Research Article

Received: 3 September 2020 Revised: 11 November 2020 Accepted article published: 21 December 2020 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11030

Chitosan stimulates secondary metabolite production and nutrient uptake in medicinal plant *Dracocephalum kotschyi*

Samaneh Kahromi and Jalil Khara*

Abstract

BACKGROUND: A wide variety of secondary metabolites are synthesized from primary metabolites by plants which have a vast range in pharmaceutical, food additive and industrial applications. In recent years, the use of elicitors has opened a novel approach for the production of secondary metabolite compounds. *Dracocephalum kotschyi* is a valuable herb due to pharmaceutical compounds like rosmarinic acid, quercetin and apigenin. In the current study, foliar application of chitosan (0, 100, 400 mg L^{-1}) as an elicitor was used.

RESULTS: After chitosan treatment, the amounts of hydrogen peroxide (H_2O_2) increased and the plant was able to increase the activities of enzymatic (guaiacol peroxidase, catalase and phenylalanine ammonium lyase) and non-enzymatic (total phenols and flavonoids) defensive metabolites. Also, foliar spray of chitosan promoted nutrient absorption which led to the accumulation of macroelements in the plant.

CONCLUSIONS: Chitosan was found to be a very effective elicitor for improving rosmarinic acid and quercetin content (up to 13-fold). Also, the content of apigenin (anticancer flavonoid) showed 16-fold enhancement compared to the control. Therefore, the treatment of *D. kotschyi* leaves with chitosan caused a very large increase in the induction and production of important pharmaceutical compounds such as rosmarinic acid and quercetin.

Keywords: chitosan; *Dracocephalum kotschyi*; elicitor; pharmaceutical compounds; secondary metabolites; nutrient absorption

INTRODUCTION

Medicinal plants are an important source of nutrition and organic metabolites for maintaining human health.[1] *Dracocephalum kotschyi* Boiss. is one of eight endemic species of *Dracocephalum* in Iran, and a prominent medicinal plant, the leaves of which are utilized as an additive to improve the taste and scent of tea and yogurt.[2,3] Also, the essential oil of this plant could be used as a food additive and safe curative agent for food purposes.[4] *D. kotschyi* using the IUCN grouping criteria is one of the vulnerable species in Iran. Extra harvesting of wild plants, limited distribution areas and lack of cultivation and domestication are the principal reasons why *D. kotschyi* is now listed as an endangered plant.[5,6] This plant is used as a traditional medicine for the treatment of liver and stomach diseases and fever. Some medicinal properties of this plant have been confirmed such as antioxidant, anti-nociceptive, antispasmodic, anti-inflammatory, antimicrobial, antitumor, cytotoxic and immunomodulatory effects.[7,8] Research has revealed that all parts of *D. kotschyi* have active molecules like essential oils, monoterpene glycosides, trypanocidal terpenoids and flavonoids.[3] Essential oils of *D. kotschyi* include citral, caryophyllene, terpinyl, acetate, limonene, α-terpineol, δ-3-carene, α-pinene, terpinen-4-ol, geranial, limonene-10-al and 1,1-dimethoxydecane.[9] This plant has anticancer properties due to its content of methoxylated flavones such as xanthomicrol, apigenin, isokaempferid, cirsimaritin and penduletin. Spinal-Z (anticancer drug) is produced by Iranian researchers, which has an effect against leukemia, and is extracted from *D. kotschyi* leaves and *Peganum harmala* seeds.[8,10] Phenolic acids such as cinnamic, chlorogenic and caffeic acids are found in *D. kotschyi*. Phenolic acids can be used as preventive and therapeutic agents for many diseases that are related to oxidative effects.[11] Also, rosmarinic acid (RA) is one of the efficient natural antioxidants in this plant, which exhibits various pharmacological activities including astringent, antibacterial, antiviral, anti-inflammatory and antiallergic action.[12]

The enhancement of secondary metabolites of medicinal plants by elicitors is one of the few strategies that have recently been commercially used. Elicitors are signal compounds that increase the synthesis of secondary metabolites by inducing pathways in response to exogenous stresses.[12] The use of elicitors effectively promotes the production of a range of secondary plant metabolites, both *in vivo* and *in vitro*. During elicitor treatment, the elicitor binds to the receptors on the plasma membrane or endomembrane. Then elicitor signal perception initiates signal transduction that results in activation or *de novo* biosynthesis of transcription

* *Correspondence to: J Khara, Department of Biology, Faculty of Science, Urmia University, PO Box 57561-51818, Urmia, Iran. E-mail: j.khara@urmia.ac.ir*

Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

factors (TFs), which regulate the expression of some biosynthetic genes involved in plant secondary metabolism.[13] Elicitors can be classified according to their origin into two types: biotic and abiotic. Both biotic and abiotic elicitors are molecules within the elicitation pathways and can induce a similar plant defensive reaction against pathogen infections or herbivore attack.[14]

The application of both types of elicitors is a strategy that has been very beneficial in reducing the processing time required to gain high product concentrations and increase product quantity.[15] Biotic elicitors include polysaccharides derived from insects and fungi, such as chitin and chitosan that are mostly applied for induction of secondary metabolites.[16]

Chitosan is a natural, nontoxic, inexpensive compound and has antibacterial properties, which has been widely used in medicines, foods and cosmetics. Besides, chitosan has agricultural applications, used to promote plant growth, improve crop yield and control plant diseases.[17] Chitosan can induce plant stress response with the consequent enhancement in phytoalexin and reactive oxygen species (ROS) accumulation. Low amounts of ROS are signal molecules that trigger series of cellular responses from the expression of certain genes to production of secondary metabolites, but higher ROS levels cause damage to membranes and other essential macromolecules, such as oxidation of proteins, DNA and lipids.[11] Also, chitosan stimulates other systems; for example, it can activate mitogen-activated protein kinase (MAPK) signaling cascades (triggering the production of ROS, inducing the expression of MPK genes, and boosting the transcription of defense-related genes) in some plants.[18] Chitosan is used both *in vivo* and *in vitro* as well as being applied on plant aerial organs, and as elicitor could enhance secondary metabolites of plants, such as terpenoids and phenolics.[19]

The low levels of secondary metabolites of *D. kotschyi* can be enhanced by the overproduction of these metabolites using simple methods such as spraying of elicitors to stimulate the synthesis of these metabolites. In the study reported here, an attempt was made to investigate the effect of chitosan on the content of shoot elements and plant phytochemical responses, including the content of phenol, flavonoid and secondary metabolites, antioxidant enzyme activity and phenylalanine ammonia lyase (PAL) activity. The main purpose of the study was to increase the secondary metabolites of *D. kotschyi* using chitosan.

MATERIALS AND METHODS

Plant materials and chitosan treatment

Chitosan (CAS no. 9012-76-4; degree of deacetylation degree of 85%) was purchased from Sigma-Aldrich (Shanghai, China). Chitosan was dissolved in 5% acetic acid and diluted in distilled water to the required concentrations (100 and 400 mg L^{-1}). The pH of chitosan solutions was adjusted to 6.5 with 2 mol L^{-1} NaOH.

Seeds for the experiment were obtained from Pakan Bazr Company, Isfahan, Iran. Seeds were soaked in 98% sulfuric acid for 10 min to remove the external germination inhibitor that was previously established for *D. kotschyi*.[20] Seeds were sterilized using 5% sodium hypochlorite for 5–10 min and washed with sterile distilled water three times. Seeds were put in Petri dishes and, after germination, seedlings were planted in plastic pots, containing a mixture of Peat Mas, sandy soil and Perlite (3:2:1). After 5 weeks, foliar application of chitosan solutions on plants was performed. The chitosan treatment was sprayed using a hand sprayer on the aerial parts until complete wetness; untreated control plants were sprayed with an equivalent volume of distilled water. Treatment was performed three times at intervals each week. After 1 week from the last treatment, the plants were harvested. After finishing the experiment, growth parameters were measured including the fresh and dry weight of roots and shoots. The total amount of some elements, hydrogen peroxide (H_2O_2) content and activities of antioxidant enzymes and PAL were measured. Also, phytochemical responses including total phenolic and flavonoid contents, contents of RA, some phenolic acids and methoxy flavonoids were determined.

The pot culture experiment was conducted in a glasshouse of the Department of Biology, Urmia University. The temperature in the greenhouse was maintained at 18–25 °C with a photoperiod of 16 h day/8 h night.

Elemental analysis

Samples of shoots were dried at 65 °C for 48 h in an oven. After drying, samples (500–800 mg) were weighed and ground samples of the plants were mineralized in a mixture of nitric and perchloric acids for 24 h. The total amounts of calcium (Ca), potassium (K), magnesium (Mg), zinc (Zn), phosphorus (P), manganese (Mn) and nickel (Ni) were determined directly from the extract using atomic absorption spectrometry (Thermo Scientific iCE 3000 series).[21]

Total contents of organic sulfur (S) and nitrogen (N) were determined in dried ground shoots (50–80 mg) using a CHNS analyzer (Elementar Vario Macrocube).[22]

Determination of H_2O_2 content

Fresh leaves and roots (500 mg) were homogenized in an ice bath with 5 mL of trichloroacetic acid (0.1% w/v) and centrifuged for 15 min at 10 000 × *g*. Then 0.5 mL of the supernatant was added to 0.5 mL of 10 mmol L^{-1} potassium phosphate buffer (pH 7) and 1 mL of 1 mol L^{-1} KI. The absorbance of the samples was spectrometrically measured at 390 nm.[23] The H_2O_2 content was calculated by comparison with a standard calibration curve prepared using various concentrations of H_2O_2.

Enzyme assays

Roots and leaves (150 mg, FW) were homogenized in 2 mL of 50 mol L^{-1} Tris–HCl buffer (pH 7) containing 3 mmol L^{-1} $MgCl_2$ and 1 mmol L^{-1} EDTA and centrifuged at 12 000 × *g* for 10 min at 4 °C. The supernatant was used for catalase (CAT) and guaiacol peroxidase (GPX) assays.[24]

Catalase activity

CAT activity was assayed in accordance with Aebi[25] with minor modification. The assay contained 3 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.0; potassium salts), 25 µL of H_2O_2 solution (1%) and 25 µL of the prepared extract, and the absorbance was measured at 240 nm. One unit of CAT activity was described as the amount of enzyme required to decompose 1 mmol of H_2O_2 per minute. For the calculation of CAT activity, an extinction coefficient of 0.026 L $mmol^{-1}$ cm^{-1} was used.[25]

Guaiacol peroxidase activity

GPX activities were assayed according to the method of Chance and Maehly.[26] The test mixture contained 1.5 mL of phosphate buffer (50 mol L^{-1}, pH 6.8), 1 mL of guaiacol (1%) and 200 µL of enzyme extract. The reaction was initiated by adding 1 mL of H_2O_2 and the changes of absorption were recorded spectrophotometrically at 470 nm for 1 min. One unit of GPX activity was defined the amount of enzyme that causes the formation of

Table 1. Effects of different concentrations of chitosan on fresh and dry weight of root and shoot in *D. kotschyi*

Chitosan (mg L^{-1})	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)
0	0.196 ± 0.009^a	0.022 ± 0.002^a	0.524 ± 0.051^a	0.082 ± 0.001^a
100	0.207 ± 0.025^a	0.024 ± 0.005^a	0.540 ± 0.039^a	0.098 ± 0.002^a
400	0.195 ± 0.016^a	0.021 ± 0.003^a	0.541 ± 0.057^a	0.091 ± 0.001^a

Values within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's multiple range test. Values are the average of three replicates ± standard deviation.

1 mmol of tetraguaiacol per minute. The GPX activity was calculated using an extinction coefficient of 26.6 L $mmol^{-1}$ cm^{-1}.[26]

PAL activity

Extraction and testing of PAL were performed using the method of Ke and Saltveit[27] with some modifications. Briefly, 0.3 g of the fresh material was homogenized in 6 mL of sodium borate buffer (0.1 mol L^{-1}, pH 8.8) including β-mercaptoethanol (5 mm), EDTA (2 mm) and 1% (p/v) insoluble poly(vinyl pyrrolidone). The extract was centrifuged at 10 000 × *g* for 20 min. Then 5 mL of supernatant was added to 1 mL of 100 mol L^{-1} phenylalanine and incubated at 40 °C for 60 min. Finally, the absorbance of the samples was read at 290 nm using a spectrophotometer.[27]

Total phenolic content

Leaf and root samples (100 mg) were homogenized in 5 mL of 80% methanol and centrifuged at 10 000 × *g* for 15 min. Then the total phenols of the methanolic extract were measured with Folin–Ciocalteu reagent at 760 nm.[28] Briefly, 1 mL of each sample was mixed with 9 mL of distilled water and 1 mL of Folin–Ciocalteu phenol reagent and was kept for 5 min at room temperature. Finally, 10 mL of Na_2CO_3 solution (7% w/v) was added and all test tubes were incubated for 90 min at room temperature. The absorbance of samples was measured at 750 nm against a reagent blank using a spectrophotometer and expressed as gallic acid equivalents with reference to a calibration curve.

Total flavonoid content

Total flavonoid contents of root and leaf extracts were determined using a method described as the aluminium chloride assay by Zhishen *et al.*[29] with some modifications. Firstly, 0.25 mL of methanolic extracts was mixed with 0.15 mL of a 5% $NaNO_2$ solution. After 5 min, 0.3 mL of a 10% $AlCl_3$ solution and 2 mL of a 1 mol L^{-1} NaOH solution were added and the solution was mixed well. After incubation of the samples for 15 min at 25 °C, the absorbance was measured at 510 nm and expressed as quercetin equivalents (QUE) with reference to a calibration curve.[29]

Extraction of phenolic compounds

Frozen leaves (1 g) were homogenized with 80% methanol (3 mL) under sonication for 25 min. The samples of extract were centrifuged at 2000 rpm for 10 min. After filtration, the supernatant was used for high-performance liquid chromatography (HPLC) analysis.

HPLC analysis

Isolation, characterization and quantification of biochemical compounds in the plant were performed using an HP 1100 series HPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with a 20 µL injection loop, quadruple gradient pump, degassing system, column oven (set to 25 °C) and diode array detector, set at wavelengths of 250, 272 and 310 nm.

Isolation of octadecylsilane was done with a ZORBAX Eclipse XDB column (particle size: 5 µm; inner diameter: 4.6 mm; length: 250 mm). Chemstation software was used for data processing. To better isolate the compounds, a washing gradient program was used. Firstly, the mobile phase started with a ratio of 10% acetonitrile (solvent A) and 90% solution of acetic acid (1% v/v in water) (solvent B). The gradient elution program was as follows: 10–25% (v/v) A at 0–5 min, 25–65% (v/v) A at 5–15 min. The separation time was 15 min.[30] The flow rate used for column elution was 1 mL min^{-1}.

HPLC chromatograms were obtained using a photodiode array detector at various wavelengths according to the absorption maxima of analyzed compounds. Each compound was identified by its retention time (RT) and by spiking with standards under the same conditions. Quantification of the samples was done by measurement of the integrated peak area and the contents were calculated using the calibration curve by plotting the peak area against the concentration of the respective standard sample. Identification of RA (RT 11.5, $\lambda_{max} = 250$ nm), gallic acid (RT 4.52, $\lambda_{max} = 272$ nm), caffeic acid (RT 8.54, $\lambda_{max} = 310$ nm), chlorogenic acid (RT 9.3, $\lambda_{max} = 250$ nm), coumaric acid (RT 10.85, $\lambda_{max} = 310$ nm), cinnamic acid (RT 14.79, $\lambda_{max} = 272$ nm),

Table 2. Effect of different concentrations of chitosan on total content of C, S, N, Ca, Mg, K, Mn, Fe, Cu and Zn in *D. kotschyi*

Chitosan (mg L^{-1})	S (%)	N (%)	Ca (g kg^{-1} DW)	Mg (g kg^{-1} DW)	K (g kg^{-1} DW)	P (g kg^{-1} DW)	Ni (mg kg^{-1} DW)	Zn (mg kg^{-1} DW)	Mn (mg kg^{-1} DW)
0	0.43 ± 0.06^a	3.41 ± 0.05^a	4.69 ± 0.2^b	0.53 ± 0.1^b	12 ± 1^b	5.7 ± 0.6^a	6.7 ± 0.1^a	48.7 ± 2^a	155.1 ± 7^a
100	0.48 ± 0.09^a	3.52 ± 0.2^a	28.3 ± 3^a	6.4 ± 0.5^a	40 ± 2^a	4.9 ± 0.5^a	6.4 ± 0.2^a	51.5 ± 3^a	157.3 ± 9^a
400	0.47 ± 0.07^a	3.53 ± 0.1^a	25.3 ± 2^a	6.5 ± 1^a	42.1 ± 3^a	5 ± 0.6^a	5.3 ± 0.3^b	38.4 ± 1^b	137.8 ± 5^b

Ca, calcium; K, potassium; Mg, magnesium; Mn, manganese; N, nitrogen; Ni, nickel; P, phosphorus; S, sulfur; Zn, zinc. Values within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's multiple range test. Data are means of three replicates.

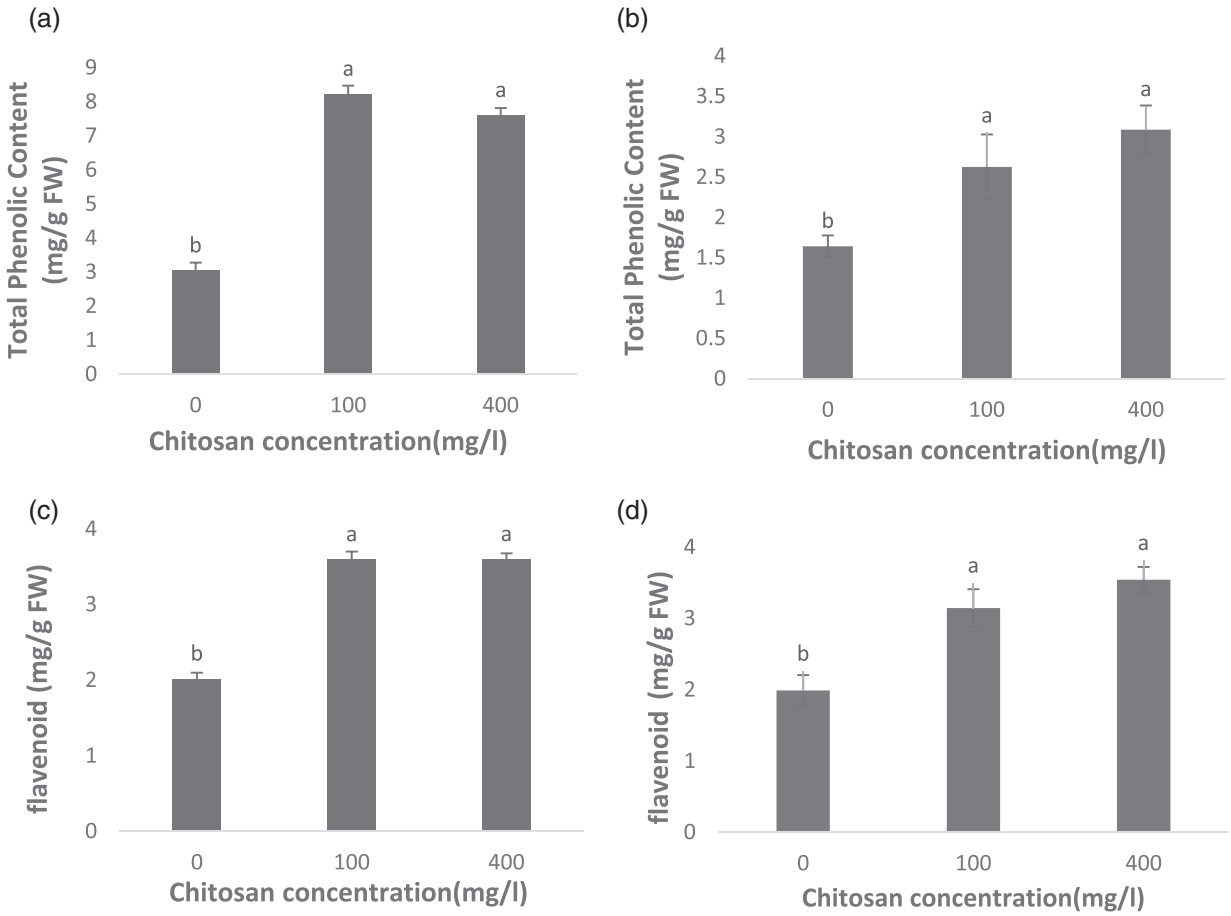

Figure 1. Effects of different concentrations of chitosan on total phenol content in leaves (a) and roots (b) and flavonoid content in leaves (c) and roots (d) of *D. kotschyi*. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Table 3. Content of H_2O_2 and enzymatic activities of GPX and CAT in roots and leaves of *D. kotschyi* treated with different concentrations of chitosan

Chitosan (mg L^{-1})	Root content of H_2O_2 (mg g^{-1} FW)	Leaf content of H_2O_2 (mg g^{-1} FW)	Root GPX activity (U mg^{-1} protein)	Leaf GPX activity (U mg^{-1} protein)	Root CAT activity (U mg^{-1} protein)	Leaf CAT activity (U mg^{-1} protein)
0	0.19 ± 0.02^{b}	0.36 ± 0.02^{ab}	0.037 ± 0.003^{b}	0.009 ± 0.001^{c}	4.64 ± 0.57^{b}	8.9 ± 0.9^{a}
100	0.21 ± 0.01^{ab}	0.50 ± 0.02^{a}	0.039 ± 0.002^{b}	0.013 ± 0.001^{b}	6.53 ± 0.63^{a}	11.2 ± 1.3^{a}
400	0.25 ± 0.01^{a}	0.52 ± 0.04^{a}	0.054 ± 0.002^{a}	0.019 ± 0.003^{a}	8.34 ± 0.92^{a}	9.4 ± 0.5^{a}

Values within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's multiple range test. Values are the average of three replicates ± standard deviation.

quercetin (RT 13.31, λ_{max} = 250 nm) and rutin (RT 10.47, λ_{max} = 310 nm) was done by comparing their RT and UV λ_{max} values with those of the standard compounds. The calibration curves were prepared using six assays with the standard for each compound.

The surface flavonoids were separated using the mentioned Agilent 1100 HPLC system equipped with a Knauer analytical column (125 cm × 4 mm inner diameter) packed with 5 µm Spherimage 80 and octadecylsilane as end-capping sorbent (E. Merck) according the procedure of Fattahi *et al.*[31]

Statistical analysis

All data were analyzed using SPSS software (Version 22) and were introduced as the mean and standard deviation of three replications (n = 3). The experimental design was based on completely randomized factorial and one-way analysis of variance was used. Significant differences were determined among the means using the Duncan test at $P < 0.05$.

RESULTS AND DISCUSSION

Growth

In this study, foliar application of chitosan had no significant effect on the biomass of *D. kotschyi* (Table 1). Similar to our results, previous studies have also shown that foliar spray of chitosan did not impact the growth of plants.[32,33] There are various feasible reasons for different impacts on plant growth, including differences in application methods, application time and the use of different

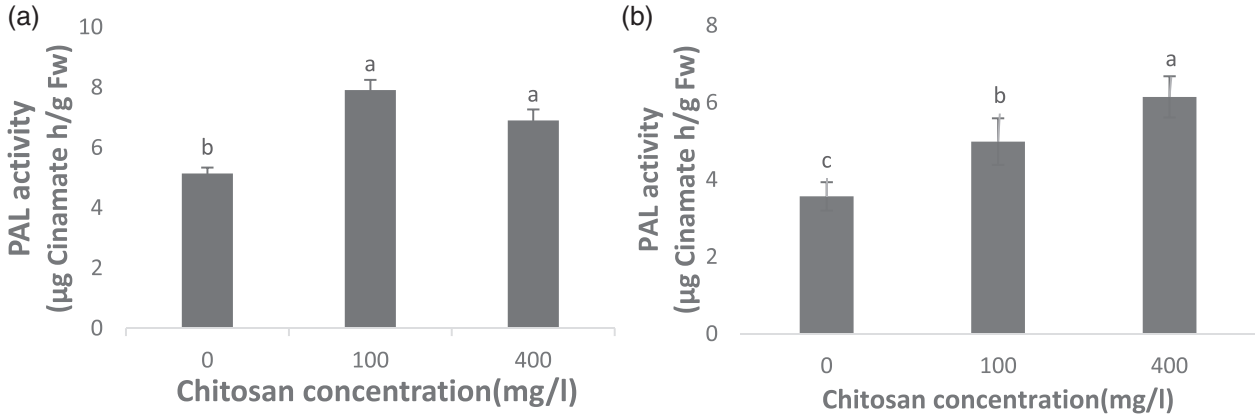

Figure 2. Effects of different concentrations of chitosan on PAL activity in leaves (a) and roots (b) of *D. kotschyi*. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

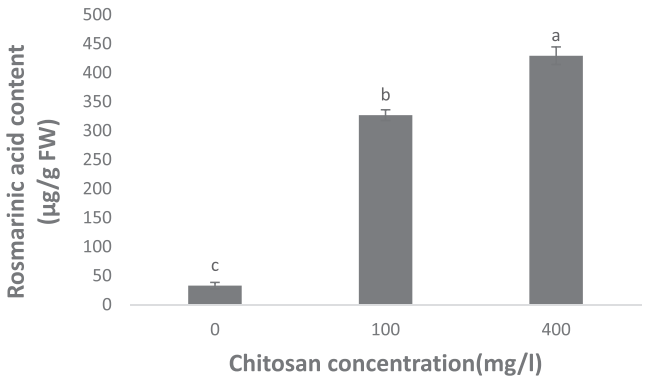

Figure 3. Effects of different concentrations of chitosan on RA content in leaves of *D. kotschyi*. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

plant species.[34] In contrast with our results, significant effects of chitosan on the growth of many plants have been observed.[35,36]

Elemental analysis

Table 2 reveals that chitosan increased the total content of most of the macroelements in *D. kotschyi*. A significant increase was observed in the contents of Ca and Mg, as well as the K content, compared to the control plants. The Ca content was found to increase sixfold with a treatment of 100 mg L^{-1} compared to the control. The K and Mg contents were found to increase 3.5-fold and 12.3-fold, respectively, with a treatment of 400 mg L^{-1}. Previous studies demonstrated that plants treated with chitosan exhibited an increased accumulation of various mineral nutrients such as N, K, Mg and Ca.[37,38] Ahmad *et al*. stated that increases in N and K contents of peppermint leaf were evident after foliar application of chitosan. Positive effects of chitosan may come from its providing some amino compounds required for plant growth.[39] Winkler *et al*. reported that plants may be capable of degrading chitosan and releasing small fragments of chitin that may be used as a source of nutrients.[40] In our research, chitosan had no significant effect on the content of P and microelements in *D. kotschiy* (Table 2).

Foliar exposure to chitosan and chitosan oligosaccharides leading to an enhanced level of antioxidants might be involved in maintaining the nutrient status through membrane stabilization resulting in protection of plasma membranes from oxidative damage and enhancing plant cell permeability that finally stimulates nutrient absorption.[19]

Total phenolic and flavonoid contents

Our results indicated that plants sprayed with chitosan showed an increase in total phenolic and flavonoid contents (Fig. 1). Phenolic compounds are well known to have antioxidant properties.[41] Various authors have reported that chitosan treatment efficiently enhanced the total amount of phenolic compounds in some plants.[42,43] Chitosan may be involved in the signaling pathway for the biosynthesis of phenolics. It has been shown that chitosan can induce chitinase and chitosanase, which are members of a group of plant pathogenesis-related proteins. These pathogenesis-related proteins can degrade the cell walls of some phytopathogens and consequently may play a role in host plant defense systems.[44] Similarly, it has been reported that the application of chitosan enhances phenolic compounds.[45]

Synthesis of flavonoid and phenolic compounds is initiated very quickly after elicitation. Overall, elicitors like chitosan are involved in some signal transduction systems that induce gene expression of enzymes of the secondary metabolic pathway, such as PAL.[46] A positive correlation was observed between PAL activity and the total content of phenols and flavonoids which shows that chitosan treatment can regulate the accumulation of phenolic compounds through stimulating PAL activity.

Flavonoids are water-soluble polyphenolic molecules that exhibit health-boosting effects, are antioxidants and free radical scavengers and have anticancer, anti-inflammatory and antidepressant activities. An increase in flavonoid level indicates an enhancement in the amount of plant nutrients[47] and a positive relationship was observed between the contents of flavonoids and macroelements in the current study. Udomsuk *et al*. reported that chitosan at 150 mg L^{-1} enhanced isoflavonoid production in *Pueraria candollei*.[48]

H_2O_2 content

The results of this study indicated that chitosan spray treatments had a significant impact ($P \leq 0.05$) on the H_2O_2 content (Table 3). It was reported chitosan stimulates the oxidative burst and the induction of the ROS scavenging system, especially the production of H_2O_2.[11,33,49] Also, Chamnanmanoontham *et al*. stated that chitosan increased production of H_2O_2 by expression of NADPH oxidase in rice.[50] A plant's NADPH oxidases regulate the signaling cascades in response to abiotic stresses. ROS are involved in intra-

Table 4. RA, phenolic acid and surface flavonoid contents (μg g^{-1} FW) in *D. kotschyi* treated with different concentrations of chitosan

Chitosan (mg mL^{-1})	Phenolic acids							Methoxylated flavonoids			
	Cin	Gal	Caf	Chl	Rut	Cou	Que	Api	Iso	Cir	Pen
0	0.57 ± 0.2^{b}	19.8 ± 1^{b}	7.65 ± 2^{b}	41.2 ± 2^{c}	15.8 ± 2^{a}	44.4 ± 2^{a}	5.37 ± 1^{c}	1.5 ± 0.3^{c}	6.29 ± 1^{a}	0.15 ± 0.04^{a}	0.85 ± 0.2^{a}
100	29.19 ± 4^{a}	57.5 ± 3^{a}	14.1 ± 3^{a}	108 ± 7^{a}	4.2 ± 1^{b}	132 ± 6^{b}	71.7 ± 6^{a}	54.2 ± 5^{a}	—	0.072 ± 0.02^{b}	0.10 ± 0.04^{b}
400	4.65 ± 2^{b}	54.9 ± 4^{a}	2.94 ± 1^{c}	75.7 ± 3^{b}	3.4 ± 0.5^{b}	118 ± 4^{b}	14.46 ± 3^{b}	24.9 ± 3^{b}	0.07 ± 0.01^{b}	0.033 ± 0.01^{c}	0

Api, apigenin; Caf, caffeic acid; Chl, chlorogenic acid; Cin, cinnamic acid; Cir, cirsimaritin; Cou, coumaric acid; Gal, gallic acid; Iso, isokaempferid; —, not detected; Pen, penduletin; Que, quercetin; Rut, rutin. Values within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's multiple range test. Data are means of three replicates.

and extracellular cell signaling.[51] Generation of these ROS molecules acts as a signal for the synthesis of defensive chemicals, such as phenolics, flavonoids and phytoalexin, defense enzymes, etc., in many chitosan-treated plant species.[52]

Activities of antioxidant enzymes

In the present experiment, chitosan enhanced the GPX activity of *D. kotschyi* roots and leaves. The activity of CAT increased in both leaves and roots, but no significant effect of chitosan on CAT activity was observed in *D. kotschyi* leaves (Table 3). Chitosan stimulates the activities of ROS-scavenging enzymes, including CAT and peroxidase, as well as defense-related enzymes such as PAL and chitinase.[51] Our findings indicated that chitosan induces the accumulation of H_2O_2 in *D. kotschyi*. Apparently, increased H_2O_2 accumulation in chitosan-treated plants may have activated antioxidant enzymes, to counteract the increase in ROS induced by chitosan. The activity of GPX increased more than twofold in the 400 mg L^{-1} chitosan treatment; thus the increased GPX activity appears to compensate the superoxide accumulation under high concentrations of chitosan. Similar to our results, the exogenous application of chitosan increased CAT and peroxidase activities in some plants.[17,49] Safikhan *et al.* demonstrated that chitosan-sprayed plants had greater CAT and peroxidase activity in leaves of *Silybum marianum*.[34] Also, it has been reported that chitosan has a potential for scavenging systems such as peroxidase, polyphenol oxidase, superoxide dismutase and CAT.[53]

PAL activity

The application of chitosan increased the activities of PAL in *D. kotschyi* (Fig. 2). Similar results have been previously reported.[54,55] PAL is the key enzyme involved in the production of phenylpropanoid compounds. In the current study, the highest PAL activity was found for leaves treated with 100 mg L^{-1} chitosan, which was also associated with an increase in phenolic compounds such as cinnamic acid. Chitosan stimulates PAL activity which produces cinnamic acid in the phenylpropanoid pathway.[47] Links between increased PAL activity by chitosan and accumulation of phenolic compounds have been observed in various plant species such as tomato[56] and spinach.[47] In the current experiment, we observed that an increase in PAL activity is associated with an increase in H_2O_2 content. In signal transduction pathways, H_2O_2 is considered as a secondary messenger producing defense signals by inducing genes of the phenylpropanoid pathway which also greatly increases the activity of PAL.[57]

RA content

As shown in Fig. 3, RA production in *D. kotschyi* treated with chitosan was significantly greater than that of the control. The foliar application of 100 mg L^{-1} chitosan increased production of RA 10-fold compared with the control value and the highest value of RA (429.1 μg mL^{-1} FW) was obtained at 400 μg mL^{-1} chitosan (13-fold increase).

Identification of some phenolic acids and a bioactive flavonoids

HPLC analysis of methanol extracts of leaves identified and quantified five different phenolic acids and quercetin and rutin as two bioactive flavonoids. As evident from Table 4, the highest amount of all polyphenols was obtained for a concentration of chitosan of 100 mg L^{-1}, which doubled the production of

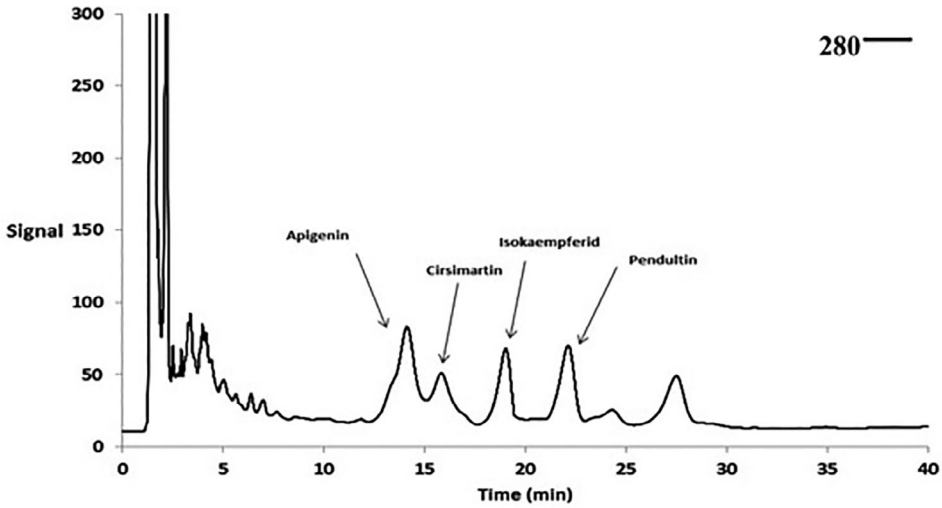

Figure 4. HPLC-DAD chromatogram peaks corresponding to apigenin, cirsimaritin, isokaempferid and penduletin.

caffeic acid and chlorogenic acid and increased threefold the production of gallic acid and coumaric acid. A concentration of chitosan of 100 mg L^{-1} increased production of quercetin and cinnamic acid 13-fold and 51-fold, respectively. Also, foliar application of 400 mg L^{-1} chitosan significantly increased the polyphenol content. However, for our results, the maximum amount of rutin was detected in control plants.

Yin *et al*. stated that chitosan stimulates the accumulation of polyphenols in *Origanum vulgare*, indicating that chitosan as a significant signal molecule can play a prominent role in various plant processes such as plant growth and production of secondary metabolites.[58] The mentioned research revealed that concentrations of chitosan of 50 and 200 ppm led to an increase in the polyphenol content of oregano, by 38% and 29%, respectively. Also, Ahmad *et al*. revealed that foliar application of chitosan increased menthol content by 8%.[39]

Signaling induced by chitosan molecules involves specific cellular receptors, e.g. chitosan binding glycoprotein (family of lectins) isolated from mustard leaves. Besides, chitosan can induce receptor kinase-like genes, the MAPK pathway and lysine motif receptor-like kinase, such as chitin elicitor receptor kinase 1 (CERK1), which can bind with chitin and chitosan.[32] On the other hand, Povero *et al*. reported that chitosan signaling is perceived through a CERK1-independent way in *Arabidopsis thaliana* seedlings. Thus, the involvement of chitosan binding receptors is still unclear.[59] In any case, signaling transduced by secondary messengers such as ROS, H_2O_2, Ca^{2+}, nitric oxide and phytohormones in the cell triggers physiological responses. Responses elicited by chitosan include an increase in cytosolic H^+, oxidative bursts and synthesis of phytoalexins. In a narrow sense, phytoalexins fall into several classes, including terpenoids, isoflavones, alkaloids and phenolics.[60]

Concentrations of chitosan of 0.25 and 0.50 g L^{-1} resulted in the highest increase in the contents of α-thujone, β-thujone and camphor of *Salvia officinalis*.[61] Moreover, chitosan doses of 200 and 400 µL L^{-1} enhanced the thymol content in Iranian thyme.[36] Chitosan can play a main signaling role in the activation of many plant defense responses and may affect gene regulation that leads to the activation of enzymes in the metabolic pathway linked with the biosynthesis of special secondary metabolites.[62]

In this research, an increase in PAL activity was linked with a higher synthesis of cinnamic acid. The first step of the phenylpropanoid pathway is the conversion of L-phenylalanine into cinnamic acid by PAL and cinnamic acid takes different routes for the synthesis of other phenylpropanoids such as phenolic acids and flavonoids.[47]

Chitosan also causes the accumulation of aromatic amino acids (phenylalanine and tyrosine) and phenylpropanoid derivatives which serve as a substrate for secondary metabolite biosynthesis such as phenolic acids.[19] Our results suggest a relationship between chitosan-induced H_2O_2 and the accumulation of polyphenols. H_2O_2 is a signal molecule, which plays an important role in many plant activities such as plant growth and secondary metabolite production.[58]

Methoxylated flavonoid content

The HPLC chromatogram with peaks relevant to the standard of each flavonoid is shown in Fig. 4, which demonstrates the accumulation pattern and RT for each compound at 280 nm. The flavonoid content in leaves of plants is presented in Table 4. The highest value of apigenin was obtained at 100 µg mL^{-1} (54.2 µg g^{-1} FW) which increased 36-times more than control treatment. However, the maximum amounts of cirismartin, isokaempferid and pendultin were detected in control plants in our study.

Chitosan also stimulates the expression of genes encoding various TFs which are considered to be the most important regulators of transcription of DNA to mRNA. Chitosan may activate plant responses through various signaling pathways, including various secondary messangers and TFs. It appears that chitosan may act as a biostimulator to transfer signals through MAPKs of cell walls and hydrogen peroxide.[19] Ji *et al*. demonstrated that a concentration of chitosan of 150 mg L^{-1} induced the expression of TF AabHLH1 followed by the induction of amorpha-4,11-diene synthase gene which enhanced biosynthesis of artemisinin in *Artemesia annua*.[63] Furthermore, it has been reported that treatment of plants with chitosan efficiently enhanced the synthesis of RA of *Ocimum basilicum*[62] and oleanolic acid in *Calendula officinalis*,[64] and anthocyanin and phenolic acid in *Vitis vinifera*.[15] Besides, Xoca-Orozco *et al*. observed that chitosan application induced a high expression of some genes related to the

production of secondary metabolites. When plants are treated with chitin-based compounds, they activate their defense mechanisms by secreting such secondary metabolites.[65]

Chitosan and nano-chitosan cause a high accumulation of flavonoids in plants, which can be a direct result of upregulation of PAL and flavanone 3-hydroxylase (F3H) genes. F3H is involved in the pathway of flavonoid biosynthesis which encodes a large family of flavonoid compounds with different biological activities. In addition, F3H catalyzes the production of dihydroflavonolols such as (2*R*,3*R*)-dihydrokaempferol and (2*R*,3*R*)-dihydroquercetin, which act as mediators for the biosynthesis of various flavonoids. Also, high levels of flavonoid accumulation may be a sign of increased resistance in plants. Exposure of plants to various biotic or abiotic stresses leads to regulation or disruption of the electrical transport chain, resulting in the production of ROS.[66]

Chitin elicitor binding proteins have been isolated in various crops, which induce defense responses. Subsequently, they affect the expression of various chitosan-responsive genes that can react with chromatin or may bind to specific receptors.[67] Chitosan enhances defense responses such as activation of H_2O_2 through the octadecanoid pathway and nitric oxide in chloroplasts, MAPK activation, oxidative burst and hypersensitive responses.[32] Then they lead to cascading events involving TFs for plant growth and biosynthesis becomes secondary to metabolites.[19]

Conclusively, in the current study application of chitosan led to increased production of valuable metabolites, particularly the content of RA and quercetin that increased 13-fold in *D. kotschyi*. Quercetin (3,39,49,5,7-pentahydroxyflavone) inhibits the growth of human mammary, ovarian, colorectal, leukemic and lung tumor cells.[68] RA is widely used in the pharmaceutical industry due to its antioxidant, antimicrobial, anti-inflammatory and antiviral properties. So our study suggested that chitosan is an efficient elicitor for the biosynthesis of valuable secondary metabolites and anticancer drugs.

CONCLUSIONS

Our results indicated that aerial spraying of *D. kotschyi* by 100 and 400 mg L^{-1} chitosan improves macroelement uptake, particularly the enhancement of Ca (up to 6-fold) and Mg (up to 13-fold). Also, production of secondary metabolites (phenolic compounds and flavonoids) is increased, associated with PAL activity. Defensive mechanisms of cells play an important role in oxidative stress caused by chitosan through the increase in antioxidant enzyme (CAT and GPX) activities. The results revealed that chitosan at concentrations of 100 and 400 mg L^{-1} could be considered as an effective abiotic elicitor for improvement of the production of valuable secondary metabolites, particularly RA and apigenin (13- and 16- fold increase compared to control, respectively). Apigenin suppresses the occurrence and development of autoimmune diseases, such as lupus and asthma. Our results are directly related to nutrient supplementation and level of secondary metabolites. It could be concluded that exposure to chitosan as an appropriate factor is a simple method for the production of anticancer compounds, particularly RA, in *D. kotschyi*. Nevertheless, molecular-level investigations are needed to determine the function of chitosan as a stimulator and further studies of several genotypes are required to confirm this proposition.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING

This work was supported by Vice Chancellor for Research, Urmia University.

AUTHORSHIP

JK and SK contributed to the study conception and design. Material preparation was performed by JK. SK conducted the tests and analyzed the data. The first draft of the manuscript was written by SK and JK commented on previous versions of the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

The authors are grateful to Department of Agriculture and Biology, WULS-SGGW, Poland for providing facilities for performing elemental analysis.

REFERENCES

1 Mazzei R, De Marco EV, Gallo O and Tagarelli G, Italian folk plant-based remedies to heal headache (XIX-XX century). *J Ethnopharmacol* **210**: 417–433 (2018).
2 Rechinger H, *Flora Iranica, Lamiaceae*, Vol. **150**. Akad Druck Verlagsantalt, Graz, Austria, 360-1 (1986).
3 Fattahi M, Nazeri V, Torras-Claveria L, Sefidkon F, Cusido RM, Zamani Z *et al*., Identification and quantification of leaf surface flavonoids in wild-growing populations of *Dracocephalum kotschyi* by LC–DAD–ESI-MS. *Food Chem* **141**:139–146 (2013).
4 Ashrafi B, Ramak P, Ezatpour B and Talei GR, Investigation on chemical composition, antimicrobial, antioxidant, and cytotoxic properties of essential oil from *Dracocephalum kotschyi* Boiss. *Afr J Tradit Complementary Altern Med* **14**:209–217 (2017).
5 Ghavam M, Study of antioxidant activity and some herbal compounds of *Dracocephalum kotschyi* Boiss. in different ages of growth. *Biotechnol Rep* **25**:e00408 (2019).
6 Jalili A and Jamzad Z, Red data book of Iran; a preliminary survey of endemic, rare & endangered plant species in Iran; Research Institute of Forest & Rangelands; Ministry of Jahad-e Sazandegi. *Tech* **215**:748 (1999).
7 Sodeifian G, Sajadian SA and Honarvar B, Mathematical modelling for extraction of oil from *Dracocephalum kotschyi* seeds in supercritical carbon dioxide. *Nat Prod Res* **32**:795–803 (2018).
8 Jahaniani F, Ebrahimi SA, Rahbar-Roshandel N and Mahmoudian M, Xanthomicrol is the main cytotoxic component of *Dracocephalum kotschyi* and a potential anti-cancer agent. *Phytochemistry* **66**: 1581–1592 (2005).
9 Heydari P, Yavari M, Adibi P, Asghari G, Ghanadian S-M, Dida GO *et al*., Medicinal properties and active constituents of *Dracocephalum kotschyi* and its significance in Iran: a systematic review. *Evidence-Based Complement Altern Med* **2**:1–14 (2019).
10 Moghaddam G, Ebrahimi SA, Rahbar-Roshandel N and Foroumadi A, Antiproliferative activity of flavonoids: influence of the sequential methoxylation state of the flavonoid structure. *Phyther Res* **26**: 1023–1028 (2012).
11 Kamalipourazad M, Sharifi M, Maivan HZ, Behmanesh M and Chashmi NA, Induction of aromatic amino acids and phenylpropanoid compounds in *Scrophularia striata* Boiss. Cell culture in response to chitosan-induced oxidative stress. *Plant Physiol Biochem* **107**:374–384 (2016).
12 Sahu R, Gangopadhyay M and Dewanjee S, Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Solenostemon scutellarioides*. *Acta Physiol Plant* **35**:1473–1481 (2013).
13 Zhao J, Davis LC and Verpoorte R, Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* **23**: 283–333 (2005).
14 Bayraktar M, Naziri E, Akgun IH, Karabey F, Ilhan E, Akyol B *et al*., Elicitor induced stevioside production, in vitro shoot growth, and biomass accumulation in micropropagated *Stevia rebaudiana*. *Plant Cell Tissue Organ Cult* **127**:289–300 (2016).
15 Cai Z, Riedel H, Saw NMMT, Mewis I, Reineke K, Knorr D *et al*., Effects of elicitors and high hydrostatic pressure on secondary metabolism of

Vitis vinifera suspension culture. *Process Biochem* **46**:1411–1416 (2011).
16 Brasili E, Pratico G, Marini F, Valletta A, Capuani G, Sciubba F *et al*., A non-targeted metabolomics approach to evaluate the effects of biomass growth and chitosan elicitation on primary and secondary metabolism of *Hypericum perforatum* in vitro roots. *Metabolomics* **10**:1186–1196 (2014).
17 Xu D, Li H, Lin L, Liao M, Deng Q, Wang J *et al*., Effects of carboxymethyl chitosan on the growth and nutrient uptake in *Prunus davidiana* seedlings. *Physiol Mol Biol Plants*. **26**:661–668 (2020).
18 Gai Q-Y, Jiao J, Wang X, Liu J, Wang Z-Y and Fu Y-J, Chitosan promoting formononetin and calycosin accumulation in Astragalus membranaceus hairy root cultures via mitogen-activated protein kinase signaling cascades. *Sci Rep* **9**:1–11 (2019).
19 Ahmed KBM, Khan MMA, Siddiqui H and Jahan A, Chitosan and its oligosaccharides, a promising option for sustainable crop production: a review. *Carbohydr Polym* **227**:115331 (2020).
20 Fattahi M, Nazeri V, Sefidkon F, Zamani Z and Palazon J, The effect of pre-sowing treatments and light on seed germination of *Dracocephalum kotschyi* Boiss: an endangered medicinal plant in Iran. *Hortic Environ Biotechnol* **52**:559–566 (2011).
21 Jones JB Jr and Case VW, Sampling, handling and analyzing plant tissue samples. *Soil Test Plant Anal*. **3**:389–427 (1990).
22 Dhaliwal GS, Gupta N, Kukal SS and Kaur M, Standardization of automated Vario EL III CHNS analyzer for total carbon and nitrogen determination in soils. *Commun Soil Sci Plant Anal* **42**:971–979 (2011).
23 Alexieva V, Sergiev I, Mapelli S and Karanov E, The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ* **24**:1337–1344 (2001).
24 Kang H and Saltveit ME, Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid. *Physiol Plant* **115**:571–576 (2002).
25 Aebi H, Catalase in vitro. *Methods Enzymol* **105**:121–126 (1984).
26 Chance B, Maehly AC. [136] Assay of catalases and peroxidases (1955).
27 Ke D and Saltveit ME Jr, Effects of calcium and auxin on russet spotting and phenylalanine ammonia-lyase activity in iceberg lettuce. *HortScience* **21**:1169–1171 (1986).
28 Marinova D, Ribarova F and Atanassova M, Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *J Univ Chem Technol Metall* **40**:255–260 (2005).
29 Zhishen J, Mengcheng T and Jianming W, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* **64**:555–559 (1999).
30 Seal T, Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves *Sonchus arvensis* and *Oenanthe linearis* of north-eastern region in India. *J Appl Pharm Sci* **6**:157–166 (2016).
31 Fattahi M, Nazeri V, Torras-Claveria L, Sefidkon F, Cusido RM, Zamani Z *et al*., A new biotechnological source of rosmarinic acid and surface flavonoids: hairy root cultures of *Dracocephalum kotschyi* Boiss. *Ind Crops Prod* **50**:256–263 (2013).
32 Hidangmayum A, Dwivedi P, Katiyar D and Hemantaranjan A, Application of chitosan on plant responses with special reference to abiotic stress. *Physiol Mol Biol Plants* **25**:313–326 (2019).
33 Lei C, Ma D, Pu G, Qiu X, Du Z, Wang H *et al*., Foliar application of chitosan activates artemisinin biosynthesis in *Artemisia annua* L. *Ind Crops Prod* **33**:176–182 (2011).
34 Safikhan S, Khoshbakht K, Chaichi MR, Amini A and Motesharezadeh B, Role of chitosan on the growth, physiological parameters and enzymatic activity of milk thistle (*Silybum marianum* (L.) Gaertn.) in a pot experiment. *J Appl Res Med Aromat Plants* **10**:49–58 (2018).
35 Anjum SA, Ashraf U, Tanveer M, Khan I, Hussain S, Shahzad B *et al*., Drought induced changes in growth, osmolyte accumulation and antioxidant metabolism of three maize hybrids. *Front Plant Sci* **8**:1–12 (2017).
36 Bistgani ZE, Siadat SA, Bakhshandeh A, Pirbalouti AG and Hashemi M, Interactive effects of drought stress and chitosan application on physiological characteristics and essential oil yield of *Thymus daenensis* Celak. *Crop J* **5**:407–415 (2017).
37 Farouk S and Amany AR, Improving growth and yield of cowpea by foliar application of chitosan under water stress. *Egypt J Biol* **14**: 14–16 (2012).
38 Dzung NA, Khanh VTP and Dzung TT, Research on impact of chitosan oligomers on biophysical characteristics, growth, development and drought resistance of coffee. *Carbohydr Polym* **84**:751–755 (2011).
39 Ahmad B, Jaleel H, Shabbir A, Khan MMA and Sadiq Y, Concomitant application of depolymerized chitosan and GA3 modulates photosynthesis, essential oil and menthol production in peppermint (*Mentha piperita* L.). *Sci Hortic* **246**:371–379 (2019).
40 Winkler AJ, Dominguez-Nuñez JA, Aranaz I, Poza-Carrión C, Ramonell K, Somerville S *et al*., Short-chain chitin oligomers: promoters of plant growth. *Mar Drugs* **15**:40 (2017).
41 He Y, Bose SK, Wang W, Jia X, Lu H and Yin H, Pre-harvest treatment of chitosan oligosaccharides improved strawberry fruit quality. *Int J Mol Sci* **19**:2194 (2018).
42 Rahman M, Mukta JA, Sabir AA, Gupta DR, Mohi-Ud-Din M, Hasanuzzaman M *et al*., Chitosan biopolymer promotes yield and stimulates accumulation of antioxidants in strawberry fruit. *PLoS One* **13**:e0203769 (2018).
43 Emami Bistgani Z, Siadat SA, Bakhshandeh A, Ghasemi Pirbaluti A and Hashemi M, Influence of chitosan concentration on morpho-physiological traits, essential oil and phenolic content under different fertilizers application in *Thymus daenensis*. *J Herb Drugs* **7**: 117–125 (2016).
44 Katiyar D, Hemantaranjan A and Singh B, Chitosan as a promising natural compound to enhance potential physiological responses in plant: a review. *Indian J Plant Physiol* **20**:1–9 (2015).
45 Khan T, Khan T, Hano C and Abbasi BH, Effects of chitosan and salicylic acid on the production of pharmacologically attractive secondary metabolites in callus cultures of *Fagonia indica*. *Ind Crops Prod* **129**: 525–535 (2019).
46 Pirbalouti AG, Malekpoor F, Salimi A, Golparvar A and Hamedi B, Effects of foliar of the application chitosan and reduced irrigation on essential oil yield, total phenol content and antioxidant activity of extracts from green and purple basil. *Acta Sci Pol Hortorum Cultus* **16**:177–186 (2017).
47 Singh S, Enhancing phytochemical levels, enzymatic and antioxidant activity of spinach leaves by chitosan treatment and an insight into the metabolic pathway using DART-MS technique. *Food Chem* **199**: 176–184 (2016).
48 Udomsuk L, Jarukamjorn K, Tanaka H and Putalun W, Improved isoflavonoid production in *Pueraria candollei* hairy root cultures using elicitation. *Biotechnol Lett* **33**:369–374 (2011).
49 Pichyangkura R and Chadchawan S, Biostimulant activity of chitosan in horticulture. *Sci Hortic* **196**:49–65 (2015).
50 Chamnanmanoontham N, Pongprayoon W, Pichayangkura R, Roytrakul S and Chadchawan S, Chitosan enhances rice seedling growth via gene expression network between nucleus and chloroplast. *Plant Growth Regul* **75**:101–114 (2015).
51 Shabani L and Razavizadeh R, Chitosan enhances rosmarinic acid production in shoot cultures of *Melissa officinalis* L. through the induction of methyl jasmonate. *Bot Stud* **60**:1–10 (2019).
52 Malerba M and Cerana R, Chitosan effects on plant systems. *Int J Mol Sci* **17**:996 (2016).
53 Lian-Ju M, Yue-Ying L, Lan-Lan W, Xue-Mei L, Liu T and Bu N, Germination and physiological response of wheat (*Triticum aestivum*) to presoaking with oligochitosan. *Int J Agric Biol* **16**:766–770 (2014).
54 Uthairatanakij A, Teixeira da Silva JA and Obsuwan K, Chitosan for improving orchid production and quality. *Orchid Sci Biotechnol* **1**: 1–5 (2007).
55 Dang QF, Yan JQ, Li Y, Cheng XJ, Liu CS and Chen XG, Chitosan acetate as an active coating material and its effects on the storing of *Prunus avium* L. *J Food Sci* **75**:S125–S131 (2010).
56 Badawy MEI and Rabea EI, Potential of the biopolymer chitosan with different molecular weights to control postharvest gray mold of tomato fruit. *Postharvest Biol Technol* **51**:110–117 (2009).
57 Hao X, Shi M, Cui L, Xu C, Zhang Y and Kai G, Effects of methyl jasmonate and salicylic acid on tanshinone production and biosynthetic gene expression in transgenic salvia miltiorrhiza hairy roots. *Biotechnol Appl Biochem* **62**:24–31 (2015).
58 Yin H, Fretté XC, Christensen LP and Grevsen K, Chitosan oligosaccharides promote the content of polyphenols in Greek oregano (*Origanum vulgare* ssp. *hirtum*). *J Agric Food Chem* **60**:136–143 (2012).
59 Povero G, Loreti E, Pucciariello C, Santaniello A, Di Tommaso D, Di Tommaso G *et al*., Transcript profiling of chitosan-treated Arabidopsis seedlings. *J Plant Res* **124**:619–629 (2011).
60 Xing K, Zhu X, Peng X and Qin S, Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. *Agron Sustainable Dev* **35**:569–588 (2015).
61 Vosoughi N, Gomarian M, Pirbalouti AG, Khaghani S and Malekpoor F, Essential oil composition and total phenolic, flavonoid contents, and

antioxidant activity of sage (*Salvia officinalis* L.) extract under chitosan application and irrigation frequencies. *Ind Crops Prod* **117**: 366–374 (2018).
62 Kim H-J, Chen F, Wang X and Rajapakse NC, Effect of chitosan on the biological properties of sweet basil (*Ocimum basilicum* L.). *J Agric Food Chem* **53**:3696–3701 (2005).
63 Ji Y, Xiao J, Shen Y, Ma D, Li Z, Pu G *et al*., Cloning and characterization of AabHLH1, a bHLH transcription factor that positively regulates artemisinin biosynthesis in *Artemisia annua*. *Plant Cell Physiol* **55**: 1592–1604 (2014).
64 Wiktorowska E, Długosz M and Janiszowska W, Significant enhancement of oleanolic acid accumulation by biotic elicitors in cell suspension cultures of *Calendula officinalis* L. *Enzyme Microb Technol* **46**:14–20 (2010).
65 Xoca-Orozco L-Á, Cuellar-Torres EA, González-Morales S, Gutiérrez-Martínez P, López-García U, Herrera-Estrella L *et al*., Transcriptomic analysis of avocado hass (*Persea americana* mill) in the interaction system fruit-chitosan-colletotrichum. *Front Plant Sci* **8**:956 (2017).
66 Chandra S, Chakraborty N, Dasgupta A, Sarkar J, Panda K and Acharya K, Chitosan nanoparticles: a positive modulator of innate immune responses in plants. *Sci Rep* **5**:15195 (2015).
67 Hadwiger LA, Anatomy of a nonhost disease resistance response of pea to *Fusarium solani*: PR gene elicitation via DNase, chitosan and chromatin alterations. *Front Plant Sci* **6**:373 (2015).
68 Caltagirone S, Rossi C, Poggi A, Ranelletti FO, Natali PG, Brunetti M *et al*., Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int J Cancer* **87**:595–600 (2000).

