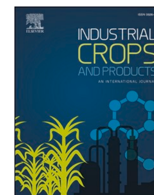
Industrial Crops & Products 214 (2024) 118508

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Biostimulation of *Calendula officinalis* with a soy protein hydrolysate induces flower and plant biomass and flower count by reversibly altering the floral metabolome

Gregorio Peron [a,b,*], Clizia Franceschi [c], Chiara Da Dalt [a], Irene Ferrarese [a], Stefania Sut [a], Stefano Dall'Acqua [a,**]

[a] *Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via Marzolo 5, Padova 35131, Italy*
[b] *Department of Molecular and Translational Medicine (DMMT), University of Brescia, viale Europa 11, Brescia 25123, Italy*
[c] *Fomet SpA, Via Vialarga 25, San Pietro di Morubio, Verona 37050, Italy*

ARTICLE INFO

Keywords:
Calendula officinalis
Soy-protein hydrolysate
Biostimulants
Plant growth
Metabolomics

ABSTRACT

Protein-hydrolysates represent the most studied and widely used biostimulants in agriculture, although limited information on their use in the cultivation of medicinal plants has been published so far. In this study, *Calendula officinalis* plants were treated in greenhouse with a soy-protein hydrolysate (SPH) at different dosages (0, 1, 2.5, 5, and 10 g/L) for 21 days. SPH was applied through different methods, namely soil drenching (SPH-S) and foliar spraying (SPH-F), and its effects on flower count and plant yield (i.e., flower and plant biomasses), and on floral metabolome were documented. SPH-S induced both flower and total plant biomasses, while SPH-F had no significant effects. The effect on plant biomass was dose-dependent, while the highest effect on flower biomass was observed with the application of the lowest (1 and 2.5 g/L) SPH-S dosages. SPH-S at 1 g/L resulted also the best treatment to induce the average flower count *per* plant (n = 14.30 for SPH-S 1 g/L, and n = 8.00 for control). Conversely, no significant effects were observed for SPH-F. Both SPH-S and SPH-F induced changes to the flowers' metabolome: hexadecanoyl- (16:0) and linoleoyl- (18:2) lysophosphatidylethanolamines, known as plant growth regulators, were induced, together with dipeptides, diglycerides, and saponins, while the amount of several flavonoids was decreased. Importantly, these effects were reversible, since floral metabolome resembled that of untreated plants after the suspension of SPH application. Carotenoids were significantly induced only by 10 g/L SPH in flowers collected 7 days after the suspension of treatment. Overall, this work is the first to show the efficacy of SPH in inducing the growth and metabolism of calendula plants, indicating that these products can be efficiently used to enhance the biomass and phytochemical content of medicinal and edible plant species. Furthermore, metabolomics data contribute to partially elucidate the molecular mechanisms underlying the effects of SPH on plants. These results may be useful for researchers and practitioners to optimize the application of SPH to medicinal plants in order to increase their productivity.

1. Introduction

Pot marigold (*Calendula officinalis* L.) is a herb with long tradition of medicinal use. It has been used to treat skin complaints, wounds and burns, conjunctivitis and poor eyesight, menstrual irregularities, varicose veins, hemorrhoids, and duodenal ulcers (Arora et al., 2013). The leaves are claimed as resolvent and diaphoretic, while the flowers as stimulant, antispasmodic and emmenagogue. All these uses are nowadays in large part abandoned, nevertheless flower extracts are still important in phytotherapy for their anti-inflammatory, antimicrobial,

Abbreviations: DAT, days after transplantation; ESI, electrospray ionization; HPLC, High performance liquid chromatography; LPE, lysophosphatidylethanolamine; LPEAT, lysophosphatidylethanolamine acyltransferase; MS, Mass Spectrometry; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; QToF, Quadrupole-time of flight; SPH, soy-protein hydrolysate; SPH-F, foliar spraying of soy-protein hydrolysate; SPH-S, soil drenching of soy-protein hydrolysate; UPLC, Ultra-high performance liquid chromatography.

* Corresponding author at: Department of Molecular and Translational Medicine (DMMT), University of Brescia, viale Europa 11, Brescia 25123, Italy.
** Corresponding author.
E-mail addresses: gregorio.peron@unibs.it (G. Peron), stefano.dallacqua@unipd.it (S. Dall'Acqua).

https://doi.org/10.1016/j.indcrop.2024.118508
Received 9 October 2023; Received in revised form 7 March 2024; Accepted 4 April 2024
Available online 13 April 2024
0926-6690/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

and hypoglycemic effects (Shahane et al., 2023). Furthermore, due to its anti-inflammatory, wound healing, and UV-B protecting properties, calendula represents a common component of cosmetic products for topical applications (Silva et al., 2021).

The phytochemical composition of the plant related to secondary metabolites is very rich and comprises flavonoids, carotenoids, triterpenoid esters, and saponins (Shahane et al., 2023), i.e. a series of compounds that have been largely documented for their anti-inflammatory, antioxidant, and antimicrobial properties. Carotenoids are considered important constituents of pot marigold flowers because they are also the main responsible of the yellow/orange colour of the petals (Bragueto Escher et al., 2019). Thanks to the high content in these constituents (1110–2760 μg total carotenoids/g fresh flowers), extracts from pot marigold flowers are used also as food dyers (Kljak et al., 2021). Polyphenols are also of interest for food applications. Recently, polyphenol-rich calendula extracts encapsulated in calcium alginate microparticles have been developed as novel natural food additives with antioxidant functions (Savic Gajic et al., 2022). Also, calendula extracts have been proposed as novel natural food preservatives, being toxic to microrganisms such as *Aspergillus niger* and *Penicillium* sp. (Podgórska-Kryszczuk and Pankiewicz, 2023).

Currently, cultivation is the most important source of calendula. The cultivation of medicinal plant species can be challenging due to the need of high quality plant materials with standardized chemical composition and high content of active constituents (Alamgir, 2017), but biostimulants can represent valuable aids. Biostimulants are defined as organic products that can enhance "plant performance" when used in small quantities (du Jardin, 2015), and nowadays they find a wide application in agriculture and horticulture. The EU Regulation 2019/1009 gives a more accurate definition of biostimulants, i.e. "products stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, availability of confined nutrients in soil or rhizosphere" (Huygens et al., 2019). During the last years, the efficacy of these products in inducing plant growth and increasing plant yields has been largely documented (de Andrade et al., 2023; Li et al., 2022; Sible et al., 2021). Nevertheless, the molecular mechanisms leading to their effects on plants are still partially unknown, hence further research is needed. In a recent paper, our group focused on the medicinal plant *Acmella oleracea* and compared different biostimulant treatments using a comprehensive approach, considering agronomic parameters and content in secondary metabolites (alkylamides). Results showed significant differences in plant yields, suggesting that the application of biostimulant can increase the yield in term of collection of plant material without reducing the content in secondary metabolites, at least in the considered species (Sut et al., 2020). However, also in this work the mechanisms of action underlying the positive effects of the biostimulant were not investigated.

C. officinalis was considered in this study, given its importance in several sectors related to human health. To the best of our knowledge, only few reports regarding the effects of biostimulant application on plant growth and inflorescence production of *C. officinalis* have been published previously (Emam et al., 2016; Machado et al., 2014; Russo et al., 1993). These works were aimed at studying the effects of seaweed extracts or biotimulants containing antioxidants (L-ascorbic acid) and mixtures of plant-growth promoters like cytokinin, auxin, gibberellic acid, and humic acids. Rogowska et al. (2023) used chitosan for the same purpose but negative effects on calendula growth were observed. In these studies, metabolome changes associated to treatment were not explored except for the determination of few chemical compounds such as flavonoids, carotenoids, steroids, and triterpenoids.

In our work, plant treatment was performed by using a commercial soy-protein hydrolysate (SPH), that was applied to plants either through foliar spraying (SPH-F) and soil drenching (SPH-S). Currently, plant-derived protein hydrolysates (PH) are among the most studied and used biostimulants. They are produced by enzymatic hydrolysis of plant (e.g., soybean and alfalfa) proteins, usually isolated from agricultural by-products (Malécange et al., 2023). Their wide use in crop cultivation is due mainly to their eco-sustainability and low costs, as well as their proven efficacy in increasing plant germination, productivity and quality, and in reducing abiotic plant stress (Colla et al., 2017b, 2017a). Until now, mechanisms of action of PH have been correlated mainly with variations induced to carbon and nitrogen metabolisms, nutrient uptake and nutrient-use efficiency, and with interference with plant hormonal activity (Colla et al., 2017b, 2017a). Significant effects on soil microbiota have also been observed, and they were also associated to positive effects on plants (Hellequin et al., 2020).

In this study, different dosages of SPH were tested, and the effects of biostimulant application on calendula plants were monitored by assessing morphological changes (e.g., flower count and plant yield in term of flower and whole plant biomass) and exploring the the metabolic fluctuatuions occurring in inflorescences.

2. Materials and methods

2.1. Plant material and SPH application protocol

Plant cultivation and biostimulation tests were carried out in a glass greenhouse located in Northern Italy (San Pietro di Morubio, Verona), during the springer-summer period. On May 15th 2020, *C. officinalis* seedlings were transplanted from 128 plug trays into 15 cm diameter plastic pots and placed onto ebb-and-flow aluminum growing tables. Each pot was filled with 2.0 L of potting soil with composition 10% perlite and 90% substrate mixture. Substrate features were: pH = 6 ± 0.5, EC = 0.40 ± 0.04 dS m^{-1}, density = 140 kg m^{-3}. Pests and pathogens were controlled based on standard IPM (Integrated Pest Management). During the experimental trial, plants were exposed to natural photoperiod, air humidity and air temperature (Supplementary Figure S1).

The experiment was designed as a factorial combination of two treatments (foliar spraying and soil drenching) and four dosages (1, 2.5, 5, and 10 g/L), and a control treatment in distilled (milli Q) water. The treatments were arranged in a randomized complete-block design with ten replicates *per* treatment, amounting to a total of 90 experimental unit plots. Each experimental unit consisted of 1 plant (n = 90 plants).

Plants were uniformly treated four times with SPH during the experiment at 8-day intervals (Fig. 1), using an electric backpack sprayer (Stocker) for the foliar application. SPH was obtained by the enzymatic hydrolysis of a soy (*Glicine max* L.) protein extract. The physicochemical properties of SPH are reported in Table S1 of the Supplementary Material.

Water and nourishing elements (Hoagland solution) were provided throughout a fertigation system. During the 5 days following the transplant, plants were daily supplied only with water according to the usual management. Once seedlings were properly developed and root tips reached the internal surface of the pot, the treatment program was started. The plants were fertigated, depending on the growing and microclimate conditions. When not supplied either with nutrient solution, plants were irrigated with water once as needed during the whole growing cycle. Electric conductivity of the water used during the whole cycle was 0.22 dS m^{-1} and the pH was 7.82.

2.2. Flowering and plant yield analysis

The plant growth analysis was performed using both biomorphometric and fresh and dry weight measurements on epigeal organs (flowers, stems and leaves). For each plant, fully developed inflorescences (Figure S2 of the Supplementary Material) were counted and manually harvested in order to estimate the effect of SPH application on flowering. Counting was performed visually at 24, 28, 31, 33, 45, 52, 59, and 71 days after transplantation (DAT), while harvesting at 45,

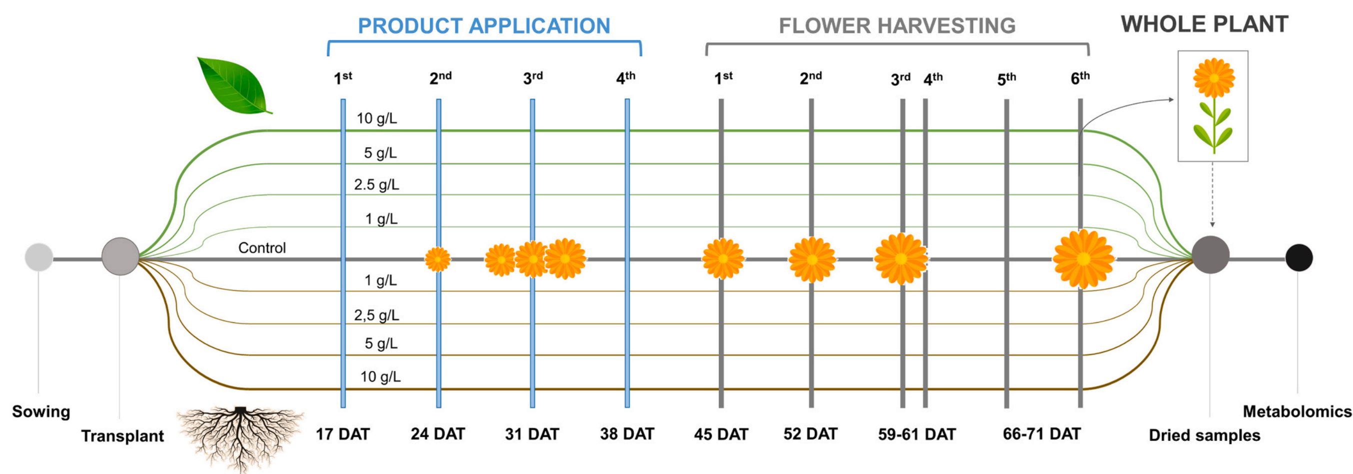


Fig. 1. Procedures of SPH application test. Seventeen days after transplant (DAT) of seedlings into plastic pots, the application of SPH started, and it was performed 4 times during a 21-day period. Groups of n = 10 plants received different SPH dosages (1, 2.5, 5, and 10 g/L) by either foliar spraying (green lines on the upper part of the image) and soil drenching (brown lines on the lower part of the image). In the period between 45 and 71 DAT, six flower harvesting were performed, and after drying and extraction, they were used for further metabolomics analyses. At 71 DAT, whole plants were harvested and weighted, both before (fresh biomass) and after drying (dry biomass). Flowers were counted during treatment and after the application of SPH, namely at 24, 28, 31, 33, 45, 52, 59, and 71 DAT. Flowers in the scheme represent the counting schedule.

52, 59, 61, 66, and 71 DAT. Samples from each collection were maintained separately and they were dried in a forced-air oven at 80 °C for 72 h. The dried flowers were then used for metabolomics analysis.

In order to estimate the plant biomass development, at the end of the experiment (27th July, 71 DAT), the plants were harvested and weighted (fresh biomass). All the samples were dried in a forced-air oven at 105 °C. Dry biomasses were determined once the weight of samples was constant.

2.3. Untargeted metabolomics of calendula flowers

Samples were prepared by weighing 500 mg of pulverized dry calendula flowers, which were then extracted with 10 mL of methanol by ultra-sound treatment for 30 min. Subsequently, 1 mL of solution was withdrawn and centrifuged at 13,300 rpm for 10 minutes, and supernatants were transferred in 0.30 mL polypropylene vials for UPLC-QToF analysis. This latter was performed by using a Waters Acquity UPLC system coupled to a Waters Xevo G2 QToF mass spectrometric (MS) detector. As stationary phase, an Agilent Zorbax Eclipse Plus C18 (2.1 ×50 mm, 1.8 µm) column was used, and column temperature was maintained at 40 °C. A mixture of water + 0.1% formic acid (A) and methanol + 0.1% formic acid (B) was used as the mobile phase. The elution gradient was as follows: 0–1 min, 98% A; 11 min, 15% A; 16 min, 0% A; 20 min, 0% A; 21 min, 98% A; 24 min, 98% A. Flow rate was 0.3 mL/min, and the injection volume was 2 µL. MS data were acquired in negative ionization mode (ESI-) in the mass range 50–2000 Da. The sampling cone voltage was adjusted at 40 V, the source offset at 80 V. The capillary voltage was adjusted to 3.5 kV. The nebulizer gas used was N_2 at a flow rate of 800 L/h. The desolvation temperature was 450 °C. The mass accuracy and reproducibility were maintained by infusing lock mass (leucine–enkephalin, $[M-H]^-$ = 554.2620 m/z) thorough Lockspray at a flow rate of 20 µL/min. The m/z value of all acquired spectra was automatically corrected during acquisition based on lock mass. A MS^e experiment was simultaneously performed to collect structural information, setting the collision energy to 30 V.

Centroided and integrated chromatographic mass data were processed by MarkerLynx Applications Manager version 4.1 (Waters) to generate a multivariate data matrix. An appropriate method for data deconvolution, alignment and peak detection was created. The parameters used were retention-time range 0.5–15 min, mass range 50–2000 Da, mass tolerance 0.01 Da. Noise elimination level was set to 7, minimum intensity was set to 15% of base peak intensity, maximum masses per RT was set to 6 and, finally, RT tolerance was set to 0.01 min. Isotopic peaks were excluded from analysis. A list of the ion intensities of each peak detected was generated, using retention time and the m/z data pairs as the identifier for each ion. The resulting three-dimensional matrix contains arbitrarily assigned peak index (retention time-m/z pairs), sample names (observations), and ion intensity information (variables). Variables with >80% of missing data in both groups were excluded. Data were sum-normalized, Pareto scaled and log-transformed before Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA), performed using Metaboanalyst v.5.0 and v.6.0 platforms (https://www.metaboanalyst.ca/). Discriminant metabolites for the effects of SPH application on floral metabolome were selected using Volcano plots, where FDR-adjusted p-values and Fold Change thresholds were set at 0.05 and 2, respectively. Finally, putative biomarkers were identified calculating the molecular formula by high-resolution m/z values of deprotonated pseudomolecular ions ($[M-H]^-$) and $[M+FA\text{-}H]^-$, $[M-H_2O-H]^-$, $[M-H_2O-H]^-$, and $[M-2H]^{2-}$ adducts, using MassLynx Elemental Composition tool and searching for both the calculated molecular formula and accurate m/z in web databases [i.e. Human Metabolome Database (https://hmdb.ca/), Metlin (https://metlin.scripps.edu/), and KNApSAcK (http://www.knapsackfamily.com/knapsack_core/top.php)]. Possible candidates were then screened evaluating the MS^e fragmentation spectra.

To assess which metabolic pathways were affected in calendula flowers by SPH application, the dataset containing the identification parameters was used to perform a chemical similarity enrichment analysis using the ChemRICH platform (Barupal and Fiehn, 2017). This provided chemical class-based information of significantly altered metabolites in each sample type analysed. ChemRICH identifies highly impacted compound classes through the generation of metabolite clusters based on chemical similarity and ontologies that are not defined by organism-specific metabolic pathways which can be inherently flawed (Barupal and Fiehn, 2017).

2.4. HPLC-UV-Vis analysis of total carotenoid content in calendula flowers

Samples were prepared by weighing 500 mg of pulverized dry calendula flowers, which were then extracted with 10 mL of acetone by

ultra-sound treatment for 30 min. Subsequently, 1 mL of solution was withdrawn and centrifuged at 13,300 rpm for 10 minutes, and supernatants were transferred in 2 mL glass vials for HPLC-UV-Vis analysis. This latter was performed on a Waters Alliance HPLC system equipped with UV-Vis detector operating at $\lambda = 450$ nm, the wavelength of maximum absorbance of carotenoids. A YMC Carotenoid C30 column (250 mm × 4.6 mm, 5 μm) maintained at 35 °C was used as stationary phase, while a mixture of methanol (A) and methyl-*tert*-butyl ether (MTBE)/methanol 90:10 (B) was used as mobile phase. The elution gradient was as follows: 0 min, 0% A; 30 min, 90% A; 32 min, 90% A; 34 min, 0% A; 39 min, 0% A. Flow rate was 1.3 mL/min, and the injection volume was 10 μL.

The amounts of single carotenoids were quantified by interpolating the AUC values of their chromatographic peaks in a calibration curve made by analysing standard 1.05–105 μg/mL beta-carotene solutions at $\lambda = 450$ nm. The equation of the calibration curve was: $y = 55240x - 12145$ ($R^2 = 0.9997$). Total carotenoids quantification was obtained by summing the amounts of every single carotenoid detected in the chromatograms.

2.5. Statistical analysis

The statistical analysis of plant growth and yield was performed by means of one-way analysis of variance (one-way ANOVA) with Duncan Test (p) = 0.05. For all the models, homogeneity of data was assessed through the Levene's test, and normality of the residuals was visually inspected through Q-Q plots. Significance levels were expressed with alphabetical values. For each trait, at least one letter in common indicates no significant difference according to the Duncan test. Statistica (StatSoft) and IBM SPSS Statistics 25 (IBM, USA) softwares were used.

Metabolomics data were analysed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test, using the SPSS software (IBM; version 25.0). P-values <0.05 were considered as statistically significant. All experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD) values.

3. Results

3.1. Application of SPH to calendula induces plant yield, expressed as biomass of aerial part

As a first result, the application of SPH to calendula plants led to different effects on plant growth and yield, depending on the application route and biostimulants' dosages. In particular, plant treatment by radical application resulted to be the most efficient route to boost plant yield, and the data indicate a dose-dependent effect (Table 1 and Figure S3 of Supplementary Material). The effects of 2.5, 5 and 10 g/L dosages were statistically significant if compared to control and denoted a clear trend of the response. The 10 g/L dosage increased the production of fresh biomass by 36% if compared to the untreated control (Table 1). On the other hand, the foliar treatment did not exert a significant effect on the production of fresh biomass. On the contrary, the highest dosage (10 g/L) induced a lower performance in plants if compared to the control. The same trends were also observed with regard to dry biomass, with overlapping trends between the results for fresh and dry biomass.

Table 1
Effect of SPH application on the yield of whole calendula plants, expressed as plant biomass (g). Whole plants were harvested at the end of the study, and were weighed in fresh and dried states (fresh and dry biomasses, respectively). The results of SPH treatment by soil drenching (SPH-S) and foliar spraying (SPH-F) are compared, as well as the different dosages tested (1–10 g/L). CTRL: untreated control. Superscript letters indicate the significance of results.

Treatment	Plant biomass (g)	
	Fresh	Dry
CTRL	40.03^{bc}	8.44^{ab}
SPH-S 1 g/L	40.88^{cd}	8.81^{bc}
SPH-S 2.5 g/L	46.37^{de}	9.75^{cd}
SPH-S 5 g/L	48.85^{ef}	10.13^{de}
SPH-S 10 g/L	54.43^{f}	11.08^{e}
SPH-F 1 g/L	34.45^{ab}	7.56^{ab}
SPH-F 2.5 g/L	35.19^{abc}	7.86^{ab}
SPH-F 5 g/L	36.35^{abc}	7.89^{ab}
SPH-F 10 g/L	33.47^{a}	7.21^{a}

3.2. Effects of SPH on calendula flowers, expressed as flower biomass and count

Regarding the efficacy of SPH on boosting the calendula fresh flowers' biomass, no effects were observed until 45 DAT (Table 2). Conversely, significant differences with controls were detected from 52 DAT. In particular, a more pronounced effect was observed for the SPH-S application at all dosages. At 59 DAT, significant effects on flower biomass were observed only in plants receiving the SPH-S application, regardless of the dosage. Similar results were obtained at 61 DAT, although the differences with the control became less marked. From 66 DAT, a gradual decrease of flower yield was observed in plants receiving either foliar or radical SPH application (Table 2). Considering the sum of the total flower biomass collected during the study (from 45 to 71 DAT), significant differences were observed only between controls and plants treated by SPH-S application, while the foliar application did not lead to significant results. On average, the SPH-S at 2.5 g/L led to an 84% increase of flower biomass in the treated plants.

Regarding flower count, significant differences between controls and plants treated by SPH-S were detected from 31 DAT (SPH-S 1 g/L; Table 3). At 33 and 39 DAT, significant differences with controls are still observed only in plants treated with SPH-S at the lowest dose, while at 52 DAT the highest flower count was observed in plants treated with SPH-S at 2.5 g/L. Overall, the cumulative count of calendula flowers throughout the 73 days of cultivation show that the highest amount was reached by plants treated with SPH-S at 1 g/L (78.8% increase; Table 3).

As regards the SPH-F treatment, no significant results were obtained, except for the flower count at 52 DAT in plants treated with a 5 g/L dose. Also the cumulative counts for all the tested dosages show that, overall, the foliar SPH application is not effective in inducing calendula flowering.

3.3. Application of SPH induces metabolic fluctuations in calendula flowers: identification of putative markers

"Control vs. treated" PCA models were used to explore the effects of SPH application on the metabolome of calendula flowers collected at 45 DAT, namely seven days after the suspension of treatment (Harvesting 1). Harvesting 1 was chosen for the evaluation of the effects of SPH on floral metabolome because a dose-dependent effect was not observed. Hence, flower extracts were divided in two groups, i.e. control and treated groups. In this latter, all treated samples were grouped in a single cluster, without discriminating for biostimulant dosages. The resulting PCA score plots for SPH-S and SPH-F applications are reported in Fig. 2 (panels a and c, respectively), where a clear separation between control and treated groups is shown. An intra-group variability in the "treated" cluster can be observed from the plots, however its contribution to the variability of the whole system is lower than that of inter-group variability (and hence of the effects of biostimulation on metabolome), as indicated by the higher values of Principal Components (PCs) 1 than PCs 2. This result indicates that SPH led to measurable effects on the metabolome of calendula flowers by both application routes.

Metabolites significantly associated to treatment were selected by using the Volcano plots reported in Fig. 2 (panels b and d for SPH-S and SPH-F, respectively). Only metabolites whose variation was significantly (FDR-adjusted p <0.05) associated to treatment and with a Fold Change

Table 2
Effect of SPH application on the yield of calendula flowers, expressed as fresh flower biomass (g). Flowers were collected at six timepoints along the study, respectively at 45, 52, 59, 61, 66, and 71 days after transplant (DAT). The results of SPH treatment by soil drenching (SPH-S) and foliar spraying (SPH-F) are compared, as well as the different dosages tested (1–10 g/L). CTRL: untreated control. Superscript letters indicate the significance of results.

Fresh flower biomass (g)							
	Harvest						
Treatment	1st (45 DAT)	2nd (52 DAT)	3rd (59 DAT)	4th (61 DAT)	5th (66 DAT)	6th (71 DAT)	TOTAL
CTRL	2.09^{a}	1.37^{a}	0.90^{a}	2.45^{ab}	1.84^{bc}	0.95^{ab}	9.60 ± 2.71^{a}
SPH-S 1 g/L	2.85^{a}	4.14^{c}	3.48^{c}	2.97^{b}	1.56^{abc}	1.03^{ab}	16.01 ± 4.90^{bc}
SPH-S 2.5 g/L	2.95^{a}	4.40^{c}	3.64^{c}	3.15^{b}	2.36^{c}	1.47^{b}	17.95 ± 3.92^{c}
SPH-S 5 g/L	2.94^{a}	3.19^{bc}	2.28^{b}	2.73^{ab}	1.66^{abc}	1.23^{ab}	13.99 ± 3.94^{b}
SPH-S 10 g/L	2.07^{a}	3.67^{bc}	2.76^{bc}	2.34^{ab}	1.96^{bc}	1.74^{b}	14.54 ± 4.18^{bc}
SPH-F 1 g/L	2.71^{a}	2.83^{abc}	0.31^{a}	1.73^{ab}	0.71^{a}	0.56^{a}	9.00 ± 3.02^{a}
SPH-F 2.5 g/L	2.68^{a}	2.78^{abc}	0.35^{a}	1.09^{a}	1.42^{abc}	0.4^{a}	8.80 ± 3.04^{a}
SPH-F 5 g/L	2.97^{a}	3.39^{bc}	0.46^{a}	1.07^{a}	1.56^{abc}	0.63^{a}	10.06 ± 2.88^{a}
SPH-F 10 g/L	2.30^{a}	2.06^{ab}	0.27^{a}	2.58^{ab}	1.18^{ab}	0.48^{a}	8.82 ± 2.34^{a}

Table 3
Effect of SPH application on the yield of calendula flowers, expressed as flower count. Flowers were counted at eight timepoints along the study, respectively at 24, 28, 31, 33, 39, 52, 59, and 73 days after transplant (DAT). The results of SPH treatment by soil drenching (SPH-S) and foliar spraying (SPH-F) are compared, as well as the different dosages tested (1–10 g/L). CTRL: untreated control. Superscript letters indicate the significance of results.

Flower count (n ± SD)										
	Days after transplant (DAT)									
Treatment	0	24	28	31	33	39	52	59	73	SUM
CTRL	ND^{a}	ND^{a}	0.1 ± 0.3^{a}	0.4 ± 0.7^{a}	1.0 ± 0.7^{a}	2.5 ± 0.8^{ab}	0.6 ± 0.7^{a}	2.2 ± 2.7^{ab}	1.2 ± 1.3^{ab}	8.0^{a}
SPH-S 1 g/L	ND^{a}	ND^{a}	0.2 ± 0.6^{a}	1.3 ± 0.8^{b}	2.1 ± 1.1^{b}	3.0 ± 1.2^{b}	3.5 ± 2.3^{cd}	3.0 ± 0.9^{b}	1.2 ± 1.1^{ab}	14.3^{c}
SPH-S 2.5 g/L	ND^{a}	ND^{a}	ND^{a}	0.4 ± 0.5^{a}	1.3 ± 0.5^{a}	2.1 ± 0.7^{a}	3.8 ± 2.3^{d}	2.8 ± 1.9^{ab}	2.0 ± 1.1^{bc}	12.4^{bc}
SPH-S 5 g/L	ND^{a}	ND^{a}	ND^{a}	0.6 ± 0.5^{ab}	1.4 ± 1.1^{ab}	2.6 ± 0.8^{ab}	3.0 ± 1.3^{bcd}	3.1 ± 1.4^{b}	1.5 ± 1.0^{ab}	12.2^{bc}
SPH-S 10 g/L	ND^{a}	ND^{a}	0.1 ± 0.3^{a}	0.5 ± 0.5^{a}	0.8 ± 0.6^{a}	2.3 ± 1.2^{ab}	3.0 ± 1.1^{bcd}	2.6 ± 1.7^{ab}	2.8 ± 2.3^{c}	12.1^{bc}
SPH-F 1 g/L	ND^{a}	0.1 ± 0.3^{a}	0.2 ± 0.4^{a}	1.0 ± 1.1^{ab}	1.0 ± 0.7^{a}	1.7 ± 0.8^{a}	1.6 ± 1.1^{ab}	1.7 ± 1.2^{ab}	0.8 ± 1.1^{ab}	8.1^{a}
SPH-F 2.5 g/L	ND^{a}	ND^{a}	0.2 ± 0.4^{a}	0.7 ± 0.6^{ab}	1.2 ± 0.8^{a}	1.8 ± 0.6^{a}	1.6 ± 1.4^{ab}	1.3 ± 1.9^{a}	0.6 ± 0.7^{a}	7.4^{a}
SPH-F 5 g/L	ND^{a}	ND^{a}	0.4 ± 0.7^{a}	0.8 ± 0.9^{ab}	1.1 ± 0.9^{a}	2.0 ± 0.9^{a}	2.2 ± 1.4^{bc}	1.2 ± 1.0^{a}	0.9 ± 0.9^{ab}	8.6^{a}
SPH-F 10 g/L	ND^{a}	ND^{a}	0.1 ± 0.3^{a}	0.9 ± 0.7^{ab}	1.5 ± 0.7^{ab}	1.9 ± 0.7^{a}	1.6 ± 1.1^{ab}	2.3 ± 1.1^{ab}	0.8 ± 1.0^{ab}	9.1^{ab}

>2 were considered as candidates. These are reported in Tables S2 and S3 of Supplementary Material, together with their putative identification, performed on the basis of high resolution MS data.

Among the identified metabolites, two of particular interest were hexadecanoyl- (16:0) and linoleoyl- (18:2) lysophosphatidylethanolamines (LPEs), whose amounts in flowers were induced by SPH application. LPEs have been already described as growth regulators in plants (Peng et al., 2019), and the amounts of both (18:2) and (16:0) LPEs have been reported to be altered in *Arabidopsis thaliana* presenting mutations of the lysophosphatidylethanolamine acyltransferase (LPEAT) genes (Jasieniecka-Gazarkiewicz et al., 2017). The MS spectra of the two metabolites obtained by MS^{e} are reported in Fig. 3, where the characteristic fragments related to the free fatty acids that were used for putative identification are shown.

The chemical similarity enrichment analysis in ChemRICH indicated that the SPH-S application led to an induction of dipeptides, diglycerides and saponins in calendula flowers, while the amounts of flavonoids were decreased (Fig. 4a). Regarding phosphatidic acids, metabolites belonging to this chemical class were induced by treatment [namely, 1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphoethanolamine and PA(15:0/22:5(4Z,7Z,10Z,13Z,16Z))], while the amounts of 1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, PGP(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:4(8Z,11Z,14Z,17Z)), 2-linoleoyl-sn-glycero-3-phosphoethanolamine, PA(18:1(12Z)-O(9 S,10 R)/24:1(15Z)), PG(20:4(5E,8Z,12Z,14Z)-OH(11 R)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), and PA(15:0/22:5(4Z,7Z,10Z,13Z,16Z)) were decreased (Tables S2 and S3). On the other hand, SPH-F application led to an increase in the amounts of both saturated and unsaturated fatty acids, and saturated diglycerides (Fig. 4b).

3.4. Untargeted metabolomics shows that the SPH effects on floral metabolome are reversible

PLS-DA models were used to study the effects of SPH application time and dosages on the metabolome of calendula flowers. As already shown, metabolomics analysis of flowers collected at 45 DAT (harvesting 1) showed a clear effect of SPH on floral metabolome, as indicated by the grouping of control and treated samples in two separate clusters. The effects of increasing hydrolysate dosages were observable only from the 3rd harvesting (59 DAT), for both SPH-S and SPH-F (Figs. 5 and 6, respectively). These effects were of higher relevance in plants receiving the soil drenching treatment, where different effects of low (1 and 2.5 g/L) and high (5 and 10 g/L) dosages were detected.

The PLS-DA plots of both foliar spraying and soil drenching treatments indicate also that the SPH application schedule reflects on the metabolome of calendula flowers, and that the effects of treatment on metabolome are reversible. In fact, as shown in the score plots of harvesting 5 (66 DAT), the separation among samples of treated and control groups are reduced if compared to harvestings 3 and 4, while group differences in harvesting 6 appear negligible. It is important to note that these metabolic variations reflect the effects of treatment on plant growth and biomass yield, that, as described above, were highest in the 2nd and 3rd harvestings (52 and 59 DAT, respectively), and gradually decreased from 61 DAT.

3.5. SPH application alters the total carotenoid content of calendula flowers

Both SPH-S and SPH-F applications altered the total carotenoid content in calendula flowers, as shown by the data reported in Table 4. Particularly, these effects were observed in samples collected at 45 DAT

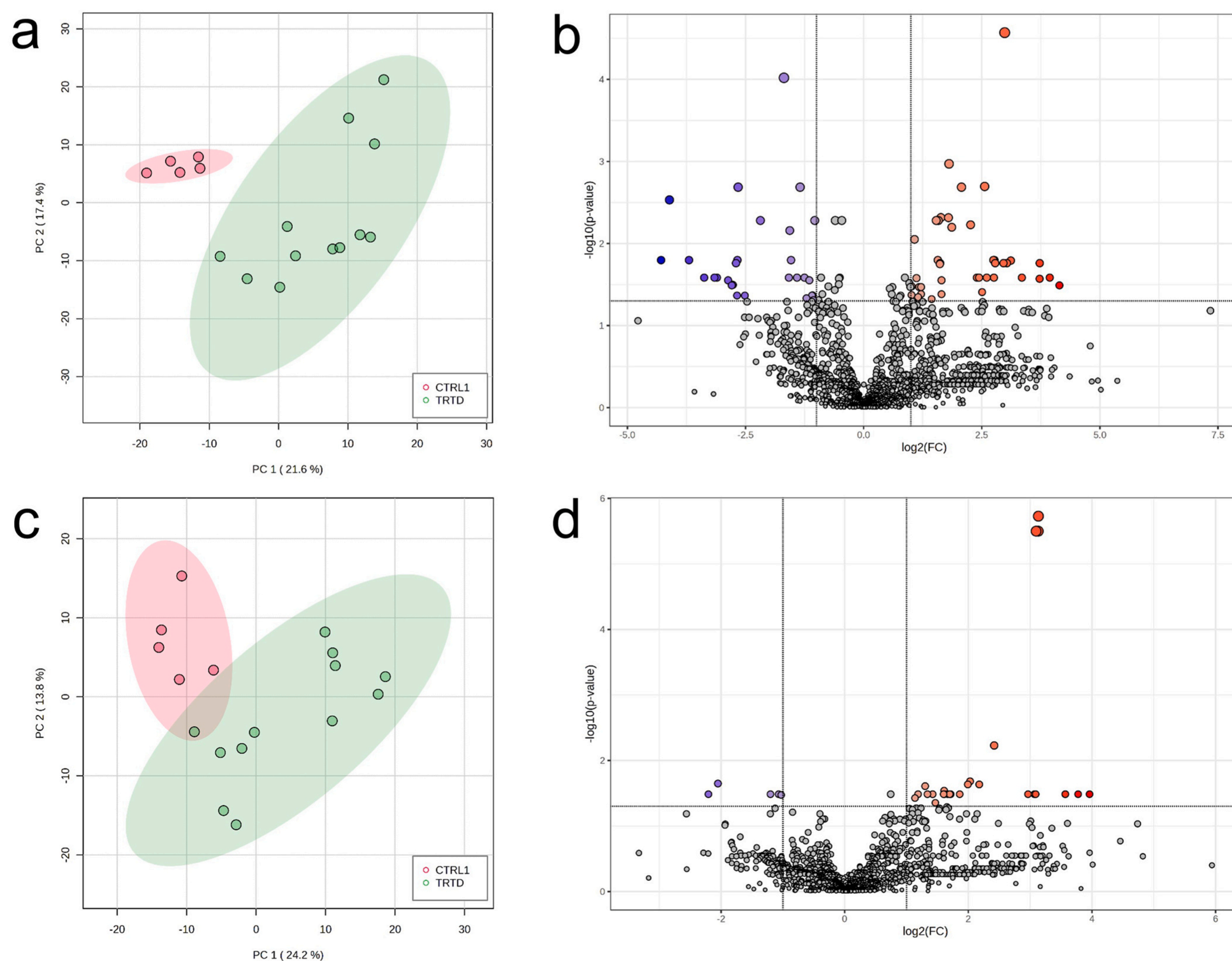


Fig. 2. Results from untargeted analysis of calendula flower extracts. In panels "a" and "c", the PCA plots show the distinction between control samples (red dots) and those collected after SPH application (green dots). In panel "a" are reported the results from SPH-S, while in panel "c" results from SPH-F are shown. Molecular features whose amounts were significantly (FDR-adjusted p <0.05) decreased (blue dots) or increased (red dots) in calendula flowers after SPH-S and SPH-F are reported in the Volcano plots in panels "b" and "d", respectively.

(Harvesting 1). For plants treated by SPH-S, an increasing trend of total carotenoid content proportional to SPH dose was observed, with the highest dose (10 g/L) leading to a significant ($p < 0.05$) increase compared to control. In the case of SPH-F application, the same trend was observed, although the treatment did not lead to significant changes (Table 4).

4. Discussion

Biostimulants, and particularly PHs, have been already reported to effectively induce the growth of common crops such as tomato and lettuce, and to alter specific metabolic pathways correlated to the morphological effects. Recently, Ceccarelli et al. (2021) showed that treatment of tomato plants with PHs derived from different botanical sources (e.g., Fabaceae, Malvaceae, Brassicaceae, and Solanaceae) can increase root length and root number, positively affecting plant growth. Metabolomics analysis of roots revealed that PHs induced variations in phytohormone profile (i.e., cytokinins and auxins), steroids, and secondary metabolites such as aliphatic glucosinolates, alkaloids, and phenylpropanoids, although the effects were not the same for all the products studied. Other recent works show that also the application route of PHs can lead to different effects on plant growth and metabolome. Choi et al. (2022) reported that the application of a PH from legume seeds to lettuce and tomato plants is more effective in inducing not only plant biomass, but also photosynthesis, water-use efficiency, chlorophyll contents, and antioxidant activities when applied by soil drenching instead of foliar spraying. Other reports show instead that the application of a legume-based PH by either roots drenching, foliar spraying or a combination of the two leads to different effects on lettuce, which differ also depending on the cultivars (Cristofano et al., 2021). In this study, we performed similar experiments on *C. officinalis*, a plant of interest for its medicinal properties and its content in compounds with food applicability, such as pigments, antioxidants and antimicrobials. Until now, the effects of PHs on the growth, morphology and metabolome of medicinal plants has been scarcely documented, in spite the potential usefulness of such products for their cultivation. Usually, medicinal plants are required to yield high contents of active constituents, which in turn need to be standardized (Sut et al., 2020). Biostimulants can represent a valuable strategy to achieve these goals, considering their potential to enhance the content of secondary metabolites in plants (Giordano et al., 2022). Furthermore, it has been already documented that their use is highly eco-sustainable, since it increases plant yield and resistance to abiotic stress allowing to reduce the use of mineral fertilizers, without affecting productivity and

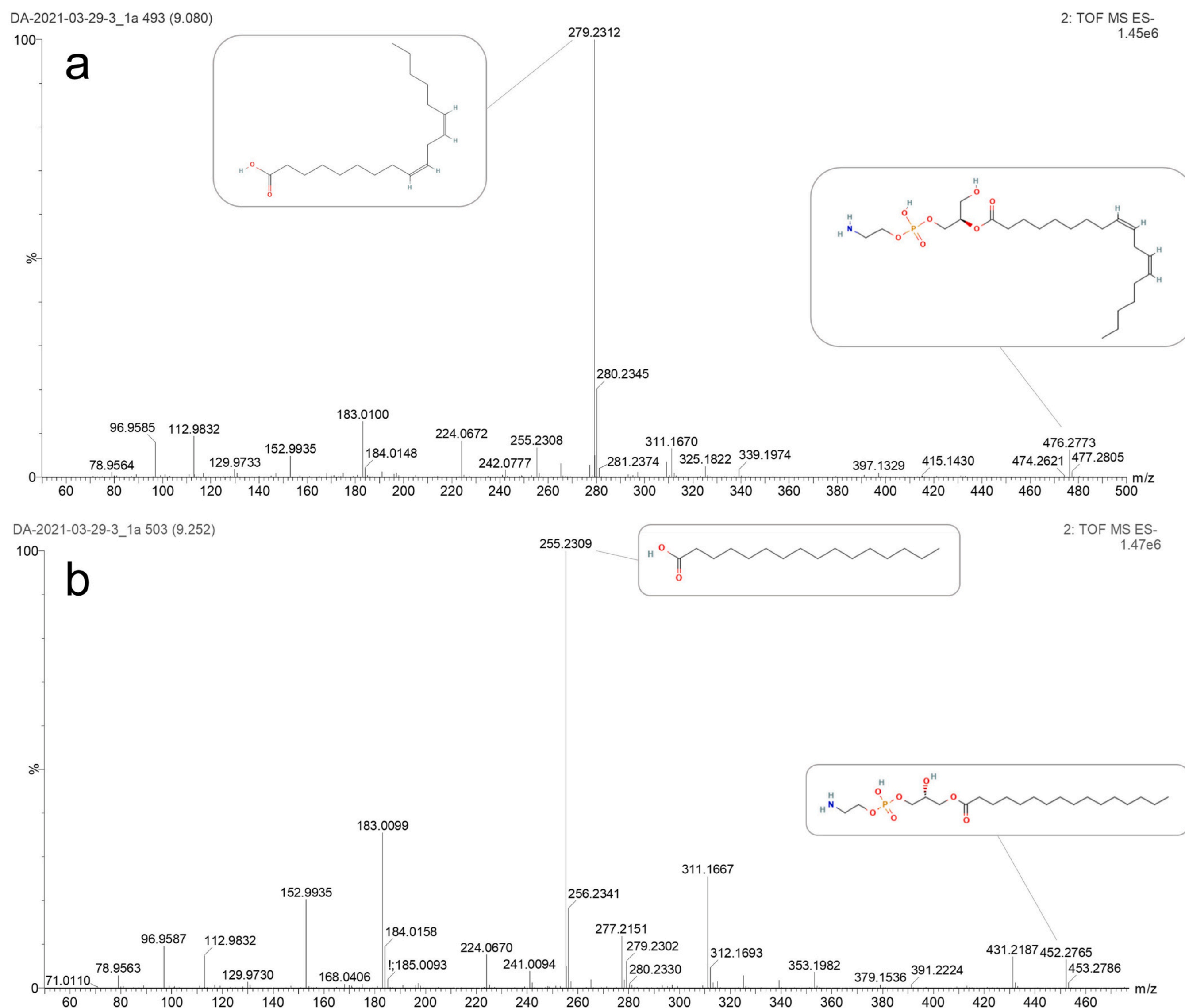


Fig. 3. Fragmentation mass spectra of the metabolites identified as LPE(18:2) (panel "a") and LPE(16:0) (panel "b"), obtained from MS^e analysis. In the Figure are reported the chemical structures of parent compounds [m/z 476.2773 and 452.2765 for LPE(18:2) and LPE(16:0), respectively] and those of the main fragments used for identification, i.e. linoleic acid (m/z 279.2312) for LPE(18:2), and palmitic acid (m/z 255.2309) for LPE(16:0).

economic output (Rajabi Hamedani et al., 2020).

Flowers of calendula represent the plant organ of medicinal, cosmetic, and food interests, since they show a chemical composition rich in secondary metabolites such as carotenoids, terpenes and terpenoids, and flavonoids (Silva et al., 2021). Here, we observed that the application of SPH by soil drenching significantly enhances plant biomass and induces flowering. A typical dose-dependent effect was observed, since increasing dosages yieleded higher increase of biomass produced. This effect was probably linked also to the amount of nitrogen supplied, in fact the 10 g/L dosage was the most effective. On the contrary, the stimulation of the floral biomass was more evident in plants supplemented with low dosages, as expected from a typical biostimulant effect. Also regarding the number of flowers, the soil drenching application was the most performing, and the trend indicated the 1 g/L dosage as the most productive, even if no significant differences among the treatments was detected. Although the carotenoid content in flowers was only partially affected by the application of SPH, floral metabolome showed significant changes after biostimulation. Moreover, the effects of treatment on floral metabolome were different between SPH-S and SPH-F, and this result is in agreement with previous observations in lettuce (Choi et al., 2022; Cristofano et al., 2021). Both SPH-S and SPH-F induced the levels of diglycerides, although variations induced by SPH-F deal more specifically with saturated derivatives. Metabolome of flowers treated by SPH-F showed also an induction of free saturated and unsaturated fatty acids. As already reported by other authors, alteration of fatty acid and lipid metabolism can be the consequence of either the remodelling of cell membranes, which naturally occurs during plant development, or being part of a signal transduction cascade (Ceccarelli et al., 2021). Regarding SPH-S, a significant increase in dipeptides was observed in treated flowers. Although it is challenging to ascribe this metabolic shift to precise mechanisms of action of SPH, we suppose that the increase of dipeptides can be associated to shifting in energetic metabolisms related to enhanced plant growth. In fact, the increase of dipeptides in the model plant *Arabidopsis thaliana* has been observed in plants under nutrient restriction, where they are involved in rewiring metabolism by regulating the activity of enzymes deputed to carbon metabolism, such as glycolysis, gluconeogenesis, TCA cycle, and pentose phosphate pathway (Calderan-Rodrigues et al., 2021). Nevertheless, further research is needed to undercover the role of these metabolites in the metabolic changes observed in calendula after SPH application.

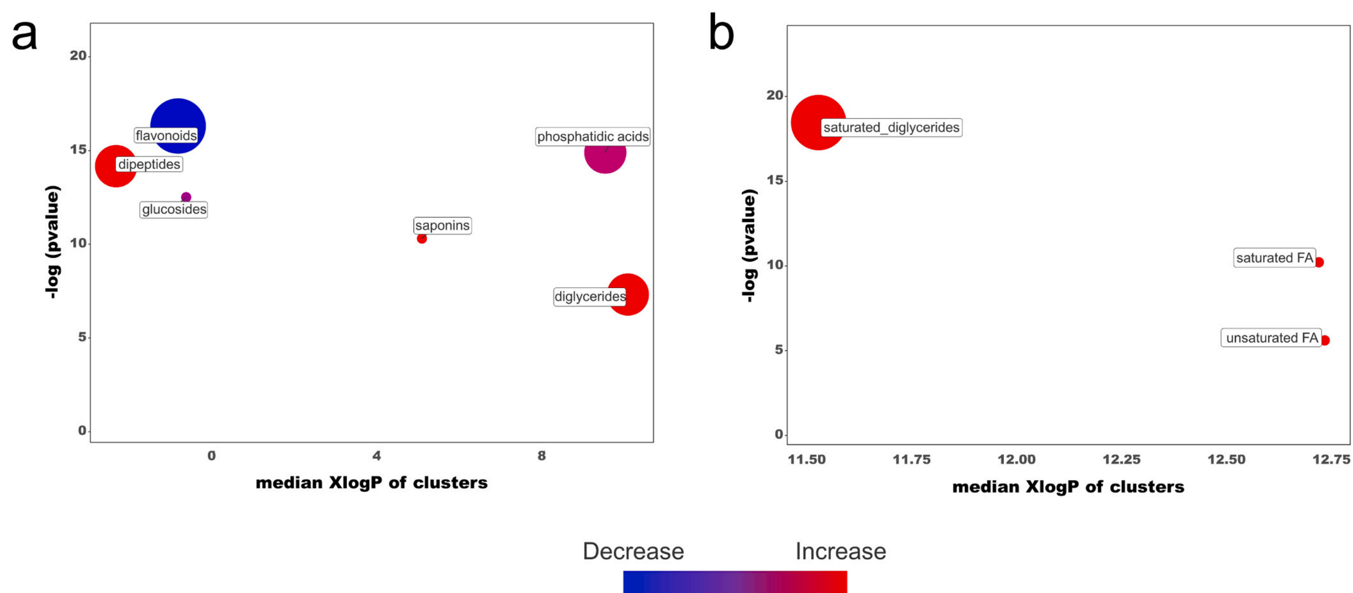


Fig. 4. Enrichment statistics plot showing the metabolic pathways involved in the effects of SPH on calendula flowers. Panel "a" refers to SPH-S application, while panel "b" to SPH-F. Each node reflects a significantly altered cluster of metabolites. Node sizes represent the total number of metabolites in each cluster set. The node colour scale shows the proportion of increased (red) or decreased (blue) compounds in treated calendula plants compared to controls. Purple-colour nodes have both increased and decreased metabolites.

Fig. 5. PLS-DA plots showing the effects of SPH application (time and doses) by soil drenching on the metabolome of calendula flowers. Plant groups receiving different dosages are reported with different colours: pink, control; red, 1 g/L; green, 2.5 g/L; violet, 5 g/L; light blue, 10 g/L.

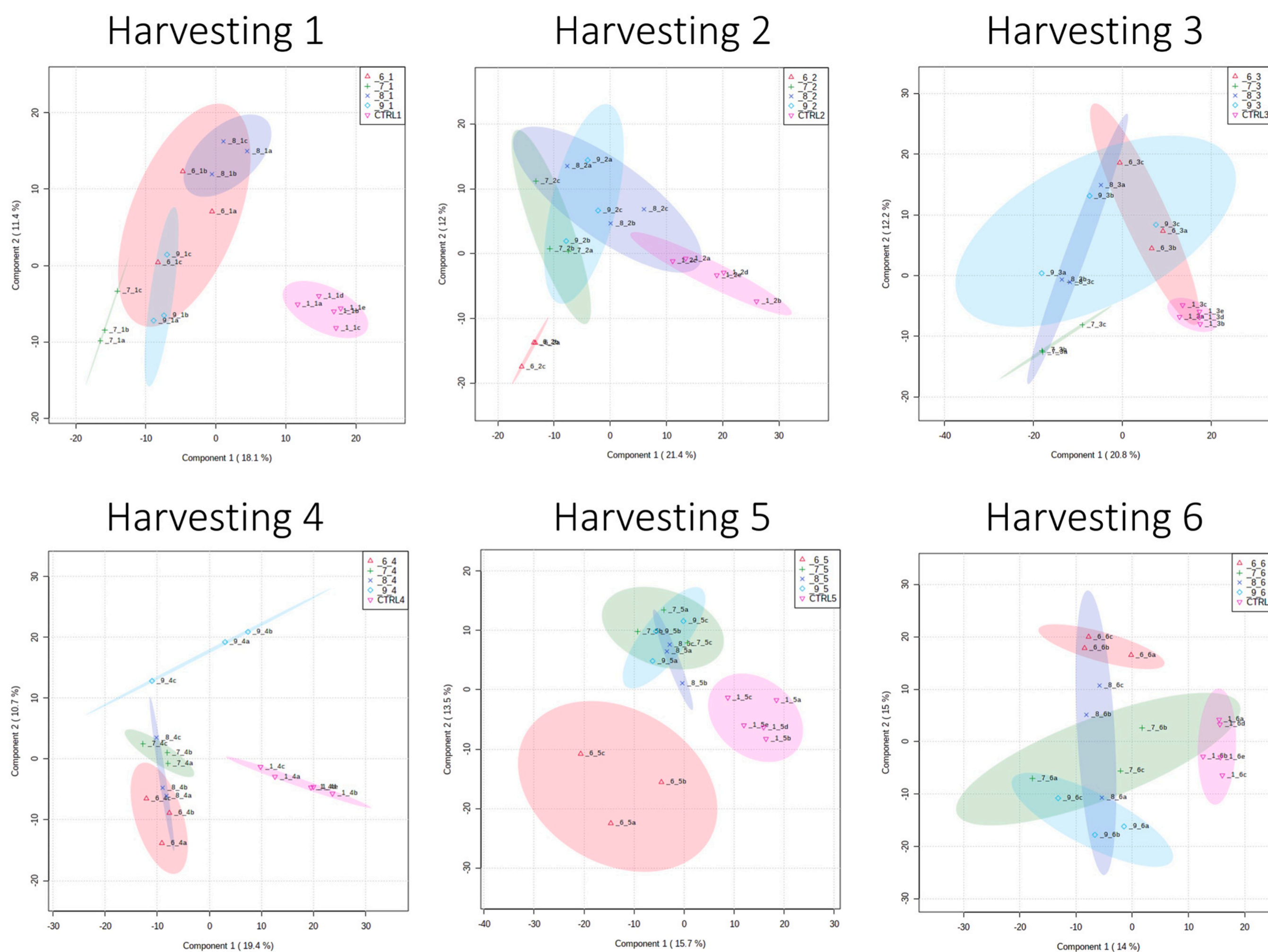


Fig. 6. PLS-DA plots showing the effects of SPH application (time and doses) by foliar spraying on the metabolome of calendula flowers. Plant groups receiving different dosages are reported with different colours: pink, control; red, 1 g/L; green, 2.5 g/L; violet, 5 g/L; light blue, 10 g/L.

After SPH-S application, shifting in the content of secondary metabolites was observed, and specifically, flavonoids were significantly reduced and characteristic calendula saponins were increased. Although plant biostimulants have been reported to induce total flavonoid content (Baltazar et al., 2021), other authors indicated that priming *A. thaliana* seedling with PH led to a reduced content of stress-related molecules in plants, among which flavonoids (Sorrentino et al., 2021). Similar effects have been observed in tomato plants after the application of a commercial product containing polyphenols and glycine betaine as bioactive ingredients for 30 days (Zuzunaga-Rosas et al., 2022). Our result is an indirect indication that plants supplemented with SPH-S are healthier compared to untreated plants, probably due to a reduced oxidative status. In fact, flavonoids are mainly accumulated in plants under stress conditions, where the production of reactive oxygen species (ROS) is increased. Generally, lower levels of ROS allow the plants to grow better (Sorrentino et al., 2021). On the other hand, the amounts of the saponins calendulosides D and H, and calendulaglycoside B were increased after SPH-S application. Triterpene (oleanane) saponins are among the most characteristic components of calendula, and have been associated to several medicinal properties of the plant such as antimicrobial (Szakiel et al., 2005) and anti-inflammatory (Lehbili et al., 2017). In plants, beside having other roles, they are also involed in growth regulation, especially through the induction of root elongation and morphology (Faizal and Geelen, 2013). The increase of specific saponins in calendula flowers after SPH application suggests that the effects on inflorescence production and biomass observed after plant treatment may be associated also to molecular pathways regulated by these compounds. Nevertheless, although these mechanisms need further investigations, this result is important since it indicates that biostimulation of calendula plants with SPH represents a valuable approach to increase the content of their main active constituents. Due to the wide range of biological properties and pharmacological applications, there is significant demand for triterpenoid saponins, particularly those applied in phytomedicines and cosmetics, or as immunoadjuvants in commercial vaccines (Alsoufi et al., 2019; Yendo et al., 2010). Hence, biostimulation with SPH can potentially increase the economic value of calendula plants.

Finally, another class of compounds that was significantly altered by SPH-S is that of phosphatidic acids and lysophosphatidic acids. Most importantly, the LPEs (18:2) and (16:0) were significantly induced, and this result undercovers another molecular mechanism potentially linked to the effects of SPH on plant growth. LPEs function as inhibitors of key enzymes acting on membrane lipid degradation, thus delaying senescence of leaves, flowers, and fruits (Ryu et al., 1997). Furthermore, some exogenous LPEs have been previously described as inducers of plant growth (Amaro and Almeida, 2013). However, data available on literature are controversial: for instance, the study by Jasieniecka-Gazarkiewicz et al. on mutant *A. thaliana* shows that total LPEs content in roots and leaves is significantly increased in plants underespressing subtypes 1 and 2 of lysophosphatidylethanolamine acyltransferase (LPEAT), which are known to be involved in plant growth (Jasieniecka-Gazarkiewicz et al., 2017). Conversely, plants overexpressing the same enzymes were growing more than controls, and

Table 4
Results from the analysis of total carotenoid content in calendula flowers harvested by plants treated by SPH-S and SPH-F, and controls. Harvests 1, 3, 3, 4, 5, and 6 correspond to 45, 52, 59, 61, 66, and 71 days after transplant (DAT), respectively. Values are reported as mg/100 g of dried material ±SD.

Sample[§]	Harvest 1st (45 DAT)	2nd (52 DAT)	3rd (59 DAT)	4th (61 DAT)	5th (66 DAT)	6th (71 DAT)	Sig.[#]
CTRL	13.34 ± 2.92	16.64 ± 2.40	14.24 ± 4.42	15.19 ± 0.62	18.20 ± 6.37	19.52 ± 7.92	NS
SPH-S 1	16.10 ± 1.97	29.28 ± 4.92	15.83 ± 1.72	15.47 ± 3.24	20.35 ± 1.75	15.74 ± 5.17	NS
SPH-S 2.5	17.33 ± 3.21	25.54 ± 1.03	12.75 ± 1.93	12.82 ± 3.23	25.06 ± 2.07	13.27 ± 5.29	NS
SPH-S 5	20.92 ± 6.19	20.24 ± 3.64	14.06 ± 1.35	17.14 ± 7.70	20.22 ± 10.64	17.51 ± 2.48	NS
SPH-S 10	27.45 ± 9.91*	37.68 ± 20.47	14.52 ± 1.16	13.81 ± 2.93	16.85 ± 6.08	16.14 ± 0.42	0.05
SPH-F 1	13.97 ± 0.26	11.68 ± 3.79	11.97 ± 3.55	14.12 ± 2.27	18.61 ± 5.00	13.02 ± 0.74	NS
SPH-F 2.5	14.34 ± 1.33	12.33 ± 2.59	17.81 ± 5.19	13.33 ± 1.28	16.15 ± 1.95	17.41 ± 4.21	NS
SPH-F 5	17.10 ± 1.38	13.15 ± 0.25	16.67 ± 4.15	15.18 ± 2.44	20.18 ± 2.64	17.47 ± 4.52	NS
SPH-F 10	16.97 ± 1.65	14.32 ± 1.90	14.30 ± 0.66	13.78 ± 1.73	17.00 ± 1.52	19.27 ± 4.22	NS

[§]SPH-S indicates the samples to whom SPH was applied by soil drenching, while SPH-F those treated by foliar spraying. "Ctrl" refers to control. Numbers indicate the SPH concentration: 1, 2.5, 5, 10 g/L. [#]Significance was assessed by using ANOVA. *Indicates a p-value <0.05 *vs*. Ctrl.

presented significantly reduced LPEs amounts. Nevertheless, changes in LPEs (18:2) and (16:0) amounts were not always consistent, since they were dependent on the mutations of either one or the other LPEAT subtypes (Jasieniecka-Gazarkiewicz et al., 2017). Overall, these literature data indicate a role of these compounds in plant growth, but a deeper investigation is required to better understand to which extent the alterations of LPEs are involved in the growth process. We suppose that alterations in the levels of the two LPEs observed in treated calendula plants are part of the molecular mechanisms underlying the positive effects of SPH on plant growth, but further research (involving also genomics and transcriptomics analyses) will be required to confirm this hypothesis.

5. Conclusion

Overall, the results of this study indicate that sustainable products such as SPH can be efficiently used to enhance the biomass and phytochemical content of medicinal plant species. To the best of our knowledge this is one of the first reports showing the efficacy of SPH on plants of medicinal, cosmetic, and food interest such as calendula. To note, doses and modes of application are important to achieve the improvement of plant growth. This aspect is of practical relevance when planning the use of SPH on plants and should be taken in account by researchers and practitioners.

This work represents one of the first insights on the variations induced by a biostimulant on the metabolome of *C. officinalis*. The metabolomics data described here partially reveal the molecular mechanisms underlying the effects of SPH on treated plants. These may be important for several aspects: 1) understanding the molecular effects of widely used plant biostimulants such as SPH is the first step to rationalize the use of these products in agriculture and, more in general, in plant cultivation; 2) these information are necessary to develop novel optimized biostimulants to be destined to the cultivation of specific plant species and crops; 3) these results will be useful to guide further investigations on the effects of SPH biostimulants on genome, proteome and transcriptome levels.

Finally, in this work the effects of SPH on the rhizosphere were not evaluated. It is reasonable to assume that at least SPH-S can influence the composition and activity of soil microbes, and these variations can consequently play a role in the positive effects observed in plants, as reported by other Authors (Costa et al., 2024). This aspect will require further investigation.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Chiara Da Dalt: Investigation, Methodology, Software, Writing – review & editing. **Irene Ferrarese:** Investigation, Writing – review & editing. **Gregorio Peron:** Conceptualization, Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. **Clizia Franceschi:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. **Stefania Sut:** Investigation, Methodology, Writing – review & editing. **Stefano Dall'Acqua:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.118508.

References

Alamgir, A.N.M., 2017. In: Alamgir, A.N.M. (Ed.), Cultivation of Herbal Drugs, Biotechnology, and In Vitro Production of Secondary Metabolites, High-Value Medicinal Plants, Herbal Wealth, and Herbal Trade BT - Therapeutic Use of Medicinal Plants and Their Extracts: Volume 1: Pharmacognosy. Springer International Publishing, Cham, pp. 379–452. https://doi.org/10.1007/978-3-319-63862-1_9.

Alsoufi, A.S., Pączkowski, C., Długosz, M., Szakiel, A., 2019. Influence of selected abiotic factors on triterpenoid biosynthesis and saponin secretion in marigold (Calendula officinalis L.) in Vitro Hairy Root Cultures. Molecules. https://doi.org/10.3390/molecules24162907.

Amaro, A.L., Almeida, D.P.F., 2013. Lysophosphatidylethanolamine effects on horticultural commodities: a review. Postharvest Biol. Technol. 78, 92–102. https://doi.org/10.1016/j.postharvbio.2012.12.011.

de Andrade, L.A., Santos, C.H., Frezarin, E.T., Sales, L.R., Rigobelo, E.C., 2023. Plant growth-promoting rhizobacteria for sustainable agricultural production. Microorganisms. https://doi.org/10.3390/microorganisms11041088.

Arora, D., Rani, A., Sharma, A., 2013. A review on phytochemistry and ethnopharmacological aspects of genus calendula. Pharmacogn. Rev. 7, 179–187. https://doi.org/10.4103/0973-7847.120520.

Baltazar, M., Correia, S., Guinan, K.J., Sujeeth, N., Bragança, R., Gonçalves, B., 2021. Recent advances in the molecular effects of biostimulants in plants: an overview. Biomolecules. https://doi.org/10.3390/biom11081096.

Barupal, D.K., Fiehn, O., 2017. Chemical similarity enrichment analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. Sci. Rep. 7, 14567 https://doi.org/10.1038/s41598-017-15231-w.

Bragueto Escher, G., Cardoso Borges, L.D., Sousa Santos, J., Mendanha Cruz, T., Boscacci Marques, M., Araújo Vieira do Carmo, M., Azevedo, L., M. Furtado, M., S. Sant'Ana, A., Wen, M., Zhang, L., Granato, D., 2019. From the field to the pot:

phytochemical and functional analyses of calendula officinalis L. flower for incorporation in an organic yogurt. Antioxidants. https://doi.org/10.3390/antiox8110559.

Calderan-Rodrigues, M.J., Luzarowski, M., Monte-Bello, C.C., Minen, R.I., Zühlke, B.M., Nikoloski, Z., Skirycz, A., Caldana, C., 2021. Proteogenic dipeptides are characterized by diel fluctuations and target of rapamycin complex-signaling dependency in the model plant arabidopsis thaliana. Front. Plant Sci.

Ceccarelli, A.V., Miras-Moreno, B., Buffagni, V., Senizza, B., Pii, Y., Cardarelli, M., Rouphael, Y., Colla, G., Lucini, L., 2021. Foliar application of different vegetal-derived protein hydrolysates distinctively modulates tomato root development and metabolism. Plants. https://doi.org/10.3390/plants10020326.

Choi, S., Colla, G., Cardarelli, M., Kim, H.-J., 2022. Effects of plant-derived protein hydrolysates on yield, quality, and nitrogen use efficiency of greenhouse grown lettuce and tomato. Agronomy. https://doi.org/10.3390/agronomy12051018.

Colla, G., Cardarelli, M., Bonini, P., Rouphael, Y., 2017a. Foliar Applications of Protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. HortScience horts 52, 1214–1220. https://doi.org/10.21273/HORTSCI12200-17.

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., Rouphael, Y., 2017b. Biostimulant Action of Protein Hydrolysates: unraveling Their Effects on Plant Physiology and Microbiome. Front. Plant Sci.

Costa, O.Y.A., Chang, J., Li, J., van Lith, W., Kuramae, E.E., 2024. Unraveling the impact of protein hydrolysates on rhizosphere microbial communities: source matters. Appl. Soil Ecol. 196, 105307 https://doi.org/10.1016/j.apsoil.2024.105307.

Cristofano, F., El-Nakhel, C., Pannico, A., Giordano, M., Colla, G., Rouphael, Y., 2021. Foliar and root applications of vegetal-derived protein hydrolysates differentially enhance the yield and qualitative attributes of two lettuce cultivars grown in floating system. Agronomy. https://doi.org/10.3390/agronomy11061194.

Emam, T.M., Hosni, A.M., Ibrahim, A.K., Hewidy, M., 2016. Response of Pot Marigold (Calendula officinalis L.) to different application methods and concentrations of seaweed extract. Arab Univ. J. Agric. Sci. 24, 581–591. https://doi.org/10.21608/ajs.2016.14428.

Faizal, A., Geelen, D., 2013. Saponins and their role in biological processes in plants. Phytochem. Rev. 12, 877–893. https://doi.org/10.1007/s11101-013-9322-4.

Giordano, M., El-Nakhel, C., Carillo, P., Colla, G., Graziani, G., Di Mola, I., Mori, M., Kyriacou, M.C., Rouphael, Y., Soteriou, G.A., Sabatino, L., 2022. Plant-derived biostimulants differentially modulate primary and secondary metabolites and improve the yield potential of red and green lettuce cultivars. Agronomy. https://doi.org/10.3390/agronomy12061361.

Hellequin, E., Monard, C., Chorin, M., Le bris, N., Daburon, V., Klarzynski, O., Binet, F., 2020. Responses of active soil microorganisms facing to a soil biostimulant input compared to plant legacy effects. Sci. Rep. 10, 13727 https://doi.org/10.1038/s41598-020-70695-7.

Huygens, D., Saveyn, H., Tonini, D., Eder, P., Delgado Sancho, L., 2019. Technical proposals for selected new fertilising materials under the Fertilising Products Regulation (Regulation (EU) 2019/1009). EU Sci. Hub. https://doi.org/10.2760/186684.

du Jardin, P., 2015. Plant biostimulants: Definition, concept, main categories and regulation. Sci. Hortic. (Amst.). 196, 3–14. https://doi.org/10.1016/j.scienta.2015.09.021.

Jasieniecka-Gazarkiewicz, K., Lager, I., Carlsson, A.S., Gutbrod, K., Peisker, H., Dörmann, P., Stymne, S., Banaś, A., 2017. Acyl-CoA:Lysophosphatidylethanolamine Acyltransferase Activity Regulates Growth of Arabidopsis. Plant Physiol. 174, 986–998. https://doi.org/10.1104/pp.17.00391.

Kljak, K., Carović-Stanko, K., Kos, I., Janječić, Z., Kiš, G., Duvnjak, M., Safner, T., Bedeković, D., 2021. Plant Carotenoids as Pigment Sources in Laying Hen Diets: effect on Yolk Color, Carotenoid Content, Oxidative Stability and Sensory Properties of Eggs. Foods. https://doi.org/10.3390/foods10040721.

Lehbili, M., Alabdul Magid, A., Kabouche, A., Voutquenne-Nazabadioko, L., Abedini, A., Morjani, H., Sarazin, T., Gangloff, S.C., Kabouche, Z., 2017. Oleanane-type triterpene saponins from Calendula stellata. Phytochemistry 144, 33–42. https://doi.org/10.1016/j.phytochem.2017.08.015.

Li, J., Van Gerrewey, T., Geelen, D., 2022. A Meta-Analysis of Biostimulant Yield Effectiveness in Field Trials. Front. Plant Sci.

Machado, V.P. de O., Pacheco, A.C., Carvalho, M.E.A., 2014. Effect of biostimulant application on production and flavonoid content of marigold (Calendula officinalis L.). Rev. Ceres 61, 983–988. https://doi.org/10.1590/0034-737×201461060014.

Malécange, M., Sergheraert, R., Teulat, B., Mounier, E., Lothier, J., Sakr, S., 2023. Biostimulant Properties of Protein Hydrolysates: recent Advances and Future Challenges. Int. J. Mol. Sci. https://doi.org/10.3390/ijms24119714.

Peng, C., Ding, L., Hu, C., Chen, X., Wang, X., Xu, X., Li, Y., Xu, J., 2019. Effect on metabolome of the grains of transgenic rice containing insecticidal cry and glyphosate tolerance epsps genes. Plant Growth Regul. 88, 1–7. https://doi.org/10.1007/s10725-019-00482-6.

Podgórska-Kryszczuk, I., Pankiewicz, U., 2023. Assessment of the Fungistatic Properties of Calendula officinalis L. Water Extract and the Effect of Its Addition on the Quality of Wheat Bread. Appl. Sci. https://doi.org/10.3390/app13127286.

Rajabi Hamedani, S., Rouphael, Y., Colla, G., Colantoni, A., Cardarelli, M., 2020. Biostimulants as a tool for improving environmental sustainability of greenhouse vegetable crops. Sustainability. https://doi.org/10.3390/su12125101.

Rogowska, A., Pączkowski, C., Szakiel, A., 2023. Modifications in steroid and triterpenoid metabolism in Calendula officinalis plants and hairy root culture in response to chitosan treatment. BMC Plant Biol. 23, 263. https://doi.org/10.1186/s12870-023-04261-4.

Russo, R., Poincelot, R.P., Berlyn, G.P., 1993. The use of a commercial organic biostimulant for improved production of marigold cultivars. J. Home Consum. Hortic. 1, 83–93. https://doi.org/10.1300/J280v01n01_05.

Ryu, S.B., Karlsson, B.H., Özgen, M., Palta, J.P., 1997. Inhibition of phospholipase D by lysophosphatidylethanolamine, a lipid-derived senescence retardant. Proc. Natl. Acad. Sci. 94, 12717–12721. https://doi.org/10.1073/pnas.94.23.12717.

Savic Gajic, I.M., Savic, I.M., Skrba, M., Dosić, A., Vujadinovic, D., 2022. Food additive based on the encapsulated pot marigold (Calendula officinalis L.) flowers extract in calcium alginate microparticles. J. Food Process. Preserv. 46, e15792 https://doi.org/10.1111/jfpp.15792.

Shahane, K., Kshirsagar, M., Tambe, S., Jain, D., Rout, S., Ferreira, M.K., Mali, S., Amin, P., Srivastav, P.P., Cruz, J., Lima, R.R., 2023. An updated review on the multifaceted therapeutic potential of calendula officinalis L. Pharmaceuticals. https://doi.org/10.3390/ph16040611.

Sible, C.N., Seebauer, J.R., Below, F.E., 2021. Plant Biostimulants: a categorical review, their implications for row crop production, and relation to soil health indicators. Agronomy. https://doi.org/10.3390/agronomy11071297.

Silva, D., Ferreira, M.S., Sousa-Lobo, J.M., Cruz, M.T., Almeida, I.F., 2021. Anti-inflammatory activity of calendula officinalis L. flower extract. Cosmetics. https://doi.org/10.3390/cosmetics8020031.

Sorrentino, M., De Diego, N., Ugena, L., Spíchal, L., Lucini, L., Miras-Moreno, B., Zhang, L., Rouphael, Y., Colla, G., Panzarová, K., 2021. Seed priming with protein hydrolysates improves arabidopsis growth and stress tolerance to abiotic stresses. Front. Plant Sci.

Sut, S., Ferrarese, I., Shrestha, S.S., Kumar, G., Slaviero, A., Sello, S., Altissimo, A., Pagni, L., Gattesco, F., Dall'Acqua, S., 2020. Comparison of biostimulant treatments in acmella oleracea cultivation for alkylamides production. Plants. https://doi.org/10.3390/plants9070818.

Szakiel, A., Ruszkowski, D., Janiszowska, W., 2005. Saponins in Calendula officinalis L. – Structure, Biosynthesis, Transport and Biological Activity. Phytochem. Rev. 4, 151–158. https://doi.org/10.1007/s11101-005-4053-9.

Yendo, A.C.A., de Costa, F., Gosmann, G., Fett-Neto, A.G., 2010. Production of plant bioactive triterpenoid saponins: elicitation strategies and target genes to improve Yields. Mol. Biotechnol. 46, 94–104. https://doi.org/10.1007/s12033-010-9257-6.

Zuzunaga-Rosas, J., González-Orenga, S., Tofei, A.M., Boscaiu, M., Moreno-Ramón, H., Ibáñez-Asensio, S., Vicente, O., 2022. Effect of a Biostimulant Based on Polyphenols and Glycine Betaine on Tomato Plants' Responses to Salt Stress. Agronomy. https://doi.org/10.3390/agronomy12092142.