International Journal of Secondary Metabolite
2023, Vol. 10, No. 1, 106–118
https://doi.org/10.21448/ijsm.01079812
Published at https://dergipark.org.tr/en/pub/ijsm
Research Article

Interactive effects of salicylic acid and jasmonic acid on secondary metabolite production in *Echinacea purpurea*

Fatemeh Rasoli[1,*], **Manoochehr Gholipoor**[1]

[1]Department of Agronomy and Plant Breeding, Shahrood University of Technology, 3619995161, Shahrood, Iran

Abstract: Secondary metabolites are highly beneficial to human health and have commercial and industrial values. So, this research aimed to study the effects of exogenous salicylic acid (SA) and jasmonic acid (JA) on some secondary metabolites in purple coneflower. A field experiment as a randomized complete block design with three replications was conducted in Shahrood, Iran. Treatments were the factorial arrangement of 3 SA (0, 0.5, and 1 millimole) and 4 JA concentrations (0, 5, 20, and 50 micromole). The non-linear regression procedure was employed to quantify the relation of these materials with each other. The results indicated that the SA effect on all ten measured secondary metabolites changed with changing the JA levels as there was the interaction between these elicitors. On average, most (7 out of 11) of the combined SA_JA levels up-regulated the production of secondary metabolites as compared to the plants not sprayed with SA and JA. In terms of average response to elicitation with 11 combined SA_JA levels, they ranked from higher to lower as the guaiacol peroxidase, hydrogen proxide (H_2O_2), polyphenol oxidase, glutathione S-transferase, superoxide dismutase, NADPH oxidase, total phenolic content, phenylalanine ammonia-lyase, anthocyanin, and flavonoid. A few secondary metabolites appeared to have a biphasic relationship with each other. For instance, over lower and medium values of NADPH oxidase activity, anthocyanin content increased linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase activity, it showed a plateau state.

ARTICLE HISTORY
Received: Feb. 27, 2022
Accepted: Feb. 01, 2023

KEYWORDS
Antioxidants,
Bioactive compounds,
Purple coneflower,
Regulatory hormones.

1. INTRODUCTION

Purple coneflower (*Echinacea purpurea) is a native plant to North America and* the most widely cultivated medicinal plant (*Kaiser et al., 2015*). Its commercial products have diversified to include capsules, tablets, powders, tinctures, teas, and other beverages, as well as personal care products (Patel *et al*., 2008). Many benefits have been reported for this plant, including chemoprevention, treating the toothache, bowel pain, snake bite, skin disorders, seizure, chronic arthritis, cancer, upper respiratory tract infections such as colds and flu, and as an immune stimulant (Patel *et al*., 2008). The *bioactive compounds of purple coneflower increase the number of white blood cells and spleen cells and improve the ability of phagocytosis by granulocytes (*Kaiser *et al.*, 2015). *The promotion of the immune system through the consumption of purple coneflower* products *has been attributed to alkamides, glycoproteins,*

*CONTACT: Dr. Fatemeh Rasoli ✉ f.rasouli86@gmail.com Shahrood University of Technology, Faculty of Agriculture, Department of Agronomy and Plant Breeding, Shahrood, Iran

ISSN-e: 2148-6905 / **© IJSM 2023**

*polysaccharides, caffeic acid, and derivatives (*Kaiser *et al.*, 2015). The phenolic compounds, as one of the most important constituents of coneflower, contain very valuable organic materials.

The synthesis of above-mentioned bioactive compounds, known as secondary metabolites, requires expensive, difficult, and time-consuming procedures; in addition, the natural combinations of these compounds are more effective than artificially made ones. Therefore, different compounds including abiotic (e.g. titanium (IV) ascorbate), biotic (e.g. yeast extract), growth regulators (e.g. gibberellic acid), herbicides (e.g. glyphosate), and stress response molecules (e.g. methyl jasmonate; the volatile derivative of JA) have been employed to elicit the secondary metabolites in the different species of coneflower (Parsons *et al*., 2018).

SA and JA are known as elicitors and key signaling compounds in the induction process, leading to the accumulation of secondary metabolites. JA and SA respond to the biotic and abiotic stresses of the plants, and induce catalytic reactions by specific enzymes involved in the biosynthesis of phenolic compounds (Mendoza *et al*., 2018). SA regulates the phenylalanine ammonia-lyase (PAL) enzyme activity, which is a biosynthetic enzyme that catalyzes biosynthetic reactions related to the formation of defensive compounds such as superoxide dismutase and peroxidase (Torun *et al*., 2020).

Generally, JA tends to be involved in physiological and molecular responses. Physiological responses include activation of the antioxidant system superoxide anion radical, peroxidase, NADPH-oxidase, called antioxidant system (Karpets *et al*., 2014), accumulation of isoleucine, methionine, and soluble sugars (Wasternack, 2014), and regulation of stomatal closing and opening. JA has been shown to interact with plant hormones like ABA, GA, and IAA (Acharya & Assmann, 2009). Under the freezing and salt stress conditions, ABA and SA together positively regulate stress tolerance response (Horváth *et al*., 2015). Exogenous SA pretreatment significantly induced freezing tolerance of wheat via enhancing biosynthesis of ABA (Wang *et al*., 2018). In salt-stressed tomato plants, SA treatment has induced ABA biosynthesis and partially recovered lowered photosynthetic activity (Horváth *et al*., 2015). Pretreatment of barley plants with SA has led to a reduction in water deficit-resulted damage (Bandurska & Stroiński, 2005). SA and methyl jasmonate foliar application has increased the phenolic compounds in plant cell suspension cultures of *Thevetia peruviana* (Mendoza *et al*., 2018). Treated *Salvia miltiorrhiza* with SA in cell culture has had more PAL activity and contained more phenolic compounds (Dong *et al*., 2010). In an experiment on callus culture of *T. peruviana,* elicitation with the combination of 100 millimole of JA and 10 millimole of abscisic acid has resulted in an increased production of phenolic compounds (Rincón-Pérez *et al*., 2016). Methyl jasmonate and SA application have had a significant effect on antioxidant enzymes activity in many plants including *Arachis hypogaea* (Kumari *et al*., 2006).

Most of the studies regarding secondary metabolite elicitation of purple coneflower have been carried out within *in vitro* culture systems. Additional studies on the application of elicitors to field-grown purple coneflower would also be useful, since effects may differ considerably under the diversity of field conditions (Parsons *et al*., 2018). Recently, Mohebby *et al*. (2021) assessed the effect of SA and methyl jasmonate on three antioxidant enzymes (catalase, peroxidase, and superoxide dismutase), chicoric acid, and chlorogenic acid content of field-grown purple coneflower. The present field experiment aimed to study the effect of SA and JA on some other secondary metabolites of purple coneflower.

2. MATERIAL and METHODS

This field experiment was conducted at the Research Farm of Shahrood University of Technology, Shahrood (36° 25' N, 55° 01' E, and 1345 m asl), Iran in 2017. The mean annual value of temperature, precipitation, and relative humidity was 14 °C, 180 mm, and 48%,

respectively. Soil texture (0-30 cm depth) was loam-silt with 0.76% organic carbon, 15.54 ppm available phosphorous, 250 ppm available potassium, 0.06% total nitrogen, pH 7.2, and EC 1.5 dSm^{-1}.

The seeds of *Echinacea purpurea* were obtained from Pakan Seeds Company, Isfahan, Iran, and were sown in the nursery on 21 March. The transplants were planted on 5 June with a 30 cm separation between plants and 60 cm separation between rows. Each plot area was 9 m^2 (3 m × 3 m) with five rows. The experiment followed a randomized complete block design with three replications per treatment. Treatments were the factorial arrangement of 4 JA (0, 5, 20, and 50 micromole), and 3 SA concentrations (0, 0.5, and 1 millimole). At the onset of reproductive growth stage, JA and SA were sprayed on plants at a 10-day interval. The foliar application was repeated two more times at the mentioned interval. One week after the last spray, plant samples (the upper fully developed leaf) were taken to measure the following secondary metabolites.

H_2O_2 assay: The concentration of this material was determined following the Nelson method as described by Snell & Snell (1971). The reaction mixture consisted of 2.5 milliliter of 0.1% titanyl sulfate in 20% sulfuric acid (v/v), and 0.75 milliliter enzyme extract. The mixture was centrifuged at 5,000 g for 15 min at 25 ºC. The absorbance readings were taken at 410 nm to quantify the intensity of the yellow color that developed in the reaction. The extinction coefficient (ε_{410}) was 0.28 micromole $^{-1}$ cm^{-1}.

Superoxide dismutase (SOD) assay: Beauchamp and Fridovich (1971) method was adopted for SOD assay. In this method, riboflavin and methionine are used to produce superoxide in the presence of light. Superoxide causes the reduction of nitro blue tetrazolium and the formation of purple formazan. The reaction mixture was incubated under fluorescent light for 10 min and the absorbance was measured against a blank at 560 nm.

Guaiacol peroxidase (GPX) assay: The activity of GPX was measured by the method of Chance & Maehly (1955). A reaction mixture of 3 milliliter contained 0.1 mole phosphate buffer (pH 6.8), 30 millimole guaiacol, 30 millimole hydrogen peroxide, and 0.3 milliliter enzyme extract. Enzyme activity began by adding hydrogen peroxide to the reaction mixture. The increase in the absorbance of the reaction solution at 470 nm was recorded for one min.

NADPH oxidase assay: NADPH oxidase activity was determined according to Van-Gestelen *et al*. (1997) method. Briefly, nitro blue tetrazolium was converted to monoformazan by two $O_2^{\bullet-}$ molecules. This reduction was ascertained for one min at 530 nm. The reaction mixture (1 milliliter) contained 50 millimole Tris-HCl buffer (pH 7.8), 1 millimole $CaCl_2$, 0.1 millimole nitro blue tetrazolium, and 0.1 millimole NADPH. NADPH oxidase activity was expressed as micromole of nitro blue tetrazolium converted per minute per gram fresh weight of tissue.

Glutathione S-transferase (GST) assay: GST activity was determined as described by Gronwald and Plaisance (1998) which is the modified procedure of Habig *et al*. (1974, cited from Gronwald and hecked, 1998). The reaction medium contained 0.1 mole potassium phosphate (pH 6.5), 1 millimole glutathione, 1 millimole 1-chloro, 2,4-dinitrobenzene (CDNB), 1% absolute ethanol, and protein in a total volume of 1 milliliter. One unit of GST activity is defined as the formation of 1 micromole product min^{-1} at the temperature 25 °C.

Polyphenol oxidase (PPO) assay: PPO activity was determined by the method of Kar & Mishra (1976). The assay mixture (3 milliliter) contained 25 millimole phosphate buffer (pH 6.6), enzymatic extract (100 microliter), 0.1 millimole pyrogallol. The absorbance of the mixture was recorded at 420 nm. Enzyme activity began by adding pyrogallol to the reaction mixture.

Phenylalanine ammonia-lyase (PAL) assay: PAL activity was determined according to Wang *et al*. (2006), which is based on the cinnamic acid (CA) production rate. One milliliter of the

extraction buffer, 0.5 milliliter of 10 millimole L-phenylalanine, 0.4 milliliter of double-distilled water, and 0.1 milliliter of enzyme extract were incubated at 37 ^{o}C (a temperature at which the peak activity of PAL takes place) for 1 hour. The reaction was terminated by adding 0.5 milliliter of 6 mole HCl; then the product was extracted with 15 milliliter ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 milliliter of 0.05 mole sodium hydroxide, and the CA concentration was quantified based on the absorbance at 280 nm.

Total phenolic content (TPC) assay*:* TPC was determined using Folin–Ciocalteu's reagent (Singleton & Rossi, 1965). Briefly, 0.5 milliliter of the diluted extract was reacted with 2.5 milliliter of 0.2 mol per liter Folin Cicoalteu reagent. After 4 min, 2 milliliter of saturated sodium carbonate (about 75 g L^{-1}) was added and then, the solution was incubated for 120 min at room temperature. The absorbance readings of the resulting blue-colored solution were taken at 760 nm. A standard curve, prepared with 100, 200, 300, 400, and 500 mg L^{-1} of gallic acid, was adopted as a reference standard and TPC was expressed as gallic acid equivalent (mg GAE)g^{-1} fresh weight of the sample.

Anthocyanin assay: Anthocyanin content was determined as described by Mita *et al*. (1997). Fresh leaf sample (20 mg) was homogenized with 3 milliliter of 1% (v/v) hydrochloric acid in methanol and then the extraction was kept under dark conditions at 4 °C for 24 h. The mixture was centrifuged at 10,000 g for 15 min and then the absorbance of the supernatant was recorded at 530 and 657 nm. One unit of anthocyanin equals one unit of absorbance [A530 - (0.25 × A657)] per milliliter of extraction solution.

Flavonoid assay: The aluminum chloride colorimetric method was applied to estimate the flavonoid content as described by Chang *et al*. (2002). Leaf sample extract (0.5 milliliter of 1:10 g $milliliter^{-1}$) in methanol was mixed with 1.5 milliliter of methanol, 0.1 milliliter of 10% aluminum chloride, 0.1 milliliter of 1 mole potassium acetate, and 2.8 milliliter of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was recorded at 415 nm. Quercetin solutions (12.5 to 100 g $milliliter^{-1}$ in methanol) were utilized to make the calibration curve.

Statistical analysis: The statistical analysis, including analysis of variance and mean comparison based on the least significant difference (LSD) method, was performed, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The following equations were applied to express the biphasic relationship between secondary metabolites, using NLIN procedure of SAS software:

$$\begin{aligned} Y &= a + b \times X && \text{If } X < X_0 \\ Y &= a + b \times X_0 && \text{If } X \geq X_0 \end{aligned} \quad (1)$$

$$\begin{aligned} Y &= a + b \times X_0 && \text{If } X \leq X_0 \\ Y &= a + b \times X && \text{If } X > X_0 \end{aligned} \quad (2)$$

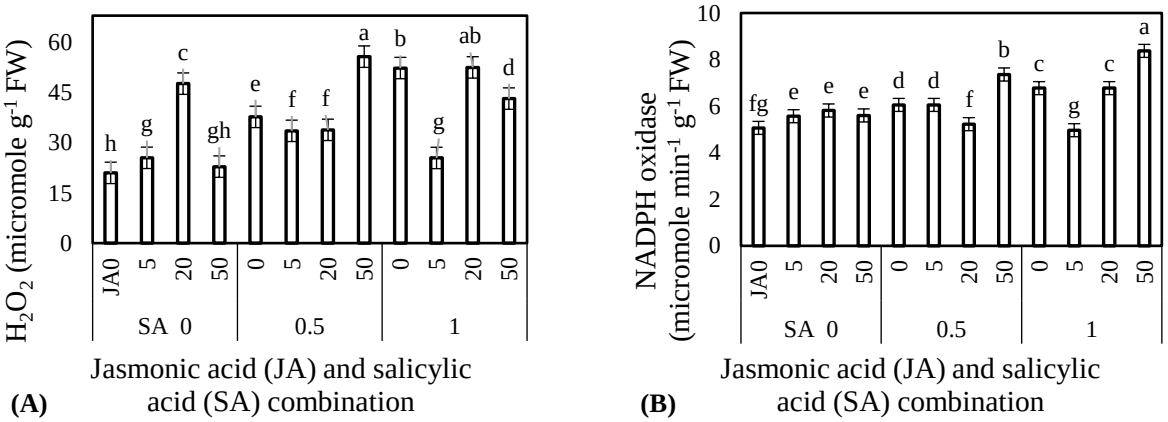
Where Y and X are secondary metabolites, a intercept, b slope, and X_0 the value of X at which the relation of Y with X shows a remarkable change.

3. RESULTS

The results of analysis of variance indicated that both simple and interactive effects of SA and JA were significant (p <0.01) on all measured secondary metabolites (data not presented). The mean values in Figure 1, pertaining to the H_2O_2 content of purple coneflower in response to varying levels of SA and JA, revealed considerable differences with each other. Under no SA (SA0) and 1 millimole SA application conditions (SA1), the higher H_2O_2 content was obtained with application of 20 millimole JA as compared to the other JA levels; while under 0.5

millimole SA spraying conditions, the higher H_2O_2 content was found upon elicitation with 50 millimole JA.

Figure 1. Interactive effects of JA (micromole) and SA (millimole) on H_2O_2 content (A), and NADPH oxidase activity (B) in purple coneflower.



In terms of NADPH oxidase activity in plants that were not sprayed with SA (SA0), treatments 5, 20, and 50 micromole JA behaved equally with non-significant differences among each other; these treatments were statistically superior to control (JA0) (Figure 1). The highest and second highest NADPH oxidase activity was found upon elicitation with 1 millimole SA and 50 micromole JA, and with 0.5 millimole SA and 50 micromole JA, respectively. As it is shown in Figure 2, the biphasic relationship was obtained between H_2O_2 content and NADPH oxidase activity; with increasing NADPH oxidase activity from 4.8 to 6.338 micromole min^{-1} g^{-1} leaf fresh weight, the H_2O_2 content was surged with a sharp slope (Table 1); with more increase in NADPH oxidase activity, it showed a plateau state.

Figure 2. Biphasic relation of H_2O_2 content with NADPH oxidase activity (A), and of anthocyanin content with NADPH oxidase activity (B).

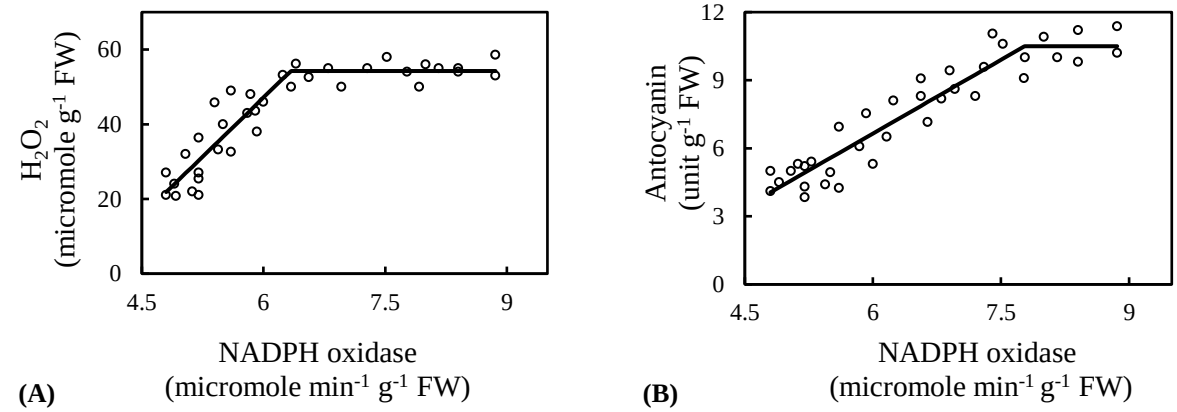


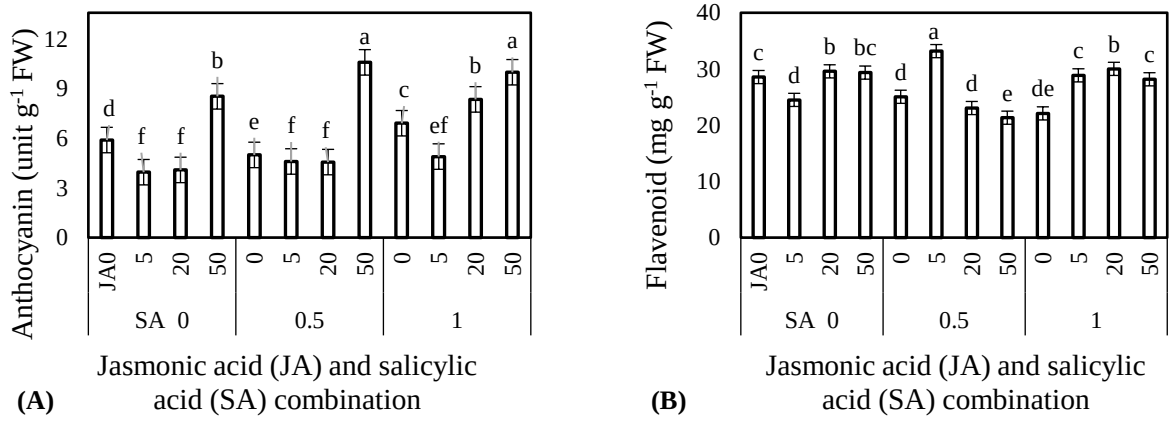
Table 1. The parameters ± standard error of non-linear-regression-based relationship between some secondary metabolites.

[a] Secondary metabolite	Intercept (a)	Slope (b)	X_0
H_2O_2 - NADPH oxidase	-79.224 ± 13.68	21.059 ± 2.52	6.338 ± 0.14
Anthocyanin - NADPH oxidase	-6.332 ± 1.07	2.164 ± 0.17	7.778 ± 0.22
Anthocyanin - GST	15.059 ± 1.21	-0.587 ± 0.09	17.924 ± 0.82
GPX - NADPH oxidase	-27.567 ± 5.12	7.117± 0.89	6.840 ± 0.18

[a] GST: Glutathione S-transferase; GPX: guaiacol peroxidase.

Regarding anthocyanin content, 50 millimole JA was superior to the other three JA levels; this was true for plants sprayed with all levels of SA (Figure 3). Surprisingly, for plants treated with all SA levels, application of 5 micromole JA decreased anthocyanin content to a level lower than control. As it is shown in Figure 2, over lower and medium values of NADPH oxidase activity, anthocyanin content increased linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase, it remained constant. The effect of JA on flavonoid content was considerably changed with changing SA levels (Figure 3). The highest and lowest flavonoid content was obtained for plants treated with 0.5 millimole SA and 5 micromole JA, and with 0.5 millimole SA and 50 micromole JA, respectively.

Figure 3. Interactive effects of JA (micromole) and SA (millimole) on anthocyanin (A), and flavonoid contents (B) in purple coneflower.



The treatment combination of 1 millimole SA and 5 micromole JA, and of 1 millimole SA and 20 micromole JA behaved equally with no significant difference between each other, and the highest SOD activity of 34.5 and 35.33 micromole g^{-1} FW were recorded in these treatment combinations, respectively (Figure 4). Averaged over JA levels, the plants elicited with 1 millimole SA consisted of lower amounts of GST activity than those elicited with no SA and 0.5 millimole SA (Figure 4). Under no SA application conditions, the value of GST activity increased linearly with increasing GA levels from 0 to 20 micromole. The treatment combination of no SA application and 20 micromole JA application got the highest position with 28.07 micromole g^{-1} FW GST activity. Over lower values of GST activity, there was an inverse relation between anthocyanin content and GST activity; over higher values of GST activity, anthocyanin content remained constant (Figure 5).

Figure 4. Interactive effects of JA (micromole) and SA (millimole) on SOD activity (A), and GST activity (B) in purple coneflower.

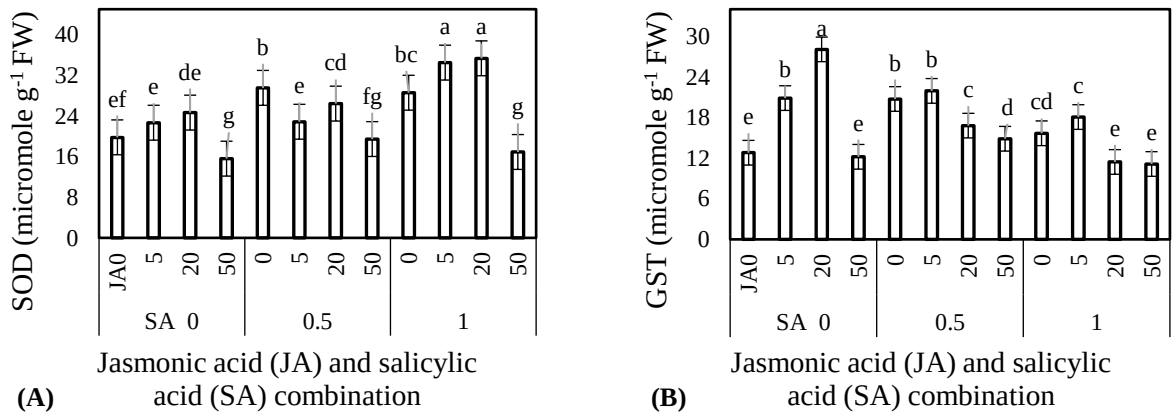


Figure 5. Biphasic relation of anthocyanin content with GST activity (A) and of GPX activity with NADPH oxidase activity (B).

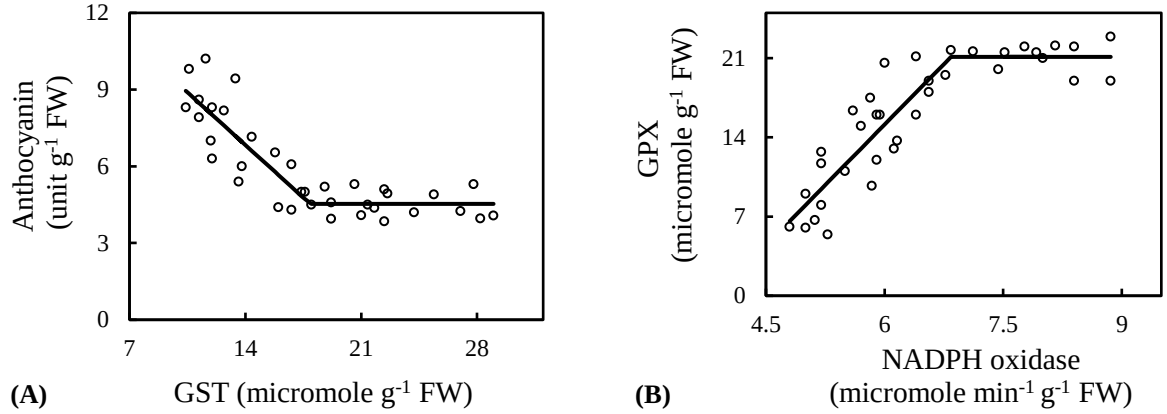
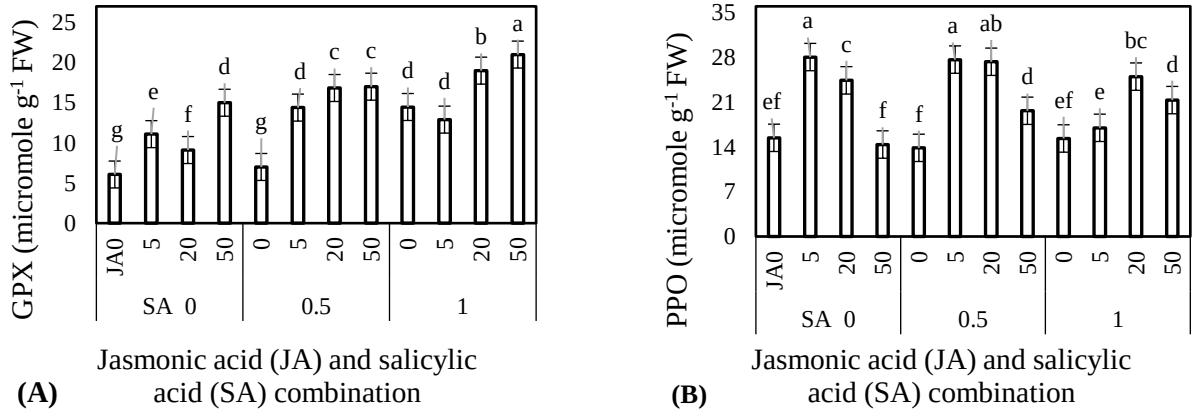
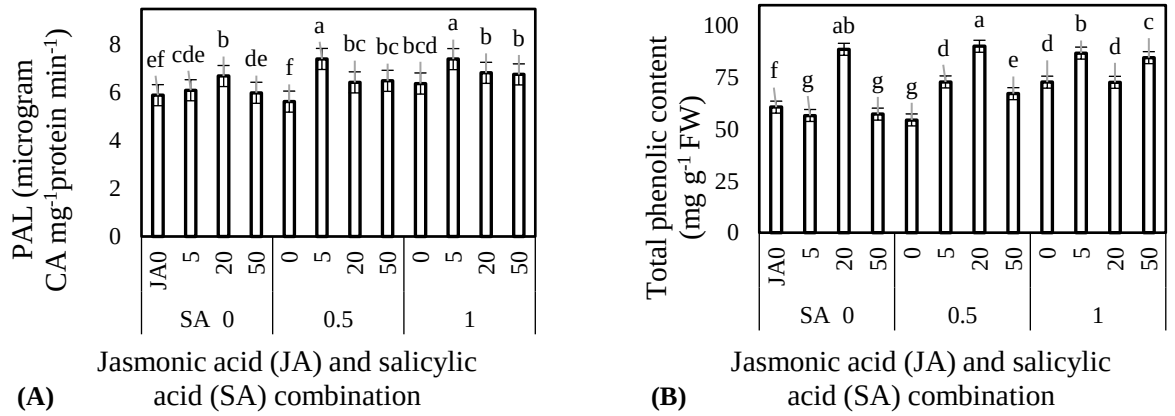


Figure 6. Interactive effects of JA (micromole) and SA (millimole) on GPX activity (A), and PPO activity (B) in purple coneflower.



All combined levels of SA_JA, except 0.5 millimole SA and no JA application, positively influenced GPX activity as compared to control (Figure 6). The highest and second highest GPX activity was obtained upon elicitation with 1 millimole SA and 50 micromole JA, and with 1 millimole SA and 20 micromole JA, respectively. Over lower values of NADPH oxidase activity, a positive relationship was found between GPX and NADPH oxidase activities; over higher values of NADPH oxidase activity, GPX activity showed a plateau state (Figure 5). In the context of PPO activity, there was no significant difference between the treatment combination of 0 millimole SA_5 micromole JA, 0.5 millimole SA_5 micromole JA, and 0.5 millimole SA_20 micromole JA; these three treatment combinations attained the greatest PPO activity (Figure 6).

Figure 7. Interactive effects of JA (micromole) and SA (millimole) on phenylalanine ammonia-lyase (PAL; CA: cinnamic acid) activity (A), and total phenolic content (B) of purple coneflower.



In terms of PAL activity, 5 micromole JA application attained superiority when combined with 0.5 and 1 millimole SA (Figure 7). The lowest PAL activity (5.63 microgram CA mg^{-1} protein min^{-1}) was obtained upon elicitation with no JA and 05 millimole SA. Under no SA application conditions, only one JA level (20 micromole) up-regulated TPC as compared to control (Figure 7); while under 0.5 and 1 millimole SA application conditions, more JA levels promoted TPC to a level higher than control.

4. DISCUSSION and CONCLUSION

The results indicated that elicitation with SA and JA induced oxidative stress as H_2O_2 content was soared (Figure 1). This induction was highly intensive (2.6-fold enhance as compared to control) upon elicitation with 0.5 millimole SA and 50 micromole JA. The change in reactive oxygen species (ROS) generation, including H_2O_2, is attributable to NADPH oxidase involvement, as Rouet *et al*. (2006) reported that the oxidative response in tobacco, induced by hypo-osmolarity, is originated from NADPH oxidase activity. In our experiment, although NADPH oxidase activity was intensified by most of SA and JA levels (Figure 1), part of the H_2O_2 may not have been originated from NADPH oxidase activity since with increasing NADPH oxidase activity from 6.338 to 8.86 micromole min^{-1} g^{-1} FW, H_2O_2 content remained constant (Figure 2). The cell wall-bound peroxidase (Bolwell *et al*., 2002), oxalate oxidase (Hu *et al*., 2003), amine oxidase (Angelini & Federico, 1989), and quinone reductase (Schopfer *et al*., 2008) might be involved in H_2O_2 generation.

Anthocyanin has nutritional value and a healing effect on cardiovascular and neurodegenerative diseases in humans (Mattioli *et al*., 2020). Its content tended to be increased considerably in response to the high level of (50 micromole) JA application (Figure 3). Five out of 11 combined levels of SA_JA promoted anthocyanin accumulation as compared to control. The accumulation of anthocyanin is triggered by biotic and abiotic stresses; ROS scavenging is one of the anthocyanin's important roles (Agati *et al*., 2020). In many other plants, including radish (Sakamoto & Suzuki, 2019), the elicitors like methyl jasmonate have also caused an increase in the accumulation of anthocyanin. It seems that anthocyanin content is not up-regulated by higher values of NADPH oxidase activity; since, as it was shown in Figure 2, over lower and medium values of NADPH oxidase activity, anthocyanin content enhanced linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase activity, it showed a plateau state.

Four out of 11 combined levels of SA_JA enhanced the flavonoids content to a level higher than control (Figure 3). The maximum increase (16%) was obtained upon elicitation with 0.5 millimole SA and 5 micromole JA. A review report by Agati *et al*. (2020) supports a remarkable

antioxidant function served by flavonoids in plants exposed to a wide range of stressors-resulted oxidative stress. Eight combinations of SA_JA levels intensified GST activity (Figure 4). These results are in tune with other reports regarding the up-regulation of GST activity by SA and even exogenous H_2O_2 application in other plants (Gong *et al*., 2005). Over lower values of GST activity, there was an inverse relation between anthocyanin content and GST activity; over higher values of GST, no relation was found between them (Figure 5). Such a relationship between these two attributes in the leaf might be due to GST-mediated transportation of anthocyanin from the leaf and accumulation in other organs like stem and/or root. GST involves diverse aspects of plant physiology, including signal transduction, regulatory functions, and transport of anthocyanin from the cytosol to the vacuole for storage (Estévez & Hernández, 2020).

GPX, a heme-containing enzyme, can effectively quench the reactive intermediary forms of O_2 and peroxy radicals under stressed conditions (Sharma *et al*., 2012). Ten combinations of SA_JA levels, especially 1 millimole SA and 50 micromole JA (more than 200% increase as compared to control), resulted in the promotion of GPX activity (Figure 6). The intensification of GPX activity upon exogenous application of SA has also been evidenced in the cell suspension culture of *Scrophularia kakudensis* (Manivannan *et al*., 2016). The SOD activity is up-regulated in response to elicitation-resulted enhance in ROS formation (Kolupaeva & Yastreb, 2021). This antioxidant enzyme has a critical role in defense mechanisms by converting $O^{\cdot-}_2$ to H_2O_2 in cytosols, chloroplasts, and mitochondria. In the present study, elicitation with five combinations of SA_JA levels intensified the SOD activity (Figure 4). The effect of 5 and 20 micromole JA on SOD activity was the highest only when combined with a high level (1 millimole) of SA. This is an interaction between SA and JA, which implies the co-potentiated production of ROS by these elicitors. Our results are in agreement with the findings that have been reported previously (Mohebby *et al*., 2021).

Many reports show a positive correlation between PPO expression and resistance/tolerance to biotic stresses (Taranto *et al*., 2017). The present study is the first report that deals with the effect of JA and SA elicitors on the activity of PPO in purple coneflower. PPO activity appeared to be up-regulated upon elicitation of plant with seven combinations of SA_JA levels (Figure 6). Therefore, the exogenous application of JA and SA mimics the response of a wound signal or a pathogen attack, which triggers a defense reaction in plants by inducing the oxidative burst. In plants, the deamination of L-phenyl alanine to produce trans-cinnamic acid and ammonia is known as the first step for phenylpropanoid skeleton biosynthesis (Bagal *et al*., 2012). This reaction is catalyzed by the PAL enzyme and is often regarded as a key step in the biosynthesis of the phenylpropanoid compounds. Eight combinations of SA_JA levels resulted in an enhanced PAL activity (Figure 7). Our results are in accordance with the previous studies regarding the impact of SA and methyl jasmonate on wheat infected by *Pratylenchus thornei* (Ketabchi *et al*., 2015).

Phenolic compounds are highly valuable as they include biochanin, homogenistic acid, and 12 more beneficial materials (Ho *et al*., 2020). In comparison with control, eight combinations of SA_JA levels, especially 0.5 millimole SA_20 micromole JA (1.47 folds of control), caused up-regulation of TPC (Figure 7). In previous studies on purple coneflower, the methyl jasmonate and SA have also increased two derivatives of phenolic compounds, including chlorogenic acid (Mohebby *et al*., 2021). Generally speaking, in the present study JA and SA elicitation activated the defense responses of purple coneflower, thereby up-regulated the production of all measured secondary metabolites which have commercial and industrial values. Given the fact that a diet rich in secondary metabolites would be beneficial to human health, the elicitation of these metabolites in all edible plants including vegetables should be a major field of research in the future.

As a conclusion, on average, 7 out of 11 combinations of SA_JA levels increased the content/activity of measured secondary metabolites as compared to control. In terms of average response to elicitation with 11 combined levels of JA and SA, the secondary metabolites ranked from higher to lower as the GPX (2.37-fold of control), H_2O_2 (1.86), PPO (1.38), GST (1.36), SOD (1.27), NADPH oxidase (1.23), TPC (1.2), PAL (1.11), anthocyanin (1.1), and flavonoid (0.94). These metabolites have direct and indirect relations with each other. Here, segmented regression could quantify the relation of a few ones which had the simple biphasic relationship (Figures 3 and 5). In future studies, the artificial neural network procedure must be employed to quantify the relation of all these metabolites together. This procedure has successfully been used to quantify complicated relationships in many other fields of research (e.g. Gholipoor *et al*., 2012; 2013; 2019; Salehzadeh *et al*., 2016).

Acknowledgments

We would thank Dr. Hamid Asghari and Dr. Kambiz Jahanbin for their valuable scientific advisements.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the Fatemeh Rasoli and Manoochehr Gholipoor.

Authorship Contribution Statement

Authors are equally participated in conducting the experiment and analyzing the results.

Orcid

Fatemeh Rasoli https://orcid.org/0000-0001-5362-6208
Manoochehr Gholipoor https://orcid.org/0000-0001-9229-4744

REFERENCES

Acharya, B.R., & Assmann, S.M. (2009). Hormone interactions in stomatal function. *Plant Molecular Biology, 69,* 451–462. https://doi.org/10.1007/s11103-008-9427-0

Agati, G., Brunetti, C., Fini, A., Gori, A., Guidi, L., Landi, M., Sebastiani, F., & Tattini, M. (2020). Are flavonoids effective antioxidants in plants? Twenty years of our investigation. *Antioxidants, 9,* 1098. https://doi.org/10.3390/antiox9111098

Angelini, R., & Federico, R. (1989). Histochemical evidence of polyamine oxidation and generation of hydrogen peroxide in the cell wall. *Journal of Plant Physiology, 135,* 212–217. https://doi.org/10.1016/S0176-1617(89)80179-8

Bagal, U.R., Leebens-mack, J.H., Lorenz, W.W., & Dean, J.F.D. (2012). The phenylalanine ammonia lyase (PAL) gene family shows a gymnosperm-specific lineage. *BMC Genomics, 13,* 1471–2164. https://doi.org/10.1186/1471-2164-13-S3-S1

Bandurska, H., & Stroiński, A. (2005). The effect of salicylic acid on barley response to water deficit. *Acta Physiol. Plant., 27,* 379–386. https://doi.org/10.1007/s11738-005-0015-5

Beauchamp, C., & Fridovich, M. (1971). Superoxide dismutase: improved assays and assay applicable to acrylamide gels. *Analytical Biochemistry, 44,* 276-287. https://doi.org/10.1016/0003-2697(71)90370-8

Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., & Minibayeva, F. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany, 53,* 1367–1376. https://doi.org/10.1093/jexbot/53.372.1367

Chance, B., & Maehly, A.C. (1955). Assay of catalases and peroxidases. *Methods in Enzymology, 11,* 764–755. https://doi.org/10.1002/9780470110171.ch14

Chang, C., Yang, M., Wen, H., & Chern, J. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, *10*, 178–182. https://doi.org/10.38212/2224-6614.2748

Dong, J., Wan, G., & Liang, Z. (2010). Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. *Journal of Biotechnology*, *148*, 99-104. https://doi.org/10.1016/j.jbiotec.2010.05.009

Estévez, I.H., & Hernández, M.R. (2020). Plant glutathione S-transferases: an overview. *Plant Gene*, *23*, 100233. https://doi.org/10.1016/j.plgene.2020.100233

Gholipoor, M., Emamgholizadeh, S., Hassanpour, H., Shahsavani, D., Shahoseini, H., Baghi, M., & Karimi, A. (2012). The optimization of root nutrient content for increased sugar beet productivity using an artificial neural network. *International Journal of Plant Production*, *6*, 429–442. https://doi.org/10.22069/IJPP.2012.758

Gholipoor, M., Rohani, A., & Torani, S. (2013). Optimization of traits to increasing barley grain yield using an artificial neural network. *International Journal of Plant Production*, *7*, 1–18. https://doi.org/10.22069/IJPP.2012.918

Gholipoor, M., & Nadali, F. (2019). Fruit yield prediction of pepper using artificial neural network. *Scientia Horticulturae*, *250*, 249-253. https://doi.org/10.1016/j.scienta.2019.02.040

Gong, H., Jiao, Y., Hu, W., & Pua, E. (2005). Expression of glutathione-S-transferase and its role in plant growth and development in vivo and shoot morphogenesis in vitro. *Plant Molecular Biology*, *57*, 53–66. https://doi.org/10.1007/s11103-004-4516-1

Gronwald, J.W., & Plaisance, K.L. (1998). Isolation and characterization of glutathione S-transferase isozymes from sorghum. *Plant Physiology*, *117*, 877-892. https://doi.org/10.1104/pp.117.3.877

Ho, T.T., Murthy, H.N., & Park, S.Y. (2020). Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *International Journal of Molecular Sciences*, *21*, 716. https://doi.org/10.3390/ijms21030716

Horváth, E., Csiszár, J., Gallé, Á., Poór, P., Szepesi, Á., & Tari, I. (2015). Hardening with salicylic acid induces concentration-dependent changes in abscisic acid biosynthesis of tomato under salt stress. *Journal of Plant Physiology*, *183*, 54-63. https://doi.org/10.1016/j.jplph.2015.05.010

Hu, X., Bidney, D.L., Yalpani, N., Duvick, J.P., Crasta, O., Folkerts, O., & Guihua, L. (2003). Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiology*, *133*, 170-181. https://doi.org/10.1104/pp.103.024026

Kaiser, C., Geneve, R., & Ernst, M. (Eds.) (2015). *Echinacea*. Cooperative Extension Service, University of Kentucky, USA.

Kar, M., & Mishra, D. (1976). Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. *Plant Physiology*, *57*, 315–319. https://doi.org/10.1104/pp.57.2.315

Karpets, Y.V., Kolupaev, Y.E., Lugovaya, A.A., & Oboznyi, A.I. (2014). Effect of jasmonic acid on the pro-/antioxidant system of wheat coleoptiles as related to hyperthermia tolerance. *Russian Journal of Plant Physiology*, *61*, 339-346. https://doi.org/10.1134/S102144371402006X

Ketabchi, S., Majzoob, S., & Charegani, H.A. (2015). Effect of salicylic acid and methyl jasmonate on phenylalanine ammonia-lyase activity and total phenol in wheat infected by *Pratylenchus thornei*. *Archives of Phytopathology and Plant Protection*, *48*, 10–17. https://doi.org/10.1080/03235408.2014.882104

Kolupaeva, Y.E., & Yastreb, T.O. (2021). Jasmonate signaling and plant adaptation to abiotic stressors (review). *Applied Biochemistry and Microbiology, 57,* 1-19. https://doi.org/10.1134/S0003683821010117

Kumari, G.J., Reddy, A.M., Naik, S.T., Kumar, S.G., Prasanthi, J., Sriranganayakulu, G., Reddy, P.C., & Sudhakar C. (2006). Jasmonic acid induced changes in protein pattern, antioxidative enzyme activities and peroxidase enzymes in peanut seedlings. *Biologia Plantarum, 50,* 219–226. https://doi.org/10.1007/s10535-006-0010-8

Manivannan, A., Soundararajan, P., Park, Y.G., & Jeong, B.R. (2016). Chemical elicitor-induced modulation of antioxidant metabolism and enhancement of secondary metabolite accumulation in cell suspension cultures of *Scrophularia kakudensis* Franch. *International Journal of Molecular Sciences, 17,* 399–412. https://doi.org/10.3390/ijms17030399

Mattioli, R., Francioso, A., Mosca, L., & Silva, P. (2020). Anthocyanins: a comprehensive review of their chemical properties and health effects on cardiovascular and neurodegenerative diseases. *Molecules, 25,* 3809. https://doi.org/10.3390/molecules25173809

Mendoza, D., Cuaspud, O., Ariasa, J.P., Ruiz, O., & Arias, M. (2018). Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana. Biotechnology Reports, 19,* e00273. https://doi.org/10.1016/j.btre.2018.e00273

Mita, S., Murano, N., Akaike, M., & Nakamura, K. (1997). Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for beta-amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant Journal, 11,* 841–851. https://doi.org/10.1046/j.1365-313x.1997.11040841.x

Mohebby, M., Mortazavi, S.N., Kheiry, A., & Saba, J. (2021). Assessment of physiological and phytochemical traits of purple coneflower (*Echinacea purpurea* L. Moench) in response to salicylic acid and methyl jasmonate application. *Medicinal Plant Research, 11,* 1–13. https://doi.org/10.5376/mpr.2021.11.0001

Parsons, J.L., Cameron, S.I., Harris, C.S., & Smith, M.L. (2018). *Echinacea* biotechnology: advances, commercialization and future considerations. *Pharmaceutical Biology, 56,* 485–494. https://doi.org/10.1080/13880209.2018.1501583

Patel, T., Crouch, A., Dowless, K., & Freier, D. (2008). Acute effects of oral administration of a glycerol extract of *Echinacea purpurea* on peritoneal exudate cells in female swiss mice. *Brain, Behavior and Immunology, 22,* 39-49. https://doi.org/10.1016/j.bbi.2008.04.124

Rincón-Pérez, J., Rodríguez-Hernández, L., Ruíz-Valdiviezo, V.M., Abud-Archila, M., Luján-Hidalgo, M.C., Ruiz-Lau, N., González-Mendoza, D., & Gutiérrez-Miceli, F.A. (2016). Fatty acids profile, phenolic compounds and antioxidant capacity in elicited callus of *Thevetia peruviana* (Pers.) K. Schum. *Journal of Oleo Science, 65,* 311–318. https://doi.org/10.5650/jos.ess15254

Rouet, M., Mathieu, Y., Barbier-Brygoo, H., & Laurière, C. (2006). Characterization of active oxygen-producing proteins in response to hypo-osmolarity in tobacco and *Arabidopsis* cell suspensions: identification of a cell wall peroxidase. *Journal of Experimental Botany, 57,* 1323–1332. https://doi.org/10.1093/jxb/erj107

Sakamoto, M., & Suzuki, T. (2019). Methyl jasmonate and salinity increase anthocyanin accumulation in radish sprouts. *Horticulturae, 5,* 62-75. https://doi.org/10.3390/horticulturae5030062

Salehzadeh, H., Gholipoor, M., Abbasdokht, H., & Baradaran, M. (2016). Optimizing plant traits to increase yield quality and quantity in tobacco using artificial neural network. *International Journal of Plant Production, 10,* 97–108.

Schopfer, P., Heyno, E., Drepper, F., & Krieger-Liszkay, A. (2008). Naphthoquinone-dependent generation of superoxide radicals by quinone reductase isolated from the plasma membrane of soybean. *Plant Physiology, 147*, 864-878. https://doi.org/10.1104/pp.108.118745

Sharma, P., Jha, A.B., Dubey, R.S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany, 2012*, 217037. https://doi.org/10.1155/2012/217037

Singleton, V.L., & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Snell, F.D., & Snell, C.T. (1971). *Colorometric Methods of Analysis*. New York, Van Nostrand Reinhold.

Taranto, F., Pasqualone, A., Mangini, G., Tripodi, P., Miazzi, M.M., Pavan, S., & Montemurro, C. (2017). Polyphenol oxidases in crops: biochemical, physiological and genetic aspects. *International Journal of Molecular Sciences, 18*, 377-393. https://doi.org/10.3390/ijms18020377

Torun, H., Novák, O., Mikulík, J., Pěnčík, A., Strnad, M., & Ayaz, F.A. (2020). Timing-dependent effects of salicylic acid treatment on phytohormonal changes, ROS regulation, and antioxidant defense in salinized barley (*Hordeum vulgare* L.). *Scientific Reports, 10*, 13886. https://doi.org/10.1038/s41598-020-70807-3

Van-Gestelen, P., Asard, H., & Caubergs, R.J. (1997). Solubilization and separation of a plant plasma membrane NADPH-O_2-synthase from other NAD(P)H oxidoreductases. *Plant Physiology, 115*, 543–550. https://doi.org/10.1104/pp.115.2.543

Wang, W., Wang, X., Huang, M., Cai, J., Zhou, Q., Dai, T., Cao, W., & Jiang, D. (2018). Hydrogen peroxide and abscisic acid mediate salicylic acid-induced freezing tolerance in wheat. *Frontiers in Plant Science, 9*, 1137. https://doi.org/10.3389/fpls.2018.01137

Wang, J., Zheng, L., Wu, J., & Tan, R. (2006). Involvement of nitric oxide in oxidative burst, phenylalanine ammonia-lyase activation and taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. *Nitric Oxide, 15*, 351-358. https://doi.org/10.1016/j.niox.2006.04.261

Wasternack, C. (2014). Action of jasmonates in plant stress responses and development-Applied aspects. *Biotechnology Advances, 32*, 31-39. https://doi.org/10.1016/j.biotechadv.2013.09.009