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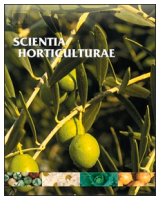

Research Paper

Exogenous gallic acid induces changes in the metabolome of *Mentha arvensis* L. regulating the expression of key genes involved in menthol biosynthesis

Alessia D'Agostino, Gabriele Di Marco, Antonella Canini, Angelo Gismondi *

Laboratory of Botany, Department of Biology, University of Rome "Tor Vergata", Rome, Italy

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ABSTRACT

Mentha arvensis L. (corn mint) is an aromatic plant of great economic interest. Nowadays, one of the most important goals of the 2030 Agenda is to identify new phytostimulants able to substitute the common fertilizers and pesticides, in promoting sustainable crop productions. Thus, the objective of the present contribution was to evaluate the potential effect of several concentrations (1–750 μM) of gallic acid (GA) as phystostimulant for corn mint plants treated for 15 and 21 days. Here, we demonstrated that GA was able to stimulate the accumulation of total phenolics, flavonoids and terpenoids. In addition, 21 phenolics were quantified by HPLC-DAD analysis, demonstrating how GA was able to increase some specific metabolites, such as kaempferol and quercetin known to exert roles in cellular signalling. By GC–MS technique, instead, the lipophilic fraction was investigated, putting in evidence that GA caused a rise in the levels of menthol, the key molecule of the mint chemotype used in this work, and a decrease of its precursor, the limonene. This result suggested a putative effect of GA on the biosynthetic steps of menthol; therefore, the gene expression of 5 enzymes involved in its biochemical pathway was measured through RT-qPCR analysis, highlighting a substantial increase in the transcript of *Menthone reductase* and a simultaneous decrease in that of *Limonene synthase*. All together these data proved that GA, especially after 21 days of treatment at a medium-low dose (50 μM), was able to exert on *M. arvensis* a positive modulation of the metabolome.

Main Conclusion

Gallic acid used as phytostimulant at a medium-low dose exerts a positive modulation on the biosynthetic steps of menthol in *Mentha arvensis*.

1. Background

Mentha arvensis L., commonly known as corn or menthol mint, is an aromatic species belonging to the Lamiaceae family and possessing a great economic interest, since its extracts are widely employed in food, agricultural, pharmaceutical, and cosmetic industry (Kumar et al., 2012; Tiwari 2016; Singh and Pandey 2018; Lal et al. 2020). To date, thousands of hectares of soils worldwide are cultivated with this herb but in the next future further fields are expected to be requested, especially after the recent pandemic, which has induced a significant increase in the demand of natural substances showing antimicrobic, antiviral and immunostimulant properties (Tiwari 2016; Patne et al. 2020; Muthumanickam et al. 2021). The biological value of the corn mint, which also justifies the use of this plant, is attributed mainly to the presence of specific phytochemicals, such as mono- and sesqui-terpenes, or rather lipophilic and volatile molecules defining the peculiar fragrance of this species (Tiwari 2016) and whose synthesis occurs in the secretory cells of the peltate glandular trichomes located on the epidermal surface (McCaskill et al. 1992). Overall, menthol can be considered one of the most typical and bioactive secondary metabolites shared among mints, even if its concentration can be minimal in some species. The principal form of this cyclic monoterpene alcohol in nature is the (−)-menthol (configuration 1R,3R,4S), although it may exist in four pairs of optical isomers (Kamatou et al. 2013). Several enzymatic reactions lead to the

* Corresponding author.
E-mail addresses: d.agostino@scienze.uniroma2.it (A. D'Agostino), gabriele.di.marco@uniroma2.it (G. Di Marco), canini@uniroma2.it (A. Canini), gismondi@scienze.uniroma2.it (A. Gismondi).

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formation of menthol, starting from geranyl pyrophosphate (GPP). Briefly, Limonene synthase (EC 4.2.3.20) brings to the cyclization of GPP in limonene, whereas the Limonene hydroxylase (EC 1.14.14.99) converts the limonene to isopiperitenol. The latter is transformed by the Isopiperitenone dehydrogenase (EC 1.1.1.223) in isopiperitenone, whose isomerization generates pulegone. This metabolite is fundamental in monoterpene metabolism as it is the precursor of menthofuran and menthone, obtained under the activity of Menthofuran synthase (EC 1.14.14.143) and Pulegone reductase (EC 1.3.1.81), respectively. Finally, menthol is produced from menthone in the presence of Menthone menthol reductase (EC 1.3.1.82) (Croteau et al. 2005; Mishra et al. 2017).

To cope with the increasing food demand and to overcome the threats posed by the climate change, the identification of new phytostimulants (in substitution of the common fertilizers and pesticides used in agriculture and showing toxicity for the environment and human health) represents one of the most important modern challenges, which is also related to the goals of the 2030 UN Agenda for Sustainable Development (e.g., *zero hunger* - Goal 2; FAO 2018). The definition of the term phytostimulant is still evolving, given the wide diversity and relative effects of the natural substances (e.g., seaweed extracts, organic acids, amino acids, biochar, nanomaterials, plant metabolites and extracts) used to enhance growth, productivity and quality of the crops, even under stressful environments (Calvo et al. 2014; Yakhin et al. 2017; Rai et al. 2021; Bano et al. 2022; Carbone et al. 2023; Wazeer et al. 2024).

Gallic acid (3,4,5-trihydroxybenzoic acid; henceforth GA), naturally synthesized in plants and extensively exploited as tanning agent, ink dyes, and reagent for paper manufacturing (Covington 1997; Eslami et al. 2010), has been proposed as a phytostimulant capable of promoting plant responses to various exogenous *stimuli*, like oxidative stress, salinity and accumulation of metals (Bhardwaj et al. 2015; Singh et al. 2017; Yetişsin and Kurt 2020; Babaei et al. 2022). In this regard, our research group has investigated the effect of GA on *Mentha spicata* subsp. *spicata* L. (spearmint), demonstrating the capability of this phytochemical to improve yield and quality of the essential oil extracted from this mint species, even in presence of drought. In detail, the results have highlighted that the treatment did not significantly alter the morphometric and physiological parameters of the spearmint, although a substantial and significant modulation of the metabolome could be registered, in qualitative and in quantitative terms. Indeed, GA administered at low doses induced, both in deficit- and well-watered conditions, the accumulation of mono- and sesquiterpenes as well as the increase in the number of peltate glandular trichomes. By contrast, all these changes were not detected in plants subjected to water stress but not exposed to GA (D'Agostino et al. 2024). Taking into consideration all this premise and that the same biostimulant can exert different biological properties on various plant species and even cultivars (Toscano et al. 2018), in the present contribution, we investigated the role of exogenous GA on the quality level of the corn mint (*M. arvensis*). To do it, the total content of the main classes of secondary metabolites was estimated by spectrophotometric assays, while liquid (HPLC-DAD) and gas (GC–MS) chromatography approaches were applied to quantify the concentration of specific phytochemicals. Finally, qPCR analyses were carried out for monitoring the expression level of some genes responsible for the synthesis of corn mint chemical markers. Among all, particular attention was paid to menthol, the metabolite which mainly characterized the chemotype of the analysed species.

2. Materials and methods

2.1. Experimental setup and growth conditions

The plant material was obtained from a guaranteed provider (Larosa Sementi SRL) and further taxonomically checked according to Pignatti (1982). Mint plants (*Mentha arvensis* L., known as corn mint) were propagated and grown in the Laboratory of Botany of the Department of Biology at the University of Rome Tor Vergata, where currently voucher samples are preserved (Voucher IDs: MA1-MA56). One-month-old specimen were randomly selected and used for the study. In detail, each experimental point consisted of 4 different plants (i.e., independent biological replicates) and each type of analysis was carried out in triplicate (i.e., independent technical replicates). Except the control (Ctrl), the mineral soil of the treated plants was enriched by blending with pure gallic acid (GA; Sigma-Aldrich) at 6 concentrations, respectively 0.17 mg per Kg of soil (henceforth, 1 μM GA), 0.85 mg per Kg of soil (henceforth, 5 μM GA), 8.50 mg per Kg of soil (henceforth, 50 μM GA), 17 mg per Kg of soil (henceforth, 100 μM GA), 85 mg per Kg of soil (henceforth, 500 μM GA), and 127.5 mg per Kg of soil (henceforth, 750 μM GA). The selection of these doses of treatment was made taking into consideration that no scientific work had investigated the effect of GA on mint plants. Thus, we preferred to apply on our model system the range of GA medium-low concentrations proposed by literature on other species. This choice, indeed, allowed to avoid potential toxic effects on plants, as we checked by preliminary laboratory tests, and to limit the accumulation of GA in the soil, in view of its possible application in sustainable agriculture practices. In addition, this aspect fit perfectly with the concept that biostimulants generally work at low doses (Vernieri et al., 2005; Ali et al., 2020). Lastly, the selected doses included also those already documented to positively modulate the phytocomplex of another *Mentha* species (D'Agostino et al., 2024). All samples were irrigated daily to maintain a water level in the soil equal to 85 % of the field capacity (FC), as suggested in Ghanbari and Ariafar (2013), Moshrefi Araghi et al. (2019) and Jahani et al. (2021), checking the weight of the pots twice per day. The treatments were performed for 15 and 21 days in presence of constant conditions (i.e., temperature: 22 °C, photoperiod: 14 h light (visible light, 400–700 nm)/10 h dark, homogeneous light intensity: 120 μmol m^{-2} s^{-1}). At the end of the experiment (Supplementary Information 0), the plant material was harvested and directly analysed; then, the leaves were powdered with pestle and mortar in presence of liquid nitrogen and then stored at −80 °C.

2.2. Biometric parameters

Stem length (from the root collar to the apical tip) and number of leaves were measured at the beginning of the experiment and at 15 and 21 days post treatment (dpt). The changes registered for these biometric parameters were reported as percentage of increase in stem dimension and percentage of number of new leaves with respect to the starting condition.

2.3. Total content of phenolics

The content of total phenolics was estimated by the spectrophotometric assay described in D'Agostino et al. (2024). Briefly, leaf powder (150 mg) was extracted with 1.5 mL of methanol:water (50:50, *v/v*) for 24 h, under agitation on a Digital Tube Rotator (DLAB, MX RD-Pro) at 50 rpm, in the dark at 4 °C. The sample was centrifuged at 12.000 g for 10 min and, then, the supernatant was collected in a new tube and used to carry out the tests. Folin-Ciocalteu colorimetric test, used for the quantitation of the total phenols (although it can react also with other peculiar molecules, such as some amino acids and reducing compounds), was performed by mixing 250 μL of Folin-Ciocalteu reagent (1:10, *v/v*), 200 μL of 0.7 M sodium carbonate and 50 μL of sample. The solution was incubated 2 h in the dark at room temperature and the absorbance was read at 760 nm by a microplate reader (Sunrise, Tecan). Blank samples were prepared, by replacing the volume of plant extract with extraction buffer and used to monitor the background signal of the reaction. Gallic acid was used as reference for the creation of a calibration curve. The results were expressed as mg of gallic acid equivalents (GAE) per g of sample fresh weight (mg GAE/g FW).

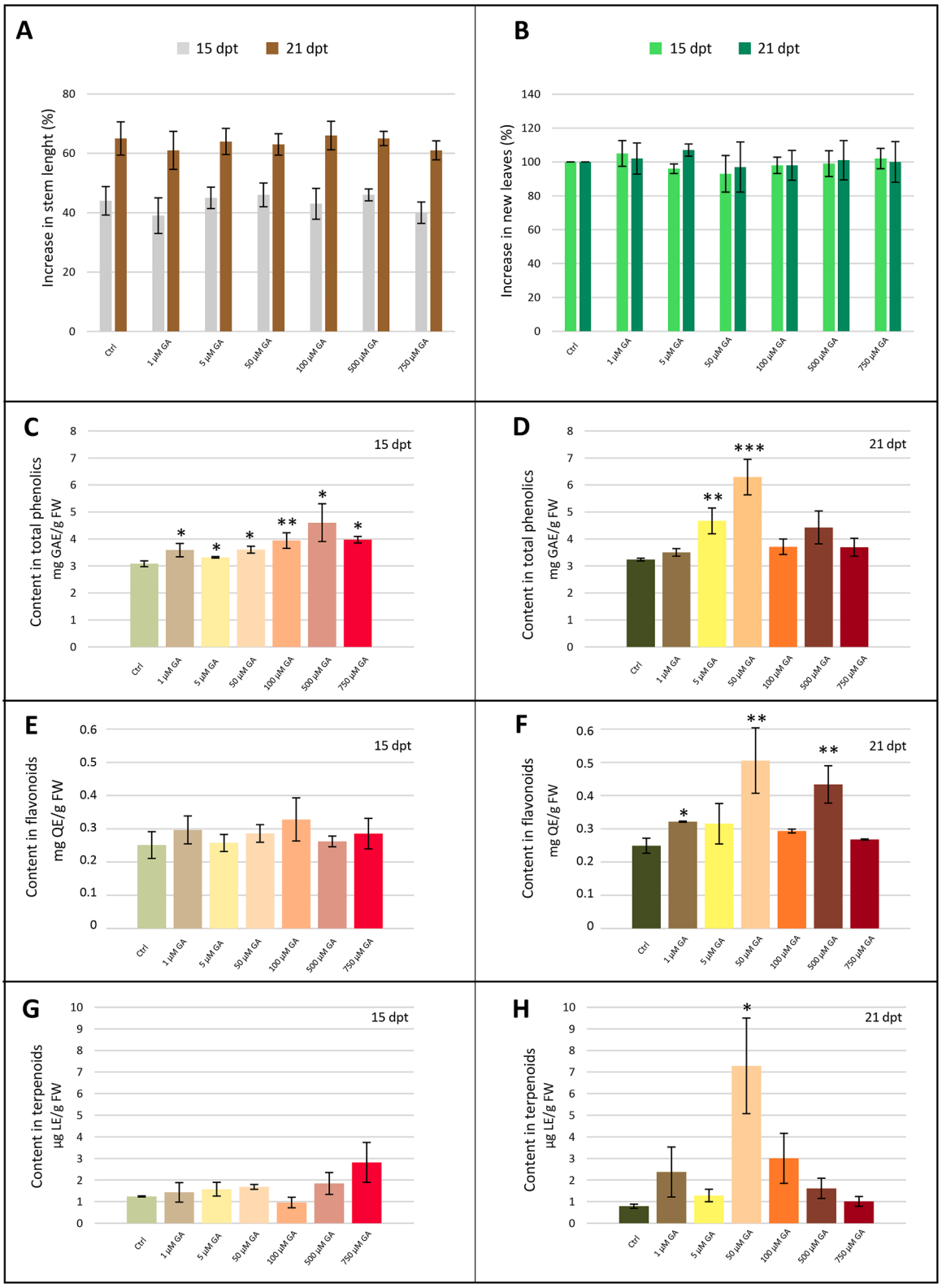

Fig. 1. Plant morphometric parameters and spectrophotometric assays. The histograms representing the percentage variation in stem length (panel A) and new leaves (panel B) after 15 and 21 days post-treatment (dpt) with GA are shown. The content of total phenolics (panel C; panel D), flavonoids (panel E; panel F), and terpenoids (panel G; panel H) measured by spectrophotometric assays is also reported. For each graph, sample labelling reflects the names specified in Materials and Methods section (where GA is gallic acid). Bars with asterisks are significantly different with respect to the respective control (vs Ctrl; one-way ANOVA: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Results are reported as mean ± standard error ($n = 6$).

2.4. Total content of flavonoids

The extract previously obtained for the quantitation of phenolics was used also for the evaluation of the total content of flavonoids. This class of phytochemicals was measured by incubating 100 μL of sample extract, 20 μL of 10 % aluminium chloride, 20 μL of 1 M potassium acetate, 300 μL of methanol, and 560 μL of ddH_2O, in the dark at room temperature for 30 min. The absorbance was read at 415 nm by a microplate reader (Sunrise, Tecan). Blank samples were prepared, by replacing the volume of plant extract with extraction buffer, and used to monitor the background signal of the reaction. Quercetin was used as a reference to produce a calibration curve and the results reported as mg of quercetin equivalents (QE) per g of sample fresh weight (mg QE/g FW).

2.5. Total content of terpenoids

The protocol of Ghorai et al. (2012) was followed for measuring the level of terpenoids. In this case, 1 g of leaf powder was immersed in 2 mL of ice-cold 95 % methanol and incubated at room temperature for 48 h in dark. The supernatant (200 μL) was mixed with 750 μL of chloroform and 50 μL sulfuric acid and incubated for 2 h in the dark. Then, the reddish-brown precipitate was resuspended in 200 μL of 95 % methanol. The absorbance was read at 538 nm with a microplate reader (Sunrise, Tecan). Blank samples were prepared, by replacing the volume of plant extract with extraction buffer and used to monitor the background signal of the reaction. Linalool was employed as standard for producing a reference curve. Data were expressed as μg of linalool equivalent (LE) per g of sample fresh weight (μg LE/g FW).

2.6. Targeted high pressure liquid chromatography analysis

The supernatant samples used for the quantitation of total phenols and flavonoids in the spectrophotometric assays were also subjected to High Pressure Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis. The instrument (Shimadzu, Kyoto, Japan) and the elution gradient applied during the run were the same indicated in D'Agostino et al. (2024). Twenty μL of each sample (150 mg/1.5 mL, *w:v*) were injected in a Luna 3 u C18(2) chromatographic column (150 mm × 4.60 mm × 3 μm) (Phenomenex, Torrance, CA, USA) whose temperature was set at 40 °C, under a flow rate of 0.95 mL per minute. Two mobile phases were employed for the analysis, that is 1 % formic acid (*v/v*) (phase A) and pure methanol (phase B), which were applied under the following gradient: 15 % B solvent maintained for 20 min, linear increase up to 35 % B solvent in 20 min, and up to 90 % in 55 min. At 68 min, the initial condition (15 % B) was restored gradually, until reaching the end of the run (70 min). The investigated molecules, that is 14 phenols and 7 flavonoids, were detected, identified, and quantified by comparing their retention times, absorption spectra, and peak areas with those of pure standards (Sigma-Aldrich, Milan, Italy). The calibration curves were produced with increasing concentrations of standard metabolites (range of $R^2 = 0.998-0.982$). The limits of detection and quantitation of the compounds were 0.75 and 1.50 g/mL, while the inter- and intra-day precision ranged from 0.57 to 2.09 % relative standard deviation (SD). The chromatographic profiles were captured at 280 nm. The LAB-SOLUTION software (Shimadzu) was used for data acquisition, while the results were expressed as μg of respective standard equivalent per g of sample fresh weight (μg SE/g FW).

2.7. Untargeted gas chromatography-mass spectrometry analysis

On the other hand, for Gas Chromatography-Mass Spectrometry (GC–MS) investigation, 150 mg of fresh leaf sample were dissolved in 1.5 mL of pure methanol for 48 h, under agitation, in the dark at 4 °C. After centrifugation at 12.000 g for 10 min, the supernatant was recovered in a new Eppendorf tube and subjected to the analysis. The separation of the molecules was performed using a QP2010 system (Shimadzu, Kyoto, Japan) equipped by a SH-Rtx-5MS capillary column (Shimadzu; length 30 m × diameter 0.25 mm × thickness 0.25 μm). Carrier gas, temperature gradient and mass spectrometer parameters were set as reported in D'Agostino et al. (2024). All peaks defining the chromatograms were analysed and each compound was identified by comparing its mass spectrum with those of pure standards registered in the NIST (National Institute of Standard and Technology) Library 14; only similarity values higher than 85 % were considered acceptable. Results (i.e., the relative abundance of each molecule) were expressed in percentage value with respect to the total mixture (100 %).

2.8. Investigation of the gene expression

Total RNA was purified from the leaf powder using the WizPrep Total RNA Kit (Wizbiosolutions, Loco Hills, United States) and following the manufacturer's guidelines. RNA was eluted in 50 μL of RNase-free water and quantified by NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesised through the WizScriptTM cDNA Synthesis Kit (High Capacity), starting from 5 μg of RNA. The obtained cDNA was quantified and stored at −80 °C until the use. Each qPCR analysis was performed by mixing 50 ng of cDNA, 1X SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Waltham, MA, USA), and 5 pM of both forward and reverse primer (Supplementary Information 1; Mishra et al. 2017). StepOnePlus Real-Time PCR System (Perkin-Elmer Applied Biosystems) was used for the amplification procedure reported here: 10 min at 95 °C followed by 45 cycles of 20 s at 95 °C and 30 s at 59 °C. The melting curve was obtained setting the apparatus at 95 °C for 15 s, 60 °C for 1 min and then by a gradient from 60 °C to 95 °C with a rate of 0.3 °C every 15 s. The amount of transcript was measured using the $2^{-\Delta\Delta Ct}$ formula, where the threshold cycle (Ct) of the target gene was normalized with that of the internal reference gene (i.e., *Actin*), selected for its stability by a preliminary screening, and then with the respective value obtained in the control sample (ΔΔCt). The $2^{-\Delta\Delta Ct}$ method was checked by ΔCt variation analysis at different template concentrations, as described in Livak and Schmittgen (2001). Results were expressed in Arbitrary Units (A.U.) with respect to the control, taken as 100.

2.9. Statistical analysis

Data were subjected to the one-way analysis of variance (ANOVA) and the differences were evaluated by the post-hoc lowest standard deviations (LSD) test, through Excel software (*p* values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Multivariate analysis of HPLC-DAD and GC–MS results was performed by an heatmapper tool (http://www.heatmapper.ca/), producing heatmaps and relative hierarchical clustering.

3. Results and discussion

Plants face stressful events at every stage of their entire life cycle but today, as climate change worsens, they must cope with them to a greater extent. Thus, several agro-chemical methods have been put in place to mitigate the adverse effects triggered by environmental pressures and, among all, one of the most promising and eco-friendly ways to achieve this goal is the application of biostimulants (Rai et al. 2021). Consequently, here, we tested the effect of several concentrations (1–750 μM) of GA on *M. arvensis*. First, *M. arvensis* plants were subjected to biometric evaluations. All samples did not show significant morphological changes, suggesting that GA was not able to induce alterations in the growth rate of the corn mint, similarly to spearmint as reported in D'Agostino et al. (2024). In detail, count of new leaves (Fig. 1, panel A) and dimension of the stem length (Fig. 1, panel B) did not differ among treated (all GA samples) and control (Ctrl) plants, as well as the total biomass.

Folin-Coicalteu assay revealed in all samples at 15 dpt a concentration of total phenolics ranging between 3.08 (in the Ctrl) and 4.60 (in the

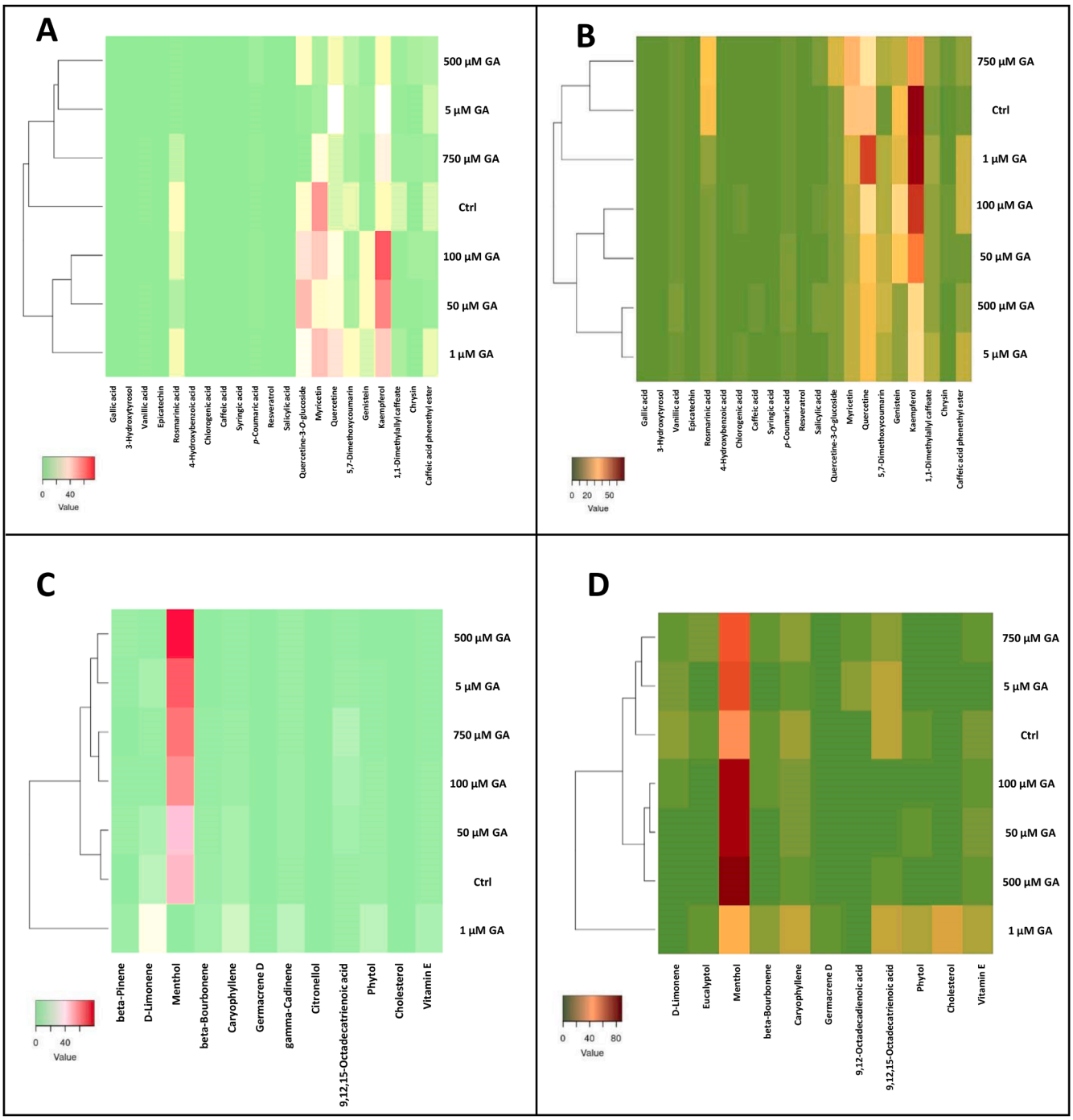

Fig. 2. Heatmaps of the analytes detected by HPLC-DAD and GC–MS analyses. The heatmaps generated starting from the metabolomic data obtained by HPLC-DAD (panels A and B) and GC–MS (panel C and D), at 15 dpt (panel A and C) and 21 dpt (panel B and D), are shown. The detected analytes were reported as columns, while the GA-treatments were indicated as rows. Minimum and maximum values were set as green and red, respectively, and shown in the scale bars. The hierarchical clustering inferred by heatmaps can be also visualized on the left of each panel.

sample 500 µM GA) mg GAE/g FW (Fig. 1, panel C). Interestingly, all GA treatments induced a significant increase of phenolics compared to the Ctrl, with the sample 100 µM GA presenting the best significance ($p < 0.01$). After 21 days (Fig. 1, panel D), the data showed the maximum value of this class of secondary metabolites in plants exposed to 50 µM GA (6.29 mg GAE/g FW), resulting highly significant ($p < 0.001$) with respect to the Ctrl (3.24 mg GAE/g FW). Together with this sample, also 5 µM GA revealed a substantial increase (+44.27 %; $p < 0.01$). This result could be justified by the fact that biostimulants seem increase yield and quality when administrated at medium-low doses (Vernieri et al. 2005; Toscano et al. 2018).

The content of total flavonoids in the series at 15 days (Fig. 1, panel E) varied between a minimum value of 0.25 mg QE/g FW corresponding to Ctrl plants and a maximum of 0.33 mg QE/g FW found in the treatments with 100 µM GA. However, none of the samples was significantly different from the Ctrl. In contrast, the level of flavonoids in plants grown for 21 days (Fig. 1, panel F) ranged from 0.25 mg QE/g FW in the Ctrl to a 0.51 mg QE/g FW in 50 µM GA, reaching significant values at 1, 50 and 500 µM GA treatments. Due to the intrinsic variability of the bioactivity that biostimulants can induce on plants, it is arduous to

provide the exact rationale underlying the effect induced by each dose of GA in this context. However, the highest dose did not show any stimulant capability, as expected, considering the premise reported above for phenolics.

In general, in all samples, total content of phenolics appeared higher in concentration than flavonoids; however, as Lamiaceae species typically accumulate lipophilic secondary metabolites in their tissue, we decided to also monitor the content of terpenes and their derivatives in the samples. At 15 days of treatment (Fig. 1, panel G), total terpenoids ranged between 0.9 μg LE/g FW (in 100 μM GA) and 2.81 μg LE/g FW (in 750 μM GA). However, this variation did not result significant. In the samples exposed to GA for 21 days (Fig. 1, panel H), the level of terpenoids varied between 0.78 μg LE/g FW in Ctrl and 7.29 μg/g FW in 50 μM GA, but only in the latter sample the result appeared significant with respect to the control ($p < 0.05$).

In summary, *M. arvensis* plants treated for 15 days with GA showed a general trend to increase the levels of the three classes of phytochemicals here investigated, although only the accumulation of phenolics might be considered significant and dependent on the concentration of phytostimulant. On the other hand, after 21 days of exposure to GA, secondary metabolites accumulated in the plant tissues in a dose-independent manner but only the treatment at 50 μM revealed the capability to induce simultaneously an increase of phenolics, flavonoids and terpenoids in a significant way.

These data are in agreement with those documented in D'Agostino and colleagues (2024), where 50 μM GA for 21 days resulted the most effective treatment in promoting *M. spicata* secondary metabolism. As already suggested by the literature (Vernieri et al. 2005; Ali et al. 2020; Carletti et al. 2021; Santini et al. 2021), this evidence would confirm that phytostimulants, including GA, exert their positive effect on plants when applied in medium-low doses. Biostimulants can modify the rhizosphere, provide nutrients and hormones, and induce plant defence with the relative gene expression; therefore, it is important to identify the best application, in terms of time and dose, to avoid that too high or low concentrations can nullify their effect, by stressing the plant system or minimizing the exogenous signalling respectively (Vernieri et al., 2005).

To better characterise the changes induced by GA on mint phytocomplex, twenty-one specific secondary metabolites, including phenols and flavonoids, were quantified by HPLC-DAD analysis in the samples of *M. arvensis* treated with phytostimulant for 15 and 21 days (Supplementary Information 2 and 3). The results were also shown as heatmaps (Fig. 2, panels A and B). In all GA samples, both after 15 and 21 days of treatment, approximately half of the detected molecules (e.g., epicatechin, syringic acid, 3-Hydroxytyrosol) did not undergo significant changes, compared to the respective Ctrl.

After 15 days (Fig. 2, panel A), 5, 500 and 750 μM GA treatments were those that up-regulated the least number of molecules, while those that induced the greatest increment in secondary metabolites were 1, 50 and 100 μM GA. Kaempferol represented the molecule which reached the highest doses at 50 and 100 μM GA (63.85 μg/g FW and 75.34 μg/g FW, respectively). Other compounds positively modulated by the same treatments were quercetin-3-glucoside (50 μM GA: 48.55 μg/g FW; 100 μM GA: 43.27 μg/g FW), quercetin (50 μM GA: 29.93 μg/g FW; 100 μM GA: 32.13 μg/g FW) and genistein (50 μM GA: 21.12 μg/g FW; 100 μM GA: 22.21 μg/g FW). Myricetin, by contrast, was abundant in the Ctrl (57.55 μg/g FW) and reduced its content in all the other samples. To date, the role of myricetin in plants is still under-investigated and the main function linked to it is the antioxidant role; therefore, understanding the peculiar trend of this metabolite after GA treatment is arduous.

At 21 days post treatment (Fig. 2, panel B), the molecules subjected to the greatest modulation were quercetin and genistein. Quercetin tended to decrease its concentration in all samples, except that at 1 μM GA (59.49 μg/g FW), with respect to the Ctrl; in parallel, genistein accumulated only in plants exposed to 50 and 100 μM GA (50 μM GA: 19.81 μg/g FW; 100 μM GA: 28.59 μg/g FW), compared to untreated samples. The highest concentration of kaempferol was detected in the Ctrl (69.63 μg/g FW), followed by the sample 1 μM GA (67.14 μg/g FW). Worthy of note is the fact that kaempferol, together with quercetin, generally increased in all plants treated with GA for 15 days, while they underwent a more pronounced reduction of concentration in all the exemplars exposed for 21 days to GA with respect to the Ctrl. The initial accumulation (at 15 dpt) of these two flavonoids could be linked to their well-known protective role against abiotic environmental stressors, such as salt, considering that GA would increase the content of solutes in the soil during the treatments (Singh et al. 2021; Jan et al. 2022). On the other hand, the phenomenon of their decrease at 21 dpt might be associated to the high level of terpenoids in the plants stimulated for that period, due to the capacity of these flavonols to work as auxin transport inhibitors (Woo et al. 2002). Indeed, it has been demonstrated that auxin promotes the synthesis of volatile phytochemicals, especially monoterpenes, like menthol, in mint species (Santoro et al. 2015; Ke et al. 2021).

The last key evidence obtained by the liquid chromatography approach was that the quantitation of GA. This substance was detected at very low levels in all samples, both at 15 and 21 dpt (maximum values: 1.08 μg/g FW in 50 μM GA at 15 dpt; 1.38 μg/g FW in the Ctrl at 21 days), as already found in *M. spicata* by D'Agostino et al. (2024). It would indicate that GA was not absorbed and internalised by the plants. Thus, the main hypothesis could be that GA acted on mints from the outside, exerting its phytostimulant effect indirectly, or rather by modifying the characteristics of the soil and/or its composition or triggering signal transduction mechanisms starting from the rhizoderma.

Overall, HPLC-DAD data confirmed the previous spectrophotometric evidence, suggesting that low doses of GA (i.e., 50 μM) modulated the metabolomic profile of the *M. arvensis* plants in the most effective and significant way.

Via GC–MS, the lipophilic fraction of the phytocomplex of the mint samples was analysed. The results were reported in Supplementary Information 2 and shown as heatmap in Fig. 2 (panels c and d for the 15 and 21 dpt, respectively). The molecules consisted of monoterpene hydrocarbons (e.g., beta-pinene; d-limonene), oxygenated monoterpenes (e.g., menthol), sesquiterpene hydrocarbons (e.g., germacrene D), fatty acids (e.g., 9,12-octadecadienoic acid) and other classes of molecules (e.g. vitamin E; cholesterol). In the metabolome of plants treated for 15 and 21 days (Fig. 2, panel c) the level of the major part of the detected compounds remained constant compared to Ctrl (among them, germacrene D, citronellol and cholesterol). Caryophyllene, gamma-cadinene and vitamin E appeared to vary minimally among the samples and without providing key information in terms of quality about the mint phytocomplex. By contrast, menthol, the main compound typifying the chemotype of the corn mint (Singh et al. 2005; Mishra et al. 2017), resulted strongly and significantly modulated by GA administration. In detail, at 15 dpt, compared to Ctrl, the level of this monoterpene increased in the samples treated with 5, 100, 500 and 750 μM GA by 45 %, 22 %, 58 % and 36 % respectively, while it lowered in the sample 1 μM GA (30 %) and remained stable in 50 μM GA. Curiously, in the same series, d-limonene, which is the precursor of menthol, increased by 17.9 % following 1 μM GA treatment, to decrease its concentration in 5, 50, 100, 500 and 750 μM GA by 36.8 %, 20.6 %, 83.76 %, 80.32 % and 73.8 %, respectively compared to the Ctrl. On the other hand, after 21 days, d-limonene decreased in all samples with respect to the Ctrl, while menthol accumulated in treated plants (i.e., 5, 50, 100, 500 e 750 μM GA by 20 %, 41 %, 42.3 %, 51.9 % and 16.65 %, in that order), except that in 1 μM GA (−56.3 %). The lowest concentration of GA was the only one able to induce a reduction of menthol at both 15 and 21 days. Although interesting, this evidence is difficult to be explained considering that plants were leafy; maybe an indirect effect on the induction of the synthesis for terpenes could be hypothesised.

The increase in menthol concentration was consistent with the spectrophotometric results obtained by the total terpenoid assay.

Unfortunately, our investigation did not detect all the typologies of terpenes usually present in mint, since we had decided to analyse the hydrophobic fraction of our samples and not only the portion of essential oil, whose isolation requires a specific procedure (e.g., steam distillation) capable to preserve all the volatile metabolites which can be lost during maceration extraction. However, our intent was to understand if GA could modulate in mint not only the terpenes but also other lipophilic substances, including fatty acids, which have been proposed as signalling molecules among plant tissues (Hou et al. 2016; He and Ding 2020; Dyall et al. 2022). Nevertheless, our data confirmed that GA was

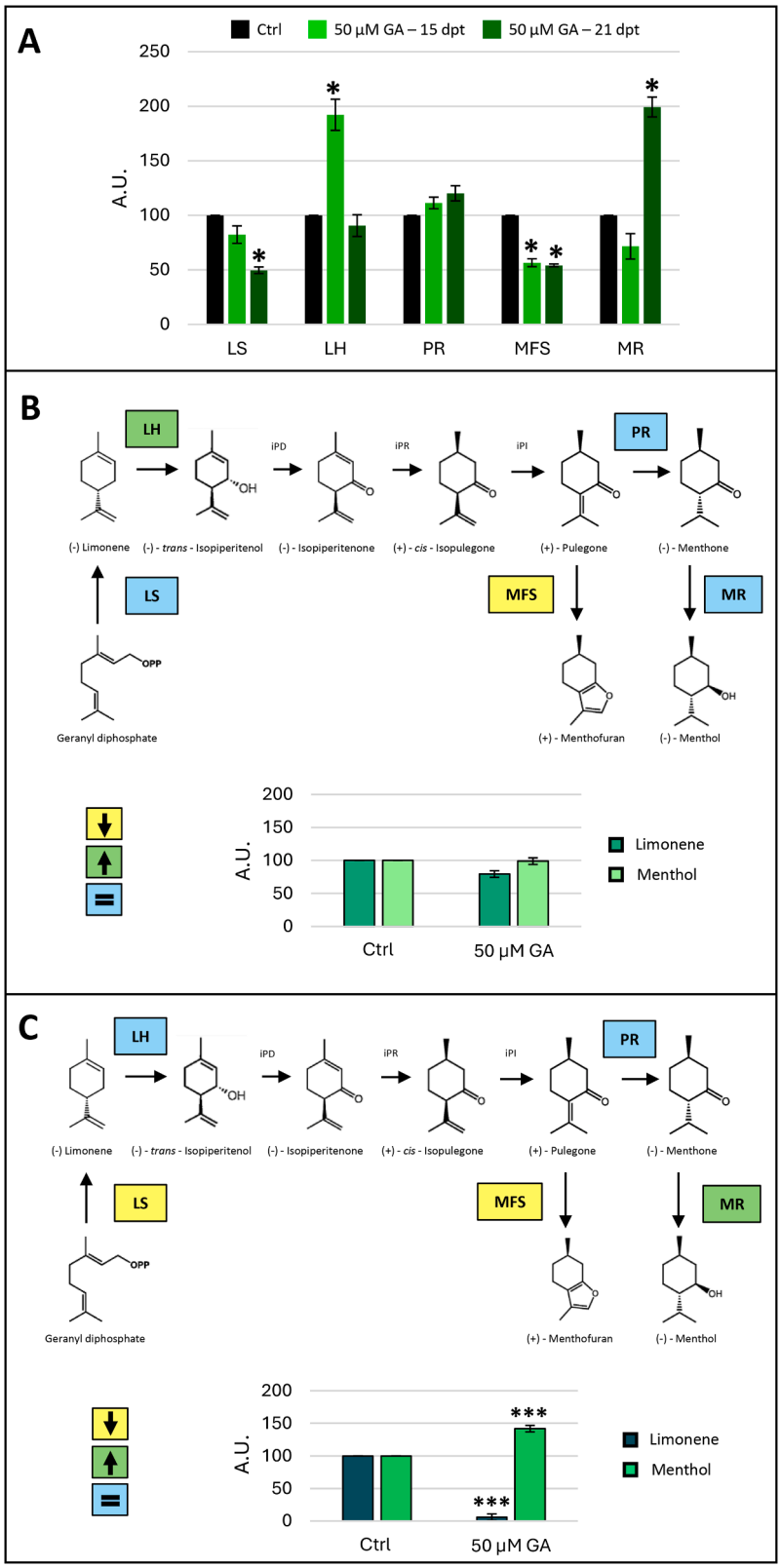

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Fig. 3. Gene expression analysis. Graphical representation of Real-time qPCR data representing the gene expression of: *Limonene synthase* (LS), *Limonene hydroxylase* (LH), *Pulegone reductase* (PR), *Menthofuran synthase* (MFS), and *Menthol reductase* (MR) (panel A). The mRNA levels were normalized with respect to β-*Actin* and expressed in arbitrary units (A.U.) compared to the Ctrl (considered as 100; black bars). Significance levels: *=$p < 0.05$, ***=$p < 0.001$. Representative schemes of the menthol biosynthetic pathway, starting from limonene and referring to samples treated with 50 μM GA for 15 days (panel B) and for 21 days (panel C) are reported. The genes shown in the biosynthetic pathway are: *Limonene synthase* (LS); *Limonene hydroxylase* (LH); *Isopiperitonol dehydrogenase* (iPD); *Isopiperitenone reductase* (iPR); *Isopulegne isomerase* (iPI); *Pulegone reductase* (PR); *Menthofuran synthase* (MFS); *Menthone reductase* (MR). In the panels B are reported the data for Ctrl and 50 μM GA sample at 15 dpt, while in panel C, those relative to Ctrl and 50 μM GA sample at 21 dpt. In both panels, the histograms indicate the level of D-limonene e menthol obtained from GC–MS analysis reported and expressed in Arbitrary Unit (A.U.) compared to control taken as 100 %, while the genes framed where those whose expression was investigated and shown in the panel A. In detail, to favour the trend of the expression for these genes, down regulated transcripts were highlighted in yellow, up-regulated ones in green, while stable mRNA levels with respect to the Ctrl were left in blue boxes.

able to stimulate the accumulation in mint of menthol, which is known to play a key role in determining the quality level of corn mint extracts, being the principal components of its chemotype.

The peculiar levels of menthol reached in GA-treated samples implied a significant modulation of the biosynthetic pathway for this terpene. Thus, the last objective of our study consisted in investigating if and whether GA was able to promote menthol production; consequently, an expression analysis for 5 key genes involved in menthol synthesis was carried out via real-time qPCR. Our results (Supplementary Information 4) revealed that, compared to the Ctrl, treated plants did not show significant changes of the investigated enzymes, except some of them from samples exposed to 5, 50 and 100 μM GA (see asterisks in SI3). However, since we observed a specific modulation in the content of total terpenoids only in exemplars treated with 50 μM GA (as shown in Fig. 1H), we focused our attention on such type of phytostimulant dose. The results for this condition, specifically reported in Fig. 3 (panel a), revealed that GA induced a time-depended reduction of *Limonene synthase* (LS) expression, which appeared significant ($p < 0.05$) only at 21 dpt (50.35 %). *Limonene hydroxylase* (LH) transcript accumulated only in plants exposed for 15 days to GA (92.18 %; $p < 0.001$), while that of *Pulegone reductase* (PR) remained unchanged after the treatment. Both at 15 and 21 days, *Menthofuran synthase* (MFS) mRNA levels significantly decreased by 43.45 % and 56 %, in that order, compared to the Ctrl. Finally, *Menthol reductase* (MR) expression significantly increased by 99.3 % only in plants treated with GA for 21 days.

To better understand the effect of GA on the biosynthesis of menthol, we joined together our GC–MS and qPCR data in the Fig. 3 (panel b and c). At 15 dpt (Fig. 3, panel b), LH transcript appeared up-regulated, with respect to LS, PR and MR mRNAs whose levels remained stable. By contrast, after 20 days of exposure to the biostimulant, the decreased expression of LS justified the extreme reduced content in Limonene (94.1 %), while the up regulation of MR gene could explain the high accumulation of menthol induced by GA (41.7 %). One could think that the opposite trend of LS and MR is weird and unexpected (being limonene the precursor of menthol) but literature has already reported that the genes involved in menthol biosynthesis are strongly correlated and able to modulate one another, influencing the yield of mint essential oil (Rahimi et al. 2017). Thus, it can be hypothesized that the hyperproduction of menthol determines negative feedback signals inhibiting LS expression. Interestingly, at both timings, MFS transcript resulted down-regulated.

Thus, the molecular analyses confirmed the biochemical results, suggesting that the best effect of GA on *M. arvensis* was exerted at 21 days of treatment by promoting the synthesis of phytochemicals, like menthol, which are responsible for the biological activity of mint essential oil and makes this plant product widely requested in food, cosmetic, pharmaceutical and medicinal field (Dhawan et al. 2018; Makkar et al. 2018; Asghar et al. 2022).

4. Conclusions

The aroma and fragrance industry is a billion-dollar world market which grows annually and that, recently, is increasing its demand for mint, due to the well-known flavouring properties of its phytochemicals. However, the interest on this type of herb is also due to the antioxidant and antimicrobial potential of its extracts. Thus, finding biostimulants able to promote yield and quality of mint species is today a target of great economic interest. The data obtained in this research propose the use of GA as phytostimulant for *M. arvensis*, due to its capacity to positively modulate the metabolome of this plant. In particular, we found that GA determined an increase in the synthesis of specific flavonoids, like quercetin and kaempferol, and some volatile compounds typical of *Mentha arvensis*, like menthol. Natural phytostimulants, like GA (which can be also isolated form plant wastes), play a substantial role in agro-environmental sustainability as their use reduces detrimental chemical inputs to fields while maintaining high crop productivity and quality levels. Finally, elucidating the possible effect of these substances on the biosynthetic pathways for phytochemicals, as done in the present work where we found a substantial increase in the transcript of *Menthone reductase,* which play a key role in the menthol biosynthesis, can provide interesting information for opening new possibilities of genetic engineering and development of *Mentha* varieties with improved economical and biological traits. In conclusion, we observed that GA, applied at a concentration of 50 μM for 21 days, induced in corn mint plants a positive modulation of the phytochemicals characterizing their aroma.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Alessia D'Agostino: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Gabriele Di Marco:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Antonella Canini:** Writing – review & editing, Supervision, Resources, Conceptualization. **Angelo Gismondi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest about the present paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2025.114282.

Data availability

All data are reported in the MS or in its Supplementary Materials.

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