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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 *Rhizophagus 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.

1. 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 (Arnold 2007). Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota (Schubler 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 (Piszczek et al., 2019).

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

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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 *Lithospermum 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, 1997; Dong and Chang 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).

2. Materials and methods

2.1. Endophytic fungi

Isolation of endophytic fungi from A. 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 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 on PDA and for each strain a single colony was gently picked 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 1990). For the non-sporulating endophytic fungi, ITS and 28 S 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, Lithuania) with 0.5 μM concentration of each primer (Sigma, Germany) and 0.2 μg 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 ×2, 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 × TAE buffer and GelRedTM Nucleic Acid Gel Stain, 10,000 × in water (Biotium, USA) with the following migration parameters: 100 V, 200 mA for 30 min 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:

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

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.

2.2. Arbuscular mycorrhizal fungal strains

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

2.3. *Echium vulgare*

Commercial seeds of *E. 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 calcined clay (Park et al., 2010) and quartz (0.4–0.8 mm) at a volume ratio 1:1.

The plants were grown in the greenhouse set at 16 h light/8 h dark photoperiod, 20 °C with 50 % relative humidity (RH) and a photosynthetic photon flux density (PPFD) of 96 μmolm^{-2}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 1–8 and once a week from week 9–12. 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 200× dilution was added weekly.

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

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

2.5. *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 (*D. 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. Five or six 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 % H_2O_2 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: H_2O, 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 10× 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.

2.6. *Quantitative and qualitative analysis of A/sd in E. vulgare roots 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,

Table 1
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	*Plectosphaerella* sp.	ITS, ITS4/ITS5
	11	*Cladosporium allicinum*	ITS, ITS4/ITS5
	13	*Cadophora* sp.	ITS, ITS4/ITS5
	14	*Clonostachys* sp.	ITS, ITS4/ITS5
	16	*Trichoderma hispanicum*	ITS, ITS4/ITS5
	17	*Cadophora luteo-olivacea*	ITS, ITS4/ITS5
	94	*Phialophora* sp.	ITS, ITS4/ITS5
	259	*Scytalidium* sp.	28 S, LROR/LR6
	265	*Fusarium acuminatum*	ITS, ITS4/ITS5
	476	*Leptosphaeria ladina*	28 S, LROR/LR6

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 10 P, Germany) followed by centrifugation for 10 min at 12,500 rpm (Hermle Z 216 MK, Germany). The supernatants were collected and subjected to HPLC-DAD analyses after filtering with 0.22 μm 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 30 A/70 B, 8 min 100 B, 13 min 100 B with 1 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-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 the Supplementary Data (Table 1 and Fig. 1).

3. 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.

4. Results

4.1. 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 2). With the exception of SFW and SDW, a significant impact of the treatments was observed for all parameters. The plants inoculated with *Cladosporium allicinum, Cadophora* sp.*, Clonostachys* sp.*, Trichoderma hispanicum* and *Leptosphaeria 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 control treatment. Overall, a significant effect of treatment was observed on the RDW. However pairwise comparisons using Tukey's HSD test did not show any differences between treatments.

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 3 and on A/Sd content in Table 2 of Supplementary Data. 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 3).

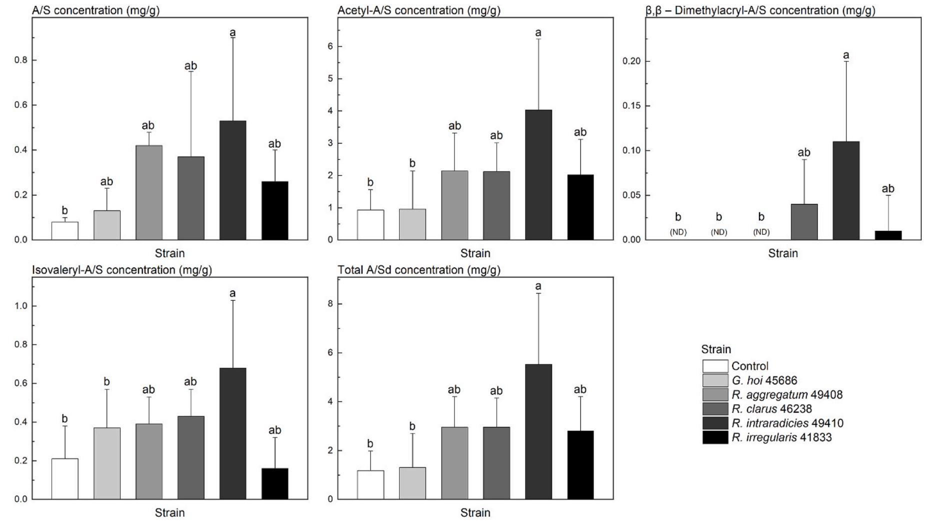

Fig. 1. A/S, acetyl-A/S, β,β – dimethylacryl-A/S (not detected (ND) in the control, *G. hoi* and *R. aggregatum* treatment and thus not reported on the figure), isovaleryl-A/S and total A/Sd concentration (mg/g) in roots of *Echium vulgare* inoculated with 5 AMF strains after three months of growth in pots under greenhouse conditions. The parameters measured are expressed as mean ± standard deviation (SD) of 5 or 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.

Table 2
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**
Plectosphaerella 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**
Cladosporium allicinum	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**
Cadophora 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**
Clonostachys 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**
Trichoderma hispanicum	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**
Cadophora 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**
Scytalidium 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**
Fusarium acuminatum	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**
Leptosphaeria ladina	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**
Phialophora 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 ($p < 0.05$).

Table 3
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
Plectosphaerella sp.	0.09 ± 0.08	1.39 ± 0.62	ND	0.26 ± 0.11	ND	0.04 ± 0.06	1.77 ± 0.71
Cladosporium allicinum	0.18 ± 0.24	0.80 ± 0.56	ND	0.10 ± 0.12	ND	0.01 ± 0.03	1.10 ± 0.81
Cadophora sp. REF040	0.08 ± 0.06	0.77 ± 0.71	ND	0.11 ± 0.11	ND	ND	0.97 ± 0.83
Clonostachys sp. G5182	0.08 ± 0.05	0.60 ± 0.27	ND	0.07 ± 0.07	ND	ND	0.75 ± 0.26
Trichoderma hispanicum	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
Cadophora sp.	0.20 ± 0.06	1.37 ± 0.76	ND	0.21 ± 0.11	ND	ND	1.77 ± 0.85
Scytalidium sp.	0.26 ± 0.21	1.23 ± 0.95	ND	0.25 ± 0.18	ND	ND	1.74 ± 1.34
Fusarium acuminatum	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
Leptosphaeria ladina	0.23 ± 0.14	0.97 ± 0.70	ND	0.10 ± 0.09	ND	ND	1.30 ± 0.89
Phialophora 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			
Prob. > F	0.3206	0.3354	0.0136*	0.0276*	–	0.3435	0.3108

ND: not detected.
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 ($p \leq 0.05$).

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 4. 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 *Glomus 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

Table 4
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 (%)	
R. irregularis MUCL 41833	63.41 ± 13.74	**a**	9.27 ± 4.79	**a**	6.85 ± 2.09	**ab**
R. clarus MUCL 46238	42.32 ± 22.90	**ab**	4.22 ± 2.81	**b**	1.28 ± 1.11	**c**
R. intraradices MUCL 49410	37.31 ± 13.28	**b**	0.42 ± 0.43	**c**	10.06 ± 6.11	**a**
G. aggregatum MUCL 49408	6.80 ± 4.64	**c**	0.58 ± 0.54	**bc**	0.67 ± 0.69	**c**
G. hoi 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 ($p < 0.05$).

Table 5
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
R. irregularis 41,833	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
R. clarus 46,238	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
R. intraradices 49,410	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
G. aggregatum 49,408	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
G. hoi 45,686	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 by different lowercase letters significantly differ according to HSD Tukey post-hoc test ($p < 0.05$).

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.

Impact of AMFs on growth of E. vulgare – The impact of AMF on plant growth are presented in Table 5. 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.

4.2.1. 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 Fig. 1 and on A/Sd content in Table 3 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 (Fig. 1) 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 3 of Supplementary Data) compared to the control plants.

5. 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 *E. 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.

E. vulgare had their RV significantly increased following inoculation with *C. 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.

5.1. 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; 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 *Trichoderma 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.

C. allicinum, Cadophora sp., *Clonostachys* sp., and *L. 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 *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 (~9 % ± 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 ± 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.

5.2. 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; Zhao et al., 2022).

In our study, we observed an overall effect of the endophytic fungi on 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, Hogekamp 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*[1] (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), 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 C_6–C_3 and C_6–C_2 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.

6. Conclusion

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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[1] It is to be noticed that the species names of AMF in this paper follow the nomenclature of today not the one at the time of publication.

Author contribution

YZ: isolation and identification of endophytic fungi from *A. tinctoria* plants, experimental set up, data collection, analysis and interpretation, drafting the work, commentaries, corrections, final approval, and agreement with all aspects of the work. NR: sample preparation for HPLC analysis, HPLC analysis and data interpretation, commentaries, corrections, final approval, and agreement with all aspects of the work. ML:sample preparation for HPLC analysis and HPLC data analysis. AN-A: provided the resources, funding acquisition, supervision, substantial contributions to the draft corrections, final approval, and agreement with all aspects of the work. IL: contribution to the development of the experiment, data analysis and interpretation, draft correction, and final approval and agreement with all aspects of the work. SD: substantial contributions to the conception and design of the experiments, interpretation of the data, draft corrections, final approval, and agreement with all aspects of the work. All authors contributed to the article and approved the submitted version.

Data availability statement

Supplementary data to this article can be found online at: https://doi.org/10.5281/zenodo.8339515.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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