Morpho-phytochemical attributes of *Echinacea purpurea* (L.) Moench exposed to salicylic acid and citric acid

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Abstract

The morpho-phytochemical responses of purple coneflower (*Echinacea purpurea* (L.) Moench) to foliar application of salicylic acid (SA) and citric acid (CA) at 0, 1, 5 and 10 mM (at four-leaf stage and two weeks later) were determined in a field experiment. Results indicated that elevated levels of SA and CA induced plant growth and biomass, including plant height, number of branches, leaves, and flowers, fresh and dry weight of aerial parts, and SPAD value. The highest flower number, flower yield/plant, and shoot fresh and dry weights were obtained under 10 mM SA combined with 5 or 10 mM CA treatment. DPPH radical scavenging activity increased about 1.9 fold under 5 or 10 mM SA combined with 10 mM CA treatments. HPLC analysis revealed that SA and CA enhanced phenolic acids (cynaric acid, chichoric acid, echinacoside, chlorogenic acid, and caftaric acid) production in plants. There was a significant positive correlation between number and yield of flowers on the one hand, and all morpho-phytochemical attributes on the other, except root weight. The data suggested that combination of SA and CA especially at 5 and 10 mM may have higher capacity to improve morpho-physiological traits and phenolic contents of *E. purpurea*.

Keywords: biostimulant, *Echinacea purpurea*, growth, HPLC, phenolic compounds

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Introduction

Purple coneflower or eastern purple coneflower (*Echinacea purpurea* (L.) Moench) is native to eastern North America and belongs to the Asteraceae family (Coelho et al., 2020; Samuel and Priyadarshoni, 2019). It is a perennial and drought-tolerant herbaceous plant growing up to 120-140 cm in height with large daisy-like flowers (Kaiser and Ernst, 2016; Lim, 2014; Samuel and Priyadarshoni, 2019). Aqueous or ethanolic extracts of the dried shoots or roots of *E. purpurea* are considered as one of the most well-known and widely used medicinal plants in the treatment of snake bites and wound infections, due to its anti-inflammatory, anti-immunosuppressant, anti-fungal, antiviral, antioxidant, and antitumor properties (Coelho et al., 2020; Samuel and Priyadarshoni, 2019). The most common phytochemicals found in *E. purpurea* are alkamides, polysaccharides, lipoproteins, betaine, sesquiterpenes, polyacetylene, saponins, and phenolic compounds (echinacoside and other caffeic acid derivatives and also chicoric acid) (Billah et al., 2019; Coelho et al., 2020; Samuel and Priyadarshoni, 2019).

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A direct and simple technique to enhance plant growth and secondary metabolites production is the exposure of the plant to elicitors such as plant growth regulators or organic acids (Ahmadi et al., 2020; Miri et al., 2015). Salicylic acid (SA) is a phenolic signaling biomolecule and a multifaceted growth regulator, distributed in a wide range of plant species (Arif et al., 2020; Asmaei et al., 2021). It plays a crucial role in the regulation of plant physiological and biochemical processes such as photosynthesis, ion uptake, membrane permeability, enzyme activities, flowering, heat production, secondary metabolites production, plant growth and development, and plant defense responses to pathogens and abiotic stresses (Arif et al., 2020; Khan et al., 2015; Miri et al., 2013; Salehi et al., 2016; Soleimani Rozbahani et al., 2018; Vafa et al., 2019; Vicente and Plasencia, 2011). The citric acid cycle, also known as the tricarboxylic acid (TCA) or Krebs cycle, is a pivotal element of carbon metabolism in higher plants (Trejo-Téllez et al., 2012). In recent years, several lines of experimental evidence have demonstrated that citrate is associated with plant tolerance to environmental and disease stresses (Mollapur et al., 2016; Trejo-Téllez et al., 2012). Indeed, current discoveries show that citric acid not only participates as an intermediary in carbon metabolism, but also as a key component in mechanisms of coping with nutrient deficiencies, metal tolerance, growth enhancement, essential oil production, and plant–microbe interactions operating at the rhizosphere (Miri et al., 2015; Trejo-Téllez et al., 2012).

To the best of our knowledge, there have been no previous scientific reports regarding the effect of SA and CA co-exposure on morpho-physiological traits and metabolic contents of *E. purpurea*. Therefore, this experiment aimed to investigate the effects of these biostimulants on growth and phytochemical accumulation of *E. purpurea*.

Material and Methods

In order to evaluate the effects of salicylic acid (SA) and citric acid (CA) on the growth and chemical composition of *E. purpurea*, a field experiment was conducted on a farm in Nazarabad (35˚ 46′ N and 50˚ 55′ E, altitude 1320 m), Alborz, Iran. Seeds of *E. purpurea* were prepared from Pakan Bazr Esfahan company and their dormancy were broken by stratification in 4 ℃ for 24 h. Seeds were sown in nursery beds in mid-February 2020, and seedlings were transplanted to the field at planting intervals of 30 cm in rows in early May 2020. Plants were sprayed twice with SA and CA (0 (distilled water), 1, 5, and 10 mM) on 12 June 2020 (four-leaf stage) and two weeks later. At full flowering stage, five plants from each treatment were randomly collected and plant height, number of branches, leaves and flowers per plant, flower yield per plant, fresh and dry weight of aerial parts and roots as well as leaf chlorophyll content (SPAD value) were recorded. Dry weight was estimated by drying the samples in an oven at 75 ℃ for 48 h. DPPH radical scavenging capacity and HPLC analysis of phenolic acids of the flower extracts were determined according to Brand-Williams et al. (1995) and Brown et al. (2011), respectively.

The experiment was conducted as a factorial arrangement based on randomized complete block design (RCBD) with 3 replications and each replicate was represented by 22 plants.

The data was subjected to two-way analysis of variance using SPSS software. Duncan's Multi Range Test was used for means comparison, and differences were considered statistically significant when P≤0.05 or P≤0.01. Relationships among variables were determined based on Pearson's correlation coefficients at P≤0.05 or P≤0.01.

Results

Plant growth and biomass

Table 1 demonstrates the positive effects of SA and CA spraying on growth and biomass characteristics of purple coneflower, and that their concomitant use at higher concentrations had more pronounced effects. In contrast to the control, increases in the plant height, branch and leaf number, and SPAD value were 65.5% (74.5 cm), 70.5% (22.0 per plant), 48.0% (110.8 per plant), and 68.7%, respectively, under application of 10 mM SA + 10 mM CA. The maximum number of flowers, flower yield/plant, and shoot fresh and dry weights were related to the treatments with

Table 1
Effect of SA and CA application on vegetative and reproductive traits of *E. purpurea*

SA (mM)	CA (mM)	Plant height (cm)	No. of branch	No. of leaf	No. of flower	Flower yield/plant (g)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	SPAD value
0	0	45.0 g	12.8 f	75.2 g	14.5 f	41.5 f	253.4 e	81.9 d	58.1 bc	20.2 abc	17.8 e
	1	47.2 fg	13.8 ef	79.4 fg	14.5 f	44.2 ef	265.4 e	82.7 d	53.6 bcd	19.8 abcd	20.3 cde
	5	50.2 efg	15.6 de	87.3 def	16.6 def	51.3 cdef	329.6 cd	99.2 c	50.4 cd	23.6 ab	20.6 cde
	10	52.2 def	16.2 cd	90.0 de	16.6 def	50.0 cdef	331.6 cd	99.5 c	55.4 bcd	18.6 cd	21.5 bcde
1	0	47.5 fg	16.3 cd	82.6 efg	15.6 ef	47.2 def	267.7 e	80.3 d	55.4 bcd	19.6 bcd	21.8 bcde
	1	47.6 fg	16.4 cd	86.4 def	16.3 def	49.2 cdef	324.6 d	97.4 c	39.3 ef	17.3 cde	22.6 bcd
	5	54.8 cde	17.2 bcd	91.3 d	17.2 cde	52.4 cde	344.6 bcd	103.4 bc	73.9 a	24.1 a	22.8 bcd
	10	55.2 cde	17.2 bcd	91.8 d	17.2 cde	53.4 cde	351.0 bcd	105.3 bc	59.7 bc	23.9 ab	22.8 bcd
5	0	53.4 cde	16.6 cd	92.8 cd	16.6 def	50.5 cdef	334.1 cd	98.2 c	54.1 bcd	15.3 def	22.9 bcd
	1	54.6 cde	17.0 cd	93.5 cd	16.7 def	52.4 cde	337.1 cd	104.6 bc	44.5 de	16.4 cdef	23.2 bcd
	5	57.3 bcd	18.0 bc	95.2 bcd	18.0 cd	56.0 bcd	355.2 bcd	106.5 bc	49.9 cd	17.5 cde	24.0 bcd
	10	58.2 bc	18.1 bc	100.9 bc	18.0 cd	55.9 bcd	358.2 bcd	107.4 bc	62.4 b	18.9 cd	24.2 bcd
10	0	58.3 bc	17.4 bcd	94.5 bcd	19.1 bc	58.5 bc	367.4 bc	110.2 b	54.7 bcd	16.9 cde	24.8 bc
	1	62.2 b	18.0 bc	94.6 bcd	19.2 bc	58.7 bc	376.5 b	112.9 b	33.3 f	13.6 ef	25.0 bc
	5	62.6 b	19.5 b	102.2 b	20.4 ab	63.5 ab	445.0 a	140.7 a	31.8 f	12.4 f	25.4 b
	10	74.5 a	22.0 a	110.8 a	22.0 a	68.7 a	465.2 a	139.5 a	53.2 bcd	19.7 bcd	30.2 a
Sig.	SA	**	**	**	**	**	**	**	**	**	**
	CA	**	**	**	**	**	**	**	**	**	*
	SA×CA	**	**	**	**	**	**	**	**	**	**
CV (%)		5.63	7.21	5.02	7.03	9.36	5.92	4.99	11.30	12.53	9.77

*, **: significant at P≤0.05 and P≤0.01, respectively

Table 2
Effect of SA and CA application on DPPH radical scavenging activity and phenolic compounds of *E. purpurea*

SA (mM)	CA (mM)	DPPH (%)	Cynaric acid (mg/g DW)	Chichoric acid (mg/g DW)	Echinacoside (mg/g DW)	Chlorogenic acid (mg/g DW)	Caftaric acid (mg/g DW)
0	0	22.2 f	0.17 hi	1.97 l	0.34 gh	0.24 g	3.33 def
	1	22.1 f	0.18 ghi	2.24 jk	0.32 h	0.29 fg	2.38 g
	5	25.3 f	0.19 gh	2.52 hi	0.46 de	0.38 def	2.86 fg
	10	29.9 de	0.21 g	2.77 gh	0.44 def	0.46 d	3.06 efg
1	0	22.2 f	0.16 hi	2.07 kl	0.32 h	0.29 fg	2.79 fg
	1	30.7 de	0.21 g	2.92 fg	0.36 fgh	0.34 ef	3.51 def
	5	34.1 c	0.30 e	3.36 de	0.45 def	0.43 de	3.92 cd
	10	34.6 c	0.36 c	3.95 c	0.61 b	0.6 c	4.53 bc
5	0	24.3 f	0.15 i	2.35 ij	0.39 efgh	0.32 fg	2.89 fg
	1	32.9 cd	0.25 f	3.12 ef	0.51 cd	0.47 d	3.95 cd
	5	34.7 c	0.38 bc	3.60 d	0.59 bc	0.59 c	4.05 cd
	10	41.3 ab	0.46 a	4.21 ab	0.93 a	0.77 b	5.39 a
10	0	29.6 e	0.17 hi	2.57 hi	0.42 defg	0.34 ef	2.99 fg
	1	35.8 c	0.33 d	3.57 d	0.62 b	0.79 b	3.81 cde
	5	39.5 b	0.41 b	3.99 bc	0.85 a	0.66 c	4.85 ab
	10	42.9 a	0.40 b	4.43 a	0.88 a	0.92 a	5.43 a
Sig.	SA	**	**	**	**	**	**
	CA	**	**	**	**	**	**
	SA×CA	**	**	**	**	**	**
CV (%)		5.83	6.32	4.71	9.31	11.04	11.85

**: significant at P≤0.01

10 mM SA + 5 or 10 mM CA, as 40.6 & 51.7%, 53.0 & 65.5%, 75.8 & 83.8%, and 71.6 & 70.2% increases compared to the control, respectively.

Regarding the root fresh and dry weights, the observed results were different from those of the traits presented above. The highest root fresh weight was obtained with 1 mM SA + 5 mM CA while the lowest root fresh weight was observed with1 mM SA + 1 mM CA and 10 mM SA + 1 or 5 mM CA. The highest root dry weight was found with 1 or 5 mM CA and 1 mM SA + 5 or 10 mM CA, which did not increase significantly compared to the control.

DPPH radical scavenging capacity and phenolic acids

Foliar application of purple coneflower with SA and CA increased DPPH radical scavenging activity and phenolic acids especially in their higher concentrations (Table 2). The best results in terms of DPPH and chichoric acid were observed in plants sprayed with 5 or 10 mM SA + 10 mM CA. The highest concentrations of echinacoside and caftaric acid were obtained with 5 mM SA + 10 mM CA and 10 mM SA + 5 or 10 mM CA. Cynaric acid and chlorogenic acid contents increased by 72.3 and 84.7%, when treated with 5 or 10 mM SA + 10 mM CA, respectively, compared to control.

Correlation among morpho-phytochemical traits

Correlation results indicated that important morpho-phytochemical traits such as plant height, number of branches, leaves and flowers, fresh and dry weights of aerial parts, SPAD value, DPPH radical scavenging activity, and phenolic acids highly correlated ($P \leq 0.01$) (Table 3). On the other hand, root fresh and dry weights had no correlation with other traits except that they had a negative relationship with shoot dry weight ($r = -0.31$ and -0.29, respectively, $P \leq 0.05$).

Discussion

This experiment was conducted to determine potential effects of SA and CA in enhancing plant growth attributes, antioxidant activity, and phenolic acids of *E. purpurea*. Results showed that SA and CA application associated with improved plant growth and biomass except fresh and dry root weights compared to the control plants. The effect of exogenous SA and CA on growth depends on the plant species, developmental stage, and the concentrations tested (Miri et al., 2015; Vicente and Plasencia, 2011). As revealed by the data presented in Table 1 and 2, it is clear that higher levels of SA combined with CA brought about a significant increase in the growth, DPPH radical scavenging activity, and phenolic acids of the plants. The increase in plant height and shoot dry weight or content of total phenolic compounds with increasing concentrations of SA and CA in the present investigation is in agreement with the findings reported by Aftab et al. (2010), Hashmi et al. (2012), and Salas-Pérez et al. (2018).

The SA-enhanced growth of the plants might be associated with its regulatory effects on a range of plant processes, including cell growth and division, ion uptake and transport, prevention of auxin oxidation and ethylene biosynthesis, maintaining of IAA and gibberellins, photosynthetic rate, protection against oxidative stress, and changes in protein synthesis associated with plant growth and metabolism (Aftab et al., 2010; Hashmi et al., 2012; Ibrahim et al., 2019; Vicente and Plasencia, 2011). In addition, the contribution of SA to flowering regulation has been well known for a long time (Vicente and Plasencia, 2011).

The role of CA in improvement of biomass and plant growth might be related to the ability of CA to enhance the nutrient uptake, photosynthesis and synthesis of phytochelatins in plants. Photosynthetic pigments play a vital role in plant life as they harvest light for biochemical reactions in leaf cells (Mallhi et al., 2019).

Our results indicated that SA and CA significantly increased the SPAD value in purple coneflower plants. Positive and significant correlation of SPAD value with growth characteristics of plant aerial parts can indicate that enhancement in photosynthetic pigments in photochemical reactions may transform the light energy more effectively under the application of SA and CA, resulting in enhancement of biomass and plant growth. SA or CA has been reported to increase vegetative growth in several medicinal plants such as *Artemisia annua* (Aftab et al., 2010), *Foeniculum vulgare* (Hashmi et al., 2012), *Thymus*

vulgaris (Miri et al., 2015), *Datura innoxia* (Ghadermazi et al., 2017), (*Tanacetum parthenium* (Ahmadi et al., 2020), and *Lippia citriodora* (Ghandom Froosh et al., 2020).

According to the correlation results, increased shoot dry weight was associated with reduced root weight. Once both root and shoot begin to grow simultaneously, their balanced growth continues to the end of the vegetative growth, and both stop growing at the same time, when reproductive growth begins. If environmental parameters change during the season, it is possible that two vegetative organs stop growing at different time points (Iwasa and Roughgarden, 1984). SA and CA stimulate growth of aerial parts and possibly due to the competition of assimilates, this increase in shoot stimulation has reduced root weight.

Phenolic acids have been considered for their potential health benefits due to their antioxidant activities. They are generated from aromatic amino acids produced via the shikimate pathway (Valanciene et al., 2020). SA has the ability to regulate enzymatic activities like phenylalanine ammonia lyase and chalcone synthase in the biosynthesis of phenolic compounds (Alamer and Fayez, 2020). Hashmi et al. (2012) assumed that exogenous applications of SA could have influenced the synthesis of major components of *Foeniculum vulgare* essential oil via gene regulation, resulting in an increase in the number of transcripts of the enzymes linked to secondary metabolic pathway of these compounds. On the other hand, CA can degrade conjugated phenols such as tannins to other simpler phenolic compounds by hydrolyzing which can accumulate in cellular vacuoles (Salas-Pérez et al., 2018). In addition, CA as an intermediary in the TCA cycle is involved in the synthesis of metabolites (Miri et al., 2015). Following the positive and significant correlations among plant growth attributes and phenolic acids, presumably, the SA- and CA-enhanced chloroplast pigments might have resulted in the enhancement of plant biomass, leading to a significant increase in phenolic acids in this study. Moreover, the higher antioxidant activity and phenolic acids contents in treated plants can be attributed to the fact that the SA and CA stimulated the defense mechanisms of *E. purpurea* plants, triggering the accumulation of antioxidant compounds.

In conclusion, we found that foliar application of SA combined with CA significantly stimulated the growth, antioxidant capacity, and phenolic acids production in *E. purpurea*. Thus, SA and CA can be utilized at higher concentrations (5-10 mM) as valuable and inexpensive sources for the cultivation of *E. purpurea* with increased phenolic acid contents and yields.

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