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β-Carotene biofortification of chia sprouts with plant growth regulators

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ABSTRACT

Chia (*Salvia hispanica*) is a native plant species from South America that is very appreciated for its oleaginous seeds in the agri-food field. Chia seeds are natural sources of many bioactive compounds which provide benefits to human health. Nevertheless, chia sprouts have better nutritional properties than seeds, such as antioxidants, essential amino acids, and phenolic compounds. Among all these beneficial compounds, β-carotene has not been studied in chia sprouts. β-carotene is a precursor of vitamin A, which contributes to maintaining our health status. In this study, to improve β-carotene content in chia sprouts, some plant growth regulators (abscisic acid, methyl jasmonate and methyl salicylate) were applied exogenously to germinating chia seeds. Gibberellins A4/A7 and cytokinin 6-benzyladenine (Promalin®) were also applied, combined with the other regulators, to antagonize a possible inhibition in the germination. Seeds were grown in darkness for 4 days, then seeds were exposed to a short light stimulus (30′) and finally to a continued light stimulus (48h). β-carotene, xanthophylls, chlorophylls, de-epoxidation status of xanthophyll cycle (DPS), germination rate, and sprouts fresh weight were analysed. The results show that sprouts treated with methyl salicylate in-creased 2,35 fold their β-carotene content when they were exposed to light for 30'+48h. Sprouts fresh weight and germination were not affected by methyl salicylate. Although more research is needed before industrial application, it is concluded that methyl salicylate can be used to improve β-carotene contents in chia sprouts.

1. Introduction

One of the most important factors of a healthy diet is the intake of plant-based food. It is recommended to daily ingest a minimum of 400 g of vegetables and fruits (World Health Organization, 2018). This helps to protect against noncommunicable (World Health Organization, 2018), chronic (Liu, 2013), and cardiovascular diseases (Patel et al., 2017). Plant-based food provides different nutrients and bioactive compounds such as vitamins, fibre, antioxidants, and minerals (Liu, 2013). This kind of food (which includes cereals, legumes, seeds, tubers, nuts, and sprouts) and its derivatives have gained popularity in the last years. Also, the vegan diet has increased in several countries, such as the U.K., Europe and U.S.A. (The Vegan Society, 2021), all this accompanied by a bigger concern in our society about healthy diets as reflected by the increase of plant-based products that currently arrive on these markets (The Vegan Society, 2021). Nowadays, food is increasingly considered something that contributes directly to maintain and improve consumers health, not only as something that provides necessary nutrients and satisfies hunger (Bigliardi and Galati, 2013).

Seeds have been gaining relevance as agri-food products. The import of oleaginous seeds in Spain has increased 13 times since 1993 to 2019 (Food and Agriculture Organization of the United Nations, 2021). Oleaginous crops are the 7th most abundant crops in the world in terms of production, producing 700 million of tonnes per year (Food and Agriculture Organization of the United Nations, 2021). Chia (*Salvia hispanica*) is a plant that produces oleaginous seeds which belongs to the Lamiaceae family (Gómez-Favela et al., 2017). It is native from northern Guatemala and southern Mexico (Gómez-Favela et al., 2017) and it has been used as food for more than 5000 years ago (Sosa et al., 2016). Its seeds are an important source of bioactive compounds with beneficial properties for human health (Ullah et al., 2016), such as their desirable content of polyunsaturated fatty acids (linolenic and linoleic acids) (Nitrayová et al., 2014), antioxidants (da Silva Marineli et al., 2014), proteins, fibre, and minerals (Melo et al., 2019). Chia seeds are used as

Abbreviations: ABA, abscisic acid; Chl, chlorophyll; CKs, cytokinins; DPS, de-epoxidation state of the xanthophyll cycle; GAs, gibberellins; HPLC, high performance liquid chromatography; MeJA, methyl jasmonate; MeSA, methyl salicylate; PAR, photosynthetically active radiation; PGRs, plant growth regulators; SA, salicylic acid.

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nutritional additives for the fortification of other foods (Katunzi-Kilewela et al., 2021). For all these reasons, chia seeds are considered "nutraceuticals" (Osorio et al., 2021) or "functional food". Nutraceuticals have a nutritional and a pharmacological component, and they are defined as food or part of food which contribute to a healthy physiological status in humans (Das et al., 2012). The demand for nutraceutical products has increased in our society the last years (Souza and Chaves, 2017) and plant-based food are a trend among this type of products (Sloan, 2014).

It is known that chia seeds contain carotenoids, but they do not have a huge amount (they contain *ca.* 0,50 μg/g) (da Silva et al., 2017). Carotenoids are the most widespread pigments in nature. They are present in plants, animals, fungi, algae, and photosynthetic bacteria (Maoka, 2020). In plants, carotenoids are the second most abundant pigment, they play an important role in photoprotection mechanisms (Cazzaniga et al., 2016) and they contribute to light absorption for photosynthesis (Cazzonelli, 2011). They are responsible for the yellow, orange, and red colouring of fruits and flowers, as well as for various aromas in plants (Domonkos et al., 2013). Carotenoids can be divided into two groups: carotenes and xanthophylls. Carotenes contain only a hydrocarbon chain without any functional group, while xanthophylls are more polar and have oxygen as a functional group (Saini et al., 2015). Neoxanthin, violaxanthin, anteraxanthin, lutein and zeaxanthin are xanthophylls, while β-carotene is a carotene, like other compounds such as lycopene. Carotenoids cannot be synthesized *de novo* by humans so they must be acquired from the diet (Fraser and Bramley, 2004). Carotenoids have antioxidant properties and contribute to the body's defence (Pechinskii and Kuregyan, 2014) as well as they help to prevent cardiovascular diseases (Maria et al., 2015) and different types of cancer (Rodriguez-Amaya et al., 2021). Lutein and zeaxanthin are essential for a good eye health in humans (Eggersdorfer and Wyss, 2018).

β-Carotene stands out among the other carotenoids in a dietary approach due to its role as a precursor of vitamin A (provitamin A). It means that vitamin A can be synthesized from β-carotene in the human body (Grune et al., 2010). It is known that β-carotene is essential for a good vitamin A intake. This usually occurs with the intake of a mixture of vitamin A itself and its precursor, with β-carotene representing a 35% of the dietary vitamin A supply (Weber and Grune, 2012). Vitamin A is a fat-soluble micronutrient (Huang et al., 2018) that has many functions in human health. It is crucial for maintaining good vision, promoting growth and development, protecting the integrity of epithelia and mucus (Huang et al., 2018) and is involved in cell differentiation and proliferation (Timoneda et al., 2018). Vitamin A is known as an anti-inflammatory vitamin, due to its role in promoting the immune function, participating in the development of the immune system and in the regulation of humoral and cellular immune responses (Huang et al., 2018). It has been shown to have a therapeutic effect in the treatment of various diseases (Huang et al., 2018), including Alzheimer's disease (Ono and Yamada, 2012). β-carotene itself also has a therapeutic effect in Alzheimer's disease (Ono and Yamada, 2012).

Chia seeds are very well known and consumed worldwide, but there is evidence that support that chia sprouts are even more interesting. Sprouts are plant-based foods that are rich in phytonutrients such as isoflavones, phenolic compounds, antioxidants, vitamins, and minerals, as well as having fewer "anti-nutrients" such as phytates, tannins and oxalates (Miyahira et al., 2021). Therefore, they have been in high demand in recent years, given their association with health benefits (Miyahira et al., 2021). Germination, a process needed to obtain sprouts from seeds, is usually a simple and quick process and it does not depend on climatic conditions in this plant species (Miyahira et al., 2021). The germination of chia increases its content of bioactive compounds, improving its antioxidant activity and its content of essential amino acids, fibre, phenolic compounds (Gómez-Favela et al., 2017) and minerals such as calcium (Calvo-Lerma et al., 2020). In general, chia sprouts show better nutritional values than seeds (Pająk et al., 2019), making them a very interesting food with a huge potential in the agri-food industry.

Previous research about the biofortification of chia sprouts had special emphasis on their antioxidant activity (Mlinarić et al., 2020; Gómez-Favela et al., 2017). The only precedent on carotenoids in chia sprouts shows that exposure to light for 48 h of germinated chia seeds increases the content of total carotenoids (Mlinarić et al., 2020), an expected result according to their role in plant photoprotection. Chia germination includes itself exposure to light if the desired final product is green sprouts. Therefore, green chia sprouts naturally contain more carotenoids than chia seeds. However, other bioactive compounds, such as phenolic compounds, are quantitatively more important in these sprouts (Mlinarić et al., 2020). Consequently, due to the importance of β-carotene in human health and the emerging interest in green chia sprouts as a food with huge nutritional potential, biofortification of this agri-food product is proposed to improve its provitamin A content.

To improve provitamin A content in chia sprouts, a non-transgenic approach is proposed here. The use of genetically modified organisms is not globally accepted so the first option for biofortifying food should be an alternative to these methods (Marques et al., 2021). Specifically, different plant growth regulators (PGRs) will be tested: abscisic acid (ABA), jasmonates applied as methyl jasmonate (MeJA) and salicylates applied as methyl salicylate (MeSA). They are all PGRs involved in stress tolerance in plants (Swamy and Smith, 1999; Yu et al., 2018; Wani et al., 2017), and we hypothesized they may be related to the antioxidant role of carotenoids. The effect of these PGRs on germination and produced biomass will be also studied in this work. The effect of PGRs will be separated in two parts: (i) when chia sprouts will have received a short light stimulus (activation of photoreceptors) for about 30' (this exposure will be done to study the effects of PGRs on the accumulation of pigments in the process of photomorphogenesis), and (ii) upon exposure to an additional prolonged light stimulus (30'+48h), so that green chia sprouts suitable for consuming are obtained, therefore representing the developmental stage when it will be interesting to do the biofortification.

2. Material and methods

2.1. Chia germination and growth

Chia (*Salvia hispanica* L.) seeds, which were bought in a local market of Barcelona (NE Spain) and stored in darkness at 22 °C and 46% of relative humidity for four months prior to the experiment, were sown on terracotta plates of 15 cm of diameter. The terracotta plates were put inside glass casseroles of 1.5 L of capacity with lids not hermetically closed. Inside each casserole there was around 680 ml of water, so that the plates were half dipped, and the porous material could absorb water. Furthermore, the plant material was moistened every 24 h by spraying it with distilled water to guarantee a homogeneous distribution of humidity and make possible a proper seed imbibition, germination and growth during the experiment.

At the start of the experiments, seeds were sown as described before and remained in the darkness for 4 days by covering all glass casseroles distributed along the lab bench. The day 5 the plant material received, all of it equally, a light stimulus of 30 min to induce photomorphogenesis by uncovering the casseroles and exposing them to light (direct light from fluorescent lamps plus indirect sunlight in the lab, PAR of 270 μmol m^{-2} s^{-1}). After this initial light stimulus, de-etiolated seedlings were exposed to a prolonged light stimulus for 48h (from day 6 to day 8 of experiment) to ensure a proper development of green sprouts. The PAR applied depended on the area previously established in the lab, but it always kept between 2 and 17 μmol m^{-2} s^{-1} during the day with a 14h:10h photoperiod (light/darkness). More specifically, the PAR ranged between 8 and 12 μmol m^{-2} s^{-1} in the morning, 9–17 μmol m^{-2} s^{-1} in the afternoon and 1–5 μmol m^{-2} s^{-1} in the evening. In the middle of this 48h light period, the replicates, which were previously randomly distributed on the lab bench, were moved to exchange their positions to

increase homogeneity. Temperature was kept between 20 °C and 22 °C and relative humidity between 48% and 55% during the experiment. PAR was measured with a sensor of PAR (Full-Spectro Quantum Sensors, Apogee Instruments, Logan, UT, USA), while temperature and relative humidity were measured with an HMP35AC thermohygrometer (Väisala, Finland). A summary of the experimental plan with a graphical representation of chia germination, from seeds to green sprouts is shown in Suppl. Fig. 1.

2.2. Treatments and samplings

The 27 casseroles were divided into nine treatments, each one with three replicates (plates). The treatments were water solutions containing various PGRs, as follows: (i) combination of cytokinins (CKs) and gibberellins (GAs) applied with the commercial product Promalin® (P). This product is composed by GA_4/GA_7 1,9% w/v + 6-benzyladenine 1,9% w/v; (ii) ABA, (iii) MeJA, (iv) MeSA, (v) P + ABA, (vi) P + MeJA, (vii) P + MeSA, (viii) P + ABA + MeJA + MeSA, and (ix) controls. The commercial product Promalin® was used to promote germination, as CKs and especially GAs, promote germination, while ABA has been widely studied as a germination inhibitor (Nambara et al., 2010; Linkies and Leubner-Metzger, 2012; Yang et al., 2018; Wang et al., 2011; Chen et al., 2008). The treatments were applied twice (days 2 and 5 of the experiment), following the same procedure as the distilled water application to moisten the seeds. On the days that the treatments were applied, no additional distilled water was applied to the plant material. A concentration of 100 μM of each PGR was applied (Suppl Table 1). In the case of Promalin®, the sum of the two GAs was 100 μM. Tween 80 was used as a surfactant to ensure the correct absorption of the PGRs, and methanol (MeOH) was used as solvent for the different PGRs. The surfactant concentration was 0.1% and the concentration of MeOH were different in each case depending on the need to dissolve the corresponding PGR (Suppl Table 1). Promalin®, as a commercial product, did not require any extra additives and was applied by diluting the commercial product 570 times. All solutions were made with distilled water. No solution was applied for controls.

Five samplings were performed at various developmental stages, including day 0 (seed before germination), day 2 (imbibed seed), day 3 (germinating seed, 24h after the first treatment application), day 6 (de-etiolated seedling, 30 min light exposure, 24h after the second treatment application), and day 8 (green sprout, 30 min + 48 h light exposure). It is understood that by day 8 of the experiment, the sprouts have reached an optimal stage of development for consumption. The plant material that was taken for each sampling was randomly chosen, except in cases where germination was affected (such as the ABA treatment), where representative individuals of the growth stage of each sampling were chosen. After putting samples into small aluminium foil envelopes, they were frozen with liquid nitrogen and stored at −80 °C. All samples were freeze-dried before biochemical analyses.

2.3. Analysis of β-carotene, xanthophylls and chlorophylls

β-carotene, neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll (Chl) a and Chl b were analysed by high performance liquid chromatography (HPLC). For this purpose, samples (each one representing at least 30 seedlings or sprouts) were extracted with 100% MeOH. They were subjected to ultrasounds for 30 min and centrifuged for 10 min at 10,000 rpm. The supernatant was recovered and transferred to another tube. Three rounds of extraction were performed for each sample. Afterwards, they were filtered with 1 mL single-use syringes with a pore size of 0.45 μm. Then, 300 μl of the extracts were transferred to vials with insert and analysed by reverse-phase HPLC, as described previously (Munné-Bosch and Alegre, 2000; Thayer and Björkman, 1990), using an Agilent 1100 Series HPLC system with a Zorbax ODS-5 μm column (4.6 mm × 250 mm). The mobile phases were acetonitrile:methanol (85:15, v/v) and methanol:ethyl acetate (68:32, v/v) and quantification was made by calculating the pmol/area ratios for each compound at 445 nm. The de-epoxidation state of the xanthophyll cycle (DPS) is a measure that functions as an indicator of how excess energy is dissipated and was calculated according to the following formula, DPS=(Z+0.5A)/(V + A + Z), where Z is zeaxanthin, A is anteraxanthin and V is violaxanthin.

2.4. Biomass and germination

The produced biomass in fresh weight was measured by weighing 20 randomly selected sprouts from each replicate on the last day of the experiment (day 8). The germination percentage was calculated on seeds germinated on day 3 of the experiment (considering all seeds from a plate, *ca.* 650 seeds, for each replicate). They were calculated using ImageJ 1.53c software.

2.5. Statistical analysis

The mean and standard error (SE) were calculated using Excel (2012 version). To detect significant differences among treatments, the statistical software STATGRAPHICS (version 1.18.13) was used. Analysis of variance (ANOVA) was performed applying Fisher's LSD test as a post-hoc. ANOVA was accepted only in the cases in which normality of residues could be accepted (Shapiro-Wilk test). In cases where there was not normality of residues, the data were transformed with logarithms with base 10. If normality was accepted, the ANOVA of the transformed data was accepted too, otherwise the non-parametric Kruskal-Wallis test was performed and the Bonferroni test was used as a post-hoc. All statistical analyses were performed with an $\alpha = 0.05$ (95% confidence). All graphs were made with Sigma-plot 10.0 (System Software, California, USA).

3. Results

3.1. Green sprouts development and β-carotene biofortification

Significant differences (P-value = 0.004) were observed among treatments in the levels of β-carotene in green chia sprouts grown for 8 days which were exposed to light for 30 min +48 h (Fig. 1). MeSA

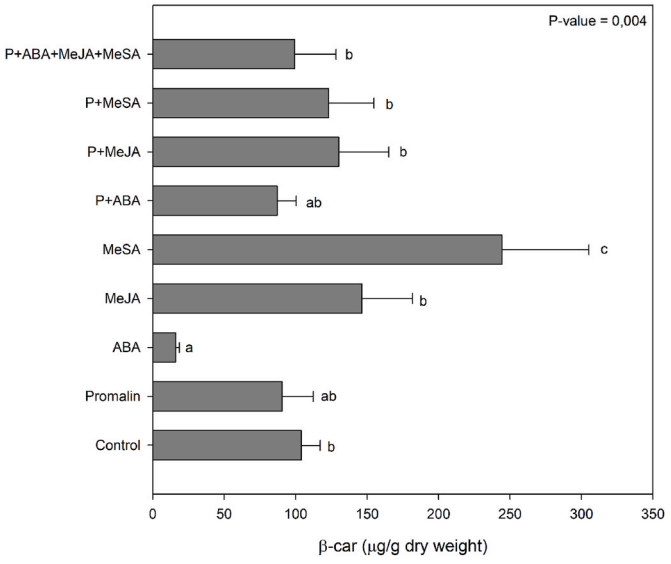


Fig. 1. β-Carotene contents (μg/g dry weight) in green chia sprouts grown for 8 days which were exposed to light for 30'+48h. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). β-Car = β-Carotene, P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate.

treatment promoted a significant increase (235%) of β-carotene compared to the control (244.54 ± 60.58 mg/g dry weight vs. 104.07 ± 13.22 mg/g dry weight). In contrast, ABA treatment caused a significant decrease of 84.7% in β-carotene content compared to the control. Differences in β-carotene contents in the MeSA did not result in an altered morphological development. Alterations in chia sprouts morphology were observed in Promalin®, ABA, P + ABA, P + MeJA, P + MeSA and P + ABA + MeJA + MeSA, where a clear reduction in sprout length was observed compared to controls (Suppl. Fig. 2). The root part seemed to be less developed in Promalin®, P + ABA, P + MeJA, P + MeSA and P +

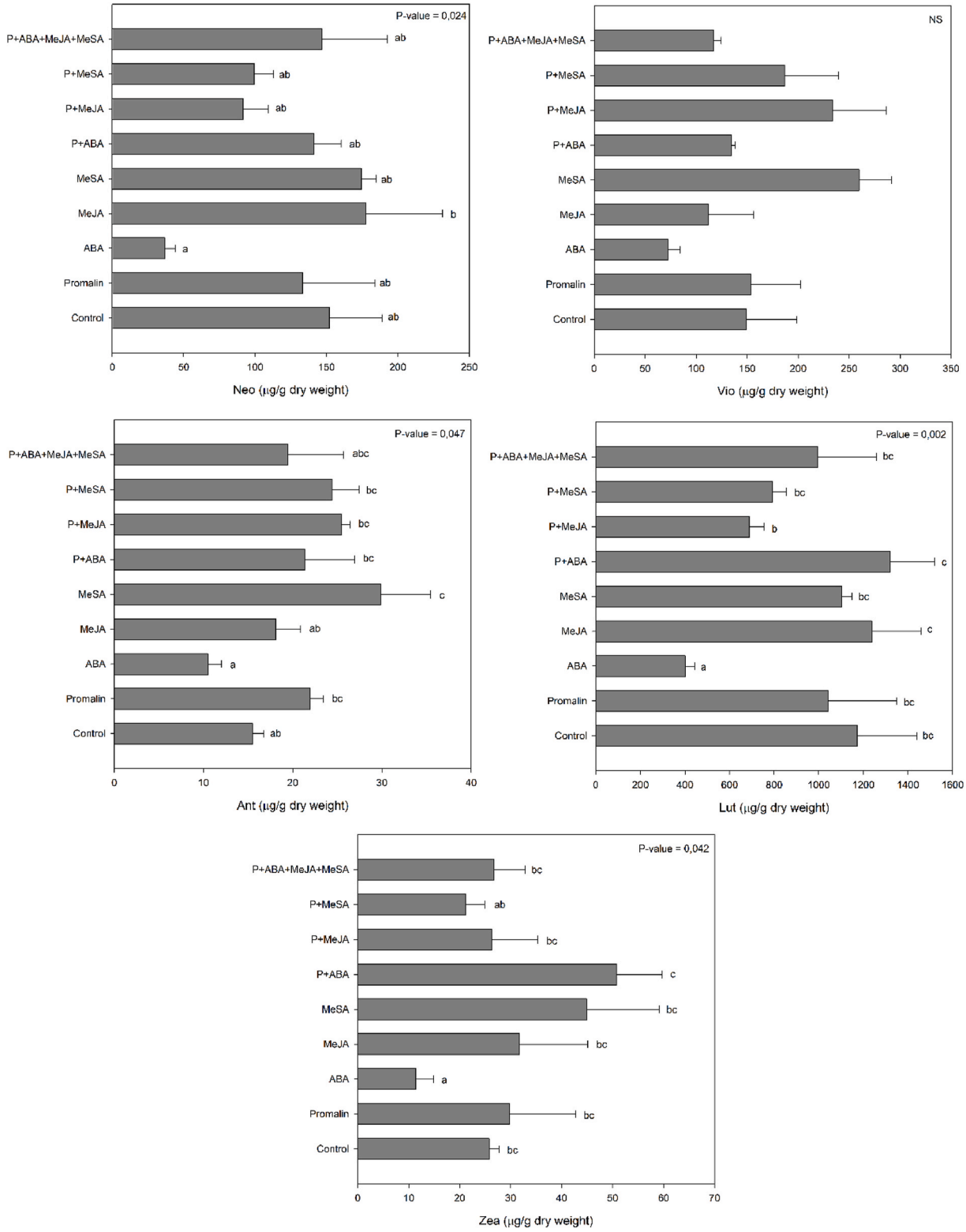


Fig. 2. Xanthophylls contents (µg/g dry weight) in green chia sprouts grown for 8 days which were exposed to light for 30'+48h. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). Neo = Neoxanthin, Vio = Violaxanthin, Ant = Anteraxanthin, Lut = Lutein, Zea = Zeaxanthin, P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate.

ABA + MeJA + MeSA.

3.2. Xanthophylls in green chia sprouts

Compared to the control, a significant increase in antheraxanthin was observed in the case of MeSA (29.88 ± 5.56 μg/g dry weight vs. 15.49 ± 1.32 μg/g dry weight). Neoxanthin content in green chia sprouts decreased in the ABA treatment compared to the controls (36.87 ± 7.45 μg/g dry weight vs. 152.22 ± 36.80 μg/g dry weight, respectively, P-value = 0.024, Fig. 2). ABA also caused a reduction in lutein and zeaxanthin contents compared to the control (Fig. 2). In the case of violaxanthin, no significant differences were observed among treatments (P-value > 0.05, Fig. 2).

3.3. Chlorophylls in green chia sprouts

Significant differences among treatments were observed in the Chl a content (Fig. 3). Compared to the control (1226.48 ± 169.46 μg/g dry weight), there is a significant reduction in the case of ABA (376.32 ± 35.17 μg/g dry weight) and P + MeJA (687.30 ± 45.91 μg/g dry weight). In this case, the effect of ABA was more intense than the effect produced by P + MeJA. Significant differences among treatments were also observed in Chl b (P-value = 0.005). ABA caused a significant reduction compared to the control (144.75 ± 9.57 μg/g dry weight vs. 474.23 ± 72.09 μg/g dry weight). In the Chl a/Chl b ratio, no significant differences were observed among treatments (P-value> 0.05, Fig. 3).

3.4. Germination and biomass

Treatments containing ABA reduced germination. Germination percentages of ABA (9.70 ± 5.74), P + ABA (25.13 ± 7.92) and P + ABA + MeJA + MeSA (35.53 ± 4.81) were much lower compared to the control (75.12 ± 4.63). No treatment resulted in a significant increase of germination (Fig. 4). Significant differences (P-value = 0.023) in fresh weight/sprout were also observed among the treatments. A significant decrease was observed in the treatments P + ABA (19.25 ± 3.17 mg) and P + ABA + MeJA + MeSA (19.45 ± 4.56 mg) compared to the control (30.54 ± 1.50 mg). No treatment caused a significant increase in the produced biomass (Fig. 4).

3.5. Carotenoids and chlorophylls during chia sprouts development

It was observed that carotenoids and chlorophylls in chia changed during its development (Fig. 5). The composition was analysed on day 0 (seed before germination), day 2 (imbibed seed), day 3 (germinating seed, 24 h after the first treatment application), day 6 (de-etiolated sprout, 30 min light exposure, 24 h after the second treatment application) and day 8 (green sprout, 30 min +48 h light exposure). Neoxanthin, violaxanthin and antheraxanthin were not detected on day 0. Both chlorophylls and carotenoids clearly increased on days 6 and 8 with light exposure. In sprouts, the most abundant chlorophyll was Chl a and the most abundant carotenoid was lutein. We observed that the levels of carotenoids and chlorophylls in seeds before germination were extremely low. When they started germinating in darkness, the levels increased a little, but were still very low. Later, light triggered pigment accumulation, even with a short exposure. And when light exposure

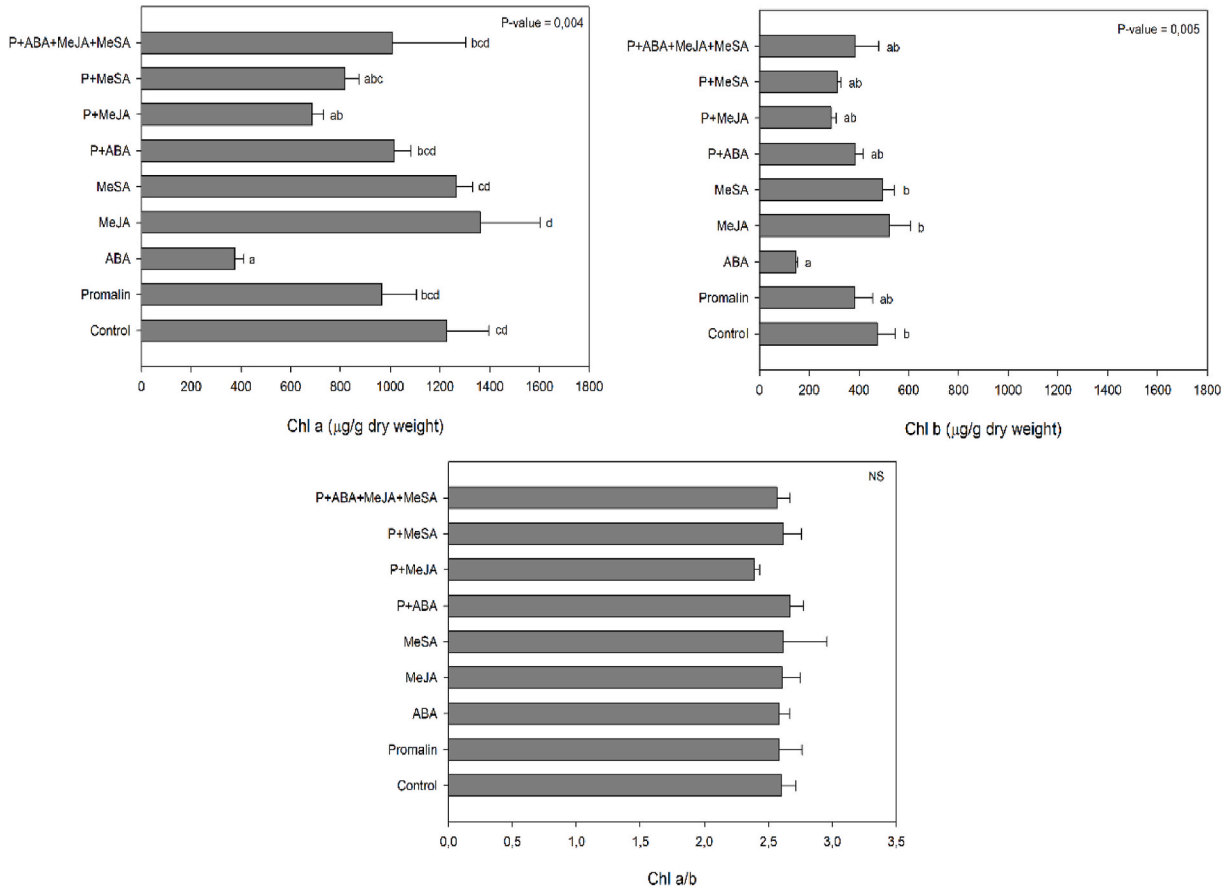


Fig. 3. Chlorophyll (Chl) a+b contents (μg/g dry weight) and Chl a/Chl b ratio in green chia sprouts grown for 8 days which were exposed to light for 30'+48h. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate.

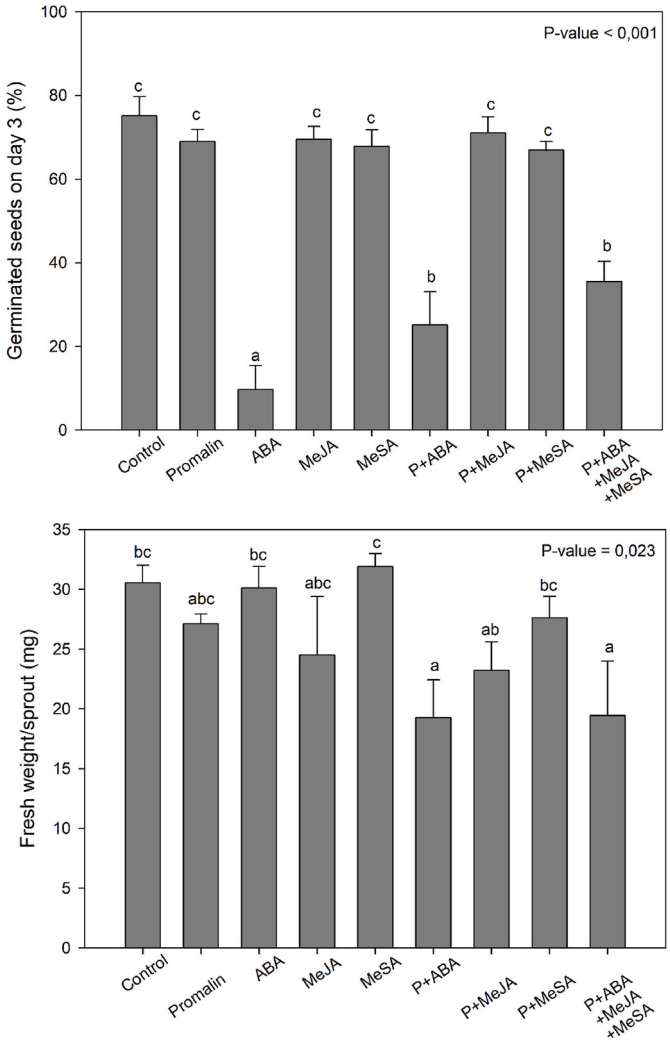


Fig. 4. Percentage of germination of chia seeds the day 3 of the experiment and biomass produced, given as fresh weight/sprout (mg), at day 8 of experiment. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate.

continued, pigment levels increased sharply (Fig. 5).

3.6. Interaction between PGRs and photomorphogenesis in xanthophylls and chlorophylls, but not β-carotene contents

β-carotene content was not affected by any of the treatments just after the first and short light stimulus of 30 min. No significant differences in β-carotene content were observed between treatments in de-etiolated chia seedlings with measurements performed at day 6 (P-value> 0.05, Suppl. Fig. 3).

In the case of neoxanthin, significant differences were observed among treatments (P-value = 0.001, Fig. 6). ABA (6.24 ± 1.14 μg/g dry weight) and P + ABA + MeJA + MeSA (16.16 ± 4.08 μg/g dry weight) treatments caused a reduction in neoxanthin compared to the control (36.07 ± 1.49 μg/g dry weight). Significant differences were also observed in violaxanthin among treatments (P-value = 0.023). MeSA (259.82 ± 31.93 μg/g dry weight) and P + MeJA (233.85 ± 52.58 μg/g dry weight) treatments promoted a significant increase compared to the control (107.52 ± 38.71 μg/g dry weight), unlike ABA (72.50 ± 11.74 μg/g dry weight), which again showed a significant reduction compared to the control. In the case of anteraxanthin, no significant differences were observed (P-value > 0.05). Moreover, significant differences (P-value = 0.010) were observed among treatments in lutein. ABA (123.60 ± 24.28 μg/g dry weight) showed a significant reduction compared to the control (391.08 ± 55.39 μg/g dry weight). For zeaxanthin and DPS levels, no significant differences were observed among treatments (P-value> 0.05) (Fig. 6).

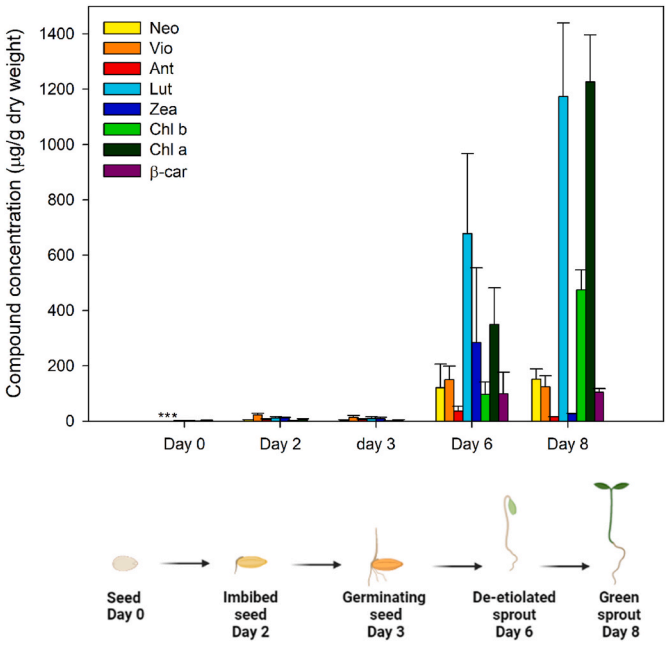


Fig. 5. Carotenoids and chlorophylls contents in chia seeds and sprouts (μg/g dry weight) during its development (control) and graphic representation (below) of the different development stages in the sampling days (created with biorender.com). The data is shown as mean ± SE of n = 3 replicates. Asterisks indicate non-detected compounds. Neo = Neoxanthin, Vio = Violaxanthin, Ant = Antheraxanthin, Lut = Lutein, Zea = Zeaxanthin, β-car = β-carotene.

Finally, significant differences were also observed between treatments in Chl a content (P-value = 0.049). ABA (64.40 ± 11.80 μg/g dry weight) and Promalin® (99.882 ± 24.42 μg/g dry weight) treatments showed a significant reduction compared to the control (226.86 ± 68.13 μg/g dry weight). No significant differences were observed (P-value> 0.05) in Chl b and Chl a/Chl b ratio (Fig. 7).

4. Discussion

The present study shows that β-carotene contents increase in response to MeSA, so that this PGR can be used as a means for the biofortification of chia seeds. MeSA can be readily converted to SA within the plant tissues thanks to the activity of esterases (Park et al., 2009). SA plays an important role in plant immunity as well as in establishing a normal root microbiome (Zhang and Li, 2019). Its role in plant defence against biotic stresses has been studied since the second half of the 20th century, when White discovered that aspirin (acetylsalicylic acid) induced resistance to tobacco mosaic virus (White, 1979). Although salicylates are well-known regulators of plant defence against pathogens, they also contribute to abiotic stress tolerance (Hara et al., 2012), such as low temperatures (Saleem et al., 2021), drought and salinity (Azad et al., 2021; Sabzmeydani et al., 2021). It is known that these stresses can alter the oxidative balance of the plant, producing oxidative stress (Saleem et al., 2021; Hussain et al., 2021), in which the plant produces too high levels of reactive oxygen species (ROS). These ROS levels cause negative effects on cell metabolism and division, membrane integrity, photosynthesis, and growth (Munns and Tester, 2008; Fatma et al., 2014; Nazar et al., 2015). SA has effects on the plant antioxidant system by enhancing the activity of enzymatic and non-enzymatic antioxidants (Saleem et al., 2021). We show here, to our

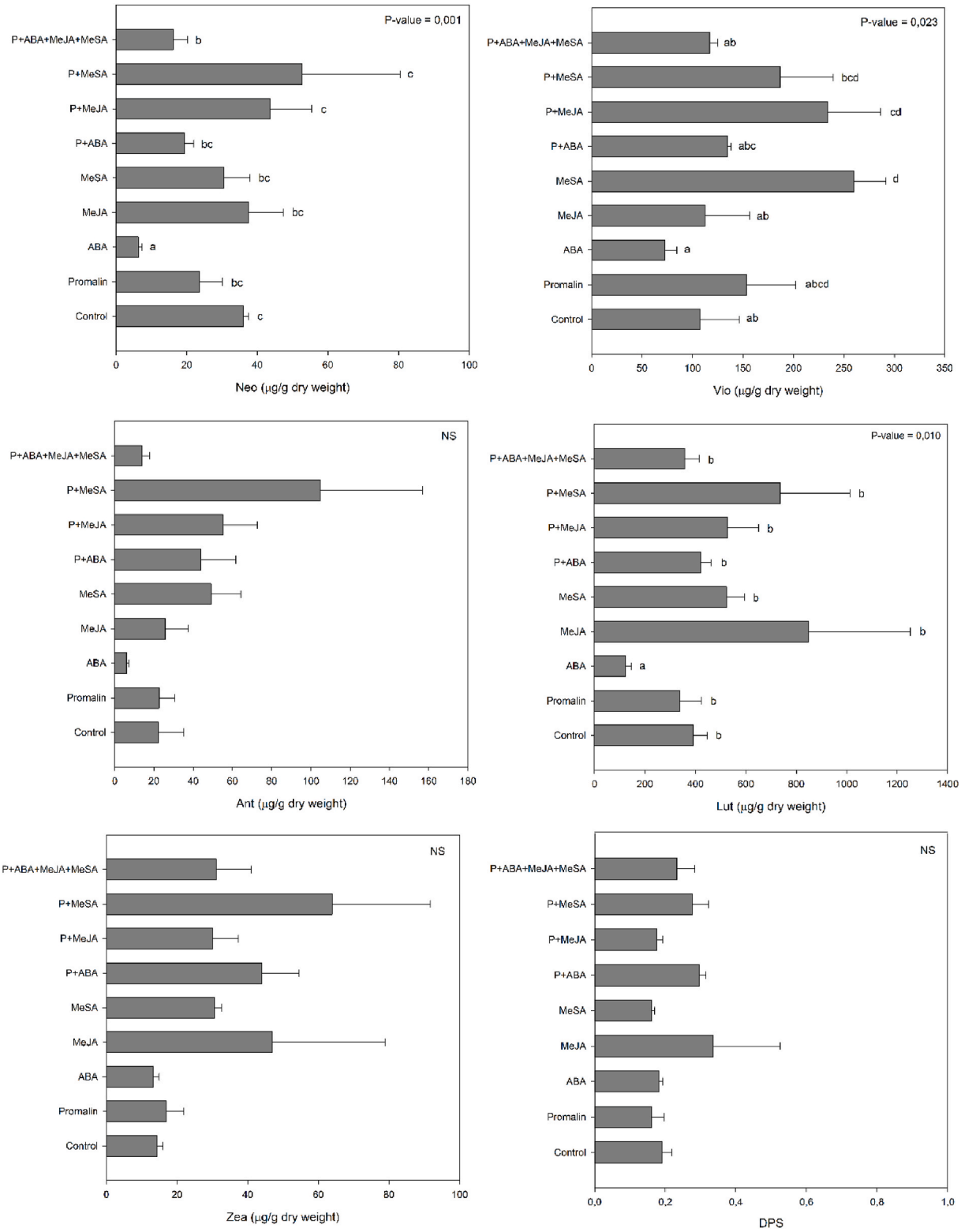


Fig. 6. Xanthophylls contents (µg/g dry weight) in de-etiolated chia sprouts grown for 6 days which were exposed to light for 30'. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate. Neo = Neoxanthin, Vio = Violaxanthin, Ant = Anteraxanthin, Lut = Lutein, Zea = Zeaxanthin, DPS = De-epoxidation state of the xanthophyll cycle, β-Car = β-Carotene.

knowledge for the first time, that MeSA can improve the contents of non-enzymatic antioxidants, and most particularly carotenoids, such as β-carotene, by more than 2.3 fold, and those of antheraxanthin by almost 2 fold, in green chia sprouts.

Carotenoids have very important functions in plants. They are involved in photosynthesis, signalling processes and photoprotection (Swapnil et al., 2021). They are non-enzymatic antioxidants that have very important functions both in plants and humans and can accumulate

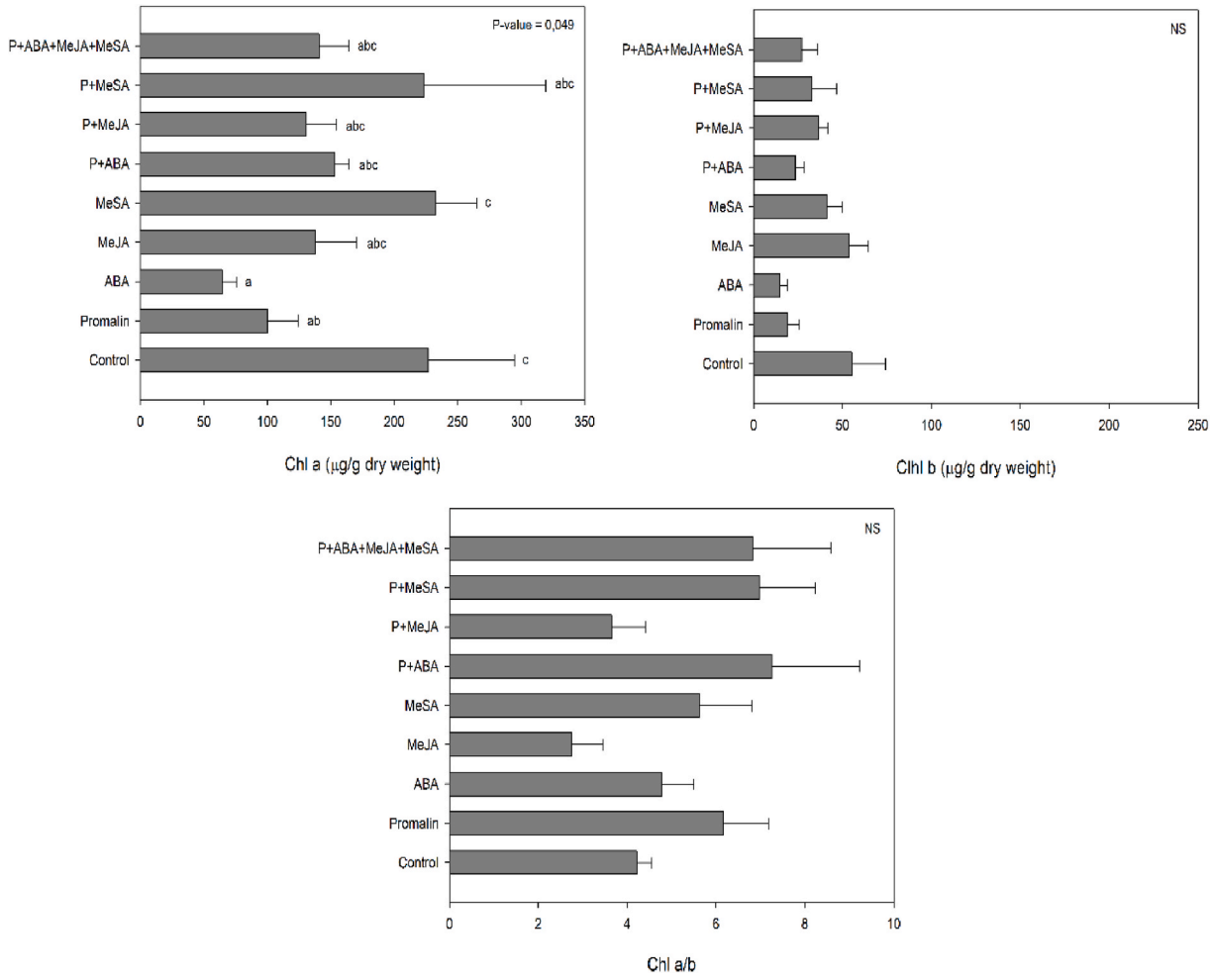
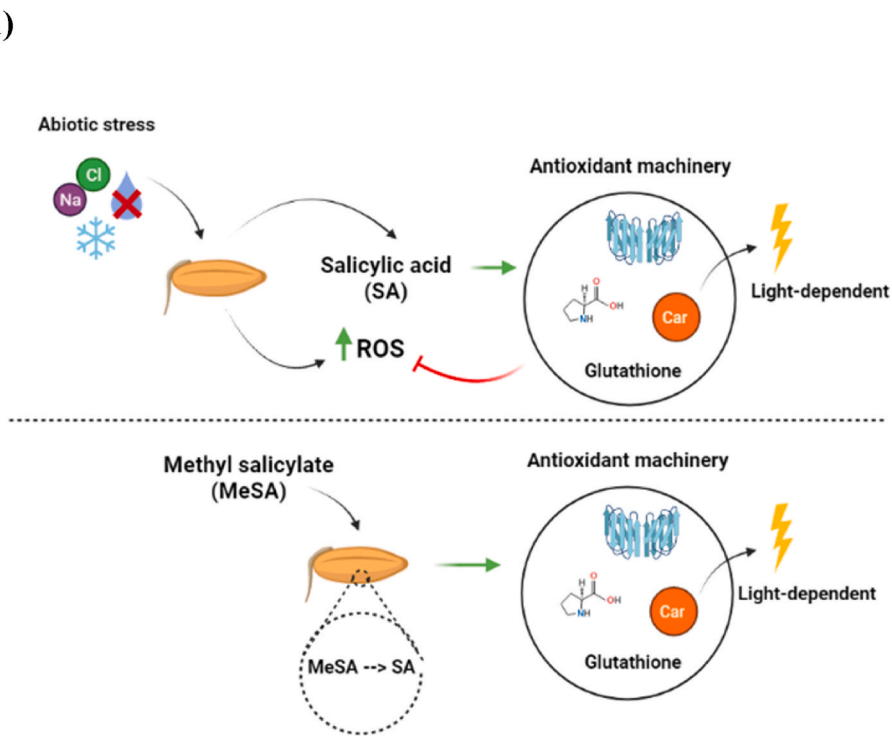


Fig. 7. Chlorophylls contents (µg/g dry weight) and Chl a/Chl b ratio in de-etiolated chia sprouts grown for 6 days which were exposed to light for 30'. The data is shown as mean ± SE of n = 3 replicates. Different letters show significant differences among treatments (P-value < 0,05). P = Promalin®, ABA = Abscisic acid, MeJA = Methyl jasmonate, MeSA = Methyl salicylate.

in plant tissues as a response to abiotic and biotic stress (Swapnil et al., 2021). In plants, they play a direct role in eliminating and reducing the damage caused by ROS under adverse conditions (Zhang et al., 2021). The relationship among abiotic stresses, exogenous SA application and the enhancement of plant antioxidant system, specifically carotenoid content, has been demonstrated to occur in other plant systems. In drought stresses, SA application has been shown to improve carotenoid levels, both when the plant is under stress, for example in wheat seeds (Singh et al., 2021) and *Mentha pulegium* (Azad et al., 2021), as well as under optimal growth conditions, in *Catharanthus roseus*, *Vicia faba* and *Impatiens walleriana* (Ababaf et al., 2021; Dawood et al., 2021; Safari et al., 2021). It has also been shown that SA application can increase the expression of the PmDXS gene, which is involved in resistance to salt and drought stress (Li et al., 2021). Overexpression of this gene in *Arabidopsis thaliana* increased carotenoids and chlorophylls contents (Li et al., 2021). Under salinity stress, an increase in total carotenoids has also been observed with SA application, in *Poa pratensis* (Sabzmeydani et al., 2021) and *Mentha pulegium* (Azad et al., 2021), and even in optimal growth conditions, again in *Vicia faba* (Dawood et al., 2021). The relationship between carotenoids increases and SA application has also been demonstrated in other studies without abiotic stress, such as in *Corylus avellana* (Khavari et al., 2021). In addition, a study demonstrated that the application of SA on wheatgrass seedlings increases its β-carotene content (Islam et al., 2021). Wheatgrass is considered a superfood so it is particularly noteworthy, as it occurs in our study with chia sprouts.

The increase in β-carotene in green chia sprouts was accompanied by an increase in antheraxanthin under prolonged (48 h) light stimulation, a carotenoid that also has a high antioxidant activity (Shimode et al., 2018). Under short (30 min) light stimulation, increases in violaxanthin, which is the precursor of antheraxanthin, was observed (Fu et al., 2011). It is known that MeSA can be metabolized to SA and vice versa in *Arabidopsis thaliana*, in one of several modifications that SA can go through (Nazar et al., 2015; Dempsey et al., 2011), thus, it appears that exogenous application of MeSA could have induced a photoprotective and antioxidant response associated with the role of this PGR against abiotic stress (Fig. 8A). This hypothesis is similar to that proposed by Azad and colleagues in their study (Azad et al., 2021). Thus, the antioxidant response induced by MeSA promoting an increase of β-carotene and antheraxanthin when the plant was exposed to enough light might be triggered as a defensive-like response to the plant. Interestingly, when the photosynthetic machinery was still not mature (light exposure for 30 min), the effect of MeSA on β-carotene was still not observed. This means that this was not a qualitative, photomorphogenesis-related effect, and that prolonged exposure to light was necessary. This is not a negative factor when using MeSA as a biofortifier of β-carotene in chia sprouts, as they are normally consumed once they are green and have received a prolonged light stimulus.

Also, we could observe that after 48h of light exposure the contents of chlorophylls and carotenoids increased in the control treatment, according with the results of Mlinarić and colleagues (Mlinarić et al., 2020). They improved the antioxidant properties of chia sprouts after exposing them to light for 48 h. Our results show that before receiving



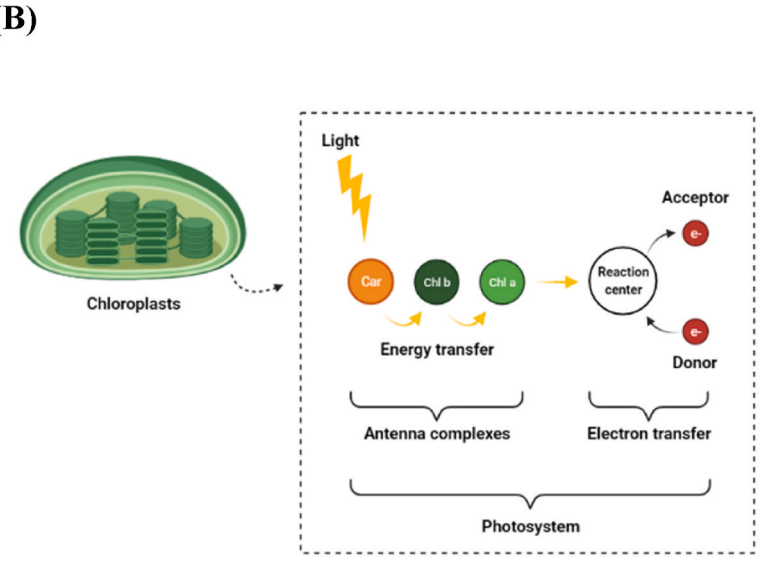


Fig. 8. **(A)** Graphical representation of the possible relationship between SA (and MeSA) and the increase of carotenoids. Above, what would happen under abiotic stress conditions, where SA would do its normal function and below, the case of this work, where MeSA is applied exogenously. The antioxidant machinery represented contains carotenoids (Car), enzymes (superoxide dismutase is represented), glutathione and proline (created in biorender.com). **(B)** Graphical representation of the photosynthetic function of carotenoids as part of the antenna complexes of photosystems. Car = Carotenoids, Chl a = Chlorophyll *a*, Chl b = Chlorophyll *b* (created in biorender.com).

light stimuli, the photosynthetic machinery of chia seedlings is not developed, so extremely low levels of chlorophylls and carotenoids during skotomorphogenesis (seedling development in darkness) are observed. Light exposure has the capacity to activate different physiological and developmental mechanisms, including the maturation of embryonic leaves, which are known as cotyledons (Arsovski et al., 2012). Thus, the short light stimulus for 30′ is enough to activate these mechanisms and make the seedling produce photosynthetic pigments, which are carotenoids and chlorophylls, causing the cotyledon to become greener. This occurs because light triggers the differentiation of photosynthetically active chloroplasts (Arsovski et al., 2012). When seedlings are exposed to light, genes encoding enzymes involved in carotenoid synthesis are strongly induced (Rodríguez-Villalón et al., 2009). This is logical considering the role of carotenoids in photosynthesis. They are part of antenna complexes (or light-harvesting complexes) that capture light in photosystems (Fig. 8B). In addition, cotyledons grow and expand, with the aim of increasing the surface area available to absorb light (Arsovski et al., 2012). This is more evident when sprouts have been exposed to light for a long period of time, where the green part is seen to be expanded. In this whole process of sprout de-etiolation, CKs are also involved and they induce the transcription of chloroplast-associated genes (Kraepiel and Miginiac, 1997). In summary, when seedlings are exposed to light, photomorphogenesis mechanisms are activated. Then, chloroplasts differentiate, and chlorophylls and carotenoids are produced and accumulated, with the aim of producing a mature photosynthetic machinery.

About the effects observed in xanthophylls in the sprouts that were exposed to a short light stimulus, it was found that ABA decreased neoxanthin and lutein compared to the control. In the case of sprouts exposed to a prolonged light stimulus, ABA decreased lutein and

zeaxanthin contents. It has been demonstrated that exogenous application of ABA has a negative effect on chia carotenoids when applied exogenously during germination. An increase in violaxanthin was also seen with the application of MeSA and P + MeJA and a decrease in neoxanthin when all PGRs were applied at the same time, under short exposure to light. These effects were related to signalling and their interaction with photoreceptors. Light is known to modulate the metabolism and signal transduction of PGRs through key transcription factors and/or other regulatory molecules (Luo and Shi, 2019). PGRs and light signalling pathways seem to be integrated at the level of photoreceptors (Luo and Shi, 2019). Endogenous ABA contents are modulated by light (Seo et al., 2009). In fact, red light decreases ABA levels through low fluence response (LFR) mediated by phytochrome B in *Arabidopsis thaliana* and lettuce leaves (Toyomasu et al., 1994; Seo et al., 2006; Oh et al., 2007). The expression level of genes responsible for ABA synthesis decreases under red light (Seo et al., 2009). This could be related to photomorphogenesis and the role of ABA in this process. Although not much is known about this, a gene involved in the response to ABA (the ABI3 gene, ABA insensitive 3) has been found to play a role in the growth of etiolated seedlings (Rohde et al., 2000). This gene encodes for a transcription factor involved in determining plastid differentiation as well as maintaining the repression of leaf development in darkness (Nemhauser and Chory, 2002). It seems that ABA could act as an antagonist of photomorphogenesis. The response to this PGR is necessary to maintain the etiolated state of the seedling, and therefore its exogenous application would negatively affect the production and accumulation of carotenoids. The same explanation could be applied in the case of chlorophylls, since they also decrease with ABA, both in short and prolonged exposure to light.

The effects on carotenoids of both P + MeJA and P + ABA + MeJA + MeSA treatments disappear under long exposure to light. Also, the effect is positive in one case (P + MeJA) and negative in the other case (P + ABA + MeJA + MeSA). What is known about the role of jasmonates in photomorphogenesis is that light promotes their accumulation to inhibit hypocotyl growth (Yi et al., 2020) and they also inhibit cotyledon expansion (Huang et al., 2017). This inhibition takes place at the same time as cotyledons development (Arsovski et al., 2012). On the other hand, GAs repress photomorphogenesis in darkness (Alabadí et al., 2004). In light, genes associated with GAs biosynthesis are inhibited so they act against the process (Arsovski et al., 2012; Nemhauser and Chory, 2002). In contrast, CKs promote photomorphogenesis (Nemhauser and Chory, 2002).

It was also found that MeSA did not affect chia germination percentage. ABA decreased seed germination. This is consistent with initial expectations given its role as a germination inhibitor (Nambara et al., 2010). It has also been observed in the results that GAs_{4+7} and 6-BA partially reversed the inhibitory effect of ABA on germination, according to what was expected before the experiment (Wang et al., 2011; Chen et al., 2008; Socolowski and Cicero, 2011). About the produced biomass in fresh weight, ABA did not reduce it, but when it is applied with GAs_{4+7} and 6-BA it reduced this parameter. Importantly, MeSA did not negatively affect the produced biomass.

In the observed effects on sprout morphology, GAs_{4+7} and 6-BA reduced the growth of the root and aerial part of the sprouts. This is a surprising result as these are regulators that are typically associated with growth enhancement (Small et al., 2019; Burke, 2011). Promalin® is normally used on fruit trees and has been found to give good results in these species (Markovic and Klett, 2021; Guo et al., 2008), although it does not always affect tree growth (Youn et al., 2000, 2001). In contrast, treatment of aubergine seeds with Promalin®, had a phytotoxic effect and negatively affected seedling germination and establishment (Neto et al., 2017). Therefore, it seems that its application on chia during seed germination, GAs_{4+7} and 6-BA, especially GAs as classical regulators of germination, do have a positive role in germination as antagonists of ABA, but they alter sprout morphology, although they do not modify its fresh weight. More research is needed to understand the mechanisms behind these results in chia. The effect on sprout morphology caused by ABA, which seemed to inhibit the aerial part growth, is consistent with what was observed in previous research on rice (Tsai et al., 1997).

In conclusion chia sprouts are a very interesting food for the agri-food industry, susceptible to greater future exploitation. It has been shown that exogenous application of MeSA (100 μM), as a PGR during the germination process, can biofortify the content of β-carotene (an important precursor of vitamin A) in green chia sprouts exposed for 30 min +48 h to light. Its application promotes a 2.35-fold increase of β-carotene in green chia sprouts. This application does not affect some essential parameters for chia sprouts production such as germination and produced biomass in fresh weight. Photomorphogenesis, a physiological process which affects carotenoids content, was not affected by MeSA application either. The increase in carotenoids content might be more related to an antioxidant response induced by MeSA which can be related to its role against stress, including molecular mechanisms that require further investigation.

CRediT authorship contribution statement

Núria F. Bermejo: designed the experiments, performed experiments, wrote the manuscript. **Ghita Hoummadi:** performed experiments. **Sergi Munné-Bosch:** designed the experiments, wrote the manuscript, supervised the work.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.10.026.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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