	0.08±0.11	0.25±0.20	ND	0.03±0.05	1.86±1.55
<i>Cadophora</i> sp.	0.20±0.06	1.37±0.76	ND	0.21±0.11	ND	ND	1.77±0.85
<i>Scytalidium</i> sp.	0.26±0.21	1.23±0.95	ND	0.25±0.18	ND	ND	1.74±1.34
<i>Fusarium acuminatum</i>	0.10±0.08	1.80±0.69	0.08±0.11	0.34±0.10	ND	0.02±0.04	2.34±0.73
<i>Leptosphaeria ladina</i>	0.23±0.14	0.97±0.70	ND	0.10±0.09	ND	ND	1.30±0.89
<i>Phialophora</i> sp.	0.23±0.23	2.15±1.68	ND	0.34±0.29	ND	0.02±0.04	2.74±2.06
Analysis of variance							

RESEARCH RESULTS-PART II-CHAPTER IV

Prob. > F	0.3206	0.3354	0.0136*	0.0276*	-	0.3435	0.3108

ND: not detected

The parameters measured are expressed as mean $\pm$ standard deviation (SD) of 5 or 6 replicates per treatment. The asterisks show the significant effect of the treatments according to one-way ANOVA ($p \leq 0.05$).

4.2 Experiment 2: Impact of AMF on the growth of *E. vulgare* and A/Sd production in roots

AMF root colonization - The impact of AMF on root colonization are presented in **Table 21**. Whatever the treatment, a significant effect was observed for %TC, %AC and %VC. The plants inoculated with *R. irregularis* MUCL 41833 had a significantly higher %TC compared to those inoculated with *R. intraradices* MUCL 49410 and *G. aggregatum* MUCL 49408, but did not differ from the plants inoculated with *G. hoi* MUCL 45686 and *R. clarus* MUCL 46238. The %TC of the plants inoculated with *G. aggregatum* MUCL 49408 was significantly lower compared to the other treatments. Similarly, plants inoculated with *R. irregularis* MUCL 41833 had a significantly higher %AC compared to those inoculated with the other AMF. No significant differences were noted between *G. aggregatum* MUCL 49408, *G. hoi* MUCL 45686 on one side and the two other AMF on the other side, while *R. clarus* MUCL 46238 had a significantly lower %AC compared to *R. irregularis* MUCL 41833. Finally, plants inoculated with *R. intraradices* MUCL 49410 had a significantly higher %VC compared to those associated with *G. aggregatum* MUCL 49408, *R. clarus* MUCL 46238 and *G. hoi* MUCL 45686, but did not differ from *R. irregularis* MUCL 41833. *R. clarus* MUCL 46238 and *G. aggregatum* MUCL 49408 had a significantly lower %VC compared to *R. intraradices* MUCL 49410 and *R. irregularis* MUCL 41833.

RESEARCH RESULTS-PART II-CHAPTER IV

Table 21: Percentages of total colonization (%TC), arbuscules colonization (%AC) and vesicles/spores colonization (%VC) of *Echium vulgare* plants inoculated with AMF strains after three months of growth in pots under greenhouse conditions.

Treatment	TC (%)	AC (%)	VC (%)
<i>R. irregularis</i> MUCL 41833	63.41±13.74 a	9.27±4.79 a	6.85±2.09 ab
<i>R. clarus</i> MUCL 46238	42.32±22.90 ab	4.22±2.81 b	1.28±1.11 c
<i>R. intraradices</i> MUCL 49410	37.31±13.28 b	0.42±0.43 c	10.06±6.11 a
<i>G. aggregatum</i> MUCL 49408	6.80±4.64 c	0.58±0.54 bc	0.67±0.69 c
<i>G. hoi</i> MUCL 45686	53.89±13.40 ab	2.30±1.42 bc	3.92±1.74 bc
Analysis of variance			
Prob. > F	<0.0001*	<0.0001*	<0.0001*

The parameters measured are expressed as mean ± standard deviation (SD) of 5 or 6 replicates per treatment.

The asterisks show the significant effect of the treatments according to one-way ANOVA ($p \leq 0.05$). Values in the same column followed by different lowercase letters differ significantly according to HSD Tukey post-hoc test ($p < 0.05$).

Impact of AMFs on growth of *E. vulgare* – The impact of AMF on plant growth are presented in **Table 22**. The plants inoculated with *G. hoi* MUCL 45686 had a significantly lower RDW compared to the control and the plants inoculated with *R. intraradices* MUCL 49410. No other differences were observed between the treatments.

RESEARCH RESULTS-PART II-CHAPTER IV

Table 22: Impact of AMF on root volume (RV), shoot fresh and dry weight (SFW and SDW, respectively), root fresh and dry weight (RFW and RDW, respectively), root water retention (RWR), plant water retention (PWR) of *E. vulgare* after three months of growth in pots under greenhouse conditions.

Treatment	RV (mL)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	RWR (g)	PWR (g)
Control	36.13 ± 9.39	16.96 ± 2.21	3.26 ± 0.37	21.43 ± 4.91	3.77 ± 1.04 a	17.66 ± 4.57	31.36 ± 5.83
<i>R. irregularis</i> 41833	35.29 ± 7.95	17.19 ± 2.75	3.13 ± 0.35	20.80 ± 4.12	3.17 ± 0.80 ab	17.63 ± 3.48	31.70 ± 5.26
<i>R. clarus</i> 46238	41.71 ± 6.47	17.19 ± 1.73	3.23 ± 0.25	21.52 ± 3.40	2.70 ± 0.67 ab	18.82 ± 2.85	32.79 ± 3.83
<i>R. intraradices</i> 49410	40.25 ± 6.58	17.30 ± 1.93	3.24 ± 0.27	22.84 ± 3.81	3.82 ± 0.89 a	19.02 ± 3.20	33.07 ± 3.96
<i>G. aggregatum</i> 49408	32.25 ± 6.61	16.6 ± 1.19	3.16 ± 0.24	20.58 ± 3.85	3.23 ± 0.48 ab	17.35 ± 3.51	30.79 ± 3.32
<i>G. hoi</i> 45686	32 ± 3.46	16.03 ± 1.09	3.07 ± 0.15	17.96 ± 1.16	2.33 ± 0.44 b	15.62 ± 0.96	28.58 ± 1.53
Analysis of variance							
Prob. > F	0.08	0.86	0.83	0.40	0.008*	0.57	0.53

The parameters measured are expressed as mean ± standard deviation (SD) of 5 or 6 replicates per treatment. The asterisks show the significant effect of the treatments according to one-way ANOVA ($p \leq 0.05$). Values in the same column, followed

RESEARCH RESULTS-PART II-CHAPTER IV

by different lowercase letters significantly differ according to HSD Tukey post-hoc test ($p < 0.05$).

RESEARCH RESULTS-PART II-CHAPTER IV

Impact of AMFs on A/Sd production in roots -The impact of AMF on the concentration of A/Sd in plant roots are presented in **Figure 32** and on A/Sd content in Table S11 of Supplementary Data. A significant effect of the treatment was observed on the concentration of A/Sd. The plants inoculated with *R. intraradices* MUCL 49410 had a significantly higher concentration of A/S, acetyl-A/S, β,β – dimethylacryl-A/S, isovaleryl-A/S and total A/Sd (**Figure 32**) compared to the control plants. Deoxy-A/S and propionyl-A/S were only detected in the plants inoculated with *R. irregularis* MUCL 41833 and *R. intraradices* MUCL 49410 (Data not shown). Moreover, the plants inoculated with *R. intraradices* MUCL 49410 had a significantly higher content of A/S, β,β – dimethylacryl-A/S, isovaleryl-A/S and total A/Sd (**Table S11** in Annex III) compared to the control plants.

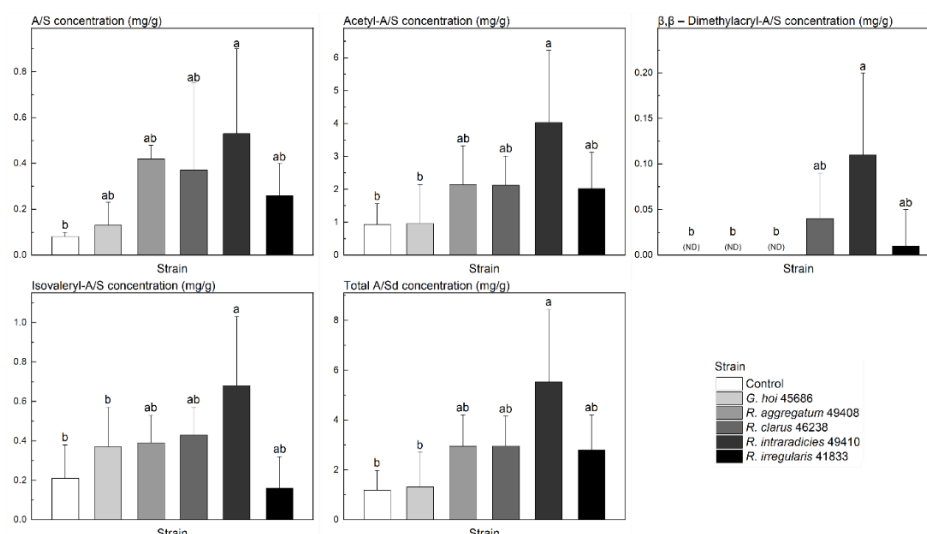


Figure 32. A/S, acetyl-A/S, β,β – dimethylacryl-A/S (not detected (ND) in the control, *G. hoi* and *R. aggregatum* treatment and thus not reported on the figure), isovaleryl-A/S and total A/Sd concentration (mg/g) in roots of *Echium vulgare* inoculated with 5 AMF strains after three months of growth in pots under greenhouse conditions.

The parameters measured are expressed as mean $\pm$ standard deviation (SD) of 6 replicates per treatment. Means followed by different lowercase letters within the same column are significantly different according to HSD Tukey *post-hoc* test ($p < 0.05$) after one-way ANOVA.

Discussion

In the present study, 10 fungal endophytes isolated from the roots of *A. tinctoria* collected in nature and 5 strains of AMF selected from GINCO, were inoculated on *Echium vulgare* in pots under greenhouse conditions. Their effects were evaluated on plant growth and A/Sd production in the roots after 3 months of cultivation.

Echium vulgare had their RV significantly increased following inoculation with *Cladosporium allicinum* (52%), *Cadophora sp.* (50 %), *Clonostachys sp.* (45%), *T. hispanicum* (66%) and *L. ladina* (62%) compared to the control. Similarly, the plants inoculated with *Plectosphaerella sp.* and *T. hispanicum* had their RFW significantly increased by 48% and 53%, respectively and their RWR by 51% and 56%, respectively. Finally, the plants associated with *T. hispanicum* had their PFW and PWR significantly increased by 27% and 30%, respectively. Unlike plants inoculated with the endophytes, no effect of AMFs was observed on growth parameters compared to the control. Conversely, the plants inoculated with AMF (i.e., *R. intraradices* MUCL 49410) had a significantly higher concentration of A/S, acetyl-A/S, β,β - dimethylacryl-A/S, isovaleryl-A/S and total A/Sd in their roots compared to the controls, while no impact was noticed with the fungal endophytes. The plants inoculated with AMF (i.e., *R. intraradices* MUCL 49410) also had a significantly higher content of these compounds except for acetyl-A/S.

Impact of endophytic fungi and AMFs on growth of *E. vulgare*

Endophytic fungi as well as AMF have been reported to provide benefits (e.g., nutrients acquisition, increased resistance against biotic and abiotic stresses) to their host plants (see review by Baron and Rigobelo 2022).

Among the 10 fungal endophytes tested, *T. hispanicum* was the most efficient, increasing significantly RV, RFW and PFW of *E. vulgare* compared to the control plants. *Trichoderma sp.* are widely described as plant growth promoting fungi (Andrzejak and Janowska 2022), establishing mutualistic relationships with many plant species (Alfiky and Weisskopf 2021). This increase in plant growth has often been attributed to a stimulation of primary and lateral roots (Cai et al., 2013; Naseby et al., 2000; Yedidia et al., 2001) via an increased production of vitamins and growth regulators by the fungus (Yedidia et al., 2001; Harman et al., 2004;

RESEARCH RESULTS-PART II-CHAPTER IV

Lorito et al., 2010). For instance, the synthesis of auxins (e.g., indole-3-acetic acid (IAA) and some of its derivatives) has been reported *in vitro* with *T. virens* Gv29.8 and *T. atroviride* IMI206040 resulting in the vigorous development of the root system and finally plant growth of *Arabidopsis thaliana* (Contreras-Cornejo et al., 2009). The production of siderophores (small molecules that present iron-chelating properties) by *Trichoderma* sp. has also been reported to induce plant growth. For example, Suebrasri et al. (2020) detected the production of siderophores by the endophytic fungus *T. koningii* ST-KKU stimulating the growth of sunchoke plants. None of these plant growth stimulation effects have been studied to date with *T. hispanicum*, suggesting that this fungus may exhibit some of these properties.

Cladosporium allicinum, *Cadophora* sp., *Clonostachys* sp., and *Leptosphaeria ladina*, significantly increased RV of *E. vulgare*. Fungi belonging to these genera were earlier reported to enhance plant growth. For example, *Cladosporium* isolates were reported to stimulate stem height and weight and root weight of tomato seedlings, compared to non-inoculated plants (Răut et al., 2021). In another study, the fungus *Cladosporium halotolerans* NGPF1 was shown to enhance shoot and root biomass in different plant species (bok choy, kimchi cabbage and broccoli) grown *in vitro* (Jiang et al., 2021). The increased plant growth has often been attributed to the production of phytohormones. For example, the application of culture filtrates of *Cladosporium* sp. MH-6 containing gibberellin markedly increased the growth of cucumber plants (Hamayun et al., 2010). Identically, *Clonostachys rosea* has been reported to promote root growth in tomato seedlings by secreting auxins (Han et al., 2022). Finally, many studies have revealed the beneficial relationships between dark septate endophyte (DSE) fungi (most DSE belong to the genera *Cadophora*) and their hosts, such as enhanced shoot growth of the blueberry varieties Brightwell and Elliott (Bizabani and Dames 2015). Besides, in the present study, plants inoculated with *Plectosphaerella* sp. showed significantly higher RFW as earlier reported with seedlings of *Noccaea caerulescens* following inoculation with *Plectosphaerella cucumerina* (Ważny et al., 2021).

None of the AMF tested increased the biomass of *E. vulgare*. A significant decrease in RDW and PDW was even noticed for the plants inoculated with

RESEARCH RESULTS-PART II-CHAPTER IV

G. hoi compared with control, while it is well known from the literature that these fungi have strong effects on plant growth and development. In fact, it is accepted that the response of plants to inoculation with an AMF may vary depending on the AMF species, and the direction (e.g., increase or decrease in plant biomass) and magnitude of the response strongly depend on the combination of plant and AMF taxa (Klironomos 2003).

It is difficult to speculate on the reasons for the absence of growth promotion in the presence of AMF. With the exception of *G. aggregatum* MUCL49408, %TC ranged between 37 and 63 %, which is quite acceptable, while %AC was overall low with values below 5% for all the AMFs, except *R. irregularis* MUCL 41833 ($\sim 9\% \pm 4.8\%$). High %TC is not necessarily correlated with increased plant biomass as mentioned by Kapoor et al. (2002), Toussaint et al. (2007) and Smith and Read (2008). For instance, Toussaint et al. (2007) showed that a relatively low level of colonization by *F. mosseae* (%TC $15 \pm 4\%$) had a considerable effect on *O. basilicum* biomass, while Kapoor et al. (2002) showed that a relatively high level of colonization by *Glomus fasciculatum* (%TC 83%) had no effect on shoot dry weight of *Trachyspermum ammi* (Linn.). Similarly, a high %AC is not necessarily associated with a visible increase in biomass. Indeed, the presence of AMF structures in a root does not automatically indicate that those structures are exchanging C for P (Fitter 1991) resulting in an increase of plant growth. In addition, it is well known that the increase in biomass of AMF-colonized plants is strongly linked to the ability of fungi to explore a volume of soil inaccessible to roots (Bowles et al., 2016). In the present study, *E. vulgare* plants were grown in 3 L pots, the volume of which was entirely occupied by the roots after three months of experiment. It is therefore not excluded that the advantages brought by a greater exploration of the hyphae were masked by the restricted size of the pots, limiting the effects of AMF on the biomass of the plants.

Impact of endophytic fungi and AMFs on the A/Sd production in *E. vulgare*

Many studies have shown that fungal endophytes and AMF can promote the accumulation of different SMs in medicinal plants (see review by Jia et al., 2016 and Zhao et al., 2022).

In our study, we observed an overall effect of the endophytic fungi on

RESEARCH RESULTS-PART II-CHAPTER IV

concentration of deoxy-AS and isovaleryl-AS. However, pairwise comparisons showed no effect of any particular endophyte compared to control plants. No difference in the production of the other A/S derivatives was noticed between the non-inoculated control treatment and the plants inoculated with the different fungal endophytes. It cannot be ruled out that the absence of stimulations is related to the fungal genotypes. Fungal endophytes are often embedded in their host's metabolic networks, which can result in alterations of metabolites production and higher amounts of active compounds in medicinal plants (Teimoori-Boghsani et al., 2020). In our study, the fungal endophytes were isolated from *A. tinctoria*. They may therefore be better suited to this specific plant in regulating the A/Sd production than with *E. vulgare*. It has been reported that various factors can influence the production of SMs in plants such as the developmental stage. For instance, in the study of Csorba et al. (2022), a significantly higher production of A/Sd was observed during the fruiting stage of *A. tinctoria*, regardless of the field soil type. Therefore, it cannot be ruled out that the production of A/Sd in *E. vulgare* may reach a peak at the harvesting time, which is much later than the harvesting time in our experiment.

Unlike the fungal endophytes, a significantly higher concentration of A/S, acetyl-A/S, β,β - dimethylacryl-A/S, isovaleryl-A/S and total A/Sd was observed in *E. vulgare* associated with the AMF strain *R. intraradices* MUCL 49410 compared with the plants in the control treatment, and an intermediate production between this strain and the other four AMF strains.

Root colonization by AMF is tightly regulated by the plant depending on its physiological and developmental status (Carbonnel and Gutjahr 2014, Gutjahr 2014). For instance, Hogeekamp and Küster (2013), have shown that genes related to secondary metabolism and possible target genes related to the synthesis of SMs (such as Gibberellin 2-beta-dioxygenase) were elicited in the pre-contact stage and in the hyphopodial stages of colonization of *Medicago truncatula* associated to *R. intraradices* (the fungal name following current taxonomy classification). The same authors further noticed that arbuscules formation induced a massive shift in gene expression patterns, leading to either preferential or specific accumulation of transcripts. For example, it has been reported that an increase in nutrients uptake via the arbuscules can lead to an enhanced production of precursor compounds, such as NADPH, ATP, acetyl-CoA (mevalonic acid pathway),

RESEARCH RESULTS-PART II-CHAPTER IV

and pyruvate glyceraldehyde and phosphate (methylerythritol-4-phosphate pathway) that are required for the biosynthesis of various SMs (e.g., terpenoids, phenolic, alkaloids) (Kapoor et al., 2017). Although the interaction between plant roots and AMF is long-lasting, at the cell level it may be ephemeral as arbuscules disappear in a matter of days after their inception (Montero et al., 2019). Then at the end, the vesicles which are terminal swellings of hyphae formed inter and intracellularly are formed (Sullia 1991). Taken together these findings may suggest that the accumulation of all the precursors leading to the biosynthesis of A/Sd correspond to the end of arbuscules life span. In fact, a significantly lower %AC and significant higher %VC in plants inoculated with *R. intraradices* MUCL 49410 at harvest time were observed which could correspond to the end of the activation of the different genes and the end of arbuscules life span. The accumulated and increased production of precursors through the active arbuscules during plant growth stage may lead to an increase biosynthesis of A/Sd concentration. Also, Tsiokanos et al. (2022), observed an effect of *R. intraradices* MUCL 49410 and *R. irregularis* MUCL 41833 on modulation of SMs (mainly C₆-C₃ and C₆-C₂ compounds) in *Anchusa officinalis* (L.) grown under a semi-hydroponic cultivation system as compared with *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408. Particularly, a few specific compounds (salicylic acid glucoside and yunnaneic acid D) were exclusively affected in plants associated with *R. intraradices*. Furthermore, it cannot be ruled out that these results are related to fungal genotypes. Though, these important soil fungi are not host specific, their affinity to a particular host can be preferential, and their proliferation and sporulation is highly dependent on plant host identity (Bever 2002; Cesaro et al., 2008). Early studies have documented that different AMF species may induce differences in SMs production in the same host or genotype (Zeng et al., 2013). For example, Toussaint et al. (2007) showed that *Funneliformis caledonium* increased rosmarinic acid and caffeic acid production in *O. basilicum* compared with non-mycorrhizal plants, whereas *F. mosseae* did not. This may explain why only *R. intraradices* MUCL 49410 inoculated *E. vulgare* plants showed the highest concentration of A/Sd.

Conclusion

RESEARCH RESULTS-PART II-CHAPTER IV

In this study, several endophytic fungi isolated from *A. tinctoria* roots significantly impacted plant growth, with *T. hispanicum* as the most promising, but without significant impact on A/Sd production in roots. Reversely, one of the five standard AMFs (i.e., *R. intraradices* MUCL 49410) from GINCO showed a significant impact on plant A/Sd production in *E. vulgare* plant roots but had no effect on plant growth. Further studies are needed to investigate the mechanisms involved in the production of A/Sd in plants associated to specific endophytic fungi /AMF and on the cultivation conditions needed for optimal production.

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GENERAL DISCUSSIONS

Since ancient times, *medicinal plants* have been a valuable source of therapeutic agents to treat various ailments and diseases, due to the production of various secondary metabolites (SMs). However, the quantity and quality of SMs produced greatly depends on the plant physiological and developmental stage (Halder et al., 2019). Moreover, their accumulation, in for instance shoots or roots, are often extremely low and for some plants, it is even necessary to wait several years before they produce SMs in marketable quantities.

For these reasons, a number of strategies have been developed aimed at increasing the production of SMs. Among these, growing plants under ***controlled greenhouse conditions*** in association with beneficial microorganisms (e.g., endophytic bacteria or fungi and arbuscular mycorrhizal fungi) represents today a potential innovative approach to improve the production of SMs (Zhao et al., 2022). However, this cultivation system also has some disadvantages, including the high demand for space, slow growth and risk of contaminations by unwanted microbes. ***Hairy root cultures*** (HRCs) of medicinal plants are another strategy that can be exploited as an alternative method for producing SMs. HRCs have many advantages: they are unique in their genetic and biosynthetic stability, grow fast and are easy to cultivate. Moreover, HRCs often accumulate phytochemicals at a higher level than cell/callus cultures that contain undifferentiated cells (Mehrotra et al., 2015). Interestingly, some studies have reported that applying abiotic and/or biotic elicitors in the HRCs of medicinal plants can enhance growth and biosynthesis of certain bioactive compounds (Sivanandhan et al., 2014; Halder et al., 2019).

Plants are associated with very diverse microbial communities comprising mainly bacteria and fungi, called the ***plant microbiome***. The microbiome plays a key role in plant health, development and metabolites synthesis (de Vries et al., 2020; Brader et al., 2014; Company et al., 2021). Indeed, in the recent decade several studies have shown that plant-associated microorganisms can influence secondary metabolism of plants and induce or stimulate the production of medicinally-relevant SMs (Zhao et al., 2022). Among these microorganisms are the ***arbuscular mycorrhizal fungi*** (AMF) and ***endophytic fungi***. AMF, are obligate root symbionts associated with nearly 72% of land plants. They have been reported to both

GENERAL DISCUSSIONS

quantitatively and qualitatively affect the production of SMs produced by their hosts (Salam et al., 2017). Endophytic fungi, colonizing internal living plant tissues, have also been reported to influence the production of metabolites in plants, affecting the quality and quantity of the crude drugs produced (Jia et al., 2016).

Alkanna tinctoria and *Echium vulgare* are major producers of alkannin and shikonin and derivatives (A/Sd). However, at the start of this thesis, no study reported the potential benefits of AMF and endophytic fungi on A/Sd production.

The thesis was separated in two parts and four chapters, the first part (with three chapters) focusing on *A. tinctoria* and the second part (with a single chapter) on *E. vulgare*.

In the first chapter, *A. tinctoria* was grown in three different soils (and thus root microbiomes) and growth stage of the plant paralleled with the root microbial composition and production of A/Sd. The highest A/Sd content was noticed in the fruiting stage and specific microorganisms influenced the production of A/Sd.

In the second chapter, AMF strains isolated from *A. tinctoria* in nature (i.e., *R. irregularis* and *S. viscosum*) and strains from GINCO (i.e., *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408) were inoculated on *A. tinctoria* in a semi-hydroponic and pot cultivation system. Their effects on the production of A/Sd and gene expression in the plants were assessed and compared. The native AMF strain *R. irregularis* increased A/Sd production in *A. tinctoria* regardless of the cultivation system, further suggesting that the inoculation of native/best performing AMF is a promising method to improve the production of important SMs.

In the third chapter, transformed HRCs of *A. tinctoria* were associated with AMF to assess whether A/Sd would be increased in these roots. The native AMF (*R. irregularis*) was more effective than the strain from GINCO (*R. irregularis* MUCL 41833) but no differences in A/Sd production was observed with the non-inoculated control.

Finally, **in the fourth chapter**, endophytic fungi isolated from *A. tinctoria* in nature and AMF selected from GINCO were evaluated on *Echium vulgare* growth and production of A/Sd. Some endophytic fungi (*Cladosporium allicinum*, *Cadophora* sp, *Clonostachys* sp.,

GENERAL DISCUSSIONS

Plectosphaerella sp, *Trichoderma hispanicum* and *Leptosphaeria ladina*) stimulated plant growth but not A/Sd, while the reverse was observed with AMF, the strain *R. intraradices* MUCL 49410 stimulating the production of alkannin/shikonin (A/S), acetyl-A/S, β,β -dimethylacryl-A/S, isovaleryl-A/S and total A/Sd, compared to the control plants.

The most important results of the PhD study are discussed below.

***Alkanna tinctoria* developmental stage and root microbiome composition impacts the production of A/Sd**

Alkanna tinctoria grown in pots under greenhouse conditions, in three different soils (and thus microbiomes) were harvested at four different growth stages and A/Sd analyzed. Plants had a higher content of A/Sd at the fruiting stage, with β,β -dimethylacrylalkannin and isovalerylalkannin representing the highest proportion of total A/Sd.

Different factors (e.g., plant developmental stage, light intensity, soil composition) have been reported to influence, both qualitatively and quantitatively, the primary and secondary metabolism of plants (Chaparro et al., 2013; Li et al., 2020). In the present study, the root metabolome of plants of the same developmental stage grown in different soils were very similar, indicating that plant maturity is the main driver of SMs production. Moreover, the microbiome composition was to a major extent influenced by the soil. Although the plants were grown in a sterilized soil substrate amended with comparable small amount of non-sterile soils obtained from different geographic regions, physico-chemical composition may have strongly influenced the roots microbiome.

Two of the most abundant A/Sd in *A. tinctoria* roots (i.e., β,β -dimethylacrylalkannin and isovalerylalkannin) were weakly or moderately correlated with abscisic acid. Since the two mentioned A/Sd represent the most abundant A/Sd in *A. tinctoria* roots, their connection to abscisic acid is noteworthy to explore. Abscisic acid has been shown to inhibit the growth of cultured cells of *Onosma paniculatum* B. F. (a Boraginaceae species), while it simultaneously reduced shikonin biosynthesis (Sun et al., 2007). However, our metabolomics data suggest this is not the case in *A. tinctoria* roots. On the contrary, an increased abscisic acid concentration was correlated with an increased content of the most abundant A/Sd in the root system. The potential role of abscisic acid in enhancing A/Sd yield in

GENERAL DISCUSSIONS

an ‘industrial’ production setting should therefore be analyzed in more detail. One key metabolite in the developmental stage network in *L. erythrorhizon* (another Boraginaceae species) is senecivernine N-oxide, a pyrrolizidine alkaloid (Ko et al., 2021). This SM was also found in our study. Pyrrolizidine alkaloids and their N-oxides serve as plant defense against herbivores and have been extensively studied due to their toxicity and risk for contamination of foods such as honey (Avula et al., 2015; Hungerford et al., 2019). Methyl jasmonate, which is known to induce metabolic changes in plants (Carlson et al., 2020; Hammerbacher et al., 2019) was also detected in our study. Besides, β , β -dimethylacrylalkannin and isovalerylalkannin, abscisic acid, maltotetraose, 6-methoxyflavanone, ostruthol and pyrogallol also had concentration peaks at the fruiting stage.

The biosynthesis of the above-mentioned metabolites would likely be positively affected by certain microorganisms herein identified as beneficial towards A/S biosynthesis. The performed correlation analyses and co-occurrence networks on the measured metabolites content and the abundance of individual bacterial and fungal taxa suggest a positive relationship between root-associated microorganisms and root metabolism. A moderate positive correlation between A/Sd levels and the bacterial genera *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Labrys* as well as fungal species of genus *Penicillium* was found. Plant rhizosphere isolates of the genus *Labrys* were previously described with indole-acetic-acid production and phosphate solubilizing capabilities (Inui et al., 2012), urea-decomposing and tryptophan-producing genes (Kim et al., 2021) which makes them potential plant growth-promoting strains. *Labrys* species were isolated several times from root nodules (Chou et al., 2007; Gerding et al., 2017; Bautist et al., 2017), but were not associated with nodulation, and at present have unknown roles in the root nodule microbial community. Species of the fungal genus *Penicillium* were found to be positively correlated with higher content of alkannins. Several species of this genus were already shown to affect plant growth and metabolism (Wakelin et al., 2007). For example, *P. digitatum* impacted the secondary and amino acid metabolism of citrus fruits (González-Candelas et al., 2010). Root exudates in *Arabidopsis thaliana* varied with developmental stage and were correlated with metabolism-specific rhizosphere microbial functions (Chaparro et al., 2013). This indicates the possibility of microbial induction of specific pathways leading to the synthesis of specific

GENERAL DISCUSSIONS

metabolites. Alternatively, these results could also pinpoint to the selection of specific microorganisms enriched by a specific metabolite.

The AMF *Rhizophagus irregularis* isolated from *A. tinctoria* in the wild is more efficient than *R. irregularis* MUCL 41833 from GINCO in stimulating A/Sd production in *A. tinctoria*

R. irregularis isolated from *A. tinctoria* in the wild significantly improved the content of shikonin and total A/Sd expressed as shikonin equivalent, in the roots of the *A. tinctoria* plants grown in pots, compared to those grown with *R. irregularis* MUCL 41833 (the most common used standard and commercial strain) or without inoculum. The native strain *S. viscosum* showed an intermediate value. It is hypothesized that native strains isolated from *A. tinctoria* in their natural habitat are better adapted to this plant species and are likely to have evolved as good associates. Indeed, the natives *R. irregularis* and *S. viscosum* are possibly more ‘tolerant’ to the presence of A/Sd, which are characterized by various effects on soil microorganisms (e.g., antifungal activities), and may thus better interact with their host plants to regulate and enhance the production of these important therapeutic metabolites (Yan et al., 2019). This observation was also made in a semi-hydroponic cultivation system in which *A. tinctoria* associated with the native *R. irregularis* showed significantly higher total A/Sd production compared with plants associated with non-native AMFs (MUCL 41833 *R. irregularis* and MUCL 49408 *R. aggregatus*) (Zhao et al., 2023).

Several factors, both nutritional (e.g., the enhanced uptake of nutrients) and non-nutritional (e.g., the alterations in phytohormones levels, signaling mechanisms between host plant and fungus, over-expression of genes coding enzymes leading to the biosynthesis of SMs), have been proposed to explain the increased production of SMs in AMF-colonized plants (Kapoor et al. 2017; Sharma et al. 2017; Dos Santos et al. 2021). Several genes, responsible for coding enzymes involved in the biosynthesis of shikonin, have been identified in Boraginaceae plant species (Wang et al., 2014; Wu et al., 2017; Takanashi et al., 2019). In the present study, 4-hydroxybenzoate-*m*-geranyltransferases (*LePGT1* and *LePGT2*) and geranylhydroquinone 3''-hydroxylase (*GHQH*) genes involved in the A/Sd biosynthesis, were analysed via RT-qPCR. No significant impact of AMF

GENERAL DISCUSSIONS

colonization was observed on the expression of *GHQH* and *LePGT1* genes (*LePGT2* target gene was not considered due to contamination by the primer dimer). This corroborates earlier study by Wang et al. (2014). Interestingly, Ahmad et al. (2022b), observed a significantly higher expression of *LePGT1*, *LePGT2*, and *LeGHQH* in roots of *Lithospermum officinale* L. treated with MeJA, but no higher accumulation of total A/Sd. The regulation and accumulation of SMs in plants is usually controlled by a complex network characterised by transcription factors (TFs), which promote or inhibit the expression of multiple genes involved in one or more biosynthetic pathways (Yang et al., 2012; Wu et al., 2021). Transcription factors can act alone or in combination with other TFs to modulate the expression of target genes, and also one TF can regulate the expression of multiple genes participating in one or more biosynthetic pathways (Pinson et al., 2009; Goossens et al., 2017; Hassani et al., 2020). In our study, different TFs might have regulated the target genes (*PGTs* and *GHQH*), yet the full landscape of the A/Sd biosynthesis pathway is not entirely understood, with co-expressed genes still to be identified (Suttiyut et al., 2022). Moreover, in a study by Andrade et al. (2013), a mismatch between alkaloid levels, another important SM group, and gene expression was reported in different *Catharanthus roseus* (L.) G. Don tissues. In this study, the AMF *Claroideoglomus etunicatum* had a greater influence (i.e., alkaloids production) in roots than in shoots, and a higher gene expression was reported in the older leaves of AMF-colonized plants as well as in the youngest leaves of non-mycorrhized plants (leaves were harvested at the same time) (Andrade et al., 2013). These results suggest a very precise phenological and spatial regulation process during alkaloid biosynthesis (Mahroug et al. 2007). In fact, A/Sd compounds are sequestered as granules in the phospholipid layer and are accumulated in the apoplastic spaces, and they can be found in the cork layer of mature roots (Brigham et al., 1999; Singh et al., 2010; Tatsumi et al., 2016). A difference in A/Sd accumulation might have occurred (i.e., higher content in the primary roots as compared to the secondary roots) upon AMF colonization and might have not been fully displayed across *A. tinctoria* root samples used for our RT-qPCR analysis (i.e., no homogenous samples analysed with mostly secondary roots utilized). A comprehensive transcriptome study should be carried out covering different plant root tissues and growth stages. The native *R. irregularis* isolated from *A. tinctoria* in the wild significantly increased the

GENERAL DISCUSSIONS

total A/Sd in roots compared to the standard AMF strain, suggesting that this native strain is better adapted to the host plant in the regulation and production of these metabolites.

Hairy root cultures of *A. tinctoria* does not produce more A/Sd in presence of AMF

Hairy root cultures (HRCs) are nowadays used for the production of SMs by plants. However, no study has reported the association of AMF to HRCs and subsequent effects of SMs production. Here, the native AMF *R. irregularis* isolated from *A. tinctoria* and *R. irregularis* MUCL 41833 (the most common commercial one) from GINCO were compared on SMs production by HRCs of *A. tinctoria*. Neither strain affected A/Sd production in the roots or in the medium after 17 months of association *in vitro*. The hairy roots died after a few months (due to the lack of carbon and minerals in the medium) and it is likely that over a period of 17 months, the SMs degraded.

A/Sd compounds are susceptible to light exposure (Cho et al., 1999; Cheng et al., 1995), heat (Cho et al., 1999) and have the tendency to polymerize in alkaline media, polar solvents and under high temperature (Assimopoulou and Papageorgiou 2004). During the summer season 2023, there was a higher temperature (around 30°C) outside and inside the incubator room. This high temperature may have accelerated the degradation of the A/Sd compounds. Finally, it has been reported by Skoneczny et al. (2019) that *Echium plantagineum* plants under water stress exhibited elevated levels of deoxyshikonin suggesting the impact of water withholding on the accumulation of A/Sd. So, we speculated that the duration of the present experiment was too long, resulting in the degradation of A/Sd compounds under high temperature and humidity in the Petri plates (the MSR medium is made of almost 99% of water). It is thus advisable to carry out further research in a shorter time-frame and with other AMFs isolated from nature on *A. tinctoria* and from culture collections. This should help to understand whether an AMF strain is able to impact the production of SMs in HRCs of *A. tinctoria*. Moreover, other solvents can be used to extract the A/Sd from the *A. tinctoria* hairy root growing in MSR medium as the one used (n-hexane) for root grown in pot may not be the best one in this study.

GENERAL DISCUSSIONS

Plant growth and A/Sd production in *Echium vulgare* are influenced by endophytic fungi and AMF

The endophytic fungi, *Cladosporium allicinum*, *Cadophora* sp., *Clonostachys* sp., *Plectosphaerella* sp., *Trichoderma hispanicum* and *Leptosphaeria ladina* (isolated from roots of *A. tinctoria*) significantly increased plant growth, with *T. hispanicum* being the most promising one. This suggest that these endophytic fungal strains have significant potential for growth of *E. vulgare*, supporting earlier studies (Răut et al., 2021; Jiang et al., 2021; Hamayun et al., 2010) with fungi belonging to the same genus. Several mechanisms have been suggested such as the increased acquisition of nutrients or phytohormones in the plants (Baron and Rigobelo 2021) or stimulation of plant defence mechanisms, promoting the activation of the induced systemic resistance (ISR). This mechanism may overlap with that of acquired systemic resistance (ASR) and both mechanisms can improve plant growth (Berg, 2009; Busby et al., 2016). To further understand the effects of endophytic fungi on the growth of *E. vulgare*, it could be interesting to analyze the accumulation of macronutrients such as phosphorus, nitrogen, potassium and magnesium, or micronutrients, such as zinc, iron, and copper and production of phytohormones such as auxins, gibberellins (GAs) and cytokinins in the plants. Curiously, none of the endophytic fungi tested affected the A/Sd production.

An opposite situation was observed with AMF. Classically, AMF are recognized for their effects on stimulating plant growth. This was not observed in our study. On the other hand, a significantly higher concentration of A/S, acetylS, β , β – dimethylacrylS, isovalerylS and totalAS was observed in the plants associated with *R. intraradices* MUCL 49410 compared with the control plants, and an intermediate production between this strain and the other AMF strains. Interestingly, *R. intraradices* MUCL 49410 and *R. irregularis* MUCL 41833 had an effect on modulation of SMs (mainly C₆-C₃ and C₆-C₂ compounds) in *Anchusa officinalis* (L.) grown under a semi-hydroponic cultivation system as compared with *R. clarus* MUCL 46238 and *R. aggregatus* MUCL 49408 (Tsiokanos et al., 2022). Particularly, a few specific compounds (salicylic acid glucoside and yunnaneic acid D) were exclusively affected in plants associated with *R. intraradices*. Interestingly, a significantly lower AC% and significantly higher VC% in plants inoculated with *R. intraradices*

GENERAL DISCUSSIONS

MUCL 49410 were observed at harvest time. Although the interaction between plant roots and AMF is long-lasting, at the cell level it may be ephemeral as arbuscules disappear in a matter of days after their inception (Montero et al., 2019). Then at the end, the vesicles which are terminal swellings of hyphae are formed (Sullia 1991). It has been reported that arbuscules formation induce a massive shift in gene expression patterns, leading to either preferential or specific accumulation of transcripts (Hogekamp and Küster 2013). For example, it has been reported that an increase in nutrients uptake via the arbuscules can lead to an enhanced production of precursor compounds, such as NADPH, ATP, acetyl-CoA (mevalonic acid pathway), and pyruvate glyceraldehyde and phosphate (methylerythritol-4-phosphate pathway) that are required for the biosynthesis of various SMs (e.g., terpenoids, phenolic, alkaloids) (Kapoor et al., 2017). Taken together these findings may suggest that the accumulation of all the precursors leading to the biosynthesis of A/Sd corresponds to the end of arbuscules life span. Furthermore, it cannot be ruled out that these results are related to fungal genotypes. Though, these important soil fungi are not host specific, their affinity to a particular host can be preferential, and their proliferation and sporulation is highly dependent on plant host identity (Bever 2002; Cesaro et al., 2008). This may explain why *E. vulgare* inoculated with *R. intraradices* MUCL 49410 had the highest concentration of A/Sd.

Overall, the metabolome and microbiome from *A. tinctoria* grown under greenhouse condition in **Chapter I** indicated that this plant produced highest concentration of A/Sd in the fruiting stage suggesting plant development plays a pivotal role in producing these A/Sd compounds. A positive relationship between microbiome and A/Sd production suggested that some bacteria and fungi may have the impact on increasing these A/Sd compounds. In **Chapter II**, the native AMF (*R. irregularis*) isolated from *A. tinctoria* had a better impact on A/Sd production in *A. tinctoria* compared with other standard strains whatever the system used (under semi-hydroponic or under greenhouse condition) suggesting that this native strain is better adapted to the host plant in the regulation and production of these metabolites. However, under *in vitro* condition (**Chapter III**), this native *R. irregularis* strain had the same impact on A/Sd compounds production with another standard strain which may be due to the extreme long experimental period that the hairy roots died after a few and the SMs

GENERAL DISCUSSIONS

degraded. Lastly, in the last **Chapter IV**, those endophytic fungi isolated from *A. tinctoria* showed significant impact on *E. vulgare* plant growth. And one AMF strain from GINCO collection significantly improved the A/Sd production on the root of *E. vulgare* plant. All together, these studies confirmed that microorganisms (especially AMF) could regulate and increase the production of certain active compounds and in our case, the A/Sd. The combination between host plant and AMF should be extensively explored to find the best combination. Moreover, more studies need to be carried out to understand the mechanisms of enhancing the production of A/Sd by associating the host plant with AMF (such as a highthrough transcriptome study).

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS AND PERSPECTIVES

The main objective of this Ph.D. thesis was to assess whether AMF and endophytic fungi can influence the biosynthesis of SMs, especially A/Sd in *A. tinctoria* and *E. vulgare*, two Boraginaceae with well-known medicinal properties. **In the first chapter**, conducted with *A. tinctoria* in pots, we hypothesized that root-associated microorganisms can influence the production of A/Sd and other SMs in the roots of *A. tinctoria*, and reversely that the plant metabolome is likely to affect the root microbiome composition. **In the second chapter**, two AMF (*R. irregularis* and *S. viscosum*) isolated from the wild on *A. tinctoria* and the standard AMFs *R. irregularis* MUCL 41833 and *R. aggregatus* MUCL 49408 were tested on the production of A/Sd in *A. tinctoria* plants grown in semi-hydroponic system and pot culture under greenhouse conditions. We hypothesized that the native strains are more efficient in stimulating the plants to produce A/Sd. The same native and standard AMF strains were tested on the production of A/Sd in hairy roots of *A. tinctoria* and the same hypothesis as in step 2 was evaluated **in the third chapter**. Finally, **in the fourth chapter** the effect of AMF (from GINCO) and endophytic fungi (isolated from *A. tinctoria* in the wild) were evaluated on the production of A/Sd in *E. vulgare* grown in pots under greenhouse conditions with the hypothesis that both groups of fungi are able to stimulate plant growth and A/Sd production.

Therefore four research questions were addressed in this Ph.D and answers summarized below.

Does the root microbiome of A. tinctoria differ with developmental stage of the plant and how does it influence the production of A/Sd?

Yes

- Plants of *A. tinctoria* grown in three different soils (and therefore microbiomes), and harvested at different growth stages showed that the developmental stage of the plant was the most important factor influencing the metabolites content of the plant. Maximum levels of alkannin/shikonin derivatives were measured in the root tissues of *A. tinctoria* at the fruiting stage.
- The metabolites production in *A. tinctoria* root tissues is correlated with the root microbiome. The bacterial genera *Allorhizobium*-

CONCLUSIONS AND PERSPECTIVES

Neorhizobium-Pararhizobium-Rhizobium and *Labrys* as well as fungal species belonging to the genus *Penicillium* were found to be positively correlated with the content of alkannins.

Perspectives:

The results suggest the potential role of individual bacteria and fungi in modulating the plant A/S biosynthesis pathway. These taxa, in particular, bacteria from *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* and *Labrys* genus as well as fungi from genus *Penicillium* may be further isolated from the root tissues of *A. tinctoria* and used to stimulate the production of A/Sd in *A. tinctoria* as single or combined inoculants. The mechanisms involved in this increase needs further to be explored more deeply and the links with plant developmental stage better understood to master the production of A/Sd in *A. tinctoria* with the help of selected microorganisms.

Are native AMFs (*R. irregularis* and *S. viscosum*) isolated from *A. tinctoria* in the wild more efficient than non-native AMF (*R. irregularis* MUCL 41833) in stimulating growth and A/Sd production in *A. tinctoria*? Do these AMFs modulate different relative gene expression involved in A/Sd biosynthesis?

Yes and no

- *A. tinctoria* root tissue associated with *R. irregularis* isolated from roots of *A. tinctoria* in the wild had the highest concentration of total A/Sd and a significantly higher concentration of shikonin as compared with *R. irregularis* MUCL 41833 (from GINCO). However, an intermediate value (total A/Sd and shikonin) was observed for the plants inoculated with another native AMF strain (*S. viscosum*).

Perspectives:

This result suggests that the selection of effective AMF species (native or not; single or combinations of different AMF strains) needs to be based on screening approaches. Indeed, it is well-known that plant growth response to AMF inoculation can vary among AMF species and that the direction (e.g., increase or decrease in plant biomass) and magnitude of the response strongly depends on the combination of plant and AMF taxa (Klironomos, 2003). It is more than likely that a similar observation can be made for SMs production. Moreover, further studies should be conducted to investigate

CONCLUSIONS AND PERSPECTIVES

the kinetic of A/Sd production in relation with the AMF development in the roots. Plant transcriptome may be a strong option to link A/Sd biosynthesis with root colonization.

Is the native AMF (*R. irregularis*) more efficient than the non-native AMF (*R. irregularis* MUCL 41833) to increase A/Sd production in hairy roots of *A. tinctoria*?

No

- Neither the native *R. irregularis* strain nor the GINCO strain *R. irregularis* MUCL 41833 impacted the A/Sd production in hairy root tissues of *A. tinctoria* or in the medium. This is probably attributed to the age of the roots and their probable death and subsequent degradation of the A/Sd in the roots and medium after 17 months of culture.

Perspectives:

Secondary metabolites (i.e., A/Sd) are light and temperature sensitive. Therefore, further research should be conducted within a shorter period of time and under more moderate temperatures to study the effects of AMF on the production of these SMs in *A. tinctoria* hairy roots. After adjusting and improvement of the HRC associating plants and AMF, this study may open new perspectives towards the application of AMF in the production of these valuable therapeutic compounds in transformed medicinal plant roots.

Does endophytic fungi isolated from *A. tinctoria* and non-native AMFs (from GINCO collection) impact growth of *Echium vulgare* and A/Sd production in roots?

Yes and no

- The endophytic fungi isolated from roots of *A. tinctoria* in the wild significantly impacted *E. vulgare* plant growth. Among the 10 fungal endophytes tested, *Trichoderma hispanicum* was the most efficient. *Cladosporium allicinum*, *Cadophora* sp., *Clonostachys* sp., and *Leptosphaeria ladina*, also increased root volume of *E. vulgare*. Conversely, none of the endophytic fungi tested impacted the plant A/Sd production.
- The AMF from GINCO did not impact plant growth parameters.

CONCLUSIONS AND PERSPECTIVES

Conversely, a significantly higher concentration of A/S, acetyl-A/S, β,β - dimethylacryl-A/S, isovaleryl-A/S and total A/Sd was observed in root tissue of *E. vulgare* associated with the *R. intraradices* MUCL 49410.

Perspectives:

Further studies should be conducted to investigate the interactions between *E. vulgare* and endophytic fungi or AMF in the context of plant growth promotion and production of A/Sd. This should lead to the pre-selection of efficient fungal strains to fully exploit the diversity of species and isolates of endophytic fungi and AMF present in roots of the medicinal plants.

ANNEXES

Annex I

Metabolite production in *Alkanna tinctoria* links plant development with the recruitment of individual members of microbiome thriving at the root soil interface

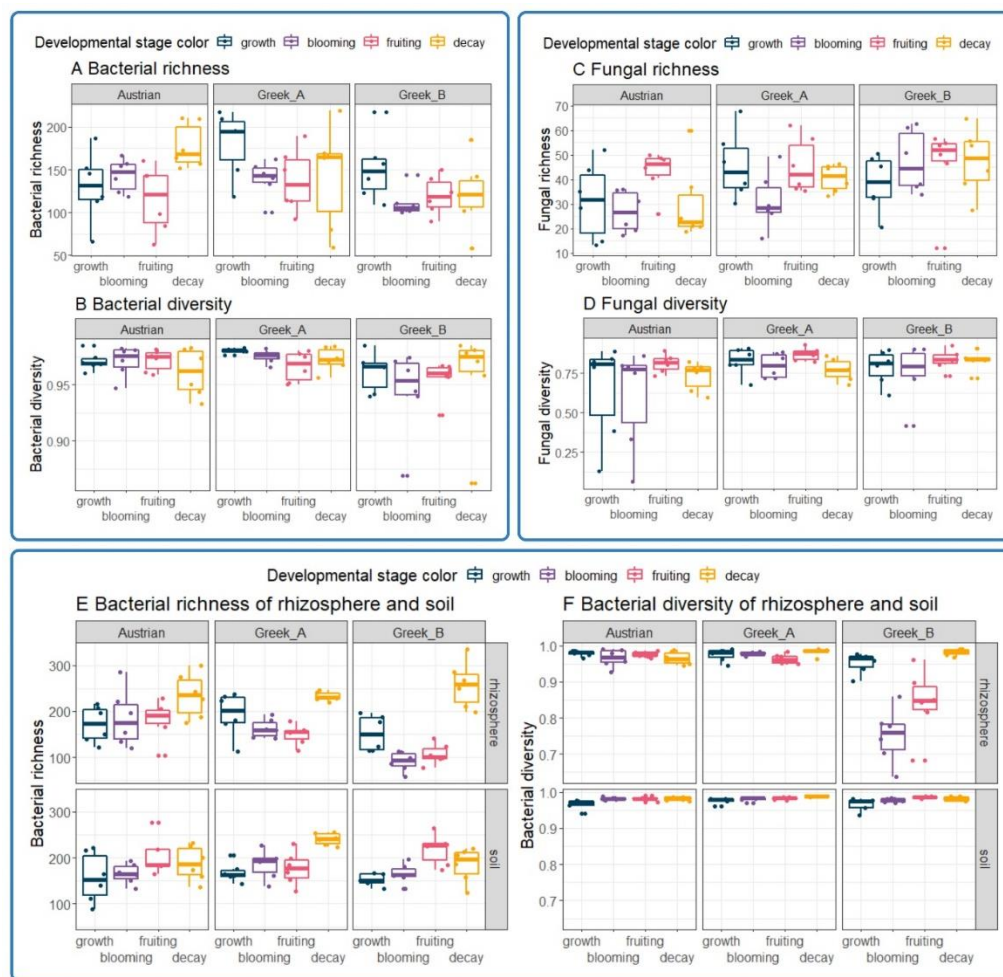


Figure S1. Alpha-diversity values across developmental stages faceted by the different soil mixes. For each box plot, $n = 6$. (A and C) Root sample richness is calculated as observed number of ASVs in all samples per group. (B and D) Root

ANNEXES

sample diversity is calculated as Simpson's diversity index values. (E and F) Bacterial alpha-diversity values of rhizosphere and soil samples. Created with BioRender.com.

ANNEXES

Table S1. Geographical and chemical characteristics of the three native soils (and source of microbiomes).

Soil name	Location	Altitude (m)	Climate	pH	Conductivity ($\mu\text{S}/\text{cm}$)	Ca	Fe	K	Mg	Mn	P
Austrian	48°08'38" 16°22'17.0"	186	Temperate-pannonic	7.91	153.3	347	1.9	11.6	9.5	6.1	0.57
Greek A	40°64' 22°96'	50-70	Mediterranean	7.81	116.0	97	1.6	1.0	6.1	2.1	0.20
Greek B	40°63' 22°97'	130	Mediterranean	7.13	105.8	86	2.4	1.2	4.4	3.3	0.35

ANNEXES

Table S2. Beta-diversity. Pairwise Adonis PERMANOVA analysis in root, rhizosphere, and soil samples based on Bray-Curtis dissimilarity values in individual subsets of each factor. Soil origin subsets are represented in green, and developmental stage subsets are represented in blue. P values are false-discovery rate adjusted.

Bacteria				Fungi			
Austrian				Austrian			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Growth vs Blooming	1.08	0.10	0.322	Growth vs Blooming	0.89	0.08	0.691
Blooming vs Fruiting	1.26	0.11	0.282	Blooming vs Fruiting	2.04	0.17	0.072
Fruiting vs Decay	1.83	0.15	0.008**	Fruiting vs Decay	1.53	0.13	0.184
Greek A				Greek A			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Growth vs Blooming	1.32	0.12	0.109	Growth vs Blooming	0.88	0.08	0.650
Blooming vs Fruiting	1.03	0.09	0.423	Blooming vs Fruiting	1.41	0.12	0.140
Fruiting vs Decay	2.42	0.19	0.022*	Fruiting vs Decay	1.37	0.12	0.140
Greek B				Greek B			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Growth vs Blooming	1.68	0.14	0.064	Growth vs Blooming	1.14	0.10	0.288
Blooming vs Fruiting	1.47	0.13	0.064	Blooming vs Fruiting	2.55	0.20	0.027*
Fruiting vs Decay	3.84	0.28	0.006**	Fruiting vs Decay	1.41	0.12	0.100
Growth stage				Growth stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value

ANNEXES

Greek A vs Greek B	1.44	0.13	0.115	Greek A vs Greek B	0.85	0.08	0.865
Greek A vs Austrian	1.89	0.16	0.054	Greek A vs Austrian	0.75	0.07	0.865
Greek B vs Austrian	1.35	0.12	0.115	Greek B vs Austrian	0.82	0.08	0.865
Blooming stage				Blooming stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	4.29	0.30	0.006**	Greek A vs Greek B	2.19	0.18	0.021*
Greek A vs Austrian	3.48	0.26	0.006**	Greek A vs Austrian	1.59	0.14	0.021*
Greek B vs Austrian	4.34	0.30	0.006**	Greek B vs Austrian	1.96	0.16	0.021*
Fruiting stage				Fruiting stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	3.20	0.24	0.009**	Greek A vs Greek B	2.06	0.17	0.012*
Greek A vs Austrian	2.02	0.17	0.074	Greek A vs Austrian	1.63	0.14	0.048*
Greek B vs Austrian	4.43	0.31	0.003**	Greek B vs Austrian	4.36	0.30	0.009**
Decay stage				Decay stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	1.58	0.14	0.021*	Greek A vs Greek B	1.13	0.10	0.249
Greek A vs Austrian	2.26	0.18	0.015*	Greek A vs Austrian	1.68	0.14	0.039*
Greek B vs Austrian	1.75	0.15	0.021*	Greek B vs Austrian	2.27	0.18	0.006**

Rhizosphere				Bulk soil			
Austrian				Austrian			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value

ANNEXES

Growth vs Blooming	1.652	0.142	0.009**	Growth vs Blooming	1.638	0.14	0.0345*
Blooming vs Fruiting	1.573	0.136	0.006**	Blooming vs Fruiting	0.941	0.095	0.468
Fruiting vs Decay	2.152	0.177	0.006**	Fruiting vs Decay	2.738	0.233	0.012*
Greek A				Greek A			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Growth vs Blooming	1.716	0.146	0.026*	Growth vs Blooming	2.09	0.173	0.007**
Blooming vs Fruiting	1.227	0.109	0.193	Blooming vs Fruiting	1.963	0.164	0.012*
Fruiting vs Decay	2.362	0.191	0.006**	Fruiting vs Decay	3.889	0.28	0.005**
Greek B				Greek B			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Growth vs Blooming	5.801	0.367	0.006**	Growth vs Blooming	1.496	0.13	0.053
Blooming vs Fruiting	1.741	0.148	0.097	Blooming vs Fruiting	1.41	0.124	0.077
Fruiting vs Decay	6.989	0.411	0.006**	Fruiting vs Decay	3.8	0.275	0.006*
Growth stage				Growth stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	2.25	0.184	0.005**	Greek A vs Greek B	3.911	0.281	0.006**

ANNEXES

Greek A vs Austrian	2.187	0.179	0.005**	Greek A vs Austrian	5.386	0.35	0.005**
Greek B vs Austrian	2.689	0.212	0.005**	Greek B vs Austrian	4.472	0.309	0.005**
Blooming stage				Blooming stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	13.257	0.57	0.003**	Greek A vs Greek B	4.193	0.296	0.006**
Greek A vs Austrian	3.858	0.278	0.003**	Greek A vs Austrian	5.275	0.345	0.009**
Greek B vs Austrian	8.714	0.466	0.003**	Greek B vs Austrian	3.599	0.265	0.003**
Fruiting stage				Fruiting stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	4.815	0.325	0.006**	Greek A vs Greek B	1.909	0.16	0.024*
Greek A vs Austrian	1.812	0.153	0.076	Greek A vs Austrian	1.371	0.132	0.132
Greek B vs Austrian	6.039	0.377	0.006**	Greek B vs Austrian	2.177	0.195	0.024*
Decay stage				Decay stage			
Pairs	F mod.	R ²	p value	Pairs	F mod.	R ²	p value
Greek A vs Greek B	2.149	0.177	0.007**	Greek A vs Greek B	2.654	0.209	0.003**

ANNEXES

Greek A vs Austrian	2.395	0.193	0.006**	Greek A vs Austrian	6.937	0.409	0.003**
Greek B vs Austrian	1.72	0.147	0.006**	Greek B vs Austrian	5.063	0.336	0.003**

ANNEXES

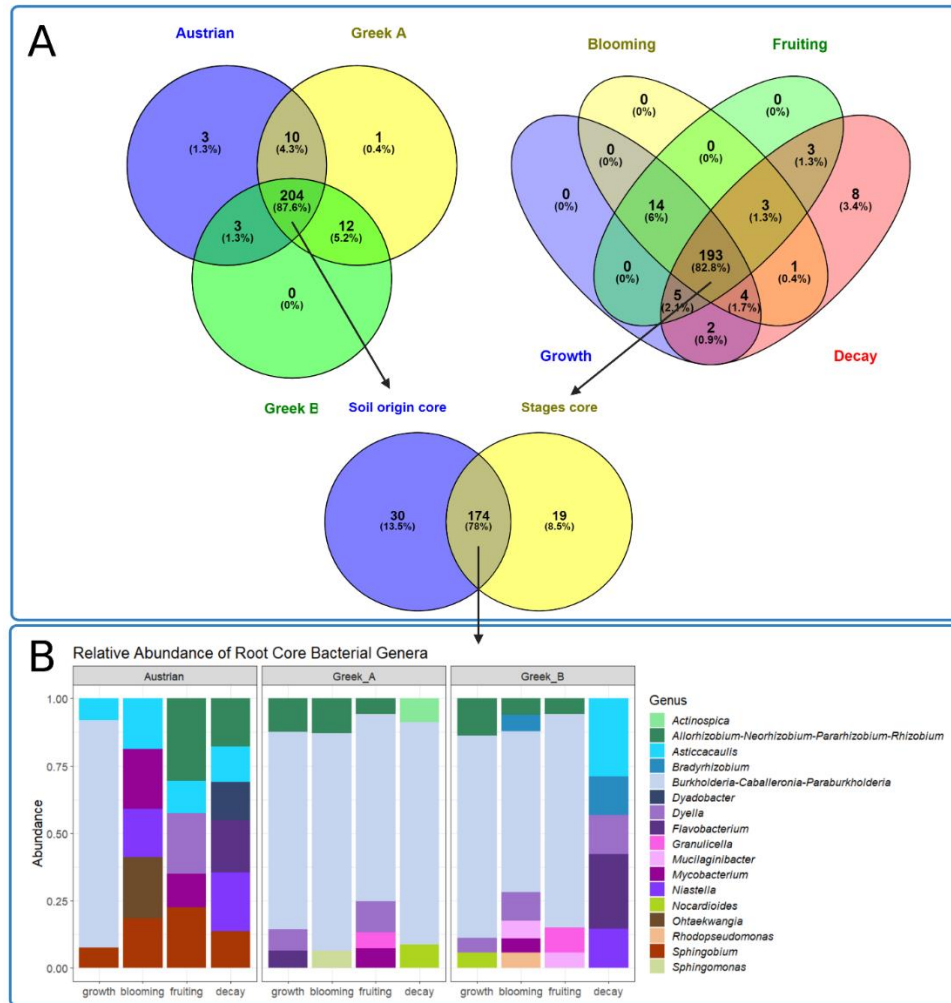


Figure S2. (A) The overlap between the bacterial rASV core Venn diagrams suggests a stable core among the main factors. (B) Relative abundance of the stable root core bacterial rASVs assigned to genera. Only the genera with the highest relative abundance were visualized by their abundance in the different groups.

ANNEXES

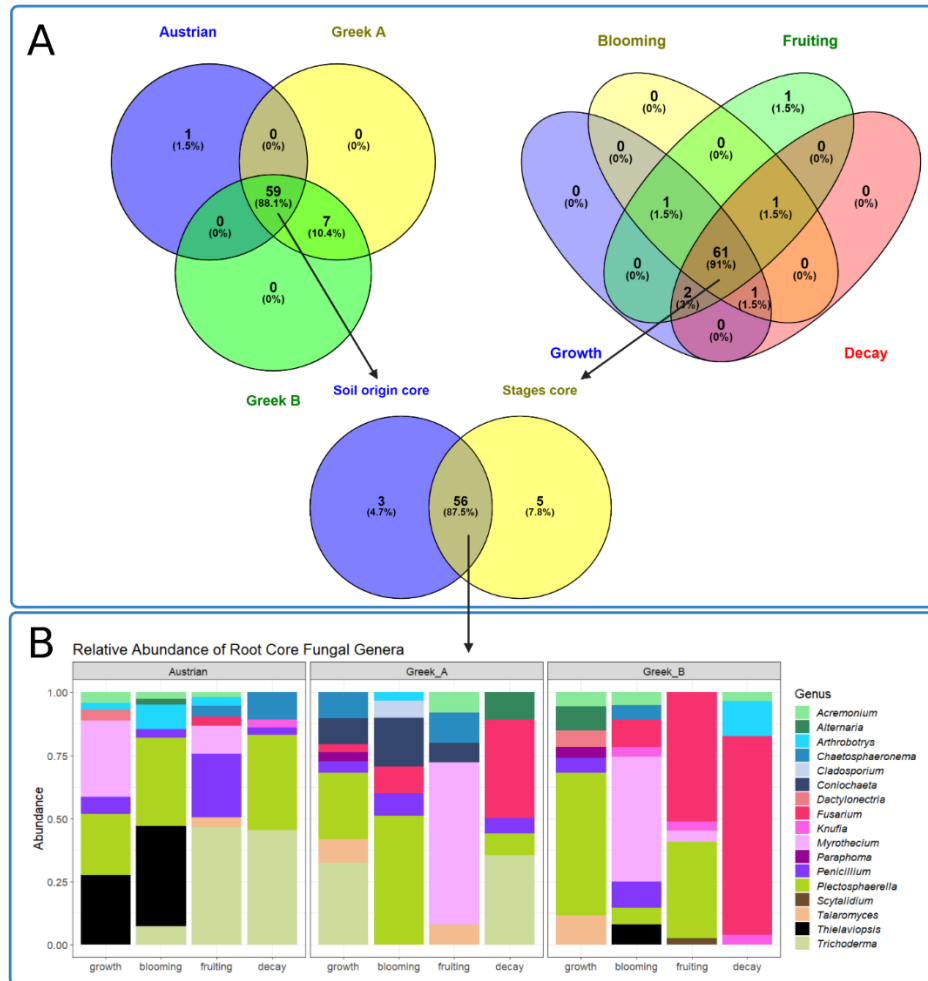


Figure S3. (A) Fungal rASV Venn diagram overlap suggests a stable core among the main factors. (B) Relative abundance of the root core bacterial rASV assigned to genera. Only the genera with the highest relative abundance were visualized by their abundance in the different groups. Created with BioRender.com.

ANNEXES

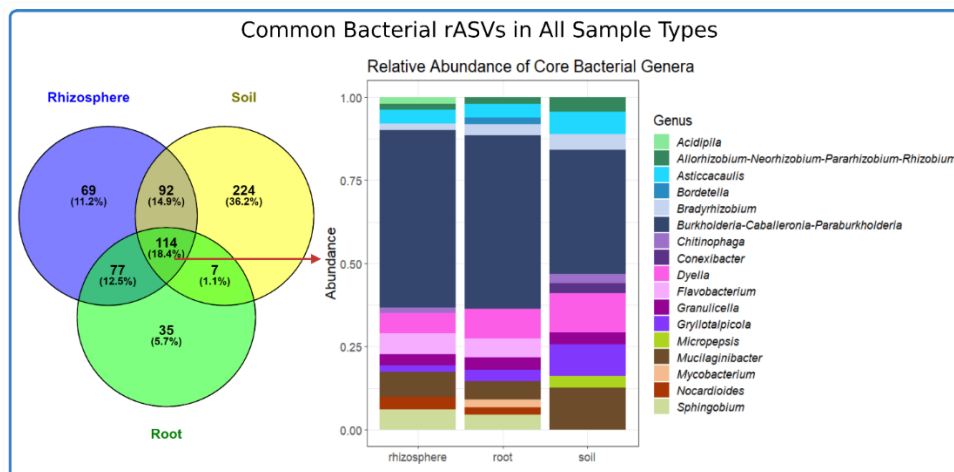


Figure S4. The stable core rASV overlap of the different bacterial sample types on the Venn diagram suggests a stable core microbiome of 114 rASVs that are present in root, rhizosphere, and soil samples. The 114 rASVs were assigned to bacterial genera and visualized by their relative abundance in the stable core microbiome dataset.

ANNEXES

Table S3. *Post hoc* analysis on the ANOVA. Total A/S content (ppm) was determined by HPLC.

Soil = Austrian					
Stage	emmean	SE	lower.CL	upper.CL	Contrast group
blooming	31.1	22.8	-27.6	89.8	a
decay	11.7	25	-52.6	76	a
fruiting	198.1	22.8	139.4	256.8	b
growth	26	22.8	-32.7	84.7	a
Soil = Greek A					
Stage	emmean	SE	lower.CL	upper.CL	Contrast group
blooming	20.1	22.8	-38.6	78.8	a
decay	28.3	22.8	-30.4	87	a
fruiting	92.3	22.8	33.6	151	a
growth	33.6	22.8	-25.1	92.3	a
Soil = Greek B					
Stage	emmean	SE	lower.CL	upper.CL	Contrast group
blooming	20	22.8	-38.7	78.7	a
decay	0	22.8	-58.7	58.7	a
fruiting	193.6	22.8	134.9	252.3	b
growth	19.8	22.8	-38.9	78.5	a

ANNEXES

Table S4. List of annotated compounds in the greenhouse *A. tinctoria* root samples by using UHPLC-HRMS.

Metabolite code	Compound	Chemical formula	Molecular weight [g/mol]	ID method	Retention time [min]	Parent ion mass [m/z]	Parent ion type	Δm [ppm]	Matched fragments (rel. int. >10%)
M1	β , β - Dimethylacrylalkannin	C ₂₁ H ₂₂ O ₆	370.3949	MS/MS (+)	9.5	393.1288	[M+Na] ⁺	-5.34	392.7, 392.1, 391.4, 375.1, 351.0, 345.5, 344.8, 344.0, 342.7, 341.3, 340.2, 328.4, 293.3, 253.1
M2	Isovalerylalkannin	C ₂₁ H ₂₄ O ₆	372.4107	MS/MS (+)	9.7	395.1443	[M+Na] ⁺	-5.57	409.3, 394.2, 377.2, 345.7, 344.8, 343.8, 343.2, 342.3, 341.1, 340.2, 339.2, 328.7, 327.9, 326.7, 292.9

ANNEXES

M3	Acetylalkan nin	C ₁₈ H ₁₈ O ₆	330.3312	MS/MS (+)	8.2	353.0988	[M+Na] ⁺	-2.27	352.8, 345.7, 342.4, 335.2, 328.3, 311.1, 293.0, 262.1, 253.0, 183.1, 111.9
M4	Alkannin	C ₁₆ H ₁₆ O ₅	288.2946	MS/MS (+)	7.4	289.1057	[M+H] ⁺	-4.84	271.0
M5	Tyrosine	C ₉ H ₁₁ NO ₃	181.1881	MS/MS (+)	2.6	182.0801	[M+H] ⁺	-6.04	164.8, 135.9
M6	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	360.3142	MS/MS (+)	3.6	378.1176	[M+NH ₄] ⁺	-1.85	162.9
M7	Phenylalani ne	C ₉ H ₁₁ NO ₂	165.1887	MS/MS (+)	2.6	166.0859	[M+H] ⁺	-2.41	119.9

ANNEXES

M8	Absciscic acid	C ₁₅ H ₂₀ O ₄	264.3161	MS/MS (+)	8.8	287.1265	[M+Na] ⁺	3.83	269.0
M9	Methyl jasmonate	C ₁₃ H ₂₀ O ₃	224.2953	MS (+)	3.3	207.1373	[M-H ₂ O+H] ⁺	-3.38	N/A
M10	Maltotetraose	C ₂₄ H ₄₂ O ₂₁	666.5760	MS/MS (+)	1.1	689.2094	[M+Na] ⁺	-2.47	527.2, 509.2, 365.0, 347.2
M11	Raffinose	C ₁₈ H ₃₂ O ₁₆	504.4358	MS/MS (+)	1.1	527.1566	[M+Na] ⁺	-3.22	365.1, 347.1
M12	6-Methoxyflavanone	C ₁₆ H ₁₄ O ₃	254.2800	MS/MS (+)	5.6	255.1006	[M+H] ⁺	-3.92	240.1, 237.0, 227.1, 213.0, 211.0, 209.0, 199.1, 185.1, 162.9, 149.9, 104.9

ANNEXES

M13	Ostruthol	C ₂₁ H ₂₂ O ₇	386.3943	MS/MS (+)	7.7	369.1329	[M-H ₂ O+H] ⁺	-1.08	269.0
M14	4-(3,4-Dihydroxyphenyl)-6,7-dihydroxy-2-naphthoic acid	C ₁₇ H ₁₂ O ₆	312.2731	MS/MS (+)	3.0	313.0699	[M+H] ⁺	-2.56	295.0, 269.0, 251.1
M15	Sinapinic acid	C ₁₁ H ₁₂ O ₅	224.2095	MS/MS (+)	4.0	207.0648	[M-H ₂ O+H] ⁺	-1.93	174.9
M16	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	267.2408	MS/MS (+)	2.2	268.1035	[M+H] ⁺	-1.86	135.9
M17	4-Hydroxy-3-(3-methylbut-2-en-1-yl)benzoic acid	C ₁₂ H ₁₄ O ₃	206.2372	MS/MS (+)	3.8	207.1005	[M+H] ⁺	-5.31	150.9
M18	2-Amino-1,3,4-octadecanetriol	C ₁₈ H ₃₉ NO ₃	317.5056	MS/MS (+)	7.8	318.2984	[M+H] ⁺	-5.97	300.2, 282.3

ANNEXES

M19	Pyrogallol	C ₆ H ₆ O ₃	126.1098	MS/MS (+)	1.2	127.0385	[M+H] ⁺	-3.94	108.8, 99.0
M20	Caffeic acid	C ₉ H ₈ O ₄	180.1571	MS/MS (+)	3.7	181.0489	[M+H] ⁺	-3.31	162.9, 138.9
M21	Methyl (7-hydroxy-11-methyl-2,9-dioxo-12,15-dioxatetracyclo[8.4.1.0 ^{1,1} _{0,0} ^{3,8}]pentadeca-3,5,7-trien-13-yl)acetate	C ₁₇ H ₁₆ O ₇	332.3041	MS/MS (+)	4.7	333.0967	[M+H] ⁺	-0.60	315.0, 297.1
N/A	12-Oxophytodienolic acid	C ₁₈ H ₂₈ O ₃	292.4120	MS/MS (+)	7.7	293.2105	[M+H] ⁺	-2.05	275.1, 257.2
N/A	1,3,6,8-Tetrahydroxy-2-[(1S)-1-methoxyhexyl]-9,10-anthraquinone	C ₂₁ H ₂₂ O ₇	386.3943	MS/MS (+)	5.7	387.1393	[M+H] ⁺	-11.62	355.0

ANNEXES

N/A	1,6,8-trihydroxy-3-(2'-hydroxypropyl)-9,10-anthraquinone	C ₁₇ H ₁₄ O ₆	314.2889	MS/MS (+)	5.3	297.0746	[M-H ₂ O+H] ⁺	-3.70	282.0, 279.0, 269.1, 268.0, 254.1, 251.0, 237.1
N/A	4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.1205	MS (+)	2.4	139.0383	[M+H] ⁺	-5.03	N/A
N/A	5-(β-D-Glucopyranosyloxy)-2-hydroxybenzoic acid	C ₁₃ H ₁₆ O ₉	316.2601	MS/MS (-)	2.5	315.0723	[M-H] ⁻	0.32	164.9, 152.9, 151.9, 108.9, 108.0
N/A	Citric acid	C ₆ H ₈ O ₇	192.1232	MS/MS (-)	1.1	191.0197	[M-H] ⁻	0.00	172.9, 110.9
N/A	Citrinin	C ₁₃ H ₁₄ O ₅	250.2467	MS/MS (+)	5.8	233.0805	[M-H ₂ O+H] ⁺	-1.29	218.0, 215.0, 205.1, 190.0, 187.0
N/A	L-(-)-Malic acid	C ₄ H ₆ O ₅	134.0872	MS/MS (-)	1.1	133.0143	[M-H] ⁻	0.75	114.9

ANNEXES

M352T3	Senecivernine N-oxide	$C_{18}H_{25}NO_6$	351.1682	MS/MS (+)	3.3	352.1736	$[M+H]^+$	-5.29	118.0, 120.0, 135.9, 153.9, 254.0
N/A	Succinic acid	$C_4H_6O_4$	118.0878	MS/MS (-)	1.7	117.0192	$[M-H]^-$	-0.85	98.9, 72.9

ANNEXES

Table S5. Absolute abundance values of selected bacterial and fungal rASVs showing significantly different abundances.

Absolute abundance values of Bacterial SDA-rASVs									
ASV 223	<i>Allorhizobium-N.-P.-Rhizobium</i>				ASV 187	<i>Phenylobacterium</i>			
Root	growth	blooming	fruiting	decay	Root	growth	blooming	fruiting	decay
Austrian	0	0	0	0	Austrian	0	12	0	1287
Greek A	39	161	295	384	Greek A	0	0	0	188
Greek B	72	80	48	90	Greek B	0	0	0	125
ASV 194	<i>Allorhizobium-N.-P.-Rhizobium</i>				ASV 37	<i>Burkholderia-C.-Paraburkholderia</i>			
Root	growth	blooming	fruiting	decay	Root	growth	blooming	fruiting	decay
Austrian	178	148	193	574	Austrian	0	93	0	327
Greek A	0	0	22	0	Greek A	748	1102	400	548
Greek B	0	0	0	0	Greek B	560	534	748	366
Rhizosphere	growth	blooming	fruiting	decay	Rhizosphere	growth	blooming	fruiting	decay
Austrian	290	310	351	379	Austrian	0	74	20	3119
Greek A	0	0	31	0	Greek A	795	1056	570	1121
Greek B	0	0	0	0	Greek B	661	349	599	1166
Soil	growth	blooming	fruiting	decay	Soil	growth	blooming	fruiting	decay
Austrian	677	467	907	510	Austrian	0	233	216	104
Greek A	0	0	250	0	Greek A	1478	1662	907	495
Greek B	0	0	189	0	Greek B	1559	1454	1561	594
ASV 1231	<i>Burkholderia-C.-Paraburkholderia</i>				ASV 625	<i>Mucilaginibacter</i>			

ANNEXES

Root	growth	blooming	fruiting	decay	Root	growth	blooming	fruiting	decay
Austrian	0	20	20	0	Austrian	0	0	0	0
Greek A	62	159	67	5	Greek A	195	153	28	55
Greek B	0	0	0	0	Greek B	0	0	0	9
ASV 553	<i>Gammaproteobacteria</i>				Soil	growth	blooming	fruiting	decay
Root	growth	blooming	fruiting	decay	Austrian	0	32	49	0
Austrian	0	0	0	367	Greek A	98	297	44	56
Greek A	0	0	0	192	Greek B	0	0	0	0
Greek B	0	0	0	49	Absolute abundance values of Fungal SDA-rASVs				
Rhizosphere	growth	blooming	fruiting	decay	ASV 41	<i>Penicillium jensenii</i>			
Austrian	0	0	0	45	Root	growth	blooming	fruiting	decay
Greek A	0	0	0	120	Austrian	0	0	455	176
Greek B	0	0	0	295	Greek A	0	0	920	277
Soil	growth	blooming	fruiting	decay	Greek B	277	1077	21272	1638
Austrian	0	0	0	18	ASV 122	<i>Helotiaceae</i>			
Greek A	0	8	34	250	Root	growth	blooming	fruiting	decay
Greek B	3	0	0	281	Austrian	63	7	0	99
					Greek A	318	20	0	0
					Greek B	939	1344	0	390

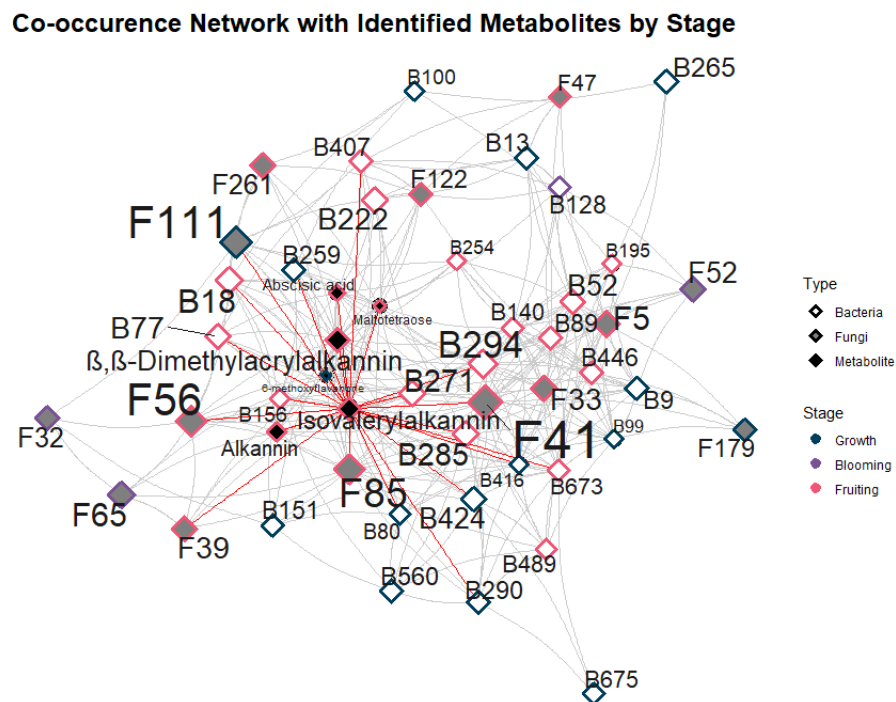


Figure S5. The interplay of abundant identified HPLC-HRMS metabolites and fungal and bacterial ASVs during plant development. In the network analysis, the size of the label shows the importance of feature based on the random forest analysis. Metabolites and microbial taxa are featured as the nodes of the network. Type of features and the corresponding stage where they occur are colored according to the figure legend. Isovalerylalkannin was identified as the key node of the network. F letters indicate the fungal rASVs on the figure, while B letters are the bacterial rASVs.

Annex II

Arbuscular mycorrhizal fungi impact the production of alkannin/shikonin and their derivatives in *Alkanna tinctoria* Tausch. grown in semi-hydroponic and pot cultivation systems

Isolation and mono-species culture of AMF

Evaluation of the presence of AMF in root/soil samples

Roots of *Alkanna tinctoria* were sampled from the wild in Greece and stained following the protocol of Vierheilig et al. (1998), and Walker (2005), to evaluate root colonization (Figure S6A). In parallel, rhizospheric soils were sampled to collect AMF spores applying a procedure adapted from Cranenbrouck et al. (2005) and Walker (2009) (personal communication) (Figure S6B). Spores were subsequently mounted on slide with polyvinyl alcohol-lacto-glycerol (PVLG) solution (Omar et al., 1979) and checked under stereomicroscope (Olympus BH2–RFCA, Japan) to confirm AMF presence.

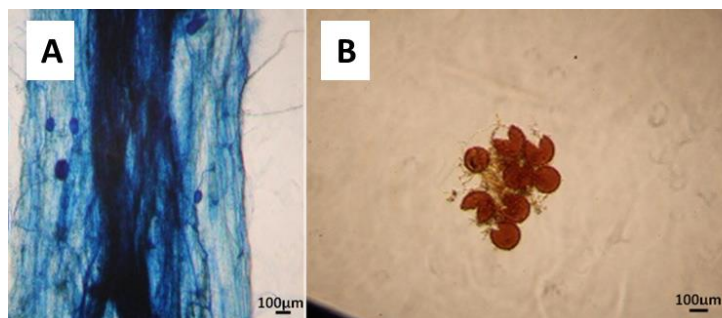


Figure S6. (A) Stained *A. tinctoria* root and (B) spores, collected from the wild in Greece (Photographs taken with Canon EOS 60D, through the lens of a bright field light stereomicroscope, Olympus BH2-RFCA, Japan).

Establishment of AMF trap cultures

Seeds of *Plantago lanceolata* L. (Ecossem, Belgium) and *Medicago truncatula* Gaertn. (SARDI, Australia) were surface sterilized by soaking in 70% ethanol for 1 min and in sodium hypochlorite (8% active chloride) for 5 min, and subsequently rinsed 3 times with deionized water. The seeds were then germinated in a 1.3 L pot (15×11.4 cm) filled with a sterilized (121°C for 15 min) substrate that consist of a mixture of calcinated clay (DCM, Belgium), quartz (0.4-0.8 mm),

ANNEXES

quartz (1-2 mm), 2:2:1 w/w.

A. tinctoria plant rhizospheric soils and roots pieces were used in trap cultures. For roots, thin fragments were placed near the root system of 15-day old seedlings of *M. truncatula* and *P. lanceolata*. Two and 5 seedlings, respectively, were placed in each pot (1.3L, 15×11.4 cm) filled with the same sterilized substrate mixture as above. For rhizospheric soils, samples were placed in individual pots between two layers of the same sterilized substrate as above, and in contact with 15-day old *M. truncatula* and *P. lanceolata* seedlings. In total 24 trap cultures were established (12 for rhizospheric soils and 12 for root samples).

To maintain the trap cultures, the pots were watered to field capacity and supplemented with slow release NPK beads (four beads per pot) (Osmocote PRO 5-6 months, 17-11-10+2MgO+TE, or Osmocote PRO 16-3-11+3 MgO, Everris, France). The pots were maintained in Sunbags (Sigma-Aldrich, Germany) closed with plastic paper clips (Laurel, Germany). They were watered with deionized water every three weeks and kept under greenhouse condition set at 20°C, 50% RH, photoperiod of 16 h day⁻¹ and PPF of 96 µmol m⁻²s⁻¹ (Figure S7).



Figure S7. Trap cultures of rhizospheric soils with *M. truncatula* and *P. lanceolata* plants, under greenhouse conditions (UCLouvain).

Establishment of AMF mono-species pot cultures

Roots and substrates from the trap cultures above were used to collect new, actively growing and healthy AMF spores. Roots and substrates were sieved through a sequence of sieves (i.e., 250, 106 and 38 µm, Euromatest Sintco, France) and supernatant cleaned with tap water. Different fractions from the sieves were collected in Petri dishes (50 mm) and observed under stereomicroscope (Olympus, SZ61) to collect healthy-looking spores with tweezers and needles. Single spores or cluster of spores attached by a common mycelium were placed

ANNEXES

at the intersection between a main root and newly growing secondary root of 15-day old *P. lanceolata* seedlings. Then, the seedlings were placed in 7x7x6 cm pots filled with sterilized substrate mixture as above. Four pots were placed in one closed Sunbag and watered with low-P Hoagland solution (Hoagland and Arnon, 1950) 100x diluted every 20 days. Sunbags were maintained under greenhouse conditions set as above (Figure S8). In total, around 220 single spores AMF pot cultures were established.

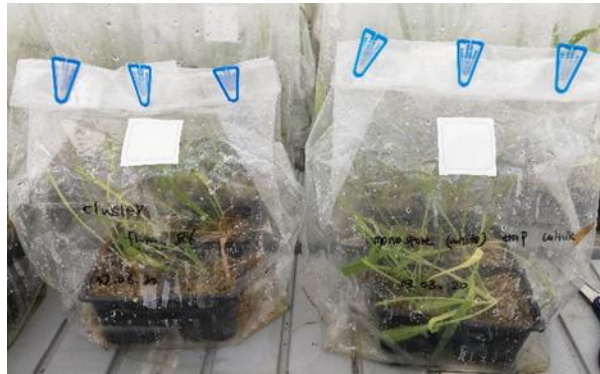


Figure S8. Single spores AMF trap cultures with *P. lanceolata* host plants maintained under greenhouse conditions (UCLouvain).

Molecular identification of isolated AMF

In total 44 cultures of AMF were successful. A selection of 9 cultures (with contrasting spores morphologies) were used for molecular identification. Three to 5 healthy spores were collected in each pot by wet sieving and picked with needle and tweezer (Vomm 113 SA). They were used for DNA extraction through sterile needle (AGANI™ NEEDLE, 0.45×23 mm, TERUMO) in 0.2 mL PCR tubes (SARSTEDT, Germany) under laminar flow hood. Five μL of PCR water was added to each sample to crush the spores and obtain the starting 5 μL of template DNA. Two-step PCR applying specific AMF primers amplifying a part of the SSU rRNA gene, the complete ITS region (including the 5.8S rRNA gene) and approx. 800 bp of the LSU rRNA gene, named SSU-ITS-LSU, were used (Krüger et al., 2009). For the first PCR, primer pairs mixtures SSUmAf – LSUmAr were used, and for the nested PCR, primer pairs mixtures SSUmCf – LSUmBr (Table S6) were used. In both amplifications, the reaction mix contained Phusion High Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Lithuania) with 0.5 μM concentration of each primer (Sigma, Germany) and 0.2 $\mu\text{g mL}^{-1}$ BSA (Albumin Bovine, AMRESCO, United States). In the first PCR reaction mix, 5 μL of template DNA (as above), 10 μL of Phusion High-Fidelity PCR

ANNEXES

Master Mix, 1 μ L of SSUmAf and LSUmAr, and 3 μ L of ultra clean water (Sigma) were used in 20 μ L of final reaction. Thermal cycling was performed in an Eppendorf Master-cycler Gradient (Eppendorf Nexus X2, Germany) using the following parameters for the first PCR: initial denaturation of 5 min at 99°C, followed by 40 cycles of denaturation (10 s at 99°C)/ annealing (30 s at 60°C)/ elongation (1 min at 72°C), and a final elongation of 10 min at 72°C. For the nested PCR, 1 μ L of the first PCR product was used as template in the same final reaction volume (20 μ L) as above, and the same thermal cycling conditions were set up with slight differences: 30 cycles and annealing temperature of 63°C. The PCR products were visualized on 1.0% agarose gel with 1 $\times$ TAE buffer (Tris/Acetic Acid/EDTA buffer 50x, Carl Roth) and stained with GelRed[®] (Biotium, USA).

ANNEXES

Table S6: Forward and reverse primers sequences used to amplify AMF following Krüger et al. (2009).

	Primer	Nucleotide sequence (5'-3')	Primer mixtures
First PCR	SSUmAf1	TGGGTAATCTTTTGAAACTTYA	SSUmAf: mixSSUmAf1-2 (equimolar)
	SSUmAf2	TGGGTAATCTTRTGAAACTTCA	
	LSUmAr1	GCTCACACTCAAATCTATCAAA	LSUmAr: mixLSUmAr1-4 (equimolar)
	LSUmAr2	GCTCTAACTCAATTCTATCGAT	
	LSUmAr3	TGCTCTTACTCAAATCTATCAAA	
	LSUmAr4	GCTCTTACTCAAACCTATCGA	
Nested PCR	SSUmCf1	TCGCTCTTCAACGAGGAATC	SSUmCf: mixSSUmCf1-3 (equimolar)
	SSUmCf2	TATTGTTCTTCAACGAGGAATC	
	SSUmCf3	TATTGCTCTTNAACGAGGAATC	
	LSUmBr1	DAACACTCGCATATATGTTAGA	LSUmBr: mixLSUmBr1-5 (equimolar)
	LSUmBr2	AACACTCGCACACATGTTAGA	
	LSUmBr3	AACACTCGCATACATGTTAGA	
	LSUmBr4	AAACACTCGCACATATGTTAGA	
	LSUmBr5	AACACTCGCATATATGCTAGA	

ANNEXES

Cloning protocol was followed as described by Krüger et al. (2009). Briefly, the 1.5 kb fragments from the nested PCR products was cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, United States) following the manufacturer's protocol. Five colonies of each sample were analysed for correct length of plasmid inserts by colony-PCR using 1x GoTaq DNA Polymerase (Promega, United States) and M13F-M13R primers. Correct clone PCR products were sequenced using M13F-M13R primers at Macrogen Inc. (Korea).

Sequences were assembled and edited with software Sequencher 5.4.6 version (Gene Codes Corporation, USA). Homologous sequences were searched by blastn at the National Center for Biotechnology Information (NCBI). An AMF freely available reference alignment from Krüger et al. (2012), was also applied to compare and establish the phylogenetic tree. Assembled sequences, homologous environmental sequences from the NCBI platform and reference alignment were aligned and performed with MAFFT online (Katoh and Standley, 2013) and then manual adjusted at the Phylogenetic Data Editor (PhyDE).

A maximum-likelihood phylogenetic tree was assembled with references using RAxML-HPC2 (Stamatakis et al., 2008) on XSEDE ver. 8.2.9 on the CIPRES Science Gateway6 with 1000 bootstrap and the GTRGAMMA model (Krüger et al., 2012). Taxonomic annotations followed the classification of Schüßler and Walker (2010). The AMF species selected in the paper were closest to *Rhizophagus irregularis* and *Septoglomus viscosum* (Figure S9).

ANNEXES

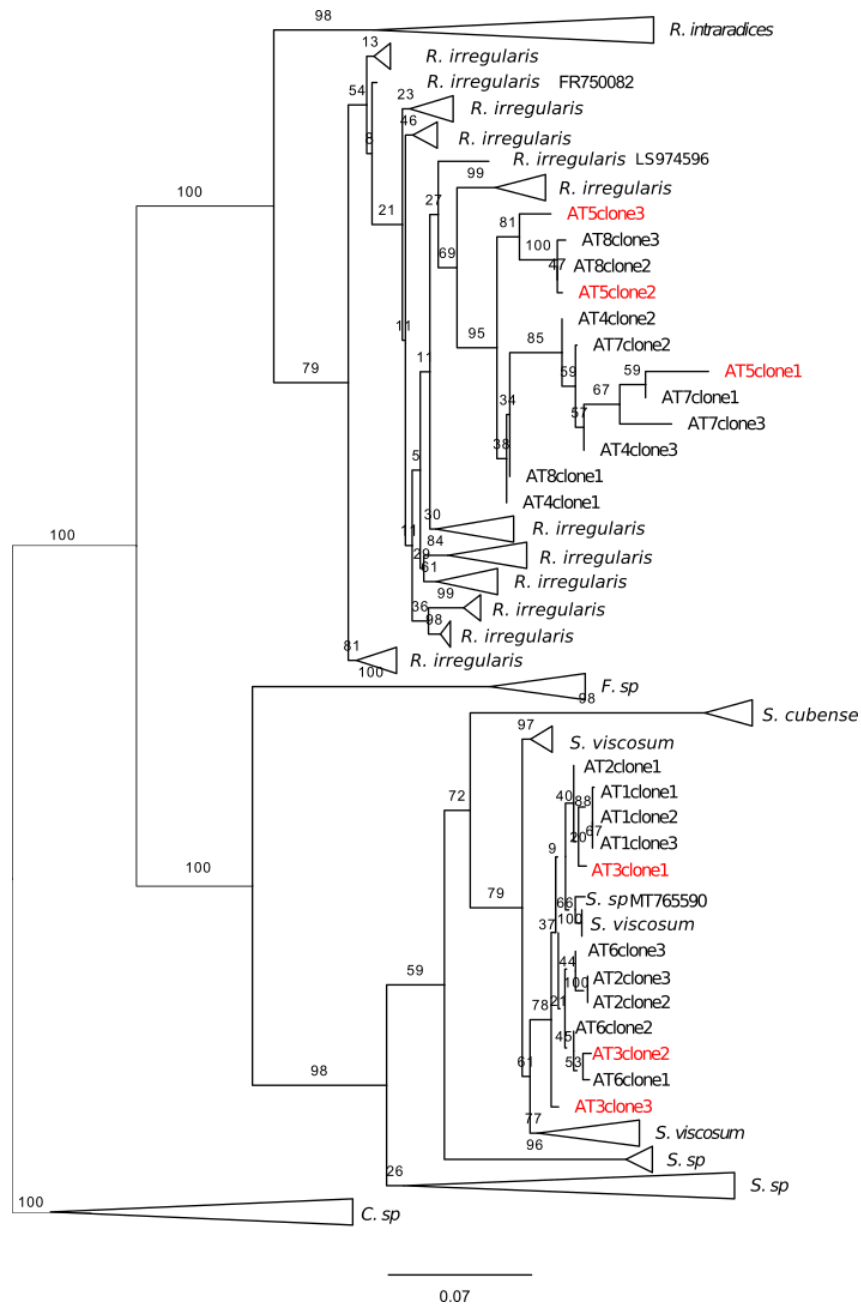


Figure S9. Phylogenetic maximum likelihood tree of the isolates AT from *A. tinctoria* and reference AMF species. Characterised *Claroideoglomus*

ANNEXES

species were used as outgroup. Size of triangles represent the sequence numbers (vertically) and distances (horizontally). Two isolates used in this paper were highlighted with red colour.

Mass-production of *Rhizophagus irregularis* and *Septoglomus viscosum*

The 2 AMF strains selected above (*Septoglomus viscosum* and *Rhizophagus irregularis*) were used to establish mass-production cultures with maize plants. Briefly, bleach soaked, and water washed maize seeds were germinated on sterilized lava substrate (120°C for 15 min) in a 5 L size pot. One-week old maize seedling was inoculated with AMF propagules of the two species (i.e., colonized *P. lanceolata* roots and substrates). Both AMF were used in association with *A. tinctoria* plants growing in the S-H system and in pots under greenhouse conditions (Experiment 2 and 3 in the main text).

***Alkanna tinctoria* acclimatization protocol**

In vitro produced *Alkanna tinctoria* plants (Cartabia et al., 2022) were acclimatized *ex vitro* as follows: plants were carefully removed from the culture medium (Figure S10a) and gently washed under running deionized water to remove the medium adhering to the roots. Subsequently, three to four rooted plants were transferred into glass pots (J. Weck GmbH u. Co. KG, Germany, 147x100 mm, size: 580 mL) closed by a cover and a cotton layer and containing a sterile (2x 121°C for 15 min) substrate mixture [3 peatmoss (DCM, Grobbendonk, Belgium)/2 compost (DCM, Grobbendonk, Belgium)/1 perlite (Perligran Premium, KNAUF GMBH, Dortmund, Germany)/1 quartz 0.4-0.8 mm (no. 4, Euroquartz, Belgium)/1 quartz 1-2 mm (no. 1, Euroquartz, Belgium)] (Figure S10b). The plants were kept constantly moist in a growth chamber set at 24°C/20°C (day/night), RH of 80%, photoperiod of 16 h day⁻¹ and PPF 130 µmol m⁻²s⁻¹. (white fluorescent light, OSRAM L36 W830 Luminux). After 2 weeks, the glass pots were opened and placed inside a closed Sunbag (Sigma-Aldrich, Taufkirchen, Germany) (Figure S10c). After 1 week, the plants were transplanted into single pots (7x7x6 cm) containing a sterile substrate mixture (2 peatmoss/2 compost/1 perlite /1 quartz 0.4-0.8 mm /1 quartz 1-2 mm) closed again under the Sunbag (Figure S10d). After 1 week, the Sunbags were gradually opened (2, 4, 6, 8 h) until the plants were completely adapted (±15 days) at the conditions in the growth chamber presented above (Figure S10e-g).

ANNEXES

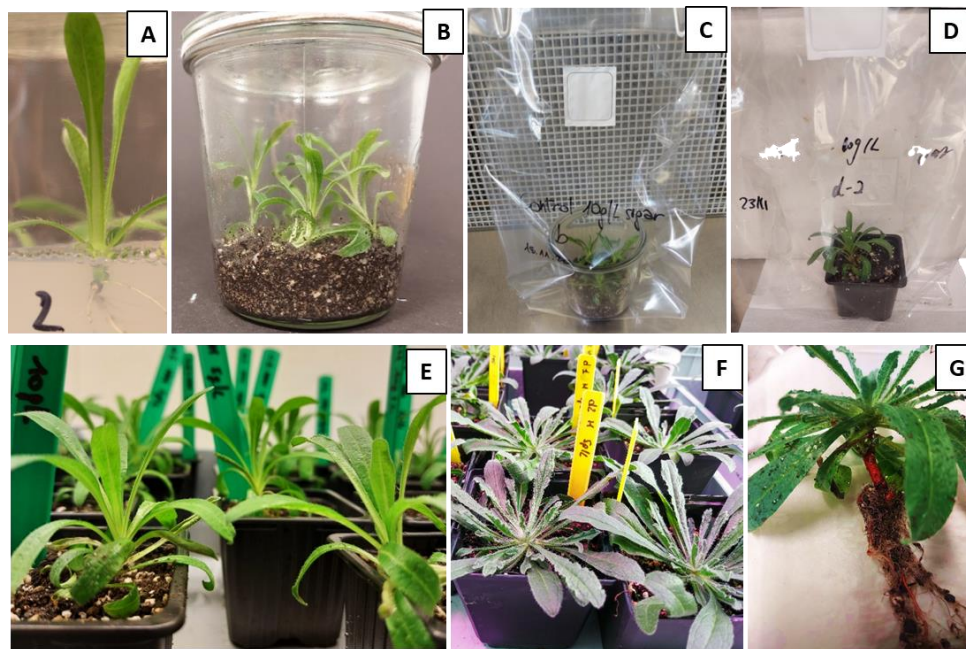


Figure S10. *Ex vitro* acclimatization and adaptation of *in vitro* produced plantlets of *A. tinctoria*: (A) rooted plantlets growing under *in vitro* conditions; (B) plantlets transferred into closed glass pot filled with mixed sterile substrate; (C) glass pot opened inside a closed Sunbag; (D) plants transplanted into an individual pot in a closed Sunbag; (E) plants adapted to the growth chamber conditions; (F) plants transplanted in bigger pots for the AMF-colonization and kept under greenhouse conditions (UCLouvain); (G) details of the characteristic roots' reddish colour during the plants harvest.

HPLC quantification: methodology validation

Response function

The most adequate linear regression was selected with 95% expectation tolerance intervals included inside the $\pm 20\%$ acceptance limits for each concentration level of the validation standards except the lowest one (Figure S11).

Trueness, precision and accuracy

Trueness was calculated at each concentration level of the validation standard and expressed in relative bias (RB). Relative bias was less than 3%, except for the lowest shikonin concentration.

Precision was evaluated intra-day (repeatability) and inter-day (intermediate

ANNEXES

precision) and expressed as relative standard deviations (RSD). The repeatability and the intermediate precision were less than 1.40% and 3.49%, respectively. All the trueness and precision results are in accordance with EMA guidelines criteria ($\leq 15\%$).

Accuracy profiles, evaluating the sum of systematic and random errors of the test values (total error), are shown in Figure S12, indicating that the relative upper and lower 95% β -expectation tolerance limits are inside the acceptance limits, set at 20%, except for the lowest concentration. The method can thus be considered as accurate between 0.1 and 0.8 mg mL⁻¹. The accuracy results are presented in Table S2.

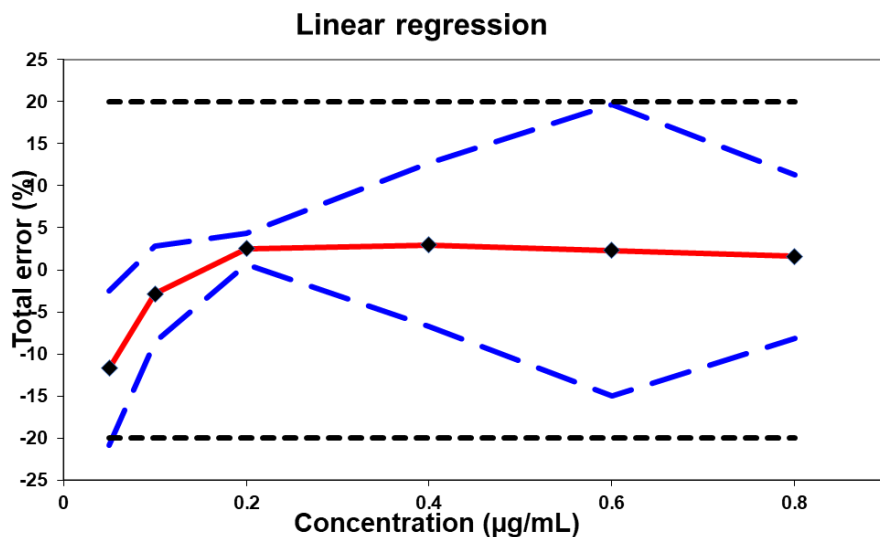


Figure S11. Accuracy profile of the mixture of shikonin obtained with linear regression. The plain line is the relative bias, dashed lines are the β -expectation tolerance limits ($\beta = 95\%$) and dotted lines represent the acceptance limits ($\pm 20\%$).

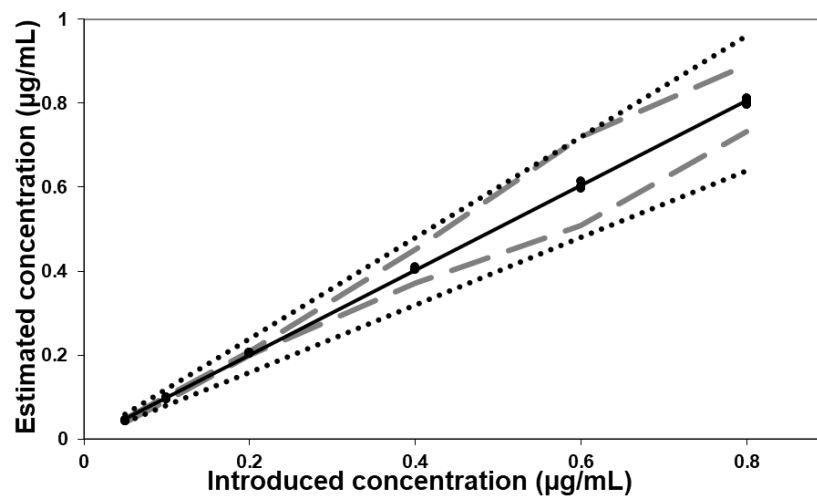


Figure S12. Linear profile of shikonin standard. The plain line is identity line ($y = x$), the dashed lines are the β -expectation tolerance limits ($\beta = 95\%$) and dotted lines represent the acceptance limits ($\pm 20\%$).

ANNEXES

Table S7. Validation results obtained for the quantification of shikonin.

Validation criteria		Concentration levels ($\mu\text{g mL}^{-1}$)					
		0.05	0.1	0.2	0.4	0.6	0.8
Response function		Linear regression					
		Calibration range (5 points)					
		0.05-0.8					
Trueness	Relative bias (%)	-11.68	-2.84	2.53	2.98	2.31	1.58
Precision	Repeatability (RSD %)	0.34	0.72	0.31	1.40	0.76	0.53
	Intermediate precision (RSD %)	1.85	1.58	0.51	2.71	3.49	1.97
Accuracy (95 % relative β -expectation lower and upper tolerance limits in %)		-20.85	-8.55	0.71	-6.76	-14.97	-8.15
		-2.52	2.85	4.36	12.71	19.6	11.31
Linearity	Slope	1.0252					
	Intercept	-0.0032					
	R ²	0.9986					
Limit of detection		0.0215					
Limit of quantification		0.0651					

ANNEXES

Analysis of A/Sd target genes expression in *A. tinctoria* roots

Alignments used to design primers

Aligned *GHQH* sequences (MH077962 = *Arnebia euchroma*; MN056184 = *L. erythrorhizon*). Coloured sequences are conserved regions that were used for primer designing.

```

                                10         20         30         40         50
60
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---|
Consensus
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MH077962
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MN056184
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120
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---|
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MN056184
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180
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---|
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MH077962
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MN056184
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240
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```

ANNEXES

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MN056184
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300
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MN056184
AAAGAAAUCCUCCAAAAACAGGACAUUUCUUCGCGGGGAAAAGAAUCCCCGACGCCUC

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----:----|----:----|----:----|----:----|----:----|----:--
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MN056184
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420
----:----|----:----|----:----|----:----|----:----|----:--
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430 440 450 460 470

480
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ANNEXES

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660
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----:-----
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670 680 690 700 710

720
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MN056184
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730 740 750 760 770

780
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MH077962

ANNEXES

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840
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MN056184
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900
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---|
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MH077962
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MN056184
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960
----:----|----:----|----:----|----:----|----:----|----:--
---|
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970 980 990 1000 1010

1020
----:----|----:----|----:----|----:----|----:----|----:--
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1030 1040 1050 1060 1070

1080

ANNEXES

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1140
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1200
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----:-----
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1260
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Consensus
UGGRAAGAUCCGUUARCAUUAACCCYAGAGGUUUCUUGACUCGGARCUUGAYUUCGGA
MH077962
UGGGAAGAUCCGUUAGCAUUAAGCCUGAGAGGUUUCUUGACUCGGAGCUUGACUUCGGA
MN056184
UGGAAAGAUCCGUUAACAUAUCCCGAGAGGUUUCUUGACUCGGAACUUGAUUUCGGA

          1270          1280          1290          1300          1310
1320
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----:-----
---|
Consensus
GGCAACMAUUUCGARAUGUUGCCUUUUGGUGCYGGRAGAAGAUCAUGCCCUGGAUUGCCU
MH077962
GGCAACAAUUUCGAGAUGUUGCCUUUUGGUGCCGGAAGAAGAUCAUGCCCUGGAUUGCCU
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ANNEXES

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MN056184
GGCAACCAUUUCGAAAUGUUGCCUUUUGGUGCUGGGAGAAGAUAUGCCUGGAUUGCCU

                                1330      1340      1350      1360      1370
1380      -----:-----|-----:-----|-----:-----|-----:-----|-----:--
---|
Consensus
AUGGCAGUUAGGAUGGUUCCUCUGMUUUUGGGGUCACUUAUCAACUCAUUUGAUUGGSWA
MH077962
AUGGCAGUUAGGAUGGUUCCUCUGCUUUUGGGGUCACUUAUCAACUCAUUUGAUUGGGUA
MN056184
AUGGCAGUUAGGAUGGUUCCUCUGAUUUUGGGGUCACUUAUCAACUCAUUUGAUUGGCAA

                                1390      1400      1410      1420      1430
1440      -----:-----|-----:-----|-----:-----|-----:-----|-----:--
---|
Consensus
CUKGAUGGUGGAAUGAAGCCUGAAGAUUURAGCAUGGAGGAAAAGGUKGGGCUYACAGCC
MH077962
CUUGAUGGUGGAAUGAAGCCUGAAGAUUUGAGCAUGGAGGAAAAGGUUGGGCUCACAGCC
MN056184
CUGGAUGGUGGAAUGAAGCCUGAAGAUUUAAGCAUGGAGGAAAAGGUUGGGGCUUACAGCC

                                1450      1460      1470      1480      1490
Consensus CAAUUGGCUCAUYCWCURAAAAUUGUYCCAAYCCCWGUAAGAAGAGUAA
MH077962 CAAUUGGCUCAUCCUCUGAAAAUUGUCCAAUCCCAGUAAAGGAAGAGUAA
MN056184 CAAUUGGCUCAUUCACUAAAAUUGUCCCAACCCCUGUAAAAGAAGAGUAA

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Aligned *LePGT2* sequences (KT991522 = *Arnebia euchroma*; AB055079.1 = *L. erythrorhizon*). Coloured sequences are conserved regions that were used for primer designing.

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                                10      20      30      40      50
60      -----:-----|-----:-----|-----:-----|-----:-----|-----
:-----|
Consensus
AKKWSYWSCAAACAAACMCAGCUMAAGAAAGGCAAGCAACCAUCAUGGAUUGAGAUKUUAU
KT991522
AGUUCCAGCAAACAAACCCAGCUCAAGAAAGGCAAGCAACCAUCAUGGAUUGAGAUUAU
AB055079.1
ATGAGTTCCAAACAAACACAGCTAAAGAAAGGCAAGCAACCATCATGGATTGAGATTTAT

                                70      80      90      100      110
120

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ANNEXES

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      -----|-----|-----|-----|-----|-----
:-----|
Consensus
UUGCCYMAAGARGUUCGRCCUUAUGCKCAYCUUGCAAGGYUAGACAAGCCUAUAGGCAGY
KT991522
UUGCCCCAAGAAGUUCGACCUUAUGCUCAUCUUGCAAGGUUAGACAAGCCUAUAGGCAGC
AB055079.1
TTGCCTAAAGAGGTTTCGGCCTTATGCGCACCTTGCAAGGCTAGACAAGCCTATAGGCAGT

      130      140      150      160      170
180
      -----|-----|-----|-----|-----|-----
:-----|
Consensus
UGGYURCUMGCUUGGCCMGCSUUYUGGUCCGUYGCAUUGRYUGCUGAUMUUGRMAGUCUA
KT991522
UGGUUGCUGAGCUUGGCCAGCCUUCUGGUCCGUCGCAUUGGCUGCUGAUUUGAAAGUCUA
AB055079.1
TGGCTACTCGCTTGGCCCGGTTTTGGTCCGTTGCATTGATTGCTGATCTTGGCAGTCTA

      190      200      210      220      230
240
      -----|-----|-----|-----|-----|-----
:-----|
Consensus
CCWAAAAUGKURGCAAUUUUGGAUGGUGGGCAGUUUGGAUCMGAGGUGCUGGAUGYACC
KT991522
CCUAAAAUGGUAGCAAUUUUGGAUGGUGGGCAGUUUGGAUCAGAGGUGCUGGAUGCACC
AB055079.1
CCAAAAATGTTGGCAATATTTGGATGGTGGGCAGTTTGGATCCGAGGTGCTGGATGTACC

      250      260      270      280      290
300
      -----|-----|-----|-----|-----|-----
:-----|
Consensus
AUUAAYGAUUACUUCGACCGGAUUUYGAYAARAARGUGGAACGUACAAAUCUAGACCW
KT991522
AUUAUGAUUACUUCGACCGGAUUUCGACAAGAAGGUGGAACGUACAAAUCUAGACCU
AB055079.1
ATTAACGATTACTTCGACCGCGATTTTGATAAAAAAGTGGAACGTACAAAATCTAGACCA

      310      320      330      340      350
360
      -----|-----|-----|-----|-----|-----
:-----|
Consensus
CUYGCUAGUGGCGUGUCUCRCCWKCMMAAGGRYUSUGGUGGCUUGCWUUUCARCURUUY
KT991522
CUUGCUGAUGGCGUGUCUCACCUUCCCAAGGAUUGUGGUGGCUUGCAUUUCAGCUGUUC
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ANNEXES

AB055079.1
CTCGCTAGTGGCGCTGTCTCGCCAGCAAAAGGGCTCTGGTGGCTTGCTTTTCAACTATTT

370 380 390 400 410

420
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----
:-----|
Consensus
AUUGGMUUGGGUGUUCUUUACCAAUUCAAAYAUCUUGACUCUUGCAUURGCUAUYKUGCAU
KT991522
AUUGGCUUGGGUGUUCUUUACCAAUUCAAACUUCUUGACUCUUGCAUUGGCUAUCUUGCAU
AB055079.1
ATTGGATTGGGTGTTCTTTACCAATTCAATATCTTGACTCTTGCATTAGCTATTGTGCAT

430 440 450 460 470

480
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----
:-----|
Consensus
GUKCCCYUUGUKUUUGCUUAYCCUCUCAUGAAAAGAAUUACMUUUGGCCUCAAGCKUUU
KT991522
GUUCCCCUUGUUUUUGCUUAUCCUCUCAUGAAAAGAAUUACCUAUUGGCCUCAAGCUUUU
AB055079.1
GTGCCCTTGTGTTTGCTTACCCTCTCATGAAAAGAATTACATATTGGCCTCAAGCGTTT

490 500 510 520 530

540
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----
:-----|
Consensus
CUUGGMGUWAUGAUMAGUUGGGGAGCUCUYUUAGGSUCCUCUGCYCUUAAAGGAAGUGUU
KT991522
CUUGGAGUAAUGAUCAGUUGGGGAGCUCUCUUAGGCUCCUCUGCUCUUAAGGAAGUGUU
AB055079.1
CTTGGCGTTATGATAAGTTGGGGAGCTCTTTTAGGGTCTCTGCCCTTAAAGGAAGTGTT

550 560 570 580 590

600
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----
:-----|
Consensus
GUYCCAAGYAKYGCCUACCCRCUYUACAUUUCGAGCUUUUUCUGGACUCUUGUUUAUGAU
KT991522
GUUCCAAGUAGCGCCUACCCGCUUUACAUUUCGAGCUUUUUCUGGACUCUUGUUUAUGAU
AB055079.1
GTCCCAAGCATTGCCTACCCACTCTACATTTTCGAGCTTTTCTGGACTCTTGTTTATGAT

610 620 630 640 650

660
-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----

ANNEXES

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:----|
Consensus
ACWAUYUAUGCACAUCAAGAUAAAGGUMGACGAYGCAAAAGCWGGRAUUAUAAUCCACWGCU
KT991522
ACUAUCUAUGCACAUCAAGAUAAAGGUAGACGAUGCAAAAGCAGGGAUUAUAAUCCACUGCU
AB055079.1
ACAATTTATGCACATCAAGATAAGGTCGACGACGCAAAAGCTGGAATTAAATCCACAGCT

              670      680      690      700      710
720
      ----:----|----:----|----:----|----:----|----:----|----
:----|
Consensus
CUAMGMUUUGGWGAUGCWACMAARAUUAGGAUUASWUGGUUCGGMRUAGGAUGCAUUGSU
KT991522
CUAAGAUUUGGAGAUGCAACCAAGAUUAGGAUUAGUUGGUUCGGAGUAGGAUGCAUUGCU
AB055079.1
CTACGCTTTGGTGATGTACAAAAATATGGATTACATGGTTCGGCATAGGATGCATTGGT

              730      740      750      760      770
780
      ----:----|----:----|----:----|----:----|----:----|----
:----|
Consensus
GCUCUWSUUMUWGGSGGGYUCAUUGUSAACAUUGGGUUKCCUUUUAYGURUUUKUGGCA
KT991522
GCUCUAGUUUUGGGGGGCUCAUUGUGAACAUUGGGUUUCCUUUUUAUGUAUUUGUGGCA
AB055079.1
GCTCTTCTTCTAGGCGGGTTCATTGTCAACATTGGGTTGCCTTATTACGTGTTTTTGGCA

              790      800      810      820      830
840
      ----:----|----:----|----:----|----:----|----:----|----
:----|
Consensus
AUYGCAACUGGUCAAUUGRYUUGGCAAAUUKUCACAGUUGAUUUUAUCAUCUCCUAUGGAU
KT991522
AUCGCAACUGGUCAAUUGGCUUGGCAAAUUGUCACAGUUGAUUUUAUCAUCUCCUAUGGAU
AB055079.1
ATTGCAACTGGTCAATTGATTTGGCAAATTTTCACAGTTGATTTATCATCTCCTATGGAT

              850      860      870      880      890
900
      ----:----|----:----|----:----|----:----|----:----|----
:----|
Consensus
UGUGGYMRGAARUUYGUKUCYAACCAAUGGUUUGGUGCUAUWAUMUUCASYGGCAUCUUR
KT991522
UGUGGUCGGAAAUCGUUUUCAACCAAUGGUUUGGUGCUAUUAUUAUUCAGUGGCAUCUUG
AB055079.1
```

ANNEXES

TGTGGCAAGAAGTTTGTGTCCAACCAATGGTTTGGTGCTATAATCTTCACCGGCATCTTA

	910	920
	----:---- ----:---- --	
Consensus	SUUGGRAGAUUGUUUMCUUAGC	
KT991522	CUUGGAAGAUUGUUUACUUAGC	
AB055079.1	GTTGGGAGATTGTTTCCTTAG-	

ANNEXES

Annex III

Effects of fungal endophytes and arbuscular mycorrhizal fungi on growth of *Echium vulgare* and alkannin/shikonin and their derivatives production in roots

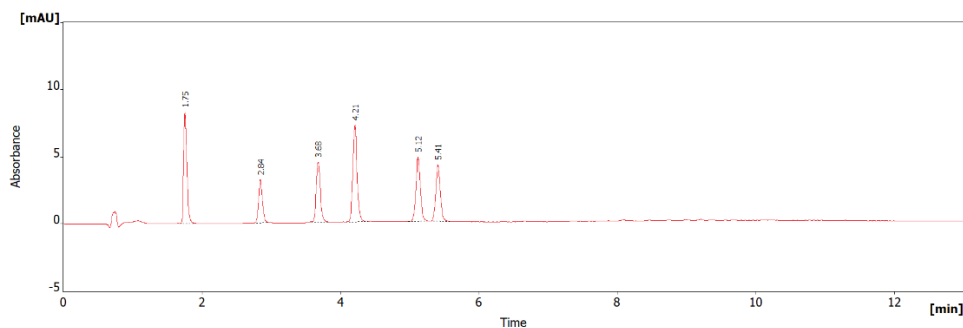
Supplementary Table S9: Standard compounds and their calibration curves, correlation coefficients, limits of detection (LOD), limits of quantification (LOQ) and HPLC method validation.

Compound	Calibration Curve (Y=concentration, ppm)	R ²	LOD (ppm)	LOQ (ppm)	Precision (%)	Intraday repeatability (%)	Interday reproducibility (%)
Shikonin	$5.3734 \times (\text{Peak Area}) - 0.9542$	0.9995	0.77	2.32	1.09	0.77	0.64
Acetylshikonin	$2.6173 \times (\text{Peak Area}) - 0.4199$	0.9985	1.37	4.15	1.83	1.03	1.39
Propionylshikonin	$3.8749 \times (\text{Peak Area}) - 0.2775$	0.9974	1.84	5.57	2.47	1.70	1.69
Deoxyshikonin	$5.9882 \times (\text{Peak Area}) + 0.9235$	0.9966	2.11	6.39	1.51	0.74	1.19
β,β -Dimethylacrylshikonin	$4.3877 \times (\text{Peak Area}) - 0.5147$	0.9993	0.93	2.81	1.35	0.96	0.91
Isovalerylshikonin	$3.8929 \times (\text{Peak Area}) + 0.0148$	0.9987	1.31	3.96	1.64	0.89	1.28

ANNEXES

Standard mixtures containing 1-80 ppm (data points 1, 2, 5, 10, 15, 20, 30, 40, 60, 80) of each of the measured compounds were prepared. The reported calibration curves are 1-15 ppm curves with lower detection/quantification limits as most compounds in most of the samples were present in a low concentration (< 15 ppm). In the case of concentrations over 15 ppm, the higher concentration data points were used for more accurate concentration estimates. Precision was calculated for the 10 ppm data point for each of the compounds. It is shown as the relative standard deviation of measurements for individual compounds. Intraday variations between measurements for the same data point were obtained by measuring the same sample three times within a day. Interday reproducibility estimates were made by measuring the same sample in three consecutive days.

Supplementary Figure S13: Chromatogram of a mixture of A/S standards.



A chromatogram (520 nm detection wavelength) of the mixture of standards containing 10 ppm of each of the measured compounds. The retention times are as follows: shikonin 1.75 min, acetylshikonin 2.84 min, propionylshikonin 3.68 min, deoxyshikonin 4.21 min, β,β – dimethylacrylshikonin 5.12 min, isovalerylshikonin 5.41 min.

ANNEXES

Table S10: A/S, Acetyl-A/S, Deoxy-A/S, Isovaleryl-A/S, Propionyl-A/S, β,β -Dimethylacryl-A/S and total A/Sd content (mg) in roots of *E. vulgare* inoculated with 10 fungal endophytes, after three months of growth in pots under greenhouse condition.

Treatment	A/S	Acetyl-A/S	Deoxy-A/S	Isovaleryl-A/S	Propionyl-A/S	β,β -Dimethylacryl-A/S	Total A/Sd
Control	0.94±1.32	3.80±4.16	ND	0.65±0.33	ND	ND	5.38±5.76
<i>Plectosphaerella</i> sp.	0.26±0.28	3.92±1.73	ND	0.71±0.25	ND	0.09±0.13	4.97±1.98
<i>Cladosporium allicinum</i>	0.55±0.71	2.77±2.01	ND	0.36±0.45	ND	0.05±0.12	3.73±2.73
<i>Cadophora</i> sp. REF040	0.34±0.29	3.10±3.06	ND	0.44±0.41	ND	ND	3.88±3.56
<i>Clonostachys</i> sp. G5182	0.29±0.27	1.98±1.31	ND	0.25±0.28	ND	ND	2.51±1.70
<i>Trichoderma hispanicum</i>	0.20±0.12	3.54±2.33	0.09±0.20	0.63±0.39	ND	0.04±0.09	4.62±3.00
<i>Cadophora luteo-olivacea</i>	0.61±0.23	4.25±2.50	ND	0.65±0.38	ND	ND	5.52±2.85
<i>Scytalidium</i> sp.	1.00±0.82	6.32±4.25	ND	0.96±0.56	ND	ND	8.05±5.14
<i>Fusarium acuminatum</i>	0.23±0.20	4.76±3.92	0.17±0.23	0.96±0.75	ND	0.04±0.09	6.72±5.45
<i>Leptosphaeria ladina</i>	0.65±0.43	4.32±1.80	ND	0.85±0.40	ND	ND	5.61±2.02
<i>Phialophora</i> sp.	0.74±0.68	2.59±1.77	ND	0.27±0.24	ND	0.03±0.07	3.51±2.29
Wilcoxon/Kruskal-Wallis Test							

ANNEXES

p-value	0,6311	0,4871	0,0271*	0,4023	-	0,3724	0,4406

The parameters measured are expressed as mean $\pm$ standard deviation (SD) of 5 or 6 replicates per treatment. P-values higher than 0.05 showed no significant impact between treatments (Wilcoxon/Kruskal-Wallis test). The asterisks show the significant effect of the treatments according to Kruskal-Wallis Test ($p \leq 0.05$).

ANNEXES

Table S11: A/S, Acetyl-A/S, Deoxy-A/S, Isovaleryl-A/S, Propionyl-A/S, β,β -Dimethylacryl-A/S and total A/Sd content (mg) in roots of *E. vulgare* inoculated with AMF, after three months of growth in pots under greenhouse condition.

Treatment	A/S	Acetyl-A/S	Deoxy-A/S	Isovaleryl-A/S	Propionyl-A/S	β,β -Dimethylacryl-A/S	Total A/Sd
Control	0.3±0.09 b	3.64±2.74 ab	ND	0.66±0.73 b	ND	ND b	4.60±3.53 b
<i>R. irregularis</i> 41833	0.93±0.40 ab	7.75±3.67 ab	0.19±0.31	1.50±0.92 ab	0.17±0.27	0.09±0.14 b	10.63±6.45 ab
<i>R. clarus</i> 46238	1.07±1.23 ab	5.52±2.86 ab	ND	1.09±0.40 ab	ND	0.11±0.13 b	7.78±4.09 b
<i>R. intraradices</i> 49410	1.89±1.28 a	14.10±7.36 a	0.24±0.34	2.55±1.51 a	0.32±0.30	0.37±0.24 a	19.46±9.89 a
<i>G. aggregatum</i> 49408	1.25±0.34 ab	6.33±3.71 ab	ND	1.13±0.36 ab	ND	ND b	8.71±4.10 ab
<i>G. hoi</i> 45686	0.50±0.62 ab	2.49±2.45 b	ND	0.58±0.41 b	ND	ND b	3.53±3.31 b
Wilcoxon/Kruskal-Wallis Test							
p-value	0,0214*	0,0179*	0.1940	0,0465*	0,0569	0.0134*	0,0101*

The parameters measured are expressed as mean $\pm$ standard deviation (SD) of 5 or 6 replicates per treatment. The asterisks show the significant effect of the treatments according to Wilcoxon/Kruskal-Wallis Test ($p \leq 0.05$). Values in the same column, followed by different lowercase letters significantly differ according to Tukey Kramer test ($p < 0.05$).

ANNEXES

OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

Scientific publications

Research articles published

Zhao Y, Cartabia A, Lalaymia I, Declerck S (2022). Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants. *Mycorrhiza* 32: 221–256.

Csorba C, Rodić N, Zhao Y, Antonielli L, Brader G, Vlachou A, Tsiokanos E, Lalaymia I, Declerck S, Papageorgiou VP, Assimopoulou AN, Sessitsch A (2022). Metabolite production in *Alkanna tinctoria* links plant development with the recruitment of individual members of microbiome thriving at the root-soil interface. *mSystems*: 26: 7(5):e0045122.

Zhao Y, Cartabia A, Garcés-Ruiz M, Herent M-F, Quetin-Leclercq J, Ortiz S, Declerck S and Lalaymia I (2023) Arbuscular mycorrhizal fungi impact the production of alkannin/shikonin and their derivatives in *Alkanna tinctoria* Tausch. grown in semi-hydroponic and pot cultivation systems. *Front. Microbiol.* 14:1216029.

Research articles submitted

Zhao Y, Rodić N, Assimopoulou AN, Lalaymia I, Declerck S. Effects of fungal endophytes and arbuscular mycorrhizal fungi on growth and alkannin/shikonin and their derivatives production of *Echium vulgare*. *Fungal biology*.

Conference participation

Zhao Y, Lalaymia I, Vincent B, Cartabia A, Csorba C, Gorfer M, Antonielli L, Krigas N, Maloupa E, Brader G, Sessitsch A, Declerck S (2019) on Root fungal community structure of *Alkanna tinctoria* differs with plant developmental stage.

International miCROPe 2019 Symposium MICROBE-ASSOCIATED CROP PRODUCTION-OPPORTUNITIES, CHALLENGE & NEEDS. (2-5, December 2019, Vienna) poster and flash talk,

National NSABS conference (31, January 2020, Gembloux);

Online 3rd International Symposium "Advances in the world of microbiomes" 18, June 2021, Ecuador.

ANNEXES

Teaching and Student supervision

International training on *in vitro* culture of Arbuscular Mycorrhizal Fungi, organized by the CESAMM laboratory, UCL, Pr. Declerck: Root disinfection training. Louvain la Neuve, Belgium (2018, 2019).

Supervision of one student (Bérengère Bostange) from the “Bioingénieurs” faculty (UCL), who performed her master thesis at the laboratory of Prof. S. Declerck (“Bioingénieurs” UCL, 2019-2020). “Influence de la composition spécifique de couverts végétaux sur les paramètres physiques, chimiques, et biologiques du système sol-plante : étude des champignons mycorrhiziens à arbuscules”.

Project training

Project Development and Horizon 2020” by RTDS Group/Stephan Webb (2017);

Summer school on “Natural products: Chemistry, Biological Activities and Technological Applications” (2018);

Network Wide Training on isolation, identification and preservation of bacteria (2019);

Winter school on Plant-associated microbiota and secondary metabolite” (Vienna);

Network wide training on *in vitro* culturing of plants and registration of microbial products (2020);

Network wide training on How to become well-trained entrepreneurially-minded and mobile researcher (2020).

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