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RESEARCH PAPER

The Effect of Non-biological Elicitors on Physiological and Biochemical Properties of Medicinal Plant *Momordica charantia* L.

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Abstract

Carla (*Momordica charantia* L.) is a medicinal plant of the Cucurbitaceae family, which has antibacterial, anticancer, antitumor, hypoglycemic, hypertensive, and cholesterol properties. In this study, the effects of zinc oxide nanoparticles (ZnO-NPs) (20, 60, and 100 ppm), jasmonate (100, 250, and 500 μM), and chitosan (10, 50, and 100 μM) were measured on the growth and some physiological and biochemical parameters of Carla plant. The results revealed the highest shoot weight of plants at 250 μM jasmonate and 10 μM chitosan, while ZnO-NPs had no significant effect on shoot weight. ZnO-NPs (20–60 ppm) and jasmonate (100 and 200 μM) significantly increased chlorophyll a content, but chitosan showed no effect on chlorophyll a content. Secondary metabolites such as phenols, flavonoids, and carotenoids as well as carbohydrate and proline content were significantly increased by all elicitors in a dose-dependent manner. Antioxidant enzyme activity showed varied responses to different concentrations of elicitors. Jasmonate increased catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidases (GPX) activity in a dose-dependent manner. Chitosan at all concentrations significantly increased CAT and APX enzymes activity, while at 100 μM concentration significantly increased GPX enzyme activity. ZnO-NPs did not affect GPX and AXP enzymes activity. Our findings confirmed for the first time that non-biological elicitors at specific levels have a significant growth promotion effect as well as increased production of valuable secondary metabolites in *M. charantia*.

Keywords *Momordica charantia* · ZnO nanoparticles · Chitosan · Jasmonate · Secondary metabolites

1 Introduction

Prescription of herbal medicine has been prevalent since ancient times (Sharifi-Rad et al. 2020). Carla (*Momordica charantia* L.) is a 1-year-old plant of the Cucurbitaceae family with long creeping vines (Joseph and Jini 2013). Several studies have reported the antidiabetic, anti-inflammatory, antimicrobial, and anticancer properties of *M. charantia* (Grover et al. 2002; Jia et al. 2017). Carla is a good source of carbohydrates, proteins, minerals such as iron, calcium, vitamins, especially vitamin C, and dietary fibers (Yibchok-Anun et al. 2006). It also contains various bioactive metabolites such as phenolic compounds, triterpenes, charantin, momorcharin, saponin, momordin, vicine, oleanolic acids, alkaloids, triterpene glycosides, and saponins (Ahmad et al. 2006; Horax et al. 2010). The researchers reported that the antioxidant activity of this plant is due to the presence of phenolic compounds (Ghous et al. 2015). Phenolic compounds have been widely reported to have high antioxidant, antidiabetic, antimicrobial, anticancer, and anti-inflammatory activities (Deshaware et al. 2017). The importance of bioactive compounds for human health shifts the agricultural practices toward their sustainable production (Björkman et al. 2011). In this regard, elicitation could efficiently induce the production of phytochemicals. Elicitation with various biotic and abiotic elicitors is a possible aid to overcome various difficulties associated with the large-scale production

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of most commercially important secondary metabolites from wild and cultivated plants (Esmaeilzadeh Bahabadi et al. 2012, 2014a). Also, elicitors could induce the production of primary metabolites and affect physiological processes, like growth and yield (Ho et al. 2018). It has been reported that elicitors can relieve reactive oxygen species (ROS) accumulation and malondialdehyde (MDA) content by induction of antioxidant enzyme activities (Esmaeilzadeh Bahabadi et al. 2014b). Jasmonic acid is one of the plant growth regulators that affects a wide range of plant physiological and developmental responses (Creelman and Mullet 1995; Kessler and Baldwin 2002). It is involved in multiple functions such as seed germination, pollen germination, senescence, seedling growth, and fruit ripening (Wang et al. 2008; Li et al. 2018). Furthermore, several studies reported that jasmonic acid could stimulate the phenylpropanoid biosynthetic pathway and increase the content of phenolic compounds in crops (Yu et al. 2002; Ku and Juvik 2013). Furthermore, this phytohormone stimulates free fatty acids, β-carotene, anthocyanin, and lignans accumulation (Esmaeilzadeh Bahabadi et al. 2011; Siddiqi and Husen 2019). Chitosan is a natural biopolymer modified from chitins which act as a potential biostimulant and elicitor in agriculture. It enhances the physiological response and mitigates the adverse effect of abiotic stresses through the stress transduction pathway via secondary messenger(s) (Hidangmayum et al. 2019). Chitosan treatment stimulates photosynthetic rate, stomatal closure, enhances antioxidant enzymes, and induces production of organic acids, sugars, amino acids, and secondary metabolites, which are required for the osmotic adjustment and energy metabolism under stresses (No et al. 2002). Increased shoot and dry root weight, germination, leaf area index, and chlorophyll content have also been reported in chitosan-affected maize and bean plants (Sheikha and Al-Malki 2011). Chitosan helps the root system of the plant absorb more nutrients from the soil, thus stimulating the plant growth (Cho et al. 2005). It has also been reported that the priming of rice seeds with chitosan increases growth, proline content, and total carbohydrate content under salinity stress (Ruan and Xue 2002). Chitosan seems to be particularly effective to increase the content of a large spectrum of phenylpropanoids and antioxidants in crops. All these compounds can be ingested by humans through daily diet, providing health benefits, such as the increase in antioxidant capacity of blood and potential prevention of cancer and cardiovascular diseases (Ferri and Tassoni 2011). Nowadays, nanoparticles (NPs) are gaining industry attention for their potential applications in agriculture to enhance crop production (Jasrotia et al. 2018). NPs can be used as a source of plant nutrients and stimulate the production of bioactive compounds (Dimkpa and Bindraban 2017). Researchers suggested that efficacy and the effect of engineered NPs on plant growth and physiology depend on the composition, physical, and chemical properties of NPs, concentration, as well as plant species and vegetative stage (Hossain et al. 2020). Earlier studies have demonstrated the potential of zinc oxide nanoparticles (ZnO-NPs) for stimulation of seed germination and plant growth as well as secondary metabolites induction (Faizan et al. 2020). There are very few reports about the effect of elicitors on physiological properties of *M. charantia*. Recently, in a research have reported 5 mg/L AgNPs as the optimum concentration for maximum accumulation of phenolics and flavonoids in cell suspension cultures of *M. charantia* (Chung et al. 2018). To our knowledge the possible effects of jasmonate, chitosan, and ZnO-NPs elicitors on production of phytochemicals and physiological responses in *M. charantia* plant has not been investigated. This research aims: (1) to evaluate whether jasmonate, chitosan and ZnO-NPs elicitors could effectively increase biomass, secondary metabolite (phenolic compounds and carotenoids) accumulation in *M. charantia* and (2) to measure the response of Carla plants to the application of possible elicitors, through the quantification of enzymatic and non- enzymatic antioxidant enzymes, malondialdehyde (MDA), and proline.

2 Methods

2.1 Plant Cultivation and Treatments

The experiments were carried out in a plant physiology research laboratory at the University of Zabol (Zabol, Iran). Seeds were planted in pots filled with 250 g of 4:2 mixture of Coco peat and soil.

In each pot, three seeds were sown at 1 cm depth. The pots were placed in a germination chamber at 28° C, 16 h light, and 8 h dark. All pots were irrigated with distilled water until elicitor treatments started. At the 4-leaf stage of growth, foliar sprays were applied of ZnO-NPs (20, 60, and 100 ppm), jasmonate (100, 250, and 500 μM), and chitosan (10, 50, and 100 μM). Each treatment included three replicates, and samples were taken after 15 days from the date of planting to estimate growth and physiological activities. The fresh biomasses were subjected to overnight heat in the oven at 70 °C for 72 h for full dehydration. Some growth-related parameters, including shoot length and shoot weight, were evaluated for all of the employed treatments.

2.2 Quantification of Chlorophyll, Carotenoid, and Anthocyanin

To measure the chlorophyll and carotenoid contents, 0.1 g of fresh leaves from each treatment was immersed in 80% acetone solvent to extract pigments. The extract was centrifuged at 2500 rpm for 10 min. The supernatants were transferred to different vials, to quantify the total content of the pigments by spectrophotometer. The content of chlorophyll *a* and *b* was measured at 663 and 645 nm, respectively, and carotenoid obtained at 440.5 nm using Eq. 1 (Lichtenthaler 1987).

$$\begin{aligned} C_a &= 0.0127 \cdot \mathrm{D663} - 0.00269 \cdot \mathrm{D645} \\ C_b\left(\mathrm{mg\ g^{-1}fresh\ leaf}\right) &= 0.0299 \cdot \mathrm{D645} - 0.00468 \cdot \mathrm{D663} \\ C_{\mathrm{car}}\left(\mathrm{mg\ g^{-1}fresh\ leaf}\right) &= 0.004695 \cdot \mathrm{D440.5} \\ &\quad - 0.000268(C_a + C_b), \end{aligned} \tag{1}$$

where C_a and C_b represent the content of chlorophyll *a* and *b* (mg g^{-1} fresh leaf), respectively. The C_{car} (mg g^{-1} fresh leaf) corresponds to the total content of carotenoid.

Total anthocyanin contents of blueberry extracts were estimated spectrophotometrically according to Wagner's method using a molar absorptivity coefficient of 133,000 mm^{-1} - cm^{-13} and reported as mg per g of FW (Bürkle et al. 2018).

2.3 Measurement of Total Phenol and Flavonoids Content

Total phenol content was determined using a spectrophotometer, following the Folin–Ciocalteu method; the gallic acid (0–1000 µM) was used as the standard to determine the results as gallic acid equivalents (mg GAE/g dry weight (McDonald et al. 2001). The total flavonoid content was measured based on the protocol adopted from Chang et al. (2002), in which the flavonoid content was calculated as mg of quercetin per gram dry weight.

2.4 Determination of Carbohydrate and Proline

Carbohydrate levels were measured by Dubois's method. Briefly, 0.5 g of fresh plant crushed in 5 ml of distilled water, then filtered and 2 ml from plant extract transferred to a test tube. Then, supernatant mixed with 1 ml of 5% phenol (v/w) and 5 ml of sulfuric acid. Finally, each tube was incubated for 1 h at 37 °C. They were left until the purple color appears and stabilized. After the appearance of the dye, the absorbance at 490 nm was measured by a spectrophotometer; glucose would be used as a standard curve to measure the sugar content (Dubois et al. 1956).

Proline content of leaves was determined according to Bates's method. 0.04 gg of leaves was homogenized in 15 mL of aqueous 3% sulfosalicylic acid and the homogenate was filtered. The filtrate (2 mL) was mixed with 2 mL of ninhydrin reagent (containing 20 ml of 6 M phosphoric acid, 30 ml of glacial acetic acid, and 1.25 g of ninhydrin). The absorbance of the colored solutions was measured at 520 nm (Bates et al. 1973).

2.5 Determination of Antioxidant Enzymes Activity

Catalase (CAT) enzyme activity was measured according to Aebi (1984). The reaction mixture consisted of 2.5 ml of 50 mM phosphate buffer (pH 7) containing 0.2 ml of 1% H_2O_2 and 0.3 ml of extract. The CAT activity was calculated as a reduction in absorbance over 1 min at 240 nm. The extinction coefficient (0.0436 m^{-1} cm^{-1}) was used to measure the activity.

Guaiacol peroxidases (GPX) enzyme activity was measured according to Upadhyaya's method. Reaction mixture consisted of 2.5 ml 50 mM phosphate buffer (pH 7) containing 1 ml of 1% guaiacol, 1 ml of H_2O_2 1%, and 0.1 ml of extract. The addition of H_2O_2 initiated the reaction, and the increase in absorbance at 420 nm was determined for 1 min. The extinction coefficient (26.6 Mm^{-1} cm^{-1}) was used to measure the activity (Upadhyaya et al. 1985). Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada, 1981. The reaction mixture consisted of 2.5 ml 50 mM phosphate buffer (pH 7) containing 0.1 mM EDTA, 0.5 mM ascorbic acid, 0.3 ml H_2O_2 1%, and 0.1 ml extract. APX activity was calculated as a decline in H_2O_2 uptake over 1 min at 240 nm.

2.6 Determination of Lipid Peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content that was determined by the Heath and Packer's method. Fresh leaves (0.15 g) were homogenized with 2 ml of ice-cold 50 mM phosphate buffer (pH 7.8) and centrifuged at 6000*g* for 5 min. Next, 4 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to 1 ml of the supernatant. The mixture was heated in a water bath shaker at 95 °C for 10 min and quickly cooled in an ice bath. The samples were centrifuged at 6000*g* for 10 min. The absorbance was measured at 532 nm (Heath and Packer 1968).

2.7 Statistical Analysis

To reduce the error, experiments were performed in 3 replications. Statistical analysis was performed according to the completely randomized block design via SPSS software using a one-way ANOVA and Duncan test at $p < 0.05$.

3 Results

3.1 Effect of ZnO-NPs, Chitosan, and Jasmonate on Growth

The results showed that the shoot length was not affected by ZnO-NPs ($p \leq 0.05$). Shoot length increased significantly at 10 µM concentration of chitosan ($p \leq 0.05$). At 250 µM and 500 µM of jasmonate, shoot length increased significantly ($p \leq 0.05$) compared to control (Fig. 1a).

The results of this study revealed that ZnO-NPs had no significant effect on the shoot weight of Carla plant ($p \leq 0.05$). The plant shoot weight at 10 µM chitosan was significantly elevated ($p \leq 0.05$), but no change was observed in the shoot weight at 50 µM and 100 µM chitosan. A significant increase in shoot length was observed at 250 and 500 µM of jasmonic acid treatment. The highest shoot weight of jasmonate-treated plant was found at 100 µM concentration ($p \leq 0.05$) (Fig. 1b).

The application of high concentrations of ZnO-NPs in wheat resulted in biomass reduction (Lin and Xing 2008). Root growth inhibition can be attributed to the high susceptibility of root apical meristem to NPs and the effect of zinc on indole acetic acid oxidase at the root level (Fiskesjo 1997). The studies showed that NPs increased growth parameters of tomato (Elmer and White 2016) and no effect on lettuce (Liu et al. 2016). Researchers suggested that efficacy and the effect of engineered NPs on plant growth and physiology depend on the composition, physical and chemical properties of NPs, concentration, as well as plant species and vegetative stage (Hossain et al. 2020).

Growth and yield of soybean improved by chitosan (Dzung and Thang 2004). In another study, researchers found that pretreatment of Ajwain plant with chitosan significantly enhanced the germination rate, root, and shoot length, as well as shoot and root weight (Mahdavi and Rahimi 2013). Foliar application of olive leaf with jasmonic acid increased the leaf area (El-Sayed et al. 2014). Jasmonate has been shown to accelerate root growth and increase plant growth parameters such as leaf area and shoots dry weight (Sairam et al. 2002). The effect of different concentrations of jasmonic acid on the growth, height, and weight of the marigold plant has been reported. Plants treated with 150 and 225 µM jasmonic acid caused the highest plant height and dry weight, respectively (Ataei et al. 2013). It has been suggested that jasmonic acid at physiological concentrations plays a crucial role in growth and metabolism expressed by the changes in the content of photosynthetic pigments and the soluble protein accumulation, while at higher concentrations promotes typical senescence symptoms (Czerpak et al. 2006).

3.2 The Effect of ZnO-NPs, Chitosan, and Jasmonate on Photosynthetic Pigments

ZnO-NPs elicitor significantly increased chlorophyll a content at the range of concentrations 20–60 ppm. Chlorophyll b content significantly increases at 20 ppm of ZnO-NPs. Chlorophyll a and chlorophyll b content decreased at 100 ppm of ZnO-NPs. Chitosan showed no effect on chlorophyll a and chlorophyll b content. The results showed that chlorophyll a content was significantly elevated at 100 and 200 µM. Jasmonate showed no effect on chlorophyll b content (Table 1).

The results showed that the amount of carotenoid significantly increased in all concentrations of ZnO-NPs and chitosan compared to control. Jasmonate at concentrations of 250 and 500 µM significantly enhanced the carotenoid content compared to control (Table 1). Exposure of plants to high concentrations of heavy metals reduces the biosynthesis of chlorophyll. It has been reported that substitution of Pb^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+} in chlorophyll instead of $Mg^{+2,}$ which is the main functional mechanism of heavy metal toxicity results in reduced chlorophyll and

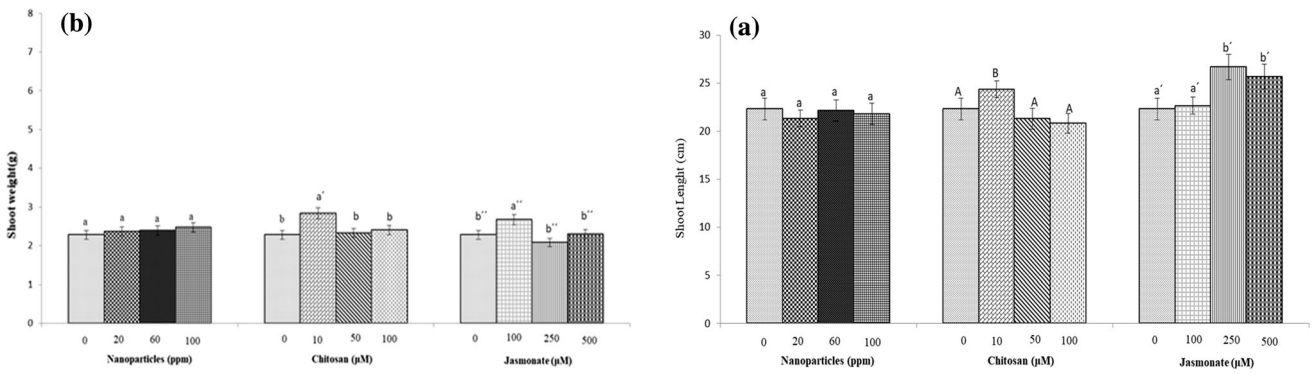

Fig. 1 Effect of different concentrations of ZnO-NPs, chitosan, and jasmonate on shoot length (**a**) and shoot weight (**b**) of Carla plant; The values shown are the mean of 3 replicates and ± SD (standard deviation). The meanings that have common words in each treatment were not statistically significant ($p \leq 0.05$)

Table 1 Effect of different concentrations of ZnO-NPs, chitosan, and jasmonate on chlorophyll a, chlorophyll b, carotenoid, anthocyanin, phenol, flavonoid, and antioxidant enzyme activity

Treatments	Control	Zno nanoparticles (ppm)		
	0	20	60	100
Chlorophyll a content (mg/g FW)	12.146 ± 0.8000	14.2 ± 0.2701	10.50 ± 1.0743	8.10 ± 0.8747
Chlorophyll b content (mg/g FW)	11.14 ± 0.800000	14 ± 0.566113	8.61 ± 1.041420	7 ± 0.459113
Carotenoid content (mg/g FW)	1.086 ± 0.103329	2.767 ± 0.370167	3.034 ± 0.200000	3.855 ± 0.204087
Anthocyanin content (mg/g FW)	208.940 ± 11.75954	232.00 ± 16.46141	221.43 ± 9.66124	227.78 ± 5.19617
Flavonoid content (mg/g DW)	7 ± 0.539569	10.13 ± 0.800000	12.34 ± 0.372118	14.32 ± 1.250288
Phenolics content (mg/g DW)	1760.6731 ± 139.54844	2023.0000 ± 186.79760	2664.0385 ± 313.50840	2678.0000 ± 78.46407
CAT activity (u/mg FW/mg protein)	0.310 ± 0.01000	0.320 ± 0.04333	0.440 ± 0.02693	0.500 ± 0.03333
GPX activity (u/mg FW/mg protein)	15.977 ± 3.32263	12.600 ± 2.15405	11.050 ± 2.56190	14.143 ± 2.89821
APX activity (u/mg FW/mg protein)	2.462 ± 0.10000	2.85 ± 0.15631	2.900 ± 0.07783	3.000 ± 0.09678

Treatments	Chitosan (μm)			Jasmonate (μm)		
	10	50	100	100	250	500
Chlorophyll a content (mg/g FW)	11.83 ± 2.003553	11.35 ± 0.7149	12.52 ± 1.1189	13.47 ± 0.3453	13.91 ± 0.2753	12.54 ± 1.0744
Chlorophyll b content (mg/g FW)	11.3 ± 0.890000	9.99 ± 0.502002	9.13 ± 1.000000	11.07 ± 1.729116	10.17 ± 1.139964	9.13 ± 1.273783
Carotenoid content (mg/g FW)	1.600 ± 0.130967	1.500 ± 0.088206	1.900 ± 0.132821	1.000 ± 0.093813	1.600 ± 0.050000	1.800 ± 0.159038
Anthocyanin content (mg/g FW)	218.54 ± 18.87674	217.60 ± 5.66177	233.54 ± 15.52625	254.12 ± 9.87158	233.01 ± 11.48823	249.22 ± 9.00000
Flavonoid content (mg/g DW)	13.29 ± 0.175588	15.52 ± 0.214969	13.5 ± 0.482539	14.78 ± 1.453471	16.50 ± 1.493023	17.64 ± 0.561732
Phenolics content (mg/g DW)	2392.0192 ± 72.75149	2854.4231 ± 188.09258	3075.9615 ± 50.60982	2001.2115 ± 81.65729	2308.0769 ± 103.08075	2734.8077 ± 118.71204
CAT activity (u/mg FW/mg protein)	0.390 ± 0.02858	0.400 ± 0.03487	0.450 ± 0.01000	1.280 ± 0.08718	3.673 ± 0.08819	4.826 ± 0.13852
GPX activity (u/mg FW/mg protein)	13.000 ± 1.05208	9.526 ± 0.98468	5.666 ± 0.81668	13.330 ± 1.43915	14.273 ± 0.43472	19.430 ± 1.20000
APX activity (u/mg FW/mg protein)	3.756 ± 0.70000	5.873 ± 0.93198	6.566 ± 0.76460	2.870 ± 0.21885	3.100 ± 0.33766	3.686 ± 0.42540

photosynthetic breakdown (Sengar et al. 2008). Inhibition of critical reactions in the chlorophyll biosynthesis pathway (5-aminolevulinic acid biosynthesis, aminolevulinic acid dehydrase, and radionuclide reductase) reduces chlorophyll storage in leaves (Vara Prasad and de Oliveira Freitas 2003).

Chitosan has been shown to increase chlorophyll content in soybeans and peanuts (Dzung and Thang 2004). Chitosan increased the amount of chlorophyll and carotenoids in coffee (Dzung et al. 2011). Methyl jasmonate also prevents the degradation of photosynthetic pigments by increasing the activity of antioxidant enzymes such as superoxide dismutase in chloroplasts and the removal of free radicals (Mckersie and Ya'acov 1994; Popova et al. 1997). Methyl jasmonate also induced the expression of key enzymes genes involved in chlorophyll biosynthesis by stimulating the formation of 5-aminolevulinic acid (Ueda and Saniewski 2006).

3.3 Effect of ZnO-NPs, Chitosan, and Jasmonate on Anthocyanin Content

The results showed that the anthocyanin content significantly increased under all concentrations of jasmonate (Table 1). Chitosan and ZnO-NPs showed no effect on anthocyanin content. Stressful environmental factors (salinity, drought, cold, UV radiation, and air pollution) cause accumulation of anthocyanin pigments in the leaves. The significant roles of anthocyanins can be attributed to the antioxidant and protective function of the photosynthetic system against photosynthesis, which plays a protective role in stressed plants (He et al. 2010). Anthocyanins are secondary metabolites of plants that are synthesized by the propanoid phenyl pathway with the ability to absorb free radicals when strained. Chitosan has been reported to increase anthocyanin in plants through the effect of crucial enzyme activity pathway on the production of phenylpropanoid derivatives (Chakraborty et al. 2009). The results of this study are consistent with findings of other researchers which observed jasmonate and salicylic acid increased anthocyanin content in alfalfa (López-Moreno et al. 2010), Arabidopsis (Jung 2004), licorice (Shabani et al. 2009), carrots (Sudha and Ravishankar 2003), sunflower (Parra-Lobato et al. 2009).

3.4 Effect of ZnO-NPs, Chitosan, and Jasmonate on Secondary Metabolites

This research showed that flavonoid and phenol content significantly increased by all elicitors used in this study in a dose-dependent manner (Table 1). Similarly, in potato plants, the amount of phenol affected by silver nanoparticles in a dose-dependent manner (Homaee and Ehsanpour 2015). Titanium dioxide increased phenol and flavonoid content in *Salvia officinalis* (Ghorbanpour and Hadian 2015). It is reported that CuO-NPs and ZnO-NPs have a positive effect on phenolic compounds accumulation (Oloumi et al. 2015). Phenolic compounds are potent inhibitors of oxidative stress, which cooperated with peroxidases in the removal of hydrogen peroxide (Kovácik et al. 2009). free hydroxyl groups in phenols are responsible for free radical removal activity (Kowalska et al. 2014). Other studies also reported increased production of phenols (Díaz et al. 2001) and flavonoids (Bota and Deliu 2011) following the application of non-biological elicitors. Chitosan treatment enhanced the phenolic compounds and activity of antioxidant enzymes in tomatoes (Liu et al. 2007).

Studies have shown that the use of naturally occurring compounds, such as methyl jasmonate, can increase secondary metabolites. The treatment of mulberry jasmine with methyl jasmonate significantly increased flavonoid content in these plants (Wang et al. 2008).

3.5 Effect of ZnO-NPs, Chitosan, and Jasmonate on Antioxidant Enzyme Activity

The results showed that the catalase enzyme activity was significantly elevated by 60 and 100 ppm of ZnO-NPs. Chitosan at all concentrations significantly increased the catalase enzyme activity.

Jasmonate significantly increased in the catalase enzyme activity in a dose-dependent manner (Table 1). ZnO-NPs did not affect GPX enzyme activity. Chitosan at 100 µM concentration significantly increased GPX enzyme activity. Jasmonate significantly increased the GPX enzyme activity in a dose-dependent manner (Table 1). The activity of AXP in ZnO-NPs-treated plants was not significantly different at all concentrations from that of control. Chitosan induced a significant rise in AXP activity at 10, 50, and 100 µM concentrations. AXP enzyme activity was significantly increased at all concentrations of jasmonate (Table 1).

Catalase catalyzes the conversion of H_2O_2 to water and oxygen and regulates H_2O_2 concentration in tissues. This is essential because H_2O_2 is a relatively long-lived ROS that has the ability to diffuse widely from the site of its generation and penetrate certain biological membranes (Sharifan et al. 2019). ZnO-NPs increased the activity of the catalase enzyme in *Fagopyrum esculentum* (Pandey et al. 2012). With increasing Zn content, antioxidant enzymes such as catalase increases (Hosseini and Poorakbar 2013). Chitosan increased the activity of antioxidant enzymes such as catalase and polyphenol oxidase in the root of eggplant (Mandal 2010). Chitosan treatment has been shown to increase the activity of antioxidant enzymes and phenolic compounds in tomatoes (Liu et al. 2007).

Furthermore, chitosan increased the activity of peroxidase and catalase enzymes in two maize species and safflower seedlings (Guan et al. 2009). Chitosan also enhanced the activity of CAT and AXP enzymes in basil (Naderi et al. 2014), which is consistent with our results which chitosan upregulated the activity of CAT and AXP at all concentration and GPX (at 100 µM) as well. Co-activation of antioxidant enzymes also reported in chitosan-treated *Trachyspermum ammi* (Naderi et al. 2016). Overall, the increase in enzyme activities could be one of the major protection mechanisms against ROS generation. Application of methyl jasmonate in peanut seedlings increased protein content and enhanced the activity of superoxide dismutase, catalase, and peroxidase enzymes (Kumari et al. 2011).

3.6 Effect of ZnO-NPs, Chitosan, and Jasmonate on Carbohydrate

The results showed that the carbohydrate content under different concentrations of ZnO-NPs, chitosan and jasmonate was significantly higher than the control (Fig. 2).

Increased content of soluble carbohydrates may be due to the osmotic adjustment mechanism in the plant. Carbohydrate accumulation is effective in maintaining cell membrane and osmotic regulation (Mckersie and Ya'acov 1994). Some research have shown that by increasing the concentration of heavy metals, the intracellular water balance is impaired, causing ultrastructural changes in cellular organelles and metabolism of sugars. Also, by increasing the concentration of heavy metals, the amount of invertase activity decreases. The increase in sugars content may be a kind of adaptive mechanism to maintain the osmotic potential under the stress of ZnO-NPs. In this study, an increase in the content of soluble sugars treated with chitosan was observed, possibly due to the hydrolysis of starch (Kovácik et al. 2009). The plant's defense mechanism against stress requires some osmotic adjustment. This osmotic adaptation can be achieved through synthesizing intracellular soluble compounds (Serrano and Rodriguez-Navarro 2001). A study found that the amount of soluble sugars increased by chitosan treatment in safflower seedlings that is similar to the results of this experiment (Mahdavi et al. 2011).

Researchers found that foliar application of rice with chitosan increased stress soluble carbohydrates under stress conditions (Boonlertnirun and Sarobol 2005), which is in line with the results of this study. Chitosan seems to have an indirect role in the biosynthesis and degradation of sugars under stress conditions. Therefore, chitosan may be useful in reducing the detrimental effects of dehydration on plants by increasing the soluble carbohydrates in plants and response to osmotic regulation and preserving the cells' water potential. It has been reported that that carbohydrate content increased with the elevation of the chitosan concentration (Khajeh and Naderi 2014). As the concentration of chitosan increases, trans-structural changes occur in cellular organelles such as tonoplast and metabolism of sugars. This is an adaptive mechanism for preserving osmotic potential under chitosan treatment.

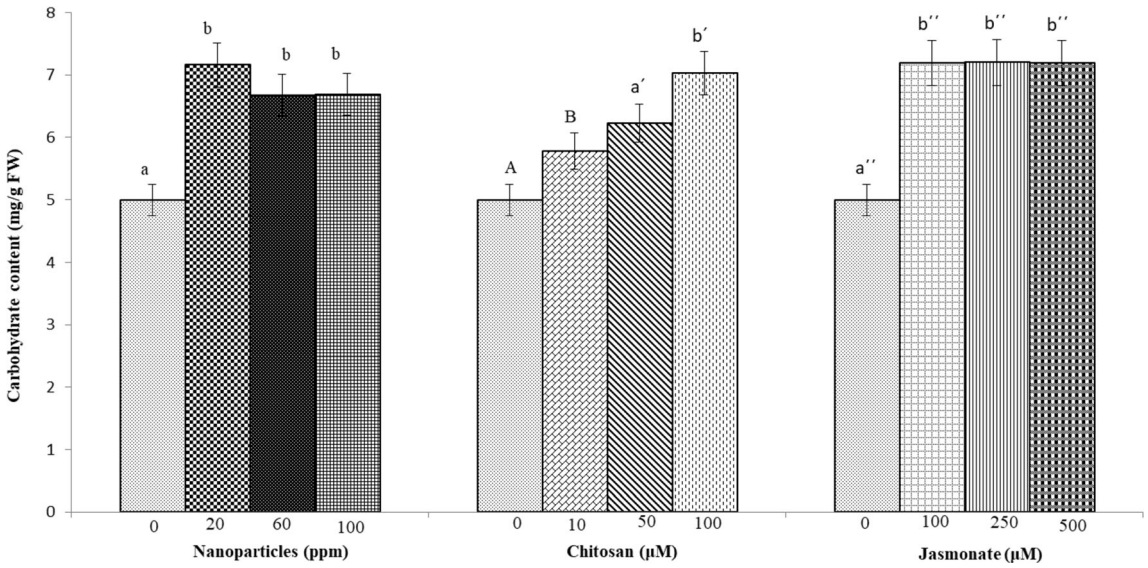

Fig. 2 Effect of different concentrations of ZnO-NPs, chitosan, and jasmonate on the carbohydrate content of Carla plant; The values are shown the mean of 3 replicates and ± SD (standard deviation). The meanings that have common words in each treatment were not statistically significant ($p \leq 0.05$)

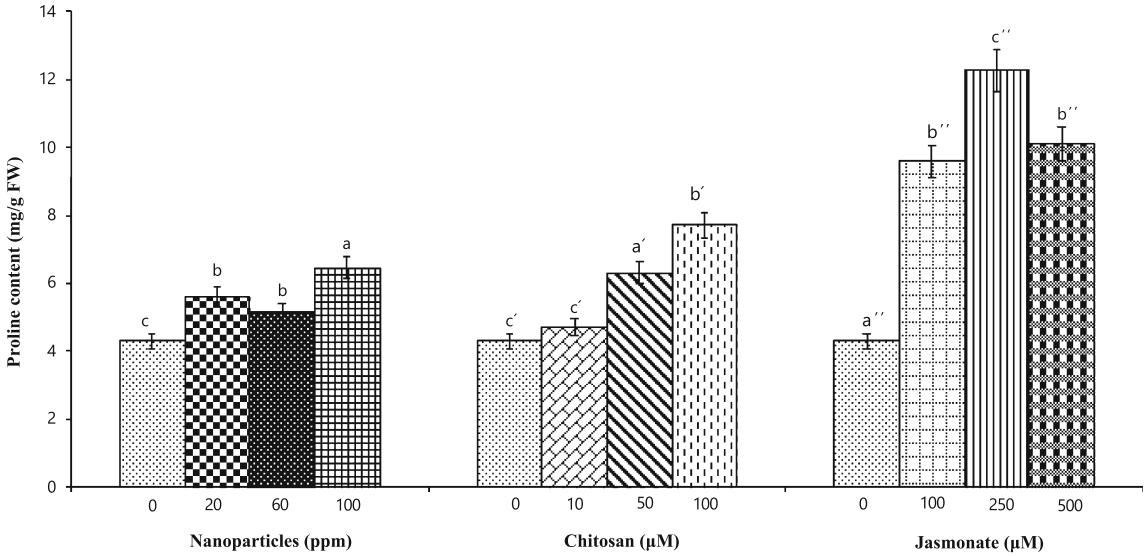

Fig. 3 Effect of different concentrations of ZnO-NPs, chitosan, and jasmonate on prolin content of Carla plant; The values are shown the mean of 3 replicates and ± SD (standard deviation). The meanings that have common words in each treatment were not statistically significant ($p \leq 0.05$)

3.7 Effect of ZnO-NPs, Chitosan, and Jasmonate on Proline Content

The results showed that the proline content increased by all concentrations of ZnO-NPs. Proline content was significantly increased under concentrations of 50 and 100 µM chitosan. All concentrations of jasmonate significantly increased the proline content compared to control (Fig. 3).

Under stress conditions, proline accumulation occurs more than other amino acids, which may contribute to osmotic regulation and possibly maintenance of plant enzymatic activity (Ashraf and Harris 2004). Proline plays a vital role in the improvement of environmental stresses, including the stresses of heavy metals in plants and microorganisms (Siripornadulsil et al. 2002). Proline stabilizes proteins and chelates metals and prevents lipid peroxidation and reactive oxygen species (Shah and Dubey 1998). Thus, during the stress of heavy metals, proline production is enhanced to protect the plant against toxicity. In addition to osmotic regulation, proline also acts as a protector against stress, thereby directly interacting with macromolecules, and further supporting the maintenance of the shape of proteins and the natural structure of stress-affected biological membranes (Kuznetsov and Shevyakova 1999). The researchers reported the increased level of proline chitosan and jasmonate (Mahdavi et al. 2011; Wasternack and Kombrink 2009).

3.8 Effect of ZnO-NPs, Chitosan, and Jasmonate on Lipid Peroxidation

The results of the study showed that all concentrations of ZnO-NPs, chitosan, and jasmonate significantly increased lipid peroxidation (Fig. 4).

Under normal growth conditions, many metabolic processes in plants produce reactive oxygen species, but plants have efficient antioxidant mechanisms to eliminate reactive oxygen species (Ma et al. 2020; Sharifan et al. 2020). Under stress conditions, this balance is disturbed, and the amount of reactive oxygen species increases. The presence of these active species is harmful to the plant and damages cellular structures such as membranes, proteins, and nucleic acids (Laspina et al. 2005). Measurement of lipid peroxidation products is one of the most common and accepted methods of measuring oxidative damage to the membrane (Shulaev and Oliver 2006). According to Mckersie and Ya'acov (1994), antioxidant enzymes are present in peroxisomes, cytosols, and mitochondria and cause H_2O_2 to H_2O and O_2 conversion. Numerous studies have shown that chitosan, as a biological elicitor, may have the potential to eliminate free radicals (Kim and Thomas 2007; Yen et al. 2008). It has been suggested that the amount of malondialdehyde increased by chitosan (Naderi et al. 2014). External application of methyl jasmonate can lead to increased production of reactive oxygen species such as superoxide and hydrogen peroxide. These species result in the peroxidation of membrane lipids by producing malondialdehyde (Charles and Simon 1990).

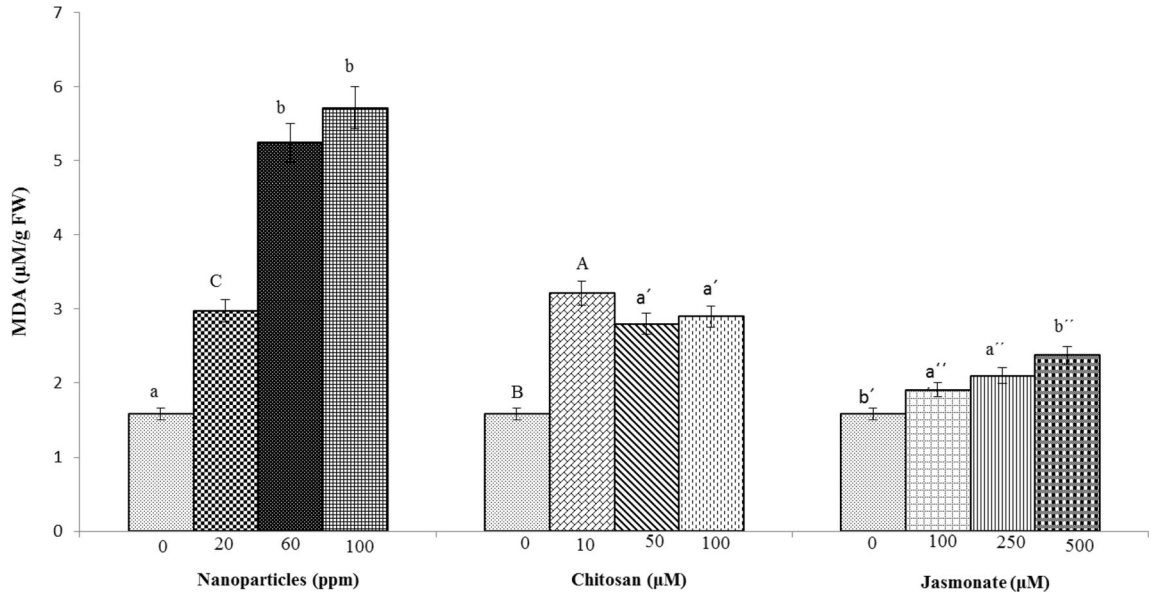

Fig. 4 Effect of different concentrations of ZnO-NPs, chitosan, and jasmonate on malondialdehyde content of Carla plant; The values are shown the mean of 3 replicates and ± SD (standard deviation). The meanings that have common words in each treatment were not statistically significant ($p \leq 0.05$)

3.9 Conclusion

In the present study, we evaluated the physiological responses of *M. charantia* in exposure to various concentrations of selected non-biological elicitors. We found that chitosan (10 μM) and jasmonate (250 μM) were acting as growth stimulators. Secondary metabolites (phenols, flavonoids, carotenoids) significantly increased by all used elicitors. Based on our result, *M. charantia* combat oxidative stress induced by elicitors through increasing in proline, carbohydrate, phenolic, malondialdehyde content, and up-regulation of antioxidant enzymes activity as well. Furthermore, the present study also suggests that the elicitors used in this study are useful for the production of bioactive compounds of *M. charantia* via metabolic engineering techniques.

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Compliance with ethical standards

Conflict of interest Authors declare that they have conflict of interest to this work.

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