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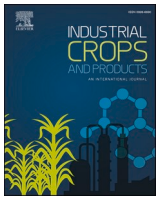

A molecular analysis in leaves of *in vitro*-cultivated commercial and non-commercial *Cannabis sativa* genotypes

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ABSTRACT

Cannabis sativa is a multipurpose crop valued for its bioactive compounds, strong and long cellulosic fibres, as well as nutritional benefits. With applications spanning different sectors, from medicine to biocomposites, it presents significant potential for pharmaceuticals, cosmetics, sustainable materials, and functional foods. This study provides a molecular characterisation of leaves from both commercial genotypes of *C. sativa* and non-commercial ones maintained in the germplasm collection of the Vavilov Institute of Plant Genetic Resources. The leaves were sampled from *in vitro*-propagated shoots cultivated on phytohormone-free medium. The targeted gene expression analysis revealed higher expression of late stage phenylpropanoid biosynthetic genes (chalcone synthase-*CHS7*, flavone synthase-*FNS6*, flavonoid 3′-hydroxylase-*F3'H3*) in non-commercial genotypes. In contrast, commercial genotypes showed increased expression of genes involved in the early steps of the pathway (phenylalanine ammonia lyases-*PAL4*, *PAL7* and 4-coumarate:coenzyme A ligase-*4CL1*). Untargeted metabolite profiling identified > 100 differentially abundant metabolites, 92 of which belonging to flavonoids, phenolic acids, cannabinoids, lignanamides and lignans. Flavonoids, their derivatives, and cannabinoids were more abundant in non-commercial genotypes, indicating prioritisation of defence compound production. These findings highlight the potential of non-commercial genotypes as natural factories of bioactive molecules, paving the way for future applications.

1. Introduction

Cannabis sativa L. is a fast-growing annual herbaceous plant which belongs to the Cannabaceae family. Native to central Asia and the Middle East, it has been cultivated for centuries because of its diverse applications (Grulichova et al., 2017). Hemp, referring to non-psychotropic *C. sativa* with a low content of Δ-9-tetrahydrocannabinol (THC content < 0.3 % on a dry weight basis; Agriculture Improvement Act of 2018) and cultivated for agricultural purposes (Malabadi et al., 2023; Montero et al., 2022; Visković et al., 2023), is considered a multi-purpose crop. Indeed, it provides straws for biocomposites/textiles (Andre et al., 2016), seeds for food/feed (Kamle et al., 2024) and oil extraction, flowers for medicinal uses (Pancaldi et al., 2025), leaves for herbal infusions (Knezevic et al., 2021), and bioactives used in pharmaceutical and cosmetic industries (Farinon et al., 2020). *C. sativa* sprouts were also shown to be rich in bioactives and to hold potential as functional food (Werz et al., 2014).

Among the bioactive compounds synthesised by *Cannabis*, cannabinoids, terpenes and phenolic compounds are particularly interesting for pharmaceutical applications (Andre et al., 2016; Erridge et al., 2020). The abundance of plant secondary metabolites depends on several endogenous and exogenous factors, namely age, genotype, organ, and environmental conditions (Flores-Sanchez and Verpoorte, 2008). In *C. sativa*, recent studies have reported the high variability in cannabinoids and phenolic compounds existing in the inflorescence of different genotypes (Izzo et al., 2020; Montero et al., 2022).

The two major cannabinoids in *Cannabis* are THC and cannabidiol (CBD) (Montero et al., 2022), while minor cannabinoids, such as cannabinol, cannabigerol, and cannabichromene are also present (Jin et al., 2020). *C. sativa* also produces a diverse array of phenolic

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compounds, which include phenolic acids, flavonoids, stilbenes, and lignans (Flores-Sanchez and Verpoorte, 2008). These compounds play a crucial role in plant defence (Erb and Kliebenstein, 2020) and offer significant health benefits to humans (Izzo et al., 2020), such as antioxidant, antimicrobial, anti-inflammatory, and antimutagenic properties (Isidore et al., 2021; Lin et al., 2016; Sauceda et al., 2017). Among phenolics, flavonoids represent a major subclass and are further categorised based on their chemical structures. These include flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, kaempferol), flavan-3-ols (e.g., catechin), anthocyanins (e.g., cyanidin), flavanones (e.g., naringenin), and isoflavones (e.g., genistein, daidzein) (Flores-Sanchez and Verpoorte, 2008; Kawser Hossain et al., 2016).

Micropropagation is a technique for the *in vitro* multiplication of plant material under sterile and controlled conditions. It allows standardisation of growth conditions while minimising those variations that would otherwise result from environmental factors and seasonal changes (Espinosa-Leal et al., 2018). Micropropagation is widely used for *C. sativa*, as it enables large-scale propagation of elite genotypes while maintaining genetic fidelity, optimising space, and reducing the risk of contamination by bacteria, viruses, and fungi (Hesami et al., 2023; Page et al., 2021; Stephen et al., 2023).

The success of micropropagation depends on selecting an appropriate culture medium, with or without plant growth regulators (PGRs), with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) being among the most commonly used for *C. sativa* micropropagation (Adams et al., 2021; Duta-Cornescu et al., 2023).

Shoot proliferation is a critical step for *in vitro* propagation: root formation can indeed be efficiently performed at later stages, either *in vitro* or *ex vitro*, with the latter being more cost-effective (Ranaweera et al., 2013) and time-saving, since rooting and acclimatisation occur simultaneously (Sharma et al., 2017).

In this study, *in vitro* shoot multiplication was conducted on both commercial and non-commercial genotypes maintained at the Vavilov Institute of Plant Genetic Resources (VIR), one of the first and largest *ex situ* collection of *Cannabis* germplasm (Clarke, 1998). The distinction between commercial and non-commercial genotypes is based on the cultivation status, as well as intended use. More specifically, commercial genotypes of *Cannabis* are selectively bred and cultivated for large-scale agricultural applications, such as straw and seed production for fibres and oil (Zheljazkov et al., 2020), as well as traits such as earliness, thousand kernel weight, precocity, lodging and broomrape resistance.

In contrast, non-commercial genotypes refer to wild accessions or landraces maintained in germplasm repositories (e.g., VIR) that have not undergone formal breeding for commercial deployment.

In this study, 5 commercial genotypes with THC content < 0.3 % and listed in the EU database of registered plant varieties (EUPVP; https://ec.europa.eu/food/plant-variety-portal/) were investigated, together with 4 non-commercial ones from the VIR collection.

The aim of this study was twofold, i.e., to develop a reliable and straightforward protocol for *C. sativa* shoot propagation from nodal explants, and to characterise the expression of genes involved in the biosynthesis of phenolic compounds, together with the phytochemical profile in the leaves of commercial and non-commercial genotypes.

A previous publication on *Cannabis* VIR genotypes focussed on cannabinoids (Small and Marcus, 2003); this study identified additional leaf secondary metabolites in non-commercial *C. sativa*.

2. Materials and methods

2.1. Media optimisation

The following genotypes were used to optimise the shoot propagation protocol, i.e., the non-commercial Bolonska and Odnodomnaya, obtained from the N.I. Vavilov Institute of Plant Genetic Resources (Russia), and the commercial Félina 32. The Santhica 27 cultivar was also included, since it was previously used as fibre-type model for molecular studies (Behr et al., 2018, 2016; Guerriero et al., 2017). The seeds of the commercial genotypes were purchased from the French cooperative Hemp-it (https://www.hemp-it.coop/).

Seeds were sown in pots with 1/3 sand and 2/3 potting soil and grown in chambers (Fitotron, Weiss Technik Belgium) for 8 weeks under controlled conditions of 16 h/8 h and 25°C/20°C (light/dark) with 60 % of humidity and light intensity of 7 kLux. These plants were used as source of explants to initiate micropropagation. Plant stems were harvested after 2 months, cut into segments used as explants (around 5 cm, containing one internode), incubated in sterile water with 5 % (v/v) NaClO and 0.05 % (v/v) Tween20 (Sigma Aldrich) for 10 min in agitation and washed three times with sterile water for 5 min each time. Then, the explants were put in culture boxes (Magenta GA-7 Plant Culture Box) containing a solid medium composed of MS basal salts supplemented with MS vitamins, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8, and grown under controlled conditions of 16 h/8 h (light/dark) photoperiod and 22°C. The plantlets were grown *in vitro* for ca. 6 months before optimising the shoot multiplication protocol.

The media chosen for the optimisation were composed of MS basal salts with MS vitamins, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8 before autoclaving, and different combinations of NAA (1-Naphthaleneacetic acid), BAP (6-Benzylaminopurine), IAA (Indole-3-acetic acid) and meta-topolin (purchased from Duchefa Biochemie). The different concentrations of PGRs are described in Table 1. The media were autoclaved (Systec 1095 VX-150 autoclave) before use, with a sterilisation cycle of 20 min at 121°C. All the hormones were added before autoclaving.

Four stem explants containing 2 nodes were transferred to Magenta boxes. For each condition, four replicates (i.e., four culture boxes) were prepared. Each box contained four explants, resulting in a total of 16 explants per condition. All treatments were arranged in a randomised design. The explants were grown under the same conditions described above. After 6 weeks, the best medium was chosen based on the following parameters: shoot length, fresh weight (FW) and number of nodes.

2.2. Micropropagation of commercial and non-commercial genotypes for molecular analyses

The genotypes studied for the molecular analyses are listed in Table 2. Seeds were germinated and explants prepared as described above. They were then transferred to culture boxes (Magenta GA-7 Plant Culture Box) containing solid MS medium supplemented with MS vitamins, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8, and grown aseptically under a 16 h/8 h (light/dark) photoperiod at 22°C. In total, 9 *C. sativa* genotypes were investigated. Among these, 5 were commercial and registered in the EU portal of agricultural plant and vegetable species, while 4 were non-commercial genotypes belonging to the VIR repository. All the commercial genotypes and Odnodomnaya were monoecious, while Bolonska, Line 43/178 and Local 14 were dioecious.

Table 1
Composition of the media used for the optimisation of shoot multiplication. The literature references are also indicated.

Medium 1 (MS0)	**Medium 2** (Grulichova et al., 2017)	**Medium 3** (Grulichova et al., 2017)	**Medium 4** (Lata et al., 2016)
MS salts	MS salts	MS salts	MS salts
MS vitamins	MS vitamins	MS vitamins	MS vitamins
30 g/l sucrose	30 g/l sucrose	30 g/l sucrose	30 g/l sucrose
8 g/l agar	8 g/l agar	8 g/l agar	8 g/l agar
	BAP 0.4 mg/l	BAP 0.4 mg/l	Meta-topolin 0.5 mg/l
	NAA 0.1 mg/l	IAA 0.1 mg/l	

Table 2
Details on the *C. sativa* genotypes micropropagated for the molecular analyses.

Genotype	Origin
Félina 32 (monoecious) Férimon 12 (monoecious) Futura 75 (monoecious) Fédora 17 (monoecious)	France, Hemp-it
Fibranova (dioecious)	Italy, Canapuglia (https://canapuglia.it/)
Bolonska (VIR, k−6; dioecious)	Former Yugoslavia (from Italy)
Line 43/178 (VIR, k−169; dioecious)	Romania
Odnodomnaya (VIR, k−475; monoecious)	Poland
Local 14 (VIR, k−485; dioecious)	Former Yugoslavia

2.3. RNA extraction and gene expression analysis

Leaves were harvested from 2 months-old *in vitro*-propagated shoots on PGR-free MS medium. Four biological replicates of each genotype were prepared, each consisting of ca. 8–10 leaves. Leaves were plunged immediately in liquid nitrogen and ground to a fine powder. One part of the ground leaves was kept at −80°C for molecular analyses and the rest was freeze-dried using a tabletop lyophilisation unit (Christ Alpha 2–4 LCS Plus) for the subsequent metabolite extractions.

Total RNA was extracted from the ground leaves using the Qiagen RNeasy Plant Mini kit with the on-column DNase treatment. The purified RNAs were quantified using a NanoPhotometer NP80 (Implen) with RNase-free water as blank, and subsequently cleaned, in case of 260/280 and 260/230 ratios < 2, using a precipitation step with ammonium acetate/ethanol and washes in ethanol, as described previously (Mangeot-Peter et al., 2016). RNA integrities were then checked by capillary gel electrophoresis with a 2100 Bioanalyzer (Agilent) according to the manufacturer's instructions, using the RNA 6000 nano chip (Agilent). All RINs were > 7.

One microgram of RNA was retrotranscribed to cDNA using the ProtoScript II RTase (NEB) and random hexamers. RT-qPCR was carried out in 384-well microplates which were prepared using a pipetting robot (epMotion, Eppendorf) and run using the Takyon Rox SYBR MasterMix dTTP blueMix in a QuantStudio™ A28569 thermal cycler. The expression was calculated using 3 reference genes (*Tip41*, *Fbox* and *eTIF4E*) which geNORM, implemented in qBASE$^+$ (Hellemans et al., 2007), identified as sufficient for data normalisation.

Primers were either previously published (Gao et al., 2018; Guerriero et al., 2017), or designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and verified with the OligoAnalyzer 3.1 tool from Integrated DNA technologies (http://eu.idtdna.com/calc/analyzer). Primer efficiencies were assessed from a serial 5-fold dilution of cDNA (25, 5, 1, 0.2, 0.04, 0.008 ng/µl).

The new primer pairs used, amplification efficiencies, and R^2 are reported in Table 3.

The principal component analysis (PCA) of the gene expression data was carried out using ClustVis (https://biit.cs.ut.ee/clustvis/) (Metsalu and Vilo, 2015). The hierarchical clustering of the expression data (presented as heatmaps) was done with Cluster 3.0 (Metsalu and Vilo, 2015) and rendered with Java Treeview (Saldanha, 2004) (available at http://jtreeview.sourceforge.net/).

2.4. Metabolomics

Finely ground and freeze-dried leaves were weighed (100 mg) and a methanol/water mixture (80/20, v/v) was added at a 1:30 ratio (w/v). The mixture was vortexed for 5 s and sonicated for 10 min at 37 Hz at room temperature. Subsequently, the samples were agitated for 1 h at 1000 rpm and 25°C (ThermoMixer F2.0, Eppendorf), and centrifuged during 20 min at 3900 rpm at 4°C.

The methanolic extracts thus obtained were placed into CentriVap (Labconco), solvent evaporation system until dryness, then dissolved in methanol/water (20/80 v/v), vortexed and filtered through 0.22 µm filters (wwPTFE, PALL).

Ten µl were injected in an Acquity UPLC I-class UPLC system equipped with a diode array detector (Waters). The separation was done on a reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7µm particle size; Waters). The solvents used were: A) water + 0.1 % v/v formic acid and B) acetonitrile + 0.1 % v/v formic acid. The column was maintained at 50°C for the whole run. The gradient was applied as follows: 99 % of A 0–16 min, 95 % of A 16–35 min, 60 % of A 35–45 min, 100 % of B 45–50 min, 99 % of A 50–54 min, and 99 % of A 54–60 min, at a 0.5 ml/min flow rate. After eluting from the column, the analytes were analysed with a TripleTOF 6600 + high resolution mass spectrometer (Sciex) in negative and positive ion mode.

Positive and negative mode electrospray ionization (ESI) was performed with a source temperature of 650°C and ion spray voltages of 4.5 and −4.5 kV respectively. The curtain gas (nitrogen) was set at 30 psi; the nebuliser gas and the turbine gas (air) at 55 psi and 50 psi. A declustering potential of 60 V and −60 V was applied in positive and negative mode. For information-dependent acquisition, MS scans (from *m/z* 100–2000) were acquired for 175 ms and the 10 most abundant, single-charged *m/z* values were selected for MS/MS scans (from *m/z* 50–2000) of 200 ms. A sweeping collision energy of 15 V below and above 15 V in positive mode and below and above -15 V in negative mode was applied to all precursor ions. An *m/z*-value was excluded for 2 s after three occurrences.

The chromatograms of the different samples were imported in Progenesis QI (v2.3, Nonlinear Dynamics, Newcastle upon Tyne, UK) for data treatment; runs were aligned, the data normalised, and LC-MS

Table 3
Primer sequences used in this study, with amplification efficiencies, amplicons' melting T (Tm) and R^2. The gene abbreviations are: *PT* (prenyltransferase), *OMT* (*O*-methyltransferase), *F3'H* (flavonoid 3'-hydroxylase) and *FNS* (flavone synthase).

Name	Sequence (5′→3′)	Amplification efficiency	Amplicon Tm (°C)	Regression coefficient R^2
CS_PT1_Fw CS_PT1_RV	TCAAACTGAGCCTCCAGAATC GAAGTTTCCAACATGCCTTCC	2.07	76.18	0.977
CsOMT21_2 FW CsOMT21_2 RV	AACAAGGCATATGGAATGACAG CATGGTAATAGTCGAGTGGTC	2.01	77.36	0.992
CsPT3_3_FW CsPT3_3_RV	ATGATCTCGACATCGACAGG AGTCAACAACCATGCCAATTC	1.99	78.48	0.99
CsPT5_FW CsPT5_RV	TGCTGGTGACCTTTCAGTTC TGGTGCCAAGGACAAGAC	1.94	81.56	0.99
CS_PT6_FW CS_PT6_RV	TTGGGATGTTGGAGGCTATC CCAACGCCAAAACTCAGAAT	1.961	81.16	0.991
CS_F3'H3_FW CS_F3'H3_RV	CCGAGATAGGCTCGTAGGTG AAGGGACAGTGGAGTCGATG	1.998	80.95	0.984
CS_FNS6_FW CS_FNS6_RV	AAGGCTACATCCACCATTGC TTCCCATGTCTTTGGGTCTC	1.989	79.98	0.982

features identified.

Identifications were done by comparison of the experimental data with different databases. A mixture of *Cannabis* (Cayman chemicals, catalogue number 37832) flavonoids was run in both ionisation modes and the acquired data added to our in-house database. The LIST-Luxembourg Institute of Science and Technology's in-house dereplication tool and associated database contains identification data (RT, MS, MS/MS, instrumental and meta-data) on all metabolites identified in the last five years at LIST and is typically used as first passage identification. Data from the analysis of standards are added for numerous commonly found plant metabolites (e.g., hydroxycinnamic acids, catechins, flavonoids, triterpenes). Derivatives of these compounds were identified by following a pseudo MS3-approach as for instance described for lupin flavonoids (Wojakowska et al., 2013). The contribution of standards to the reported identifications was indicated, with "Y" when the compound was a level 1 identification as defined by the Metabolomics Standards Initiative (MSI) supported by the RT and MS/MS of an authentic standard. The annotation "Aglc" indicates that part of the identified molecules was confirmed by MS/MS matching of an in-source fragment with an authentic standard. These were level 2 identifications as defined by MSI. All other identifications were based on MS/MS matching of the experimental data with data available in publications and databases such as Global Natural Product Social Molecular Networking (GNPS), Metlin, MZCloud, lipidmaps, and others. These must be considered tentative and level 2 identifications. Malonylation was confirmed by comparison of the positive mode *m/z* with the *m/z* in negative mode, the latter showing the characteristic loss of CO_2 (Piasecka et al., 2015). Key data for the tentative identification of the different cannabinoids were found in the literature (Berman et al., 2018; Padilla-González et al., 2023; Xu et al., 2024). The MS/MS spectra of the ditertbutylphenol derivatives allowed to identify the glycosylation, while the aglycone, the third most abundant volatile in hemp seed oil (Gulcin et al., 2024), showed the characteristic loss of 56.06 (C4H8) of butylated compounds.

The hierarchical clustering of the normalised abundances (presented as heatmaps) was done as described for the qPCR data.

2.5. Statistics

Statistics was performed with the SPSS software (IBM SPSS Statistics v20) after log10-transformation of the data. Normality and homogeneity were verified with a Shapiro-Wilk and Levene's test, respectively. Normal and homogeneous data were analysed with a one-way ANOVA followed by a Tukey's *post-hoc* test; non-parametric data were analysed with a Kruskal-Wallis test and Dunn's *post-hoc* test.

3. Results

3.1. Identification of the best medium for shoot propagation in representative commercial and non-commercial C. sativa genotypes

The first step consisted in the identification of a media for *in vitro* propagation of both commercial and non-commercial genotypes. The best shoot propagation medium was selected by evaluating different parameters, namely shoot FW (mg), length (cm) and number of nodes. These parameters were evaluated on 4 representative genotypes, 2 commercial and 2 non-commercial.

Félina 32 did not show significant differences in FW among the plants cultivated on medium 1 (devoid of any PGRs, also referred to as MS0), 3 (containing BAP and NAA) and 4 (supplemented with meta-topolin); likewise, there were no significant differences in length and number of nodes among plants grown on media 1 and 3 (Fig. 1A).

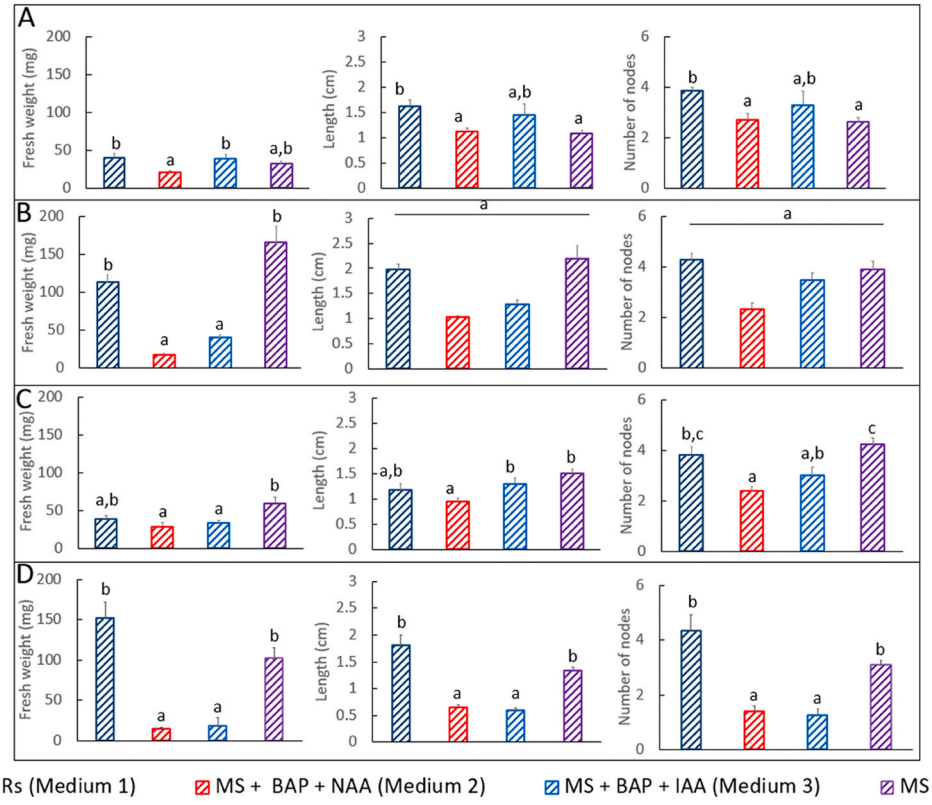

Fig. 1. Bar charts of the different parameters (FW, length and number of nodes) measured for Félina 32 (**A**), Santhica 27 (**B**), Bolonska (**C**), Odnodomnaya (**D**) grown on different micropropagation media. The error bars indicate the standard error of the mean (n = 4). Different letters indicate statistically significant differences among groups ($p < 0.05$). Medium 1: no PGRs (MS0), medium 2: BAP 0.4 mg/l and NAA 0.1 mg/l, medium 3: BAP 0.4 mg/l and IAA 0.1 mg/l, medium 4: meta-topolin 0.5 mg/l.

However, there were significant variations in FW among the plants cultivated on media 1 or 3, and 2 (with BAP and IAA), while for length and number of nodes, there were significant differences among plants grown on media 1, and 2 or 4.

Concerning Santhica 27, a significant increase in FW was observed when comparing media 1 and 4 with 2 and 3 (Fig. 1B). No significant differences were instead observed in length and number of nodes across the different media.

In Bolonska, significant differences were observed in FW and number of nodes when comparing medium 4 with 2 and 3 (Fig. 1C). Shoots grown on media 3 and 4 showed increased length compared to medium 2.

Odnodomnaya grown on media 1 and 4 showed higher FW, length and number of nodes compared to the shoots on media 2 and 3 (Fig. 1D).

The results obtained indicated that the growth parameters on the different media were genotype dependent. From the data, it can be concluded that media 1 and 4 scored, in average, better than 2 and 3.

For the subsequent analyses on other commercial and non-commercial genotypes, medium 1 (MS0) was chosen.

3.2. Gene expression analysis in commercial and non-commercial *C. sativa*

After the selection of the micropropagation medium, targeted gene expression profiling was carried out on the leaves from a wider panel of *in vitro*-cultivated genotypes, i.e., 5 commercial and 4 non-commercial.

To determine whether genotype-dependent differences existed in the expression of genes involved in phenolic compound biosynthesis, 11 genes encoding enzymes that participate in the phenylpropanoid pathway were screened. The genes were phenylalanine ammonia lyase (*PAL*), 4-coumarate:coenzyme A ligase (*4CL*), chalcone synthase (*CHS*), flavone synthase (*FNS*), flavonoid 3'-hydroxylase (*F3'H3*). Prenyltransferases (*PT*), as well as an *O*-methyltransferase (*OMT21*) known to be expressed in the leaves (Zhu et al., 2022) were also included, given their role in the synthesis of the prenylated flavonoids cannflavins (Rea et al., 2019).

The PCA of the gene expression data showed two main groups composed of commercial and non-commercial genotypes (Fig. 2A). The non-commercial genotypes grouped into a tighter cluster characterised by less scattered data points.

The hierarchical clustering of the expression values also highlighted the presence of two main groups by setting a Pearson's coefficient threshold $\geq$ 0.69 (Fig. 2B). The first grouped genes in the earlier stages of the phenylpropanoid pathway, namely *4CL1*, *PAL4*, *PAL7*, while the second included those involved in the late steps of the pathway (*CHS7, FNS6, F3'H3*), along with *OMT21*, and all the *PT*s. The genes in cluster 1 showed overall higher expression in the commercial genotypes (except for Fédora 17) compared to non-commercial ones, while those in cluster 2 were more expressed in non-commercial genotypes (except for Futura 75; Fig. 2C).

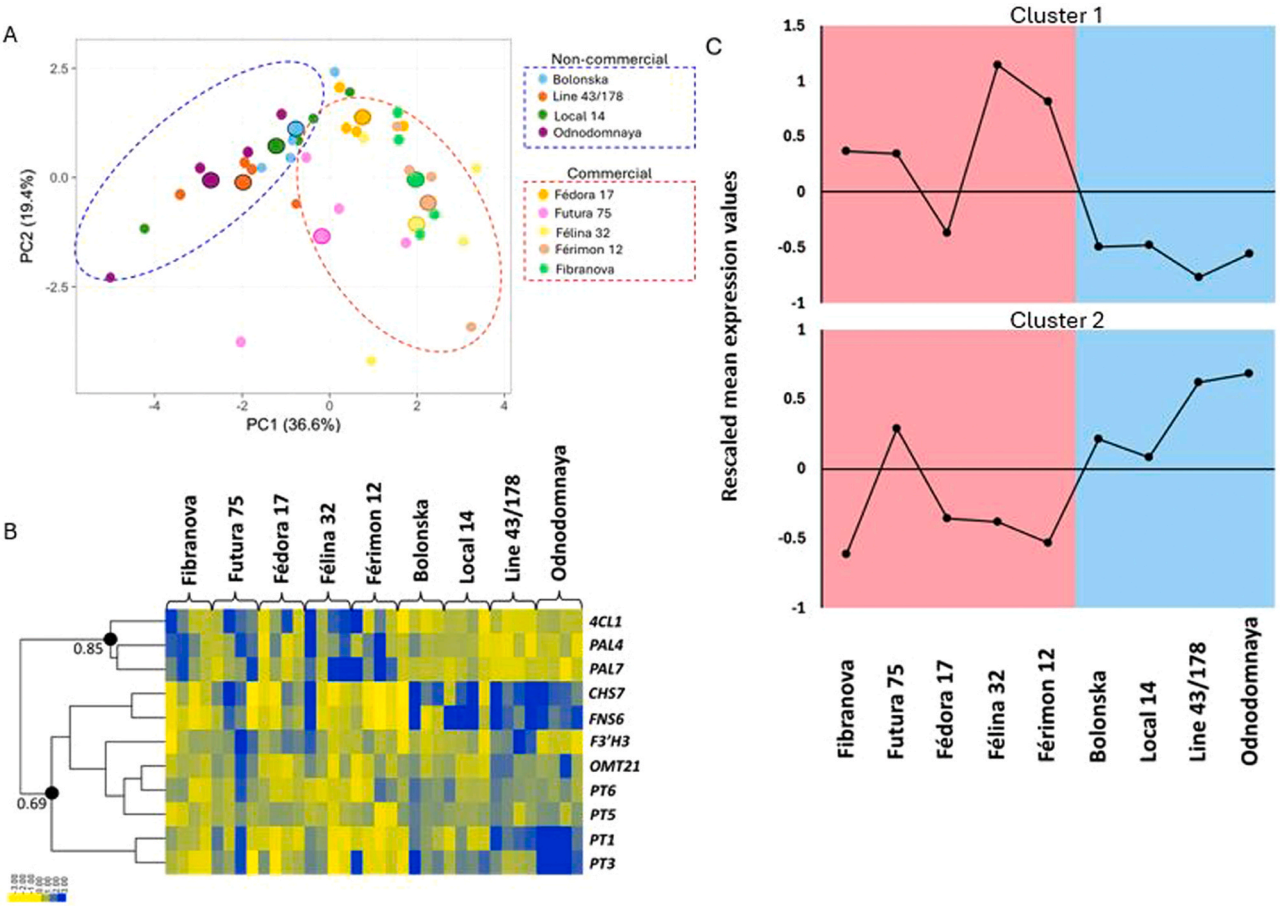

Fig. 2. Expression of genes involved in the phenylpropanoid pathway in commercial and non-commercial genotypes. (**A**) Principal component analysis (PCA) of the qPCR data. The small, coloured dots represent the 4 replicates from each genotype studied, while the 9 bigger coloured dots represent the means for each genotype. (**B**) Heatmap hierarchical clustering (HC) of the gene expression data. Rescaled expression values (**C**). In light red, commercial genotypes and, in light blue, non-commercial genotypes. The rescaled values were obtained by subtracting from each expression value the average among the genotypes and dividing by the standard deviation. The gene expression data are indicated in Supplementary File.

3.3. Untargeted metabolomics

Untargeted metabolomics was then carried out to obtain insights into the metabolomic signature of the different genotypes and to highlight any correlation with the gene expression profiles. A total of 118 metabolites were identified in negative mode and 63 in positive mode that were differentially accumulated among the genotypes (Supplementary File). The hierarchical clustering of the normalised abundances relative to 92 compounds belonging to cannabinoids and phenolic compounds showed the presence of 10 main clusters by taking a Pearson coefficient $\geq$ 0.72 (Fig. 3).

The first 2 clusters comprised the methoxyflavone acacetin and its malonylated or glycosylated derivatives whose abundance was higher in the non-commercial genotypes Local 14 and Odnodomnaya compared to all the others.

Cluster 3 grouped cannabinoids with marked higher abundance in the VIR genotypes Line 43/178 and Odnodomnaya compared to the other ones.

Cluster 4 comprised flavonoids (the flavone chrysoeriol, the flavanone eriodictyol, the dihydroflavonol taxifolin), together with cannabinoids which were more abundant in Line 43/178 and Odnodomnaya.

Cluster 5 comprised malonylated and glycosylated derivatives of chrysoeriol and scoparin, with increased abundance in Futura 75, Fédora 17, as well as Odnodomnaya.

Cluster 6 showed higher abundance of cannabinoids and phenolic acid derivatives (feruloyl gluconic acid, coumaroyl glucaric acid, coumaroyl malonyl pentoside) in Line 43/178 with respect to the other genotypes.

Flavonoid glycosides and malonylated derivatives of orientin and vitexin were instead present in cluster 7, with higher abundance in the commercial genotypes Fédora 17 and Félina 32, as well as in the non-commercial genotypes Bolonska and Local 14, compared to the other genotypes.

In cluster 8, hydroxycinnamic acid amides were found with the highest abundance in Fibranova, Férimon 12 and Bolonska.

The lignanamides cannabisin D and grossamide were found in cluster 9 with increased abundance in the genotype Férimon 12.

The lignan syringaresinol and its derivatives were grouped in the last cluster with the highest abundance in Line 43/178.

4. Discussion

In recent decades, the interest in high-value bioactive compounds produced by *C. sativa* has grown significantly (Izzo et al., 2020; Jin et al., 2020; Mkpenie et al., 2012; Pellati et al., 2018; Tomko et al., 2022); in this context, the present study investigated the gene expression and phytochemical profiles of leaves from 9 different *C. sativa* genotypes cultivated *in vitro*, after having identified the most suitable medium for rapid shoot propagation. To ensure consistency, analytical depth was prioritised over genotypic breadth, with each genotype subjected to both targeted gene expression analysis and high-throughput untargeted metabolomics.

In vitro propagation was here selected as the technique because it is cost-effective, season-independent, and allows both aseptic conditions and precise control over environmental factors such as light and temperature. Moreover, it ensures genetic uniformity, enabling large-scale commercial propagation from a single mother plant (Ioannidis et al., 2022; Máthé et al., 2015).

In this study, two widely used classes of PGRs were studied, i.e., auxins and cytokinins.

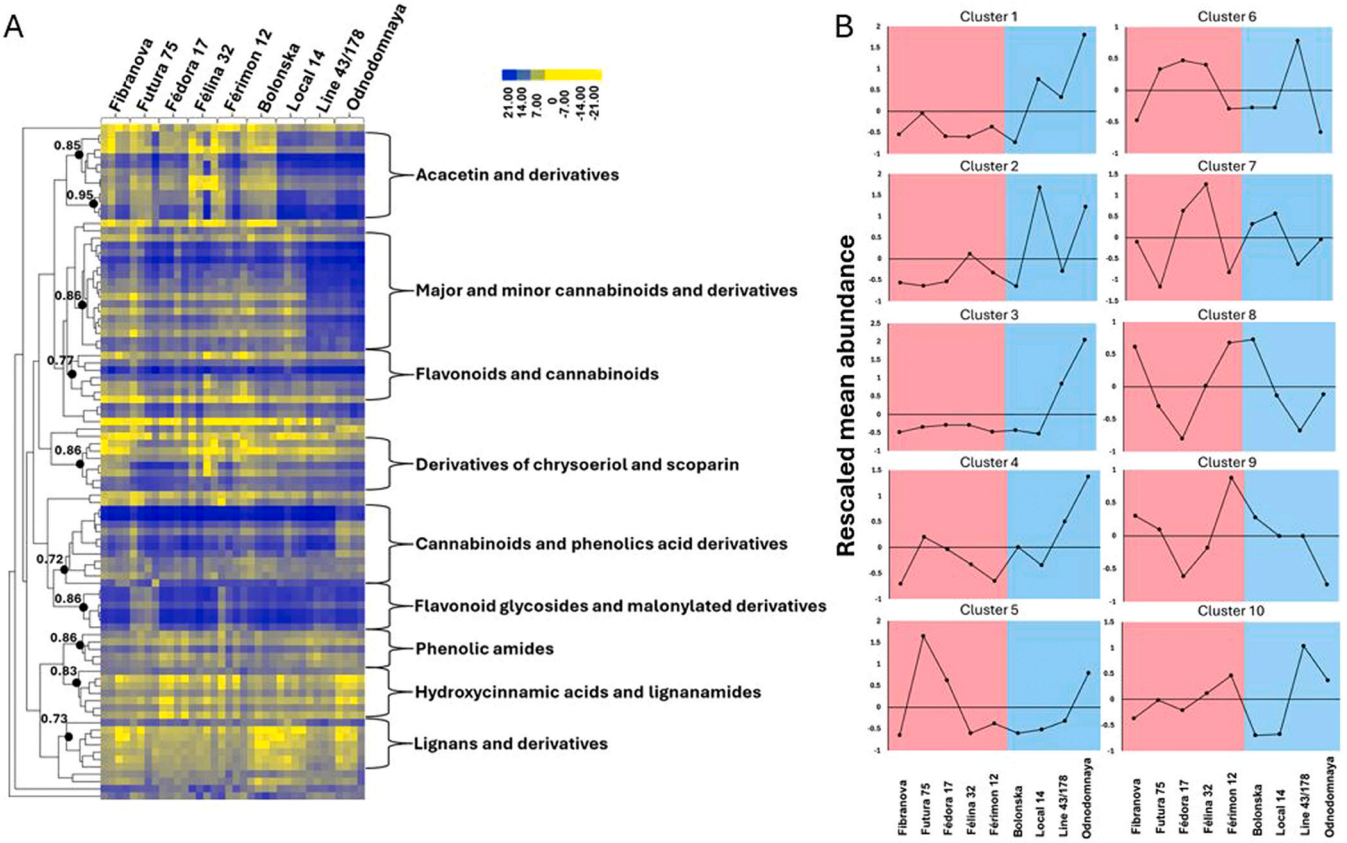

Fig. 3. Heatmap hierarchical clustering of the normalised metabolite abundances with indication of the correlation coefficients characterising each cluster (**A**). Rescaled metabolite abundances; in light red, commercial genotypes and, in light blue, non-commercial genotypes (**B**). The rescaled values were obtained by subtracting from each abundance value the average among the genotypes and dividing by the standard deviation.

Auxins influence plant development by regulating physiological processes such as cell elongation, apical dominance and roots morphogenesis (Brumos et al., 2018; Burgel et al., 2020; Pasternak and Steinmacher, 2024; Sabagh et al., 2021). Commonly used auxins include the natural IAA, and the synthetic compounds NAA and 2,4-dichlorophenoxyacetic acid (2,4-D). Depending on the ratio used, auxins can act antagonistically or synergistically with other hormones, e.g., with cytokinins, thereby regulating cell proliferation and differentiation to form new organs (Burgel et al., 2020; Su et al., 2011).

Cytokinins, such as BAP and kinetin, are involved in cell division, leaf senescence, and the response to biotic and abiotic factors (Yu et al., 2024).

As a first step, a comparison of different media was here conducted to identify a propagation system that would be both applicable across diverse *C. sativa* genotypes and minimally influencing intrinsic physiological processes and metabolic responses. The inclusion of the PGR-free medium was motivated by the growing evidence that exogenous PGRs can impact the plant's architecture and synthesis of secondary metabolites such as cannabinoids. For example, auxins and cytokinins, alone or in combination, were shown to affect parameters such as height, number of internodes and inflorescence (Burgel et al., 2020). Also, the promoters of genes involved in the synthesis of cannabinoids, namely *PT1* and *PT4*, were shown to respond to treatments with different PGRs, among which auxins and cytokinins (Sands et al., 2023).

The data obtained showed that the medium devoid of PGRs (MS0) or supplemented with 0.5 mg/l meta-topolin gave the best results in terms of FW, length of the shoot and number of nodes (Fig. 1).

Meta-topolin, an aromatic cytokinin, was first isolated from poplar leaves (Strnad et al., 1997) and has been used for the stimulation of shoot proliferation. It was shown that it avoided physiological disorders and hyperhydricity induced by BAP (Abdalla and Dobránszki, 2024; Chauhan and Taylor, 2018; Lata et al., 2016; Ptak et al., 2023). Previous research on *C. sativa* demonstrated the effectiveness of meta-topolin in a one-step regeneration protocol for the rapid shoot proliferation and *in vitro* rooting of micropropagated plantlets that successfully adapted to grow in climate-controlled growth rooms, achieving a 100 % survival rate (Lata et al., 2016). In another study focussing on two monoecious varieties of industrial hemp, Bialobrzeskie and Monoica, the medium devoid of hormones or supplemented with meta-topolin 0.5 mg/l showed the highest number of roots, together with the highest biomass FW, number of nodes and stem length (Grulichova et al., 2017).

The medium without PGRs was chosen as a practical and reproducible baseline for comparative molecular analyses. It simplifies the culture process, reduces costs and risks of somaclonal variation (Krishna et al., 2016), and it allows plants to regulate growth using their endogenous phytohormones, minimising variations in morphogenesis, gene expression and secondary metabolite biosynthesis across the genotypes.

The second part of the study focussed on molecular analyses on the leaves of micropropagated commercial and non-commercial genotypes. The decision to focus on leaves rather than flowers was both strategic and motivated by experimental constraints. While it is well established that cannabinoids are typically more abundant in *Cannabis* female flowers than leaves (ranging from 15.77 % to 20.37 % in floral tissues compared to 1.10–2.10 % in leaves; Jin et al., 2020), the flavonoid content has been reported to be higher in leaf tissue, with values between 0.34 % and 0.44 % in leaves versus 0.07–0.14 % in flowers (Jin et al., 2020). Moreover, under *in vitro* propagation conditions, floral development was not consistently observed across all genotypes, precluding the possibility of homogeneous flower sampling. Leaves, on the other hand, were produced across all genotypes, enabling a consistent and comparable tissue sampling.

It is also worth noting that evidence from the literature indicates that cannabinoids are not restricted to floral tissues alone, but are also present in leaves (Chen et al., 2024; Lorensen et al., 2023).

The expression of genes involved in the late stage of the phenylpropanoid pathway was overall higher in the leaves of non-commercial genotypes, while those involved in the earlier steps of the pathway were, instead, higher in commercial ones (Fig. 2). The higher *FNS6* expression suggests a transcriptional control favouring flavonoid accumulation in non-commercial leaves (Gagalova et al., 2024). This is confirmed by the lower expression of genes involved in earlier stages of the phenylpropanoid pathway, *PAL4*, *PAL7* and *4CL1*, which were instead expressed at higher levels in the commercial genotypes. *PAL* and *4CL* increased in expression in the hemp hypocotyl undergoing the transition from elongation to radial thickening, denoting their role in secondary cell wall biosynthesis (Behr et al., 2016).

The data here obtained therefore suggest a flavonoid-dominant metabolic shift over lignin monomers' production in non-commercial genotypes. *PAL* and *4CL* are known to be involved in lignin biosynthesis (Docimo et al., 2013; Yoon et al., 2015): their higher expression in commercial genotypes rather than non-commercial ones aligns with their selection and cultivation for vigorous growth and biomass production.

This separation between commercial and non-commercial genotypes was confirmed by untargeted metabolomics. Indeed, the hierarchical clustering of the normalised metabolite abundances revealed differences between commercial and non-commercial *C. sativa* genotypes, particularly in the accumulation of 92 compounds belonging to the categories flavonoids, phenolic acids and their derivatives, cannabinoids, lignans and lignanamides (Fig. 3 and Supplementary File).

Among flavonoids, the *O*-methyl flavone acacetin and its derivatives, synthesised from apigenin through methylation (Bassolino et al., 2024), were identified in clusters 1 and 2. Flavones, including acacetin, are well-known for their ability to protect plants against UV-B radiation (Falcone Ferreyra et al., 2012; Li et al., 2020) and to act as natural pesticides, defending against insects and fungi (Hostetler et al., 2017). Acacetin has shown relevant pharmaceutical properties, namely anti-inflammatory, anticancer, and cardiovascular protective effects (Bassolino et al., 2024; Jin et al., 2021; Wu et al., 2022).

Considering the significantly higher abundance of acacetin and its derivatives in Local 14 and Odnodomnaya, these genotypes could serve as valuable sources for future applied research and pharmaceutical applications.

Cluster 3 and 4, which comprised cannabinoids and flavonoids, displayed the highest abundance in Line 43/178 and Odnodomnaya. Among the cannabinoids identified, there was cannabichromenic acid, a minor cannabinoid and a precursor of cannabichromene (CBC), together with cannabichromevarinic acid. CBC is known to possess bioactivities, e.g., anti-inflammatory and antibacterial properties (Sepulveda et al., 2024; Zagožen et al., 2020). Cannabinoids are known to play key roles in plant protection, acting as deterrents against herbivores and pathogens, while also mitigating oxidative stress and conferring UV-B protection (Gülck and Møller, 2020; MacWilliams et al., 2023; Stack et al., 2023; Westmoreland et al., 2023). This result agrees with the growth-defence trade-off hypothesis (He et al., 2022) according to which, in the absence of selection for yield-related traits, plants prioritise the production of secondary metabolites helping in survival, particularly those involved in chemical defence.

The presence of flavonoid glycosides and malonylated derivatives of orientin, vitexin, chrysoeriol and scoparin in some commercial and non-commercial genotypes (as evidenced by cluster 5 and 7) highlighted a more widespread distribution of these compounds that is not genotype-specific. Flavonoid malonylation and glycosylation are modifications that typically enhance solubility, stability, and transport (Alseekh et al., 2020; Zhao et al., 2011): these modifications can make flavonoids more effective in long-term storage and mobile defence mechanisms, regardless of whether the specific genotype is cultivated for commercial purposes or not.

The lignanamides cannabisin D and grossamide, more abundant in the commercial genotype Férimon 12 (in cluster 9), along with syringaresinol and its derivatives in Férimon 12 and the VIR genotype Line

43/178 (cluster 10), suggest that these genotypes synthesise lignanamides and lignans, respectively, and as part of a defence strategy balancing structural and chemical defences. Lignanamides, a subclass of lignans, are natural secondary metabolites derived from oxidative coupling mechanisms with hydroxycinnamic acid amides as intermediates (Leonard et al., 2021). Hydroxycinnamic acid amides are known to act in several growth and developmental stages, e.g., tuberization, flower development and plant defence against pathogens (Liu et al., 2022).

The molecular analyses enabled a comparison between gene expression patterns and metabolite abundances, highlighting both similarities and differences across genotypes. The expression of *OMT21*, a gene coding for an *O*-methyltransferase that methylates the 3′-*O* position of luteolin to form chrysoeriol (Bautista et al., 2021) was higher in Futura 75, Line 43/178 and Odnodomnaya compared to the other genotypes (Fig. 2 and Suppl. File). This expression trend correlated with the higher normalised abundance of chrysoeriol in the same genotypes in negative mode (Fig. 3 and Suppl. File). However, the expression level of *OMT21* in Fédora 17 was among the lowest, despite the higher abundance of chrysoeriol compared to other genotypes such as Félina 32 or Férimon 12.

The expression of *PT1*, one of the prenyltransferases involved in CBGA biosynthesis (Apicella et al., 2022; Page and Boubakir, 2012), was the highest in Odnodomnaya, and this genotype also showed the highest normalised abundance of CBGA in negative mode (Figs. 2 and 3; Suppl. File). However, its expression was much lower in all the other genotypes, regardless of the differences detected in CBGA abundance. For example, in negative mode, a 10-times higher normalised abundance of CBGA was observed in Fédora 17 compared to Local 14 (Supplementary File).

The discrepancies between gene expression levels and metabolite abundances can be caused by translational/post-transcriptional regulatory layers. Indeed, unlike the relationship between transcripts and proteins (despite the presence of deviations), the link between transcripts and metabolites is not straightforward (Cavill et al., 2016). In the absence of direct enzymatic activity measurements, this correlation remains inferential, and further studies including enzymatic assays, or metabolite flux analysis will be required to prove the link between gene expression and metabolite accumulation in the VIR genotypes.

Since this study focussed on *in vitro*-propagated genotypes, future work will also be necessary to evaluate the correlation between the data here reported and the results obtained under agronomically relevant conditions.

5. Conclusions

The data obtained showed specific gene expression and phytochemical profiles in commercial and non-commercial *C. sativa* genotypes.

Commercial varieties, cultivated primarily for lignocellulosic biomass production and seeds, have been selected for traits that favour rapid growth and enhanced fibre and/or seed production. For example, the expression of *PAL* and *4CL1* in the commercial genotype Férimon 12 was among the highest (Fig. 2). In this genotype, there was also a high abundance of phenolic amides, hydroxycinnamic acids and lignanamides (Fig. 3).

Non-commercial genotypes invest instead in the biosynthesis of bioactive compounds such as flavonoids and cannabinoids, which serve as chemical defences against herbivores, pathogens, and other environmental stressors. The increased abundance of flavonoids in these genotypes was positively correlated with the overall increased expression of genes intervening in the late stages of the phenylpropanoid pathway (Fig. 2). For example, the expression of *FNS6* and *CHS7* in the non-commercial genotypes Line 43/178 and Odnodomnaya was the highest (Fig. 2). Additionally, the abundance of flavonoids was also the highest in these genotypes (cluster 1 and 4; Fig. 3).

Based on the results presented, the VIR genotypes show promising potential for further investigation due to their flavonoid and cannabinoid content and warrant future studies aimed at assessing their biotechnological applications.

CRediT authorship contribution statement

Margaux Thiry: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kjell Sergeant:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Emmanuelle Cocco:** Writing – review & editing, Methodology, Formal analysis. **Sergei Grigorev:** Writing – review & editing, Resources, Methodology. **Marcus Iken:** Writing – review & editing, Methodology. **Jean-Francois Hausman:** Writing – review & editing, Supervision, Resources, Methodology. **Jenny Renaut:** Writing – review & editing, Resources, Methodology. **Stanley Lutts:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation. **Gea Guerriero:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.121921.

Data availability

All data produced are available in the manuscript and Supplementary file

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