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Research paper

Effects of selenium nanoparticles and ancymidol on physiological responses of *Stevia rebaudiana* Bertoni colonized by *Piriformospora indica*

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Abstract
The South American plant, *Stevia rebaudiana* Bertoni, is a good source of steviol glycosides, antioxidants, all of the essential amino acids, and other important nutritional compounds. The present study was conducted to investigate the effects of 5 and 10 mg L^{-1} concentrations of selenium nanoparticles (SeNPs) and 50 mg L^{-1}ancymidol (ANC) on physiological and biochemical characteristics of *S. rebaudiana* colonized by the root endophytic fungus *Piriformospora indica* at vegetative and initial flowering stages. Results indicated that ANC decreased root colonization rate and antioxidant enzyme activities, but increased the content of H_2O_2 (vegetative stage), malondialdehyde (initial flowering stage), and phosphorus uptake (initial flowering stage) significantly and had no effect on total carbohydrate content. The adverse effects of ANC reduced after *P. indica* colonization and somewhat with SeNPs application. Our results suggest that the *P. indica* colonization and SeNPs application can alter the equilibrium between the production of free radicals and enzymatic defence reactions in *S. rebaudiana* by enhancing the scavenging capacity of free radicals and by decreasing membrane lipid peroxidation during both vegetative and initial flowering stages. Moreover, the effects of ANC, SeNPs, and *P. indica* on the measured characteristics at the vegetative stage were higher than those observed at the initial flowering stage.

Keywords: antioxidant enzymes; endophytic fungus; growth retardants; stevia; steviol glycosides

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Introduction

Stevia rebaudiana Bertoni is an important medicinal plant belonging to the Asteraceae family that is widely distributed in Paraguay and Southern Brazil. It is a perennial, photoperiod-sensitive, insect-pollinated, and self-incompatible bushy shrub. *Stevia* leaves contain steviol glycosides such as stevioside and rebaudioside, which are non-caloric and 300-350 times sweeter than sucrose (Ahmed *et al.* 2011). Gibberellin and steviol glycosides biosynthetic pathways have many common stages and intermediate metabolites for the formation of kaurenoic acid. In the branch point steviol and gibberellin are generated by hydroxylation on C_3 and C_7 of kaurenoic acid, respectively, through the activity of kaurenoic acid-3-hydroxylase enzyme and kaurenoic acid oxidase. Steviol glycosides are produced by the action of many glucosyl transferases that transfer glucose units to steviol (Karimi *et al.* 2014). *Stevia* calorie-free sweet extract is used in many countries of the world including some East Asian countries, certain countries of South America and United States as dietary supplements and sweetening soft drinks, soju, soy sauce, yogurt, and other foods (Chan *et al.* 2005). It has been reported that *Stevia* can be used as an alternate to sugar and to control some chronical diseases such as diabetes. In addition, *Stevia* leaves have other important compounds including diterpenes, triterpenes,

sterols, flavonoids, volatile oil constituents, pigments, and inorganic matters (Kinghorn 1992).

Probably the most important symbiosis in nature is between plants and arbuscular mycorrhizal fungi (AM fungi), which significantly enhances the plants' performances (Hoshyar *et al.* 2017). About 80% of plants can undergo symbiosis with these fungi at different stress conditions (Redecker *et al.* 2000). *Piriformospora indica* is an endophytic fungus, often called an arbuscular mycorrhizal-like fungus, which stimulates growth and overall biomass of plants and increases plant tolerance to biotic and abiotic stresses (Mensah *et al.* 2019). One of the mechanisms of mycorrhizal fungi that protect plants against biotic and abiotic stresses and reactive oxygen species (ROS) generation, is increasing the activities of some antioxidant enzymes (Estrada *et al.* 2013).

Selenium (Se) is an essential non-metallic mineral and a constituent of redox active enzymes such as glutathione peroxidase and glycine reductase (Hatfield *et al.* 2014). However, usefulness or toxicity of Se for many organisms depends on its concentration. Thus, Se deficiency increases oxidative stress and contributes to the development of oxidative damage. It has been shown that the Se particles in nanometre size have a high biochemical activity and improves bioavailability. Selenium nanoparticles (SeNPs) have grown more attention due to their low toxicity and strong ability to scavenge free radicals (Sieber *et al.* 2005).

Plant growth retardants are often used to control the plant height and growth habit in order to produce more marketable plants. Cycocel and ancymidol are essential growth regulators for plants that decrease the concentration of gibberellins (Seyed Sharifi and Khalilzadeh 2018). Ancymidol (α-cyclopropyl-α-[4-methoxyphenyl]-5-pyrimidinemethanol) ($C_{15}H_{16}N_2O_2$) commonly reduces or suppresses the synthesis of GAs, which in turn decreases the capacity of cells to elongate and impairs shoot growth in numerous mono- and di-cotyledonous species (Karimi *et al.* 2014). Since steviol glycosides and gibberellins share biosynthetic pathway in *Stevia*, studying the effects of ancymidol, as a gibberellins inhibitor, on steviol glycosides in the *Stevia* plants could be of interest. The objective of this study was to investigate the effect of ancymidol, as a plant growth retardant, and SeNPs, as elicitors, on physiological and biochemical attributes of *S. rebaudiana*, inoculated with *P. indica*.

Materials and Methods

Culture and growth conditions of P. indica

P. indica was grown in petri dishes with a diameter of 8 cm on Kaefer's medium and incubated at 25 °C for two weeks. For inoculation of *Stevia* roots with *P. indica*, sterile distilled water was added to the cultures of *P. indica* plates and the plates were gently scraped to loosen the spores and mycelia. The suspension was collected, vortexed for 10 min, and then centrifuged at 10,000 g for 5 min. The supernatant was discarded and the pellet was suspended in the sterile distilled water. The fungal suspension was diluted to 5×10^5 spores per mL^{-1} (Khatabi *et al.* 2012) and the roots of the plants were immersed in 100 mL of this suspension for inoculation.

Culture and growth conditions of S. rebaudiana

Seeds of *S. rebaudiana* were provided from the Pakan-Bazr Seed Production Company (Isfahan, Iran). The seeds were cultured on MS basal medium (Murashige and Skoog 1962) and

incubated under controlled climatic conditions at 28/25 °C day/night temperatures, light/dark regimes of 16/8 h, light intensity at the table height of 280 µmol $m^{-2}s^{-1}$ (Sylvania VHO cool white, 215 W lamps), and 70% relative humidity. After two weeks of culture, seedlings were obtained from the *in vitro* seed germination medium. For micropropagation, shoot tips were excised and cultured under the same conditions. Then, two-months old plantlets with similar size and number of internodes (four internodes) were transferred to plastic cups. Before transforming the plantlets to the plastic cups, the roots were washed thoroughly under tap water to remove agar particles. Then, the plants in similar size and number of internodes (10 internodes) were transferred to plastic pots (4.5 cm diameter × 7 cm height) filled with 500 g autoclaved mix of cocopeat: soil (1:3 v/v), per pot. Before the plants were transferred to the pots, the roots inoculated with *P. indica* suspension for 15 min. Potted plants were grown under controlled climatic conditions at 28/25 °C day/night temperatures, and short day conditions (8:16 h, light/dark) in the cultivation room. The plants were harvested at two stages: two months after transfer to pots (vegetative stage), and just before entering the flowering or initial flowering stage (Figure 1). SeNPs and ANC were sprayed once a week in the last three weeks of the first harvest (vegetative stage). 10 mL ANC at the concentration of 50 mg L^{-1}, and 10 mL SeNPs at the concentrations of 5 and 10 mg L^{-1} were sprayed with three replications (Table 1).

Table 1. List of the treatments used on the *Stevia* plants in this study

Set No.	Treatment
1	Control
2	Selenium nanoparticles 5 mg L^{-1} (SeNPs 5)
3	Selenium nanoparticles 10 mg L^{-1} (SeNPs 10)
4	Ancymidol (ANC)
5	Ancymidol + SeNPs (5 mg L^{-1}) (ANC + SeNPs 5)
6	Ancymidol + SeNPs (10 mg L^{-1}) (ANC + SeNPs 10)
7	*P. indica* (Pi)
8	SeNPs (5 mg L^{-1}) + *P. indica* (SeNPs 5 + Pi)
9	SeNPs (10 mg L^{-1}) + *P. indica* (SeNPs 10 + Pi)
10	Ancymidol + *P. indica* (ANC + Pi)
11	SeNPs (5 mg L^{-1}) + Ancymidol + *P. indica* (SeNPs 5 + ANC + Pi)
12	SeNPs (5 mg L^{-1}) + Ancymidol + *P. indica* (SeNPs 5 + ANC + Pi)

Root colonization rate

Roots of *Stevia* plants were washed thoroughly in the running tap water to remove soil, then they cut into 1 cm pieces. The root pieces were treated overnight with 10% KOH solution at room temperature. Thereafter, the root pieces were washed 3-5 times with the sterilized distilled water and neutralized by 1% HCl before staining with 0.05% trypan blue. Then, they were distained in glycerol: DI water (1:1 v/v) solution. Root colonization was assessed by grid line-intersect method (Giovannetti and Mosse 1980) (Figure 2) and calculated as follows:

Colonization percent = Number of roots colonized with *P. indica* /Total number of roots inspected × 100

Se content

The concentration of Se in the *Stevia* leaves was determined by inductively coupled plasma mass

spectrometry (ICP-MS) according to the method described by Liu and Gu (2009) with slight modification. After harvesting, *Stevia* leaves were washed three times with deionized water to remove the remaining Se particles on their surfaces and then dried at 60 °C for 48 h. Then, 1 g of the dried samples was digested in 5 mL of a mixture composed of 4 mL HNO_3 and 1 mL $HClO_4$ at 30 °C for 1 h. After cooling, 5 ml of the concentrated HCl was added to the mixture and incubated at 115 °C for 20 min. The solution after digestion and cooling at room temperature, was transferred to a tube and allowed to rise to 50 mL by adding the distilled water. The final solution was used to determine the Se bioaccumulation by ICP-MS (HP-4500, USA).

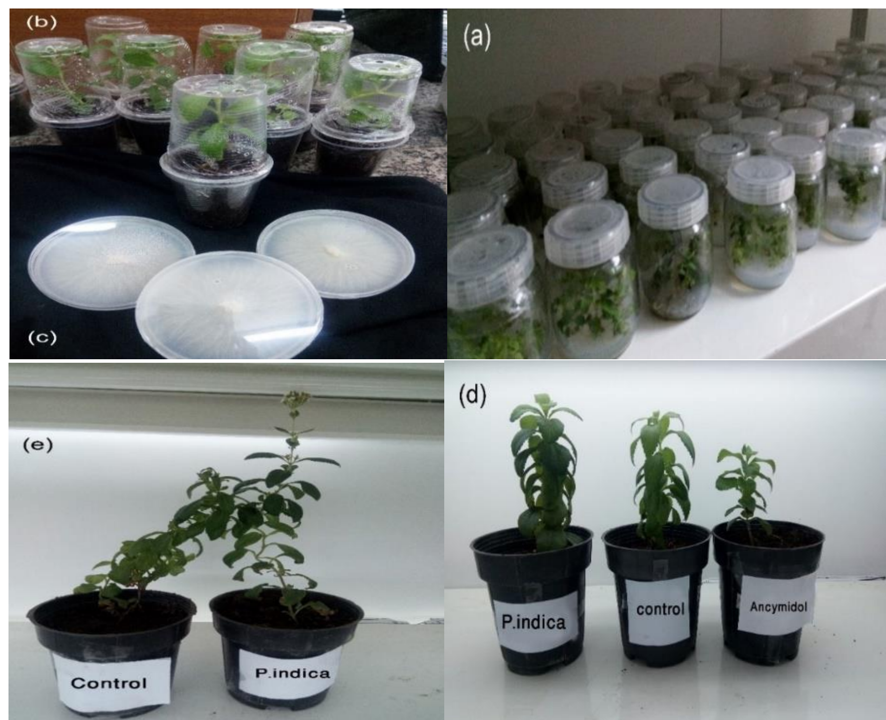

Figure 1. *Stevia rebaudiana* plants: a) *Stevia* plants in the MS culture media, b) *Stevia* plants in the plastic pots ready for inoculation with *Piriformospora indica*, c) Growth of *P. indica* on the solid media, d) *Stevia* plants at the vegetative stage, and e) *Stevia* plants at the initial flowering stage

Figure 2. *Piriformospora indica* spores on the *Stevia* root surface

Phosphorus content

The phosphorus (P) content in the *Stevia* leaves was determined using Vanadate-Molybdate-Yellow method (Chapman and Pratt 1961). After determination of the dry mass, tissues were milled and analysed for total P concentration. Samples (0.5 g) were digested in HNO_3 for 24 h. Then, 10 mL of the mixture containing ammonium monovandate (300 mL)/ammonium heptamolibedate (400 mL)/HNO_3 (250 mL) was added to 10 mL of the sample extract and its absorbance was determined by a UV-Vis spectrophotometer at 470 nm.

Total carbohydrates

Total carbohydrates content of the *Stevia* leaves was measured by the phenol-sulfuric acid method as described by Kochert (1978). Briefly, 10 mL of 70% ethanol was added to 0.1 g of dry leaf sample and kept in the refrigerator for one week. Then, 0.5 mL of the upper solution was mixed with 1 mL of 5% phenol and 5 mL sulphuric acid and kept at the lab temperature for 30 min to develop the color. Then, the absorbance of the solution was read by a UV-Vis spectrophotometer at 485 nm and results was expressed as mg g^{-1} DW.

Total phenols, flavonoids, and anthocyanins

For determination of the total phenolic and flavonoids contents, the leaves were extracted using 80% methanol, centrifuged at 5,000 rpm for 20 min and then assessed according to Miliauskas *et al.* (2004) and Chang *et al.* (2002), respectively. The total anthocyanin content was determined as described by Wagner (1979).

Flavonoids content was determined by the aluminium chloride colorimetric method. Briefly, 500 µL of each sample mixed with 2.8 mL distilled water, 100 µL potassium acetate (1 M), 100 µL 10% aluminium chloride solution and 1.5 mL methanol. After 30 min, the absorbance of the reaction mixture was measured at 415 nm. A calibration curve was constructed by preparing quercetin solution and flavonoids values were expressed as quercetin equivalents (mg g^{-1} FW).

The total phenolic content was determined by the Folin-Ciocalteu assay. The metanolic extract (500 µL) was mixed with 2.5 mL Folin-Ciocalteu reagent, and after 2 min, 2 mL sodium carbonate (7%) was added. Absorbance of samples was determined at 762 nm using an spectrophotometer. The results were calculated by the calibration curve of gallic acid and expressed as gallic acid equivalents (mg g^{-1} FW).

The method proposed by Wagner (1979) was used to estimate the total anthocyanin content. Leaf discs (100 mg) were soaked immediately in 10 mL acidified methanol (methanol: HCl, 99:1 v/v) and incubated for 24 h in darkness at 25 °C. Then, the sample was centrifuged at 4000 g for 10 min and finally the supernatant absorbance was determined by a digital spectrophotometer at 550 nm.

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

The amount of MDA was measured according to Heath and Packer (1968). In short, leaf samples (100 mg) were homogenized in 3 mL 0.1% (w/v) TCA, and centrifuged at 12,000 g for 15 min, then 1 mL thiobarbituric acid (0.5% w/v) was added.

The mixture was heated for 30 min at 90 °C and rapidly cooled in an ice bath. The absorbance of supernatant solution was read at 532 nm. The extinction coefficient (ε) of 1.55×105 /M cm was used to determine the MDA concentration.

Determination of H_2O_2 concentration was based on the method of Velikova *et al.* (2000). Leaf tissues (0.1 g) were extracted with 3 mL TCA (0.1%, w/v) at 0 °C and centrifuged at 12,000 g for 15 min. Then, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance of supernatant was read at 390 nm. Using the molar extinction coefficient of 0.28 mol^{-1} cm^{-1} (ε_{530}=0.28 mol^{-1} cm^{-1}), the concentration of H_2O_2 was calculated.

Total soluble proteins and the activities of antioxidant enzymes

The extraction procedure for determination of soluble proteins content and the activity of antioxidant enzymes was according to Gadzovska *et al.* (2007). The frozen leaf samples (1 g) were homogenized in 2 mL of 0.1 M KH_2PO_4/K_2HPO_4 buffer at pH 8.0, containing 2 mM ethylenediamine tetra-acetic acid, 1.4 mM β-mercaptoethanol, and 1% (w/v) polyvinyl pyrrolidone. The homogenate was centrifuged at 13,000 g for 20 min at 4 °C, and the supernatant was used for the protein determination and enzyme activity assay. The total proteins content was determined according to Bradford (1976) and was expressed in mg g^{-1} FW.

For determination of super oxide dismutase (SOD) activity, the procedure of Beauchamp and Fridovich (1971) was used. The SOD activity was measured by the degree of inhibition of the photochemical reduction of nitroblue tetrazolium (NBT, Sigma, USA) in the presence of riboflavin. The reaction mixture containing 50 mM potassium phosphate (pH 7.8), 9.9 mM methionine, and 57 µM NBT was placed under a 18 W light bulb for 20 min, and then stopped by placing in the dark and absorbed by spectrophotometry at 560 nm. The peroxidase (POD) activity was determined by the method of González *et al.* (1999). The reaction mixture contained 60 mM sodium phosphate buffer (pH 6.0), 28 mM guaiacol, 5 Mm H_2O_2, and the enzyme extract. Immediately after the reaction, increase in the rate of absorbance was measured at 470 nm for 1 min. The polyphenol oxidase (PPO) activity was evaluated following the method of Raymond *et al*. (1993). The reaction mixture contained 20 mM pyrogallol, 0.2 M sodium phosphate buffer (pH 6.8) and the enzyme extract. The result from the oxidation rate of pyrogallol by PPO was read at 430 nm. The SOD, POD, and PPO activities were expressed as unit g^{-1} FW.

Experimental design and statistical analysis

This experiment was conducted in a factorial arrangement based on completely randomized design with three replications. Error bars of the graphs indicated the standard error (SE) of the means. The statistical analyses, including one-way analysis of variance and comparison of means by Duncan's multiple range test at $p \leq 0.05$, were performed with the SPSS statistical software program (version 20, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Root colonization rate

In microscopic inspection, the spores of *P. indica* were observed in the inoculated root samples and not detected in the non-inoculated samples. Inoculated plants were colonized in the ranges of 35–65% with higher rates at the vegetative stage than those at the initial flowering stage, however, this difference was not significant (Figure 3). Our findings showed that the colonization rate of *P. indica* was considerably inhibited by ANC compared to the control plants. In addition, both SeNPs concentrations didn't have significant difference with the control in root colonization rate. The infectivity of *P. indica* in the inoculated roots can be predicted by the colonization rate of the host (Kumari *et al.* 2003). The extent of root colonization by *P. indica* in plants depends on growth, co-cultivation conditions, and plant species (Das *et al.* 2012). According to Wedding *et al.* (1978), in the *Arum* inflorescences during early flowering bud development, there was a mobilization of metabolites toward the developing buds, and consequently, less metabolites were available in the roots for exudation and VAM formation. It was found by Golubkina *et al.* (2019) that Se had no significant effect on the mycorrhizal colonization index in shallot plants.

Se content

The advantages of using nano-shaped Se compared to its ionic forms are greater chemical stability, high biocompatibility, faster absorption, and less toxicity (Li *et al.* 2020). The method of absorption of SeNPs is not fully understood. It is possible that both intracellular and extracellular uptake occur. It has not yet been determined that Pegone nanoparticles pass through the casparian strip, but this transfer is thought to be meristematic. The cell wall acts as a physical barrier to the passage of the materials into the cell and has pores of 5 to 20 nanometres in diameter through which small nanoparticles pass. Results from the ICP analysis of the SeNPs-sprayed *Stevia* plants confirmed Se accumulation at both developmental stages. Also, Se bioaccumulation in the *Stevia* plants was positively related to its concentration and increased after *P. indica* inoculation. In addition, our findings showed that there was no difference in Se content between ANC-treated and untreated *Stevia* plants (Figure 4). Se content of the leaves increased in the inoculated plants with *P. indica* compared to the control plants, however, some of these increases were not significant. Maximal increase in the Se content was recorded in the treated plants with 10 mg L^{-1} SeNPs. It seems that AM fungi could improve the uptake of nutrient elements. Jianheng *et al.* (2015) showed that the inoculation of AM fungi can improve the absorption of Se in the *Salvia* plants at low concentration. However, at the higher concentration of Se, both inoculated and non-inoculated plants accumulated higher amount of Se. Our findings showed no differences between ancymidol-treated plants and controls for the Se content. However, Se concentration at the vegetative stage was higher than that of the initial flowering stage.

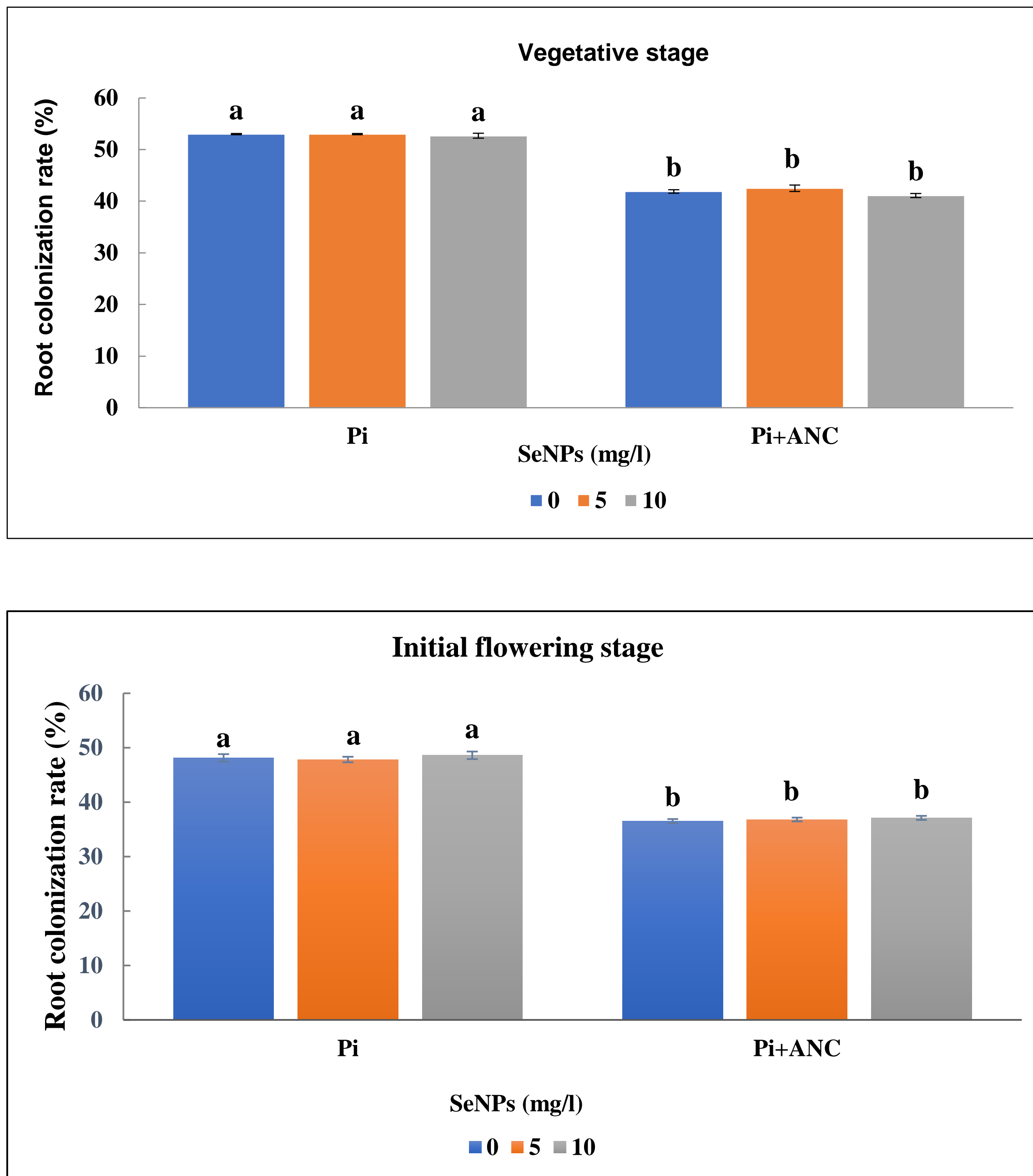

Figure 3. Root colonization rate (%) of *P. indica* in the *Stevia* plants treated with ancymidol (ANC) and selenium nanoparticles (SeNPs): a) Vegetative stage, and b) Initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

Phosphorus content

Results indicated that P content of the *Stevia* plants was higher at the vegetative stage than that of the initial flowering stage. The treated plants with ANC did not reveal an induced sizable change in the P content compared to the control at the vegetative stage, but slightly increased at the initial flowering stage (Figure 5). *Stevia* plants treated with SeNPs and inoculated with *P. indica* exhibited significant increase in P content compared to the corresponding controls. Highest amount of P content was recorded in inoculated plants with *P.*

indica. It was shown that *P. indica* colonized maize roots cortex could obtain carbon from the host plant, while assisted it by improving P and other low mobility nutrients uptake from the soil, and their translocation to the host root (Bielesk and Ferguson 1983). The low availability of phosphorus in soil can limit plant growth and metabolism due to its poor solubility and mobility in soil. The best characterized benefit of AM symbiosis for plants is the enhanced P nutrition (Vance 2003). AMF by root proliferation via indole-3-acetic acid production, provide various micro and macro-nutrients (particularly P) and water supplies for host plants. Photosynthetic assimilates are transported from the plants into endosymbiotic AMF and used for their development (Mitra *et al.* 2019). Although Se is considered a quasi-essential micronutrient, but its effects on absorption and accumulation of nutrients in plants have been little investigated. Arvy *et al.* (1995) showed that selenite or selenate treatment increased the concentration of some elements such as zinc and copper, but did not modify the levels of S, K, Ca, P, Mg, Fe, Mn, Na, and Al in the *Catharanthus roseus* plant. There has been little investigation on the effect of ANC on the nutrients absorption and accumulation. Tsujita (1979) showed that P content of the leaves and roots of *Lilium longiflorum* was not influenced by ANC. Römer and Schilling (1986) showed that phosphorus was absorbed during the early growth stages, but the cause of this phenomenon has not yet been determined. Therefore, to quantify the required P, it is necessary to pay attention to the growth stages of the plant.

Total soluble carbohydrate content

Plants inoculated with *P. indica* showed a significant increase in carbohydrate content comparing with control plants, however, this increase was lesser in the plants treated with ANC (Figure 6). The maximal increase in carbohydrate content was obtained in *Pi*+SeNPs treatment. The main phenological factor affecting the steviol-glycosides content in *Stevia* plants is flowering, and the optimal time to harvest the leaves is at the onset of flowering, when the accumulation of steviol glycosides reaches its peak. The concentration range of SVglys in *S. rebaudiana* leaves is 10-30% of their dry masses, which is affected by various factors like genotype, phenological stage, and growth conditions. The concentration of glycoside in the leaves of *Stevia* increased, when the plants are grown under long days (Yadav et al. 2011). Since glycoside synthesis is reduced at or just before flowering, delaying flowering with long days allows more time for glycoside accumulation (Yadav *et al.* 2011). Jiao *et al.* (1986) showed that ANC in the Easter lily plants resulted in low carbohydrate levels in leaves, indicating reduced plant vigour. Hajihashemi (2018) found that the carbohydrates accumulation in gibberellic acid- and paclobutrazol-treated *Stevia* plants showed no correlation with photosynthetic pigments. Moreover, there have been reports that the presence of metal nanoparticles might enhance photosynthetic activity by regulation of genes related to the light harvesting complex II, which in turn increased level of soluble sugars. It seems that the SeNPs might act as activators of photosynthetic machine

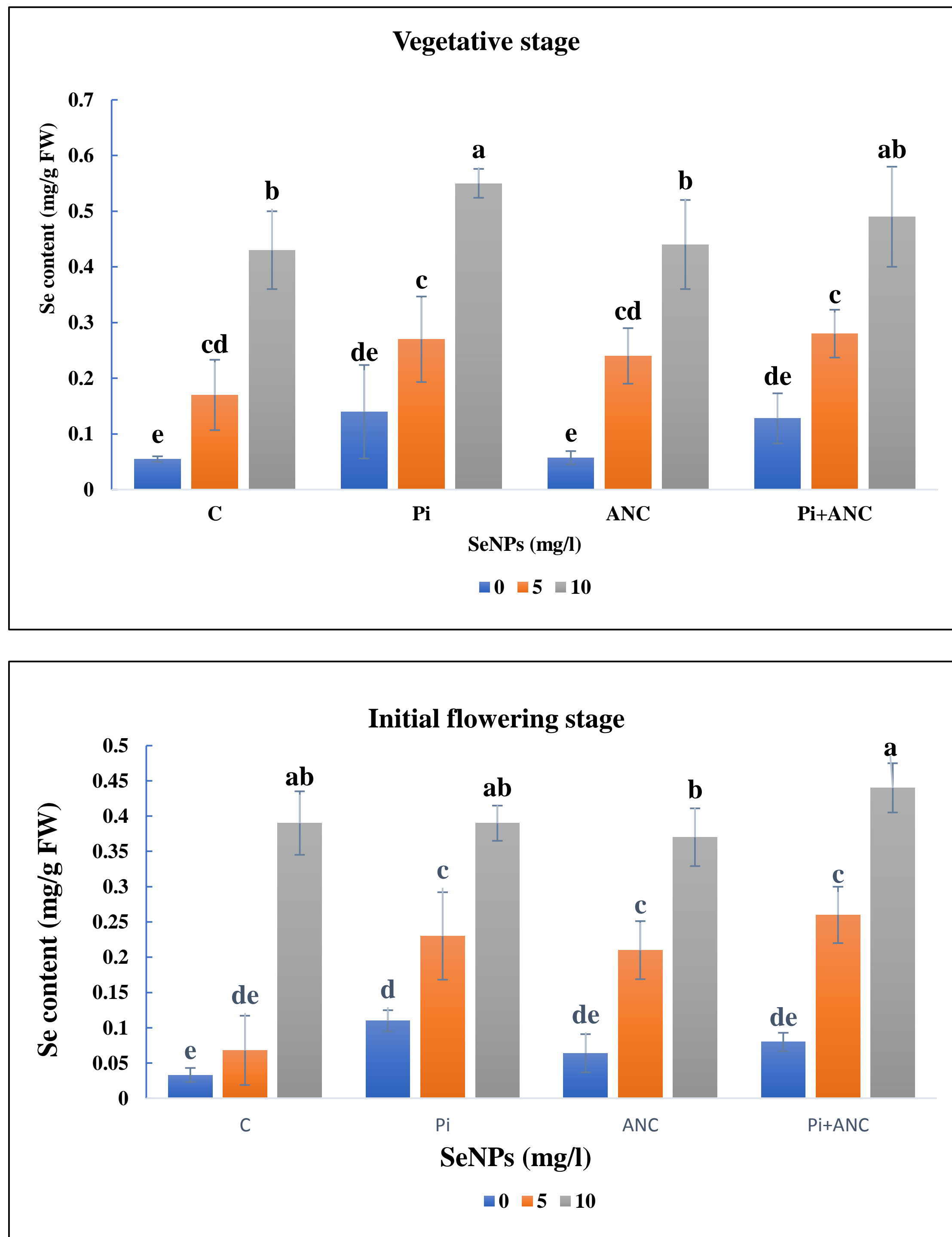

Figure 4. Selenium content of the *Stevia* plants treated with selenium nanoparticles (SeNPs) and inoculated with *P. indica* as compared with the control (C): a) Vegetative stage and b) Initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

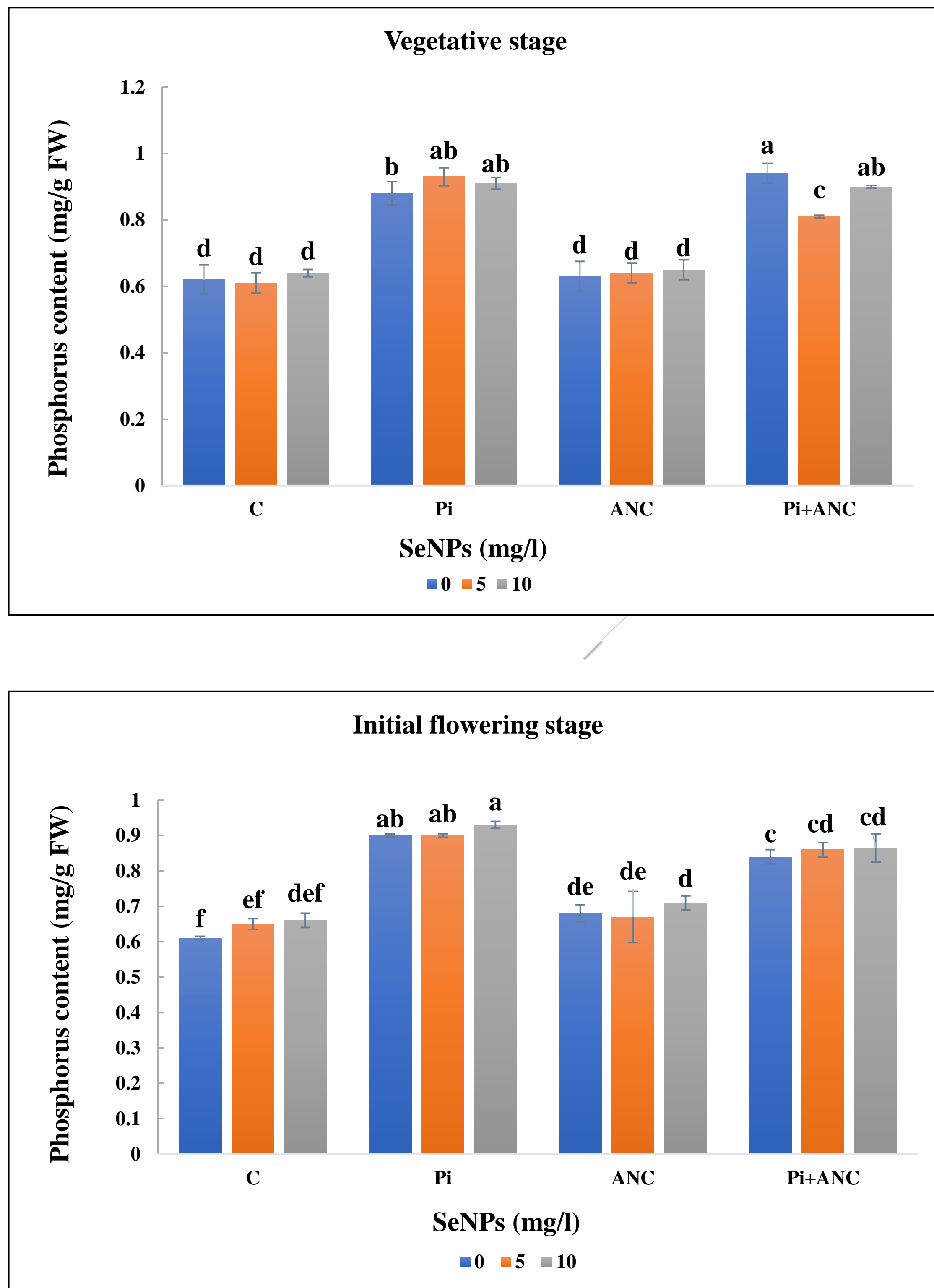

Figure 5. Phosphors content of the *Stevia* plants treated with selenium nanoparticles (SeNPs) and inoculated with *P. indica* compared with the control (C): a) Vegetative stage and (b) Initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

and promote total carbohydrate content (Smirnoff 2011). It was reported that treatment of *Stevia* plants with biofertilizers and chemical fertilizers increased growth and the content of chlorophyll, carbohydrate, and protein compared to the control (Patil 2010). In addition, there are some reports on higher carbohydrate concentration of mychorrizal-inoculated plants compared to the non-inoculated ones, because the fungi require plant carbohydrates as energy source for their growth and activity. AM symbiosis can cause an important carbohydrate gain in the host plant and up to 20% of total photosynthetic assimilates can be transferred to the fungal partner (Wu *et al.* 2011).

Total phenol, flavonoids, and anthocyanin contents

Plants treated with ANC+SeNPs revealed induced significant increase in total phenol content compared to the control except at 5 mg L^{-1} of SeNPs at the vegetative stage (Figure 7). ANC alone also increased the flavonoids content at both stages. However, ANC+SeNPs increased only the flavonoids content at 5 mg L^{-1} of SeNPs at the initial flowering stage. Total phenol, flavonoids and anthocyanin contents increased in the plants inoculated with *P. indica.* It was appeared that SeNPs induced change in the amount of flavonoids in the roots of *Stevia* plants inoculated with *P. indica*.. In all studied treatments, flavonoids and anthocyanin contents were higher at vegetative stage than those at initial flowering stage (Figure 7). ANC+SeNPs resulted in a significant increase in the anthocyanin content in the plants inoculated with *P.* indica (Figure 7). Plants are the main sources of natural antioxidants within food ingredients. Among the plant compounds, it seems that secondary metabolites such as phenols and flavonoids affect plant defence systems (Bourgaud *et al.* 2001). The amount of *Stevia* phenolic compounds in the present study was considerably lower than that reported by Ruiz *et al.* (2015), but was similar to Garcia-Mier *et al.* (2021). Khalvandi *et al.* (2019) reported that accumulation of phenol and anthocyanin in *Mentha piperita* plants inoculated with *P. indica* was positively correlated with antioxidant activity under saline condition. Plants with higher phenolic compounds had higher antiROS activity. Phenolic metabolites are involved in plant responses to different biotic stresses, and are actually vital in plant defence against pathogens (Cvikrová *et al.* 2008). Our findings were in agreement with other reports on *P. indica*-inoculated grape for phenol (Eftekhari *et al.* 2012), on *Eleusine coracana* for flavonoids (Tyagi *et al.* 2017), and on *Mentha piperita* for anthocyanin (Khalvandi *et al.* 2019) contents. The plant defence system is induced by fungi elicitors like glycoproteins and lipopolysaccharides generated by the plant hydrolase enzymes in response to *P. indica* inoculation (Gao *et al.* 2010) that consequently causes an increase of flavonoids and phenol contents (Teshome *et al.* 2015). According to Leamsamrong *et al.* (2019), Se treatment increased the phenolic content of Chinese kale, however, the mechanism was still not fully understood. The phenylpropanoid pathway is connected to the biosynthesis of lignan, lignin, and phenolic compounds, namely phenolic acids and flavonoids (Sreelakshmi and Sharma 2008).

Biosynthesis of phenylpropanoids begins with deamination of phenylalanine to trans-cinnamic acid by phenylalanine ammonia-lyase (PAL). Se increases PAL activity as a key enzyme in the anthocyanin biosynthesis and also chalcone synthase activity. In addition, Hawrylak-Nowak

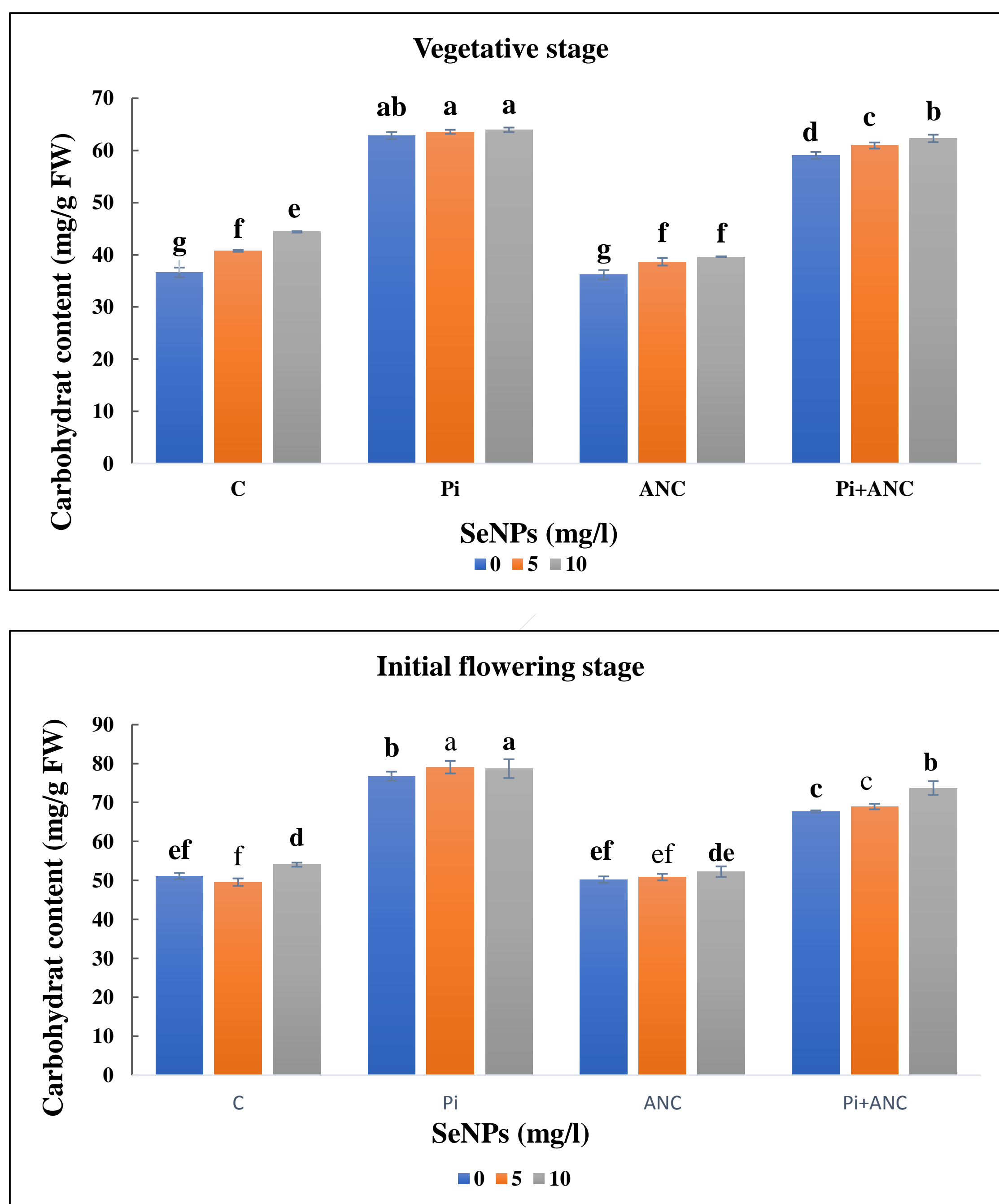

Figure 6. Total carbohydrates content of the *Stevia* plants treated with ancymidol (ANC) and selenium nanoparticles (SeNPs) and inoculated with *P. indica* compared with the control (C): a) vegetative stage and b) initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

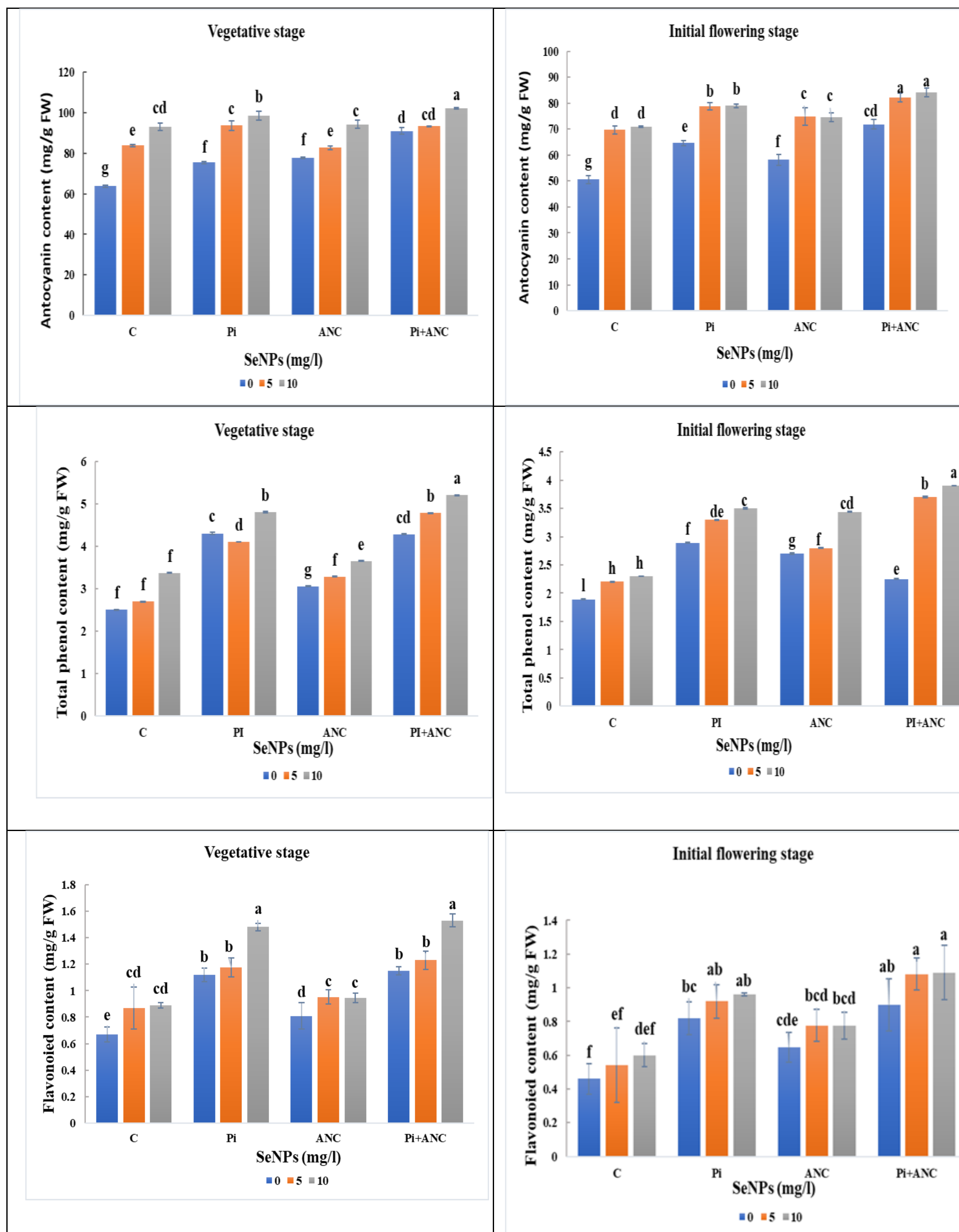

Figure 7. Total phenols, flavonoids, and anthocyanin contents of the *Stevia* plants treated with ancymidol (ANC) and selenium nanoparticles (SeNPs) and inoculated with *P. indica* compared with the control (C): a) Anthocyanin content at the vegetative stage, b) Anthocyanin content at the initial flowering stage, c) Total phenols content at the vegetative stage, d) Total phenols content at the initial flowering stage, e) Flavonoids content at the vegetative stage, and f) Flavonoids content at the initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

(2009) suggested that Se treatment increased the synthesis of anthocyanin and phenolic compounds in lettuce plants. Factors affecting anthocyanin biosynthesis and accumulation in cell cultures have been studied in several plant species and GA_3 was introduced as one of the medium components, which inhibit anthocyanin accumulation (Ozeki and Komamine 1985).

H_2O_2 and MDA contents

Results from H_2O_2 quantitative measurement showed that the exposure of *Stevia* plants to ANC slightly increased the H_2O_2 content but it was significant only at the vegetative stage. SeNPs alone at 10 mg L^{-1} of SeNPs at the vegetative stage and at both concentrations at the initial flowering stage decreased the H_2O_2 content (Figure 8). *P. indica* alone was demonstrated a significant decrease of H_2O_2 content compared to the non-inoculated plants at the vegetative stage, which showed the protecting effect of the fungus on *Stevia* plants. But a slight increase in H_2O_2 content was observed in the *Stevia* plants exposed to ANC. Lipid peroxidation, expressed as MDA concentration, decreased after SeNPs foliar application. *P. indica* inoculation alone showed a greater reduction in MDA concentration in the *Stevia* leaves. ANC+SeNPs significantly increased the MDA content compared to untreated plants. The impact of ANC was mitigated when *Stevia* plants inoculated with *P. indica* (Figure 8), however, some decreases were not significant. H_2O_2 at the vegetative stage was more affected by the applied treatments compared to the initial flowering stage. H_2O_2 is a versatile and deleterious molecule that is continuously produced during plant metabolism and involved in oxidative stress and ROS-scavenging mechanisms (Zou *et al.* 2015). Shahabivand *et al.* (2016) showed the decrease of H_2O_2 and MDA contents in wheat roots inoculated with *P. indica*. They also found that *P. indica* could prevent or retard the degradation of lipids by preventing excess ROS formation. It was shown that the H_2O_2 level in the ANC-treated leaf sections of *Narcissus* was lower than that of untreated leaf sections in liquid-culture (Chen and Ziv 2004). Djanaguiraman *et al.* (2018) showed that application of SeNPs decreased both H_2O_2 and MDA contents, and membrane damage under stress conditions. Selenium cannot directly scavenge H_2O_2, however, it can activate H_2O_2 quenchers [POD, glutathione peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX)], leading to decreased H_2O_2 content. The level of lipid peroxidation measured by the MDA content is useful for determining stress tolerance of plants. Many reports exhibited that MDA and H_2O_2 contents in the plants inoculated with *P. indica* are lower than those in the control plants, indicating the presence of *P. indica* could reduce the peroxidation of membrane lipids (Shahabivand *et al.* 2016). Decreasing MDA concentration in the AMF-inoculated plants may be due to the substantial increase in antioxidant enzymes activities and phosphate metabolism (Tang *et al.* 2009). Oprica *et al.* (2018) showed that the foliar application of SeNPs in *Ocimum basilicum* seedlings reduced MDA content compared to the control. In addition, it has been reported that selenite increased the activity of SOD, POD, and

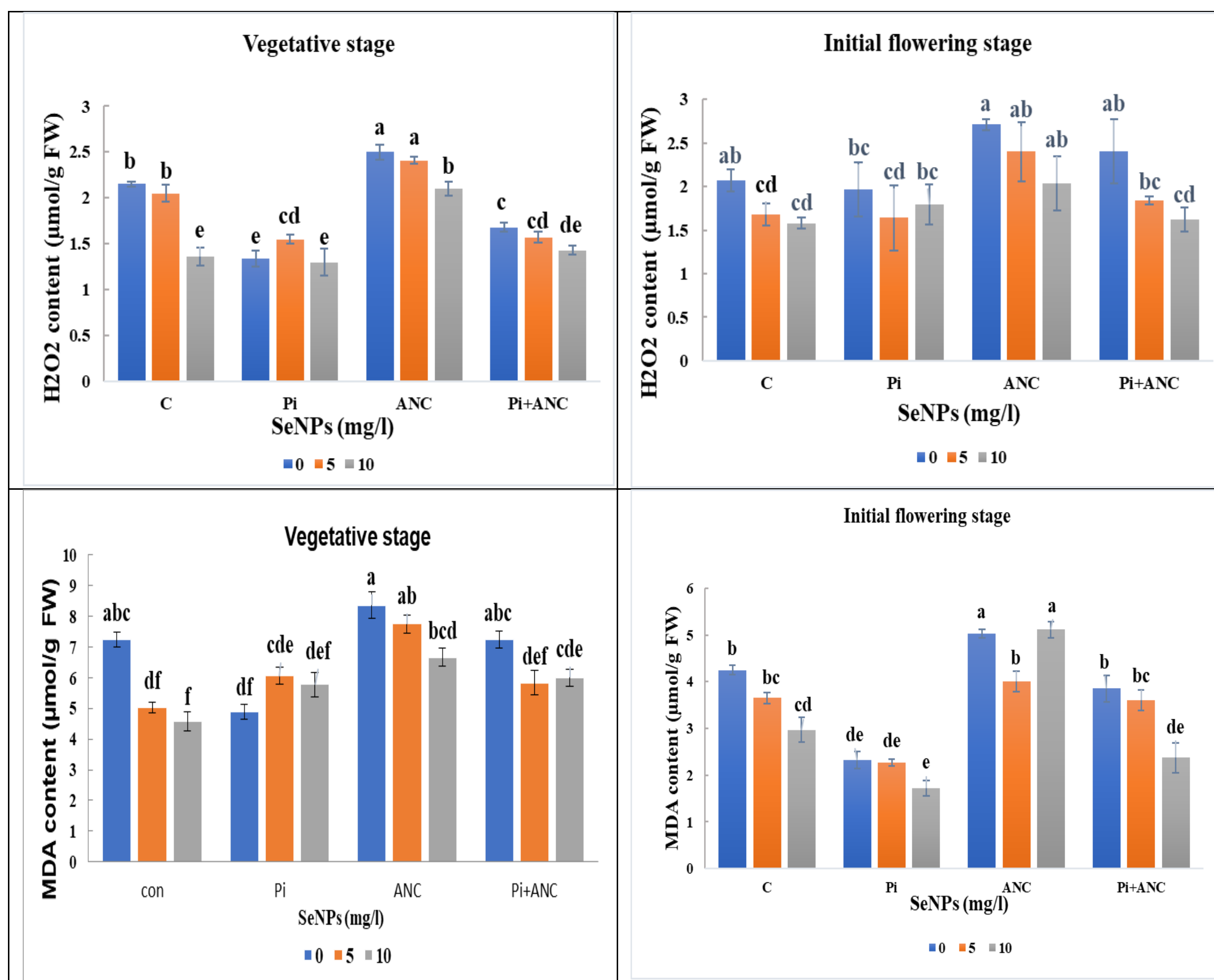

Figure 8. Hydrogen peroxide (H_2O_2) and Malondialdehyde (MDA) contents of the *Stevia* plants treated with ancymidol (ANC) and selenium nanoparticles (SeNPs) and inoculated with *P. indica* compared with control (C): a) H_2O_2 content at the vegetative stage, b) H_2O_2 content at the initial flowering stage, c) MDA content at the vegetative stage, and d) MDA content at the initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

CAT in rape root system, resulted in reduction of lipids peroxidation and MDA content (Xw *et al.* 2015).

Total protein content and antioxidant enzymes activities

Total protein content and antioxidant enzymes activities in the treated *Stevia* plants considerably changed at vegetative stage compared to initial flowering stage. A significant increase of protein content was observed in the plants exposed to SeNPs alone compared to the controls. In contrast, decrease in protein content was not significant in the plants exposed to ANC compared to control, except for ANC+SeNPs with a significant decrease at vegetative stage. Exposure of the plants to ANC led to decrease of SOD activity, but SeNPs alleviated it, however, these changes were not significant. No significant changes in the SOD activity were observed in the plants inoculated with *P. indica* alone (Figure 9). Reduction of SOD activity may be resulted from the increased production of H_2O_2. Our results also demonstrated that SeNPs+*P. indica* inoculation influenced POD

activity significantly at both stages (Figure 9). Notably, the *P. indica*-treated plants represented an elevated POD activity compared to the control. At both concentrations of SeNPs, no significant increase in SOD and POD activities were observed compared to control plants, except at 10 mg L^{-1} SeNPs at the initial flowering stage. Our results showed increased PPO activity in the SeNPs-treated plants compared to control plants but the increase was not significant. On the other hand, an increase of the PPO activity in *P. indica*-inoculated plants was observed at the vegetative stage. The activities of all enzymes, except POD, at 10 mg L^{-1} SeNPs were higher than those at 5 mg L^{-1} SeNPs (Figure 9). The treated *Stevia* plants at the vegetative stage revealed that there is a positive correlation of SOD and PPO activity with the anthocyanin and flavonoids contents of the leaves under *P. indica*-inoculation (Table 2). Our results also demonstrated that the POD activity was positively correlated with the total phenol content in the *P. indica*-inoculated plants. The opposite result was observed in ANC-treated plants. In addition, at the initial flowering stage, the POD activity showed a significant positive correlation with the flavonoids content in the *P. indica*-inoculated plants (Table 3). In the ANC-treated plants, the PPO and POD activities were positively correlated with the total phenol content, and SOD activity with the anthocyanin content. In the ANC-treated plants which inoculated with *P. indica*, only POD activity had positive correlation with the anthocyanin, total phenol and flavonoids contents.

Oxidative stress results in higher activity of

Table 2. Correlation coefficients between evaluated biochemical characteristics in the *Stevia* plants treated with selenium nanoparticles and ancymidol (ANC), and inoculated with *P. indica* at the vegetative stage.

	TP	TF	TA	SOD	POD	PPO
P. indica						
TF	0.84**					
TA	ns	0.74*				
SOD	ns	0.79*	0.82**			
POD	0.76*	ns	ns	0.76*		
PPO	ns	0.81**	0.72*	0.72*	0.67*	
CAR	0.79*	0.79*	ns	0.68*	0.80*	ns
ANC						
TF	ns					
TA	0.94**	ns				
SOD	0.68*	ns	ns			
POD	ns	0.75*	ns	ns		
PPO	0.75*	ns	0.83**	ns	ns	
CAR	0.95**	0.84**	0.95**	ns	ns	0.86**
P. indica + ANC						
TF	0.89**					
TA	0.95**	0.97**				
SOD	ns	ns	ns			
POD	ns	ns	ns	0.77*		
PPO	ns	0.72*	ns	ns	ns	
CAR	0.73*	ns	ns	ns	ns	ns

TP: Total phenol, TF: Total flavonoid, TA: Total anthocyanin, RC: Root colonization, SOD: Superoxide dismutase, POD: Peroxidase, PPO: Polyphenol oxidase, CAR: Carbohydrates. Levels of significance are: *$p \leq 0.05$, **$p \leq 0.01$, ns: non-significant.

antioxidant enzymes such as SOD, POD, and PPO. ROS that produces under normal physiological conditions and biotic and abiotic stresses, alter cellular metabolism by changing the activity of antioxidant enzymes, and also nucleic acids, proteins, and lipid peroxidation (Harinasut *et al.* 2003). Different antioxidant defence mechanisms in plants are used for ROS scavenging including non-enzymatic and enzymatic systems. Non-enzymatic antioxidant defence compounds comprise ascorbate, gluthatione, tocopherol, carotenoids, flavonoids, and antioxidant enzymatic defence system comprise CAT, POD, SOD, and PPO (Kabir *et al.* 2016). PPO is a member of the type-3 copper enzyme family that oxidizes phenolic compounds to quinones and eventually quinones are polymerized to brown pigments (Jiang and Penner 2019). POD can catalyse the oxidation of numerous organic compounds using H_2O_2 as the electron acceptor (Dawson 1992). SOD is involved in scavenging of the highly superoxide anion radicals into water and H_2O_2 (Meloni *et al.* 2003).

Initial stages of AM fungus colonization trigger intracellular ROS burst in the host plant; however, this effect is transient and is overcame by enhanced activities of antioxidant enzymes. The inductive effect of AM symbiosis on activities of the antioxidant enzymes may be the indirect result of the mycorrhizal effects on the host plant growth and procurement of phosphorus and nitrogen (Kapoor and Singh 2017). Mollavali *et al.* (2015) showed that mycorrhizal inoculation of *Allium cepa* caused increase of antioxiodant enzyme activities such as CAT and POD, but not PPO. They also showed that mycorrhizal inoculation induces the biosynthesis of antioxidant enzymes by increasing nutrient uptake or by induction of the plant defence system. Previous studies also have shown that the co-cultivation of *P. indica* with *Arabidopsis*, tobacco, and rice plants enhanced fresh weight and the content of total soluble proteins and free proline, and also the activity of antioxidant enzymes (Sherameti *et al.* 2005).

The positive role of Se on the antioxidative enzymes was reported in several studies. This antioxidant capacity was due to the inhibition of lipid peroxidation and the increased activity of GSH-PX, SOD, and PPO by Se (Djanaguiraman *et al.* 2005). Xue *et al.* (2001) showed that lettuce plants treated with Se, exhibited higher activity of H_2O_2-detoxifying enzymes. Moreover, application of SeNPs increases growth, protects the chloroplast, and improves the chlorophyll biosynthesis by increasing the activity of antioxidant enzymes in *Arachis hypogaea* L. (Hussein *et al.* 2019). The foliar application of SeNPs on sorghum plants increased the activity of SOD and CAT, which increased the tolerance of these plants (Djanaguiraman *et al.* 2018). Studies have shown that the antioxidant properties of SeNPs increase with their surface to volume ratio and decrease the particle size. In agreement with our results, Chen and Ziv (2001) suggested that APX and CAT activities in the leaf sections of *Narcissus* treated with ANC in the liquid media, were lower than those in the untreated cultures. In another report, in the ANC-treated hyperhydric sections of *Narcissus* leaves, SOD, APX, CAT activities, and also H_2O_2 level were lower than

Table 3. Correlation coefficients between evaluated biochemical parameters in *Stevia* plants treated with selenium nanoparticles and ancymidol (ANC), and inoculated with *P. indica* at the initial flowering stage

	TP	TF	TA	SOD	POD	PPO
P. indica						
TF	ns					
TA	ns	0.74*				
SOD	ns	ns	ns			
POD	ns	0.75*	ns	ns		
PPO	ns	ns	ns	ns	0.78*	
CAR	ns	ns	ns	0.68*	ns	ns
ANC						
TF	ns					
TA	0.94**	ns				
SOD	ns	ns	0.69*			
POD	0.74*	ns	ns	ns		
PPO	0.72*	ns	ns	ns	ns	
CAR	0.62**	ns	ns	ns	ns	0.70*
P. indica + ANC						
TF	0.89**					
TA	0.95**	0.97**				
SOD	ns	ns	ns			
POD	0.85*	0.73*	0.76*	0.77*		
PPO	ns	ns	ns	ns	ns	
CAR	0.86**	ns	0.70*	ns	0.79*	ns

TP: Total phenol, TF: Total flavonoid, TA: Total anthocyanin, RC: Root colonization, SOD: Superoxide dismutase, POD: Peroxidase, PPO: Polyphenol oxidase, CAR: Carbohydrates. Levels of significance are: *$p \leq 0.05$, **$p \leq 0.01$, ns: non-significant.

those in the untreated leaf sections. Plant growth retardants such as ANC have been reported to affect cell division and cell enlargement, probably by interfering with the gibberellin biosynthesis as well as to alter protein biosynthesis (Grossmann *et al.* 1986). In addition, a previous study has indicated that three wilt resistant chickpea genotypes showed significant increase in SOD, APX, GPX, and CAT activities under water-deficit stress at the vegetative stage (Dalvi *et al.* 2017). Anjum *et al.* (2008) revealed that activities of enzymatic antioxidants such as SOD, CAT, APX, and glutathione reductase differentially increased in vegetative, initial flowering, and flowering stages in *Brassica campestris* L.

Conclusions

In summary, our results from the present study on the effects of SeNPs and ANC on *Stevia* plants inoculated with *P. indica* indicated that these treatments had different effects on this plant at vegetative and initial flowering stages. SeNPs improved the *P. indica* symbiosis effects on the *Stevia* characteristics by increasing total phenol, flavonoids and anthocyanin contents, POD, and PPO activities and also decreasing H_2O_2 (vegetative stage) and MDA contents (at initial flowering stage. The *P. indica* was more efficient than SeNPs in increasing total soluble carbohydrate and phosphorus contents, which could be related to better symbiosis of fungi with

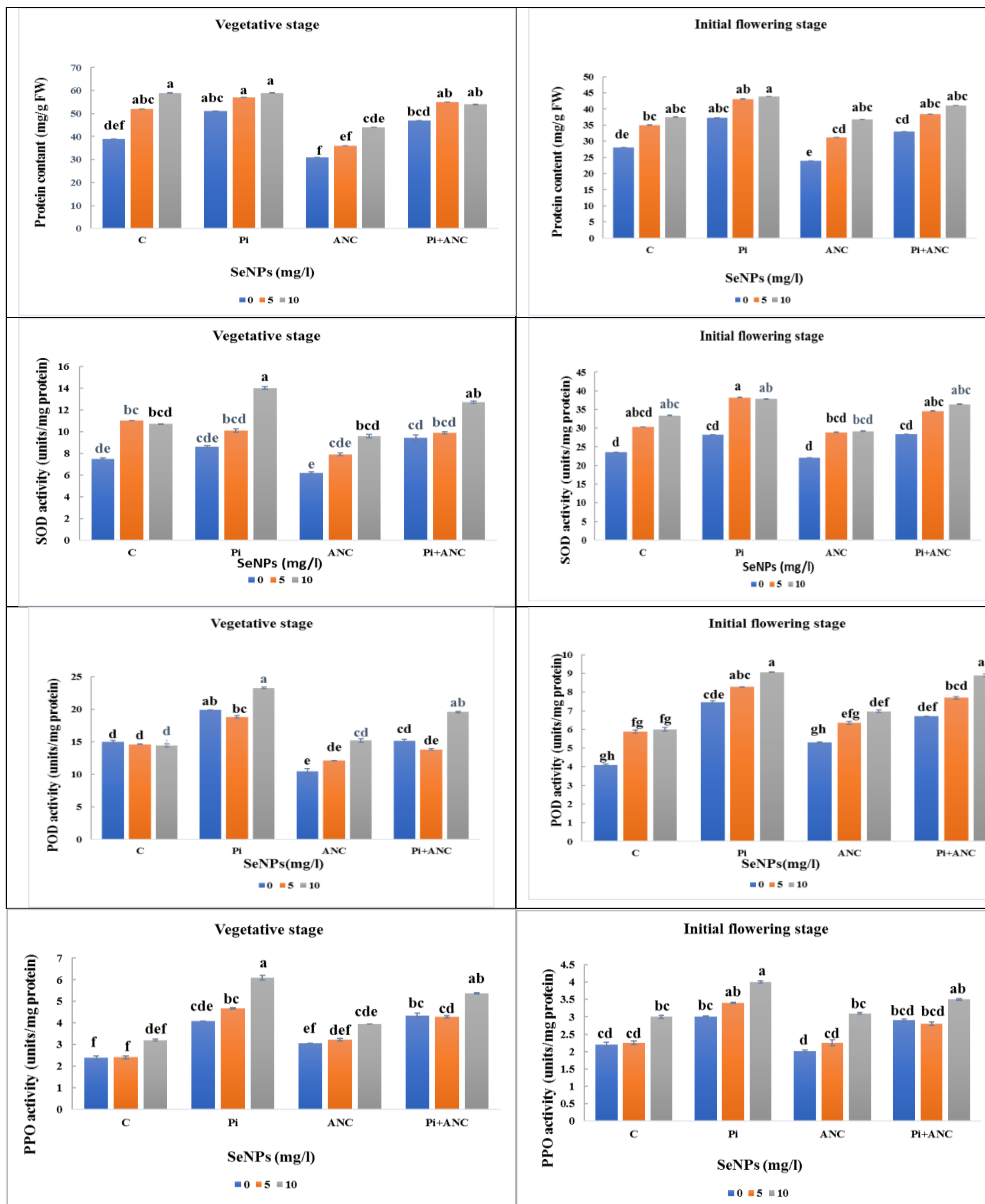

Figure 9. Total proteins content and the activities of antioxidant enzymes in the *Stevia* plants treated with ancymidol (ANC) and selenium nanoparticles (SeNPs) and inoculated with *P. indica* compared with the control (C): a) Total proteins content at the vegetative stage, b) Total proteins content at the initial flowering stage, c) Super oxide dismutase (SOD) activity at the vegetative stage, (d) SOD activity at the initial flowering stage, e) Peroxidase (POD) activity at the vegetative stage, f) POD activity at the initial flowering stage, g) Polyphenol oxidase (PPO) activity at the vegetative stage, (b) PPO activity at the initial flowering stage. Means with different letters indicate significant differences at $p \leq 0.05$ based on Duncan's multiple range test.

Stevia roots. ANC foliar spraying alone or in combination with *P. indica* enhanced positively the SeNPs effects by increasing total phenol, flavonoids and anthocyanin contents. These results suggest that SeNPs in combination with *P. indica* can serve as a nanobiofertilizer for enhancement of the growth and productivity of *Stevia*. Our study recommends further research to discover the important mechanisms in *Stevia* plants under *P. indica* symbiosis and treatment with nanoparticles and plant growth regulators at the molecular level.

According to our results, the maximum activity of antioxidant enzymes and also the maximum content of total protein at the vegetative stage likely was due to the higher rate of colonization roots by *P. indica* in this stage.

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Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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اثرات کاربرد نانوذرات سلنیوم و آنسیمیدول بر پاسخ‌های فیزیولوژیکی گیاه ***Stevia rebaudiana*** **Bertoni** تلقیح شده با قارچ ***Piriformospora indica***

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چکیده

گیاه استویا بومی آمریکای جنوبی و منبع خوبی از استویول گلیکوزیدها، آنتی‌اکسیدان‌ها، اسیدهای آمینه ضروری و سایر ترکیبات مغذی مهم است. در مطالعه حاضر اثر ۵ و ۱۰ میلی‌گرم در لیتر نانوذرات سلنیوم (SeNPs) و ۵۰ میلی‌گرم در لیتر آنسیمیدول (ANC) در دو مرحله رویشی و آغاز گلدهی، بر پویژگی‌های بیوشیمیایی و فیزیولوژیکی گیاه *Stevia rebaudiana*، تلقیح شده با قارچ اندوفیت *Piriformospora indica* مورد بررسی قرار گرفت. نتایج نشان داد که ANC، میزان کلونیزاسیون ریشه و فعالیت آنزیم‌های آنتی‌اکسیدانی را کاهش داد، لیکن محتوای پراکسید هیدروژن (مرحله رویشی)، مالون دی آلدئید (مرحله آغاز گلدهی) و جذب فسفات (مرحله آغاز گلدهی) را افزایش داد و تاثیری بر محتوای کربوهیدرات‌های کل نداشت. اثرات منفی ANC پس از کلونیزاسیون ریشه با قارچ و تا حدودی با کاربرد SeNPs تقلیل یافت. بر اساس نتایج این پژوهش، کلونیزاسیون ریشه با *P. indica* و کاربرد SeNPs قادر است تعادل میان تولید رادیکال‌های آزاد و واکنش‌های دفاع آنزیمی در گیاه استویا را با افزایش پتانسیل مهار رادیکال آزاد و کاهش پراکسیداسیون لیپیدی غشاء در هر دو مرحله رویشی و آغاز گلدهی تغییر دهد. علاوه بر این، اثرات تیمارهای SeNPs، ANC و تلقیح *P. indica* در مرحله رویشی بیشتر از مرحله آغاز گلدهی بود.

واژه‌های کلیدی: آنزیم‌های آنتی‌اکسیدانی؛ استویا؛ استویول گلیکوزیدها؛ بازدارنده رشد؛ قارچ اندوفیت

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