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Seed-priming with cold plasma and supplementation of nutrient solution with carbon nanotube enhanced carotenoid contents and the expression of *psy* and *pds* in Bitter melon (*Momordica charantia*)

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Summary

Recent studies on cold-plasma and nanotechnology in some crop species have shown a potential for application in food, medicine, and crop improvement. Here, the behaviours of *Momordica charantia* were evaluated, following supplementation of nutrient solution with multi-walled carbon nanotube (CNT) and seed priming with cold plasma treatments. The ultra-structural study of stems confirmed CNT uptake and symplastic transportation. CNT supplementation and seed-priming with plasma synergistically provoked a drastic increase in the plant's early growth and performance. Quantitative real-time PCR analysis confirmed that the applied treatments mediated variations in transcriptions of the phytoene-synthase gene (*McPSY*) and phytoene desaturase (*McPDS*). The *McPDS* and *McPSY* genes showed a similar expression trend in which the highest expression levels were observed in CNT50+Plasma 60 group. According to HPLC analysis, the CNT50+Plasma60 treatment was the most effective way to increase concentrations of β-carotene. The applied treatments dependent on dose and treatment method increased zeaxanthin concentration. Similarly, CNT50+Plasma 60 and CNT100+Plasma 60 groups had significantly higher α-carotene levels than the other treatment group. Moreover, the statistical analysis confirmed the significant positive correlations between the expression of target genes and concentrations of carotenoids. Herein, a theoretical basis was gained to exploit in the food, pharmaceutical, and agricultural industries.

Keywords: Carbon nanotube; cold plasma; carotenoid; gene expression; secondary metabolites; seed-priming

Introduction

Momordica charantia L., belonging to the Cucurbitaceae family and commonly known as bitter melon, is a medicinal plant that is widely grown in the tropical and subtropical parts of the world such as India, China, and Indonesia (Jia, 2017). Traditionally, bitter melon has been used in the treatment of diabetes and recently many scientific evidence have also verified that it is a promising antidiabetic and therapeutic substance (Joseph and Jini, 2013). Furthermore, bitter melon has some other medicinally useful properties, such as antiviral (Pongthanapisith, 2013), antibacterial (Costa, 2010), antisepsis, anticancer (Poolperm and Jiraungkoorskul, 2017), antihepatotoxic (Aparna Upadhyay, 2015), antiulcer and antioxidant (Gill, 2012). *M. charantia* contains several different types of primary and secondary metabolites in the whole plant such as phenolic, triterpene, flavonoid, and carotenoid compounds, including alpha and beta-carotene, lycopene, and zeaxanthin (Hyun Young, 2013; Jia, 2017). Among them, carotenoids have significant roles in many physiological processes in this plant and may play an important role in the prevention of some diseases (Cuong, 2017; Ishida, 2004; Lee, 2017). For example, carotenoids act as the precursors in the synthesis of abscisic acid, which is mainly involved in the plant development and stress regulations. In addition, they can prevent damaging photo-oxidative processes (photo-inhibition phenomenon) by absorption of light in photosynthetic membranes. Moreover, the associated color in flowers and fruits attracts both pollinators and seed dispersal agents (Lee, 2017; Tuan, 2011). In humans, the carotenoid derivatives have several medical applications, especially in controlling the chronic and vascular diseases (Fiedor, 2014). They are used as a precursor in vitamin A biosynthesis that is essential to prevent blindness and xerophtalmia (Tuan, 2011). In addition, carotenoids can significantly reduce the risk of cataracts, photosensitivity disease, lung cancer, prostate cancer and cardiovascular disease. Due to the inability of humans to biosynthesize essential carotenoids, it is necessary to be obtained through the food sources. Therefore, manipulating the metabolic pathway of carotenoids to enhance their synthesis can play an important role in increasing the quality and quantity of the product (Cuong, 2017; Tuan, 2011). In the carotenoid biosynthetic pathway, phytoene synthase (PSY) and phytoenedesaturase (PDS) are two key enzymes with a close relationship between the associated expression and the level of carotenoids (Fig. 1) (Cuong, 2017; Tuan, 2011).

Recently non-thermal (cold) plasma technology has been considered as an innovative eco-friendly safe standalone technology, which could be scaled up and exploited in diverse agricultural-related activities (Bourke, 2018; Iranbakhsh, 2017; Iranbakhsh, 2018). During the production of plasma phenomenon, not only UV photons are emitted, but also varieties of active nitrogen and oxygen species, like nitric oxide are generated, which all may individually or simultaneously act as an efficient epigenetic agent to activate key crucial signaling routes, contributed to modulation of plant growth, physiology, productivity, and protection (Bourke, 2018; Iranbakhsh, 2017; Iranbakhsh, 2018). Also, seed pre-treatment with cold plasma as modern eco-agricultural technology could enhance seed germination, seedling growth, however, limited studies are exploring the associated effect on the gene expression (Bourke, 2018; Iranbakhsh, 2017; Iranbakhsh, 2018).

To gain insight into diverse nanoproducts, more convincing studies are required to figure out their advantage or toxicity, and elucidate the contributed mechanisms, for further exploitation in the related industries (Rajaee Behbahani, 2020; Sotoodehnia-Korani et al., 2020). The different industrial consumptions of carbon nanotubes (CNTs), including single-wall (SWNTs) and multi-wall CNTs (MWNTs) are rapidly increasing (Cano, 2016). Based on the recent evidence, CNTs are suitable candidates to achieve various agricultural and biotechnological aims, especially as pesticides, nano-encapsulated products,

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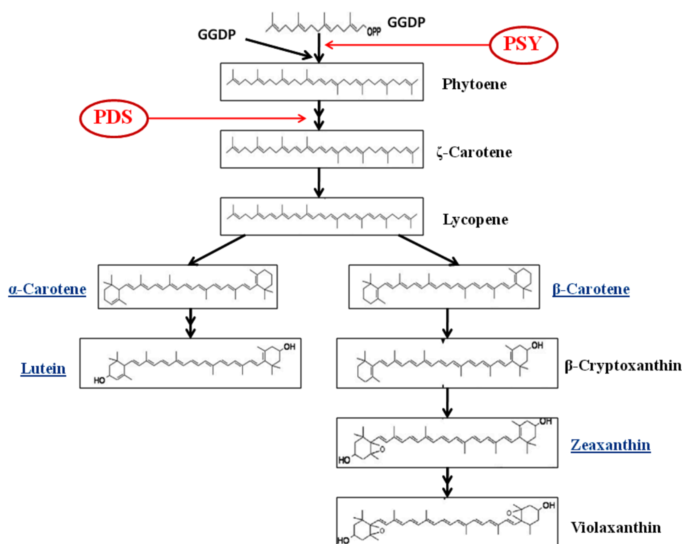

Fig. 1: Carotenoid biosynthesis pathway in plants. The activity of phytoene synthase (PSY) and phytoene desaturase (PDS) in the biosynthesis of carotenoids. Blue colour denotes the carotenoids measured in this study by HPLC analysis and red colour indicates enzymatic activities for which gene expression was monitored via real time-PCR. GGDP, geranylgeranyl diphosphate.

and fertilizers (Husen, 2014; Kah, 2015; Liu, 2015). Studies showed that the application of CNTs influenced the growth-related traits in *Triticum aestivum* (Joshi, 2018), *Zea mays* (Yan, 2013), and *Oryza sativa* (Yan, 2016). The functionalized carbon nanotubes have been demonstrated to increase the water-retaining capacity, biomass, and fruit yield in plants, which is a significant achievement of nanotechnology in recent years (Husen, 2014). However, there is limited evidence, especially at the molecular level and further studies are required. On the other hand, some recent studies on carbon nanotube (CNT) or cold plasma in several crops have shown evidence for increased germination, seedling growth, and physiological activities, including photosynthetic activity, enhancement of the level of key enzymes in the metabolic pathways. These also make positive changes in the gene expression that are essential for cell division and plant development, indicating their potential use in crop improvement [Lakshman K. Randeniya, 2015 #33;Ling, 2014 #34;Kole, 2013 #36;Husen, 2014 #19;Khodakovskaya, 2013 #38(Haghighi M, 2014). In this study, early growth and the relationship between carotenoid accumulation and the expression of *McPSY* and *McPDS*, were investigated, using two treatments of cold plasma and carbon nanotube. Furthermore, the simultaneous effect of both actions was examined in *M. Charantia*.

Materials and methods

Nanomaterial and seeds

The uniform seeds of *M. charantia*, PALEE F1, were obtained from the East-West Seed International LTD, Thailand. Laboratory and greenhouse experiments were arranged in a completely randomized design with three replications. In this study, MWCNT was purchased from US research nanomaterials, Inc (3302 Twig Leaf Lane Houston, TX 77084, USA) (Tab. 1).

Plasma experimental apparatus

The applied experimental apparatus in this study was DBD (Model: PS200, Nik Fanavaran Plasma Co., Iran). The details and schematic of the applied plasma producing device, and the plasma diagnostic

Tab. 1: Physical properties of the MWCNT-COOH.

	MWCNT-COOH
Young's modulus (GPa)	1200
Tensile strength (GPa)	150
Density (g/cm^3)	2.6
Thermal conductivity (W/m.k)	3000
Electrone conductivity (S/m)	10^5 - 10^7

data were represented in our previously published papers (Fig. 2A and 2B) (Babajani, 2018; Iranbakhsh, 2017; Iranbakhsh, 2018). Plasma at atmospheric pressure is generated between two glass plates as dielectric barriers (the gap between dielectrics: 4 mm) covering the two powered circular plate copper electrodes (radius = 5.5 cm). Argon was utilized as a functional gas between dielectrics (flow rate of 2 liters per min (l·min^{-1})). The dielectric acts as a stabilizing material when the potential across the gap reaches the breakdown voltage leading to the formation of a large number of micro-discharges. Moreover, a modified AC high voltage power supply (mp516, Nik Plasma Tech., Iran) was utilized (Fig. 2A). The voltage was quantified by a high voltage probe (Pintek HVP40) linked to an oscilloscope (Tektronix TDS1012B). Furthermore, the frequency and the voltage of the apparatus were respectively fixed at 13 kHz and 10 kV. The instrument power was 80 W, so for 94.98 cm^2 plasma treatment areas, the surface power density was equal to 0.84 W/cm^2. Besides, to provide plasma diagnostics data, the optical emission spectroscopy was carried out (the peaks were compared to the data of the NIST Atomic Spectra Database). The temperature was estimated to be between 27-29 °C. The peaks detected in 320-400 nm refer to the presence of UV, OH, and nitrogen related species, whereas the peaks between 700 and 1000 nm related to Ar (Fig. 2C).

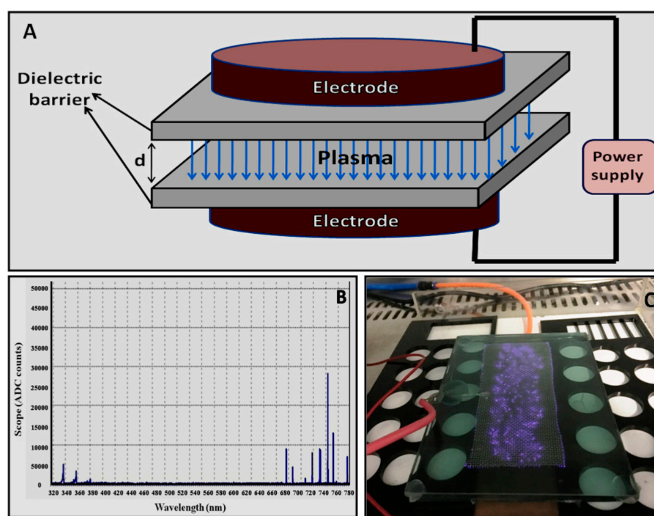

Fig. 2: The plasma experimental apparatus: (A) A schematic design of DBD. (B) The plasma experimental apparatus DBD (Model: PS200, Nik Fanavaran Plasma Co., Iran). (C) Optical emission spectroscopy-based spectrum to illustrate plasma diagnostic data.

Treatment of seeds using CNT and cold plasma and greenhouse experiment

Uniform and healthy seeds of *M. charantia* were surface-sterilized by submerging in a 1% (v/v) solution of commercial sodium hypochlorite for 15 min and rinsed twice with sterile distilled water. Some seeds were soaked in distilled water and the other in MWCNT solutions (50, 100, and 200 mg L^{-1}) for 48 h. After that, the soaked seeds

were treated with cold plasma (above described DBD; surface power density of 0.84 W/cm^2) at three different exposure times, including 0 s (control), 60 s, and 120 s. In this experiment, six seeds in three independent replications (three pots; two seeds per pot) were considered for each treatment group. In detail, treatment descriptions are presented in Tab. 2. Then, the plasma or CNT-primed seeds and the seeds treated by both plasma and MWCNTs were planted in pots filled with a cocopeat/perlite (1:1, v/v) near the substrate surface (1-2 cm deep). Each treatment was replicated three times. The plants were grown under uniform conditions of temperature (25 ± 2 °C/15 ± 2 °C day/night), relative humidity (60%), and photoperiod (16/8 h light/dark). All pots were irrigated twice a week with Hoagland nutrient solution containing different concentrations of MWCNT (0, 50, 100, and 200 mg L^{-1}) and distilled water in intervals (Tab. 2).

Tab. 2: Descriptions of treatment groups.

Group name	Number	Treatment description
Control	12 Seeds	MWCNT 0 mg L^{-1} + Plasma 0s
CNT50	6 Seeds	MWCNT 50 mg L^{-1}
CNT100	6 Seeds	MWCNT 100 mg L^{-1}
CNT200	6 Seeds	MWCNT 200 mg L^{-1}
Plasma60	6 Seeds	Plasma 60 s
Plasma120	6 Seeds	Plasma 120 s
CNT50+Plasma60	6 Seeds	MWCNT 50 mg L^{-1} + Plasma 60s
CNT100+Plasma60	6 Seeds	MWCNT 100 mg L^{-1} + Plasma 60s
CNT200+Plasma60	6 Seeds	MWCNT 200 mg L^{-1} + Plasma 60s
CNT50+Plasma120	6 Seeds	MWCNT 50 mg L^{-1} + Plasma 120s
CNT100+Plasma120	6 Seeds	MWCNT 100 mg L^{-1} + Plasma 120s
CNT120+Plasma120	6 Seeds	MWCNT 200 mg L^{-1} + Plasma 120s

Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscopy (FESEM; model: TESCAN MIRA3-FEG, Czech. Republic) was conducted using gold-plated material to investigate tracing uptake and accumulation of the nanoparticles on the 21-day old samples. Cross-sections of stems were prepared. The samples were dehydrated and fixed using a freeze dryer. Then, the prepared samples were gold-coated. A portable Microscope Camera 400x USB (Dino Lite AMH-RUT, Shenzhen, China; portable, handheld) was used to observe the effects of CNTs on imbibition.

Isolation of RNA and cDNA synthesis

Total RNA from the leaves of Bitter melon were extracted and purified by using the RNeasy Plant Mini Kit (QIAGEN, USA). After extraction, complementary DNA (cDNA) was synthesized from 1 µg of total RNA using QuantiTect Reverse Transcription Kit (QIAGEN, USA) according to the manufacturers' instructions.

Sequence analysis

Using sequence data from the sequencing of cDNA libraries obtained from bitter melon seedlings. The genes that demonstrated maximum identity and similarity were selected for further study.

Quantitative Real-time PCR analysis of McPSY and McPDS

To design primers for quantitative real-time PCR (qRT-PCR), the Bicon designer and Primer 3 program (http://frodo.wi.mit.edu/primer3) was used based on published gene sequences of the *M. charantia* PSY (GenBank Accession Number: AY494789) and PDS (GenBank Accession Number: AY494790) cDNA sequences. The *M. charantia* 18S ribosomal RNA gene (GenBank Accession Number AY900000.1), as a housekeeping gene, was used as an internal reference. The sequences of primers for these genes are presented in Tab. 3. The level of each gene expression was showed with relative expression, which is the copy number of each gene compared to that of a housekeeping gene. The qRT-PCR was conducted in 0.5 µM of each set of primers in 20 µl final reaction volume of SYBR Green Real-time PCR Premix Ex Taq™ (Takara Bio Inc, Japan) on a Rotor-Gene Q real-time PCR system (QIAGEN, USA) under the following conditions: 94 ºC for 5 min followed by 40 cycles of 94 ºC for 15 s, 56 ºC for 15 s, and 72 ºC for 20 s. All experiments were performed in duplicate and three times. Relative expression of *McPSY* and *McPSD* were calculated using equation $2^{-\Delta\Delta Ct}$ in which ΔCt was obtained by subtracting the internal control Ct value from the Ct value of *McPSY* and *McPSD* (Rajaee Behbahani et al., 2020).

Tab. 3: Sequences of specific primers used for quantitative real-time PCR.

Genes		Primer sequences	Amplicon size (bp)
McPSY	F	GCTTCATCGTTGGTTGTCTCTCT	154
	R	TGCTCCATTTCTGCCTCTTACTC	
McPDS	F	TTTGCTTGGATTACCCTAGACCA	128
	R	TGCACCAGCGATCACTACTTTTA	
Mc18S rRNA	F	ATAACTCGATGGATCGCACGG	136
	R	TCCTCCGGAATCGAACCCTA	

High-performance liquid chromatography (HPLC) analysis of carotenoids

Carotenoids were extracted from *M. charantia* leaf samples (0.1 g) in each group with 3 ml of ethanol containing 0.1% ascorbic acid (w/v). This mixture was vortexed for 20 s, and then incubated in a water bath at 85 ºC for 5 min. Subsequently, 120 µl of potassium hydroxide (80% w/v) was added to saponify any potentially interfering oils. After vortexing and incubating at 85 ºC for 10 min, the samples were placed on ice and 1.5 ml of cold deionized water and 0.05 ml of β-Apo-80-carotenal (12.5 µg ml^{-1}), an internal standard, were added. Next, the carotenoids were extracted twice with 1.5 ml of hexane and centrifuged at 1200 g each time to separate the layers. Then, the extracts were freeze-dried under a stream of nitrogen gas and resuspended in 50:50 (v/v) dichloromethane/methanol. The extraction method used for carotenoid analysis was similar to that described (Howe and Tanumihardjo, 2006). For HPLC analysis, the carotenoids were separated on an Agilent 1100 HPLC system with a Hector-M C18 column (150 × 4.6 mm, 5 µm, P/N: C18-M51001546) and detected with an array detector at 450 nm. Solvent A consisted of methanol/water (92:8 v/v) with 10 mM ammonium acetate. Solvent B consisted of 100% methyl tert-butyl ether. The flow rate was maintained at 1 ml/min and samples were eluted with the following gradient: 0 min, 83% A/17% B; 23 min, 70% A/30% B; 29 min, 59% A/41% B; 35 min, 30% A/70% B; 40 min, 30% A/70% B; 44 min, 83% A/17% B; and 55 min, 83% A/17% B. Identification and peak assignment of carotenoids were primarily based on comparison of their retention time and UV-visible spectrum data with that of standards, and with guidelines previously presented.

Statistical analysis

The obtained data were subjected to statistical analyses using SPSS software. All data were expressed as mean ± standard error (SE) values of three independent replicates. Significant mean differences between the treatments were estimated according to Duncan's multiple range test at the level of $P \leq 0.05$.

Results

Both cold plasma and CNT treatments synergistically provoked considerable changes in the early growth of the treated plants, especially the combined treatments, among which the simultaneous treatment of 200 mg L^{-1} CNT and cold plasma for 60 s has shown the best performance (Fig. 3). There was a considerable linear relationship between the applied concentrations of the CNT and the growth rate of seedlings. It should be noted that CNT supplementations have not caused any toxic effects. The ultra-structure of stems (by FESEM) was photographed to trace the uptake and transportation of MWCNT. Cellular uptake and translocation of the CNT were manifested based on the stem ultra-structure (Fig. 4).

Expression levels of the McPSY and McPDS

Quantitative real-time PCR analysis was performed to determine the expression levels of *McPSY* and *McPDS* in all treated groups of *M. charantia*. *McPSY* and *McPDS* expressed in all treated groups, were examined, and shown a similar expression pattern. The *McPSY* was expressed at the highest level in the CNT50+ Plasma 60 group (26.6-fold) and CNT50 group (6.69-fold) (Tab. 4). Between CNT treated groups, CNT50 had significantly higher expression levels (6.5-fold) than the others (*P<0.001*). Among Plasma-treated groups, *McPSY* in Plasma 60 group (3.65-fold) had significantly higher expression levels (*P<0.001*) than Plasma 120 and control groups (Tab. 4). The *McPDS* expression showed a similar expression trend to *McPSY*, and at the highest expression level in the CNT50 group (58.06-fold) and CNT50+ Plasma 60 group (37.45-fold) (Tab. 4). Between CNT- treated groups, CNT50 (58.06-fold) had significantly higher levels than the other concentrations (*P<0.001*). However, there is no significant difference between Plasma-treated groups. Finally, between the combination groups treated with Plasma and CNT, the CNT50+ Plasma 60, had significantly higher expression levels (37.45-fold) than the others (*P<0.001*) (Tab. 4).

Fig. 3: The recorded differences in plant early growth following seed priming with the plasma and the supplementation of carbon nanotubes (CNT), 8 days after the treatments. A-Control; B-CNT of 50 mgl^{-1}; C-CNT of 100 mg l^{-1}; D-CNT of 200 mg l^{-1}; E-plasma of 60 s; F- plasma of 60 s and CNT of 50 mg l^{-1}; G-plasma of 60 s and CNT of 100 mg l^{-1}; H-plasma of 60 s and CNT of 200 mg l^{-1}; I-plasma of 120 s; J-plasma of 120 s and CNT of 50 mg l^{-1}; K-plasma of 120 s and CNT of 100 mg l^{-1}; L-plasma of 120 s and CNT of 200 mg l^{-1}.

HPLC analyses of carotenoids

Carotenoids (β-carotene, zeaxanthin, α-carotene, and lutein) were identified in different treatment groups by HPLC. The carotenoid levels varied in the different treated groups of *M. charantia* (Tab. 4).

Changes in β-carotene content

Among Plasma-treated groups, the content of β-carotene in Plasma 60 was 155.5 μg g^{-1} fw and had higher levels than Plasma 120 and control groups (Tab. 4). In addition, in CNT-treated groups, the CNT50 group with 249.3 μg g^{-1} fw content of β-carotene had significantly higher levels (*P<0.001*) than CNT100, CNT200, and control groups (Tab. 4). Totally, between all treated groups, the CNT50 group and CNT50+Plasma60 group had significantly higher levels of β-carotene content than the other treated groups (Tab. 4). The lowest level of β-carotene was in Plasma 120 and CNT200 groups, with no significant difference from the control group (Tab. 4).

Changes in zeaxanthin content

The production of zeaxanthin in all groups was less than 9 μg g^{-1} fw, which is relatively low (Tab. 4). In Plasma-treated groups, the content of zeaxanthin in Plasma 60 was 3.87 μg g^{-1} fw, with significantly higher levels (*P<0.001*) than Plasma 120 and the control groups (Tab. 4). Among CNT-treated groups, the CNT50 group with 8.43 μg g^{-1} fw content of zeaxanthin had significantly higher levels (*P<0.001*) than the other CNT and control groups (Tab. 4). Finally, between all treated groups, the CNT50, CNT50+Plasma 60 group, and CNT100+Plasma 60 group had significantly higher zeaxanthin levels (*P<0.001*) than the other treated groups (Tab. 4). The lowest zeaxanthin level was in Plasma 120 and CNT100 groups and there was no significant difference with the control group (Tab. 4).

Changes in α-carotene content

The findings show that CNT50+ Plasma 60 group had significantly higher α-carotene levels (p<0.001) than the control and other groups. In CNT-treated groups, the CNT50 group had a significantly higher (p<0.001) α-carotene level than the CNT100, CNT200, and control groups (Tab. 4). However, there were no statistically significant differences in α-carotene production between plasma-treated groups. Among combination groups, treated with plasma and CNT simultaneously, both CNT50+ Plasma 60 and CNT100+ Plasma 60 groups had significantly higher α-carotene levels than the other treated groups (p<0.001) (Tab. 4). Meanwhile, the CNT200 group contained the lowest α-carotene level (Tab. 4).

Changes in lutein content

Tab. 4 shows that the highest lutein level was observed in the CNT50+Plasma 60 group with a content of 171.6 μg g^{-1} fw. Moreover,

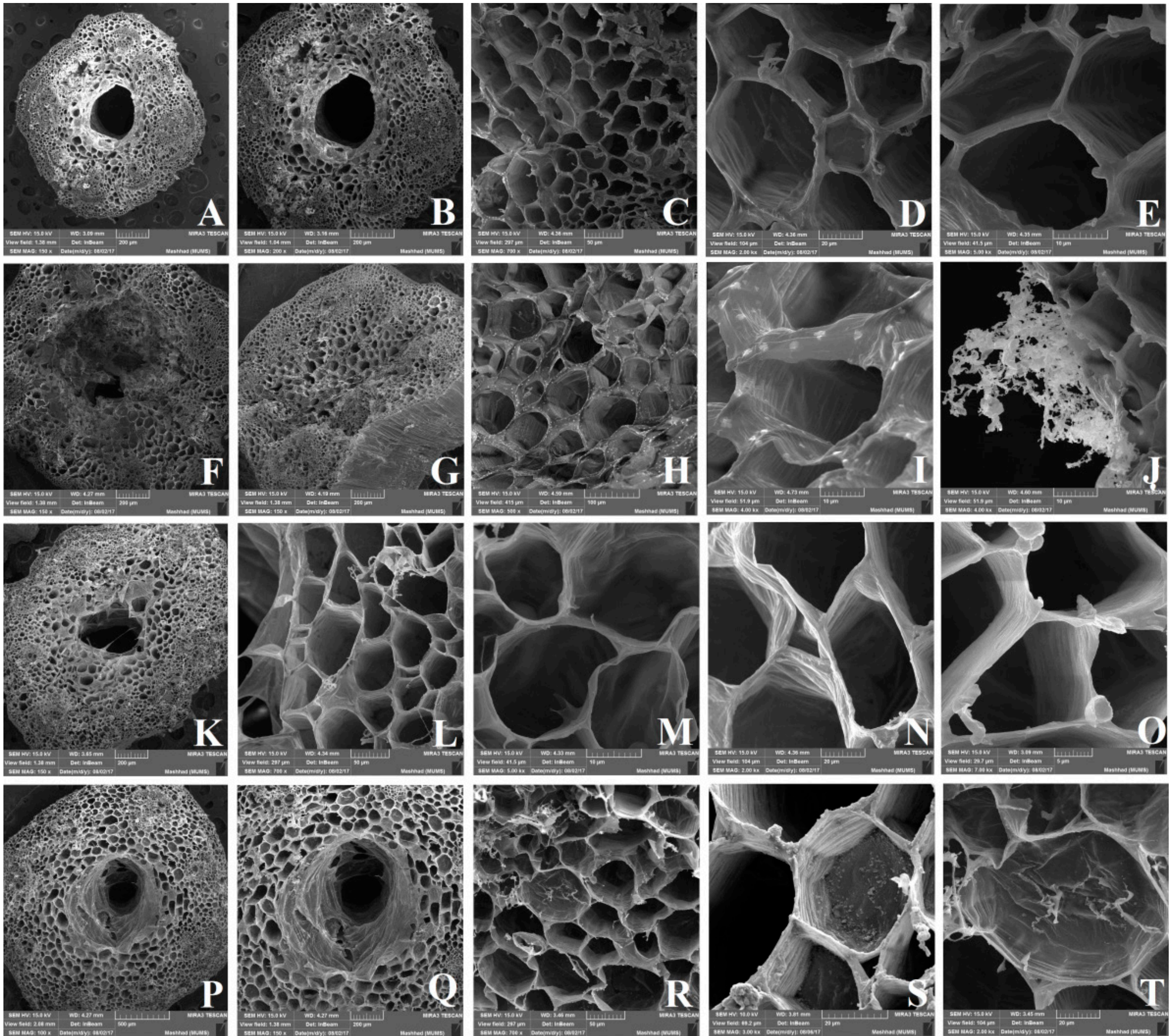

Fig. 4: The ultra-structure images are based on the electron microscopy (FESEM) for tracing the uptake of CNT in shoots of the seedlings treated by the plasma and CNT. Cross-sections of *M. charantia* main shoots after exposure to (A, B, C, D, and E) control, (F, G, H, I and J) functionalized CNT 200 mgl^{-1}, (K, L, M, N, and O) Plasma of 120 s (P, Q, R, S, and T) Plasma of 120 s + functionalized CNT 200 mgl^{-1}. From left to right panels represent general and detailed information, respectively, concerning selected areas.

Tab. 4: Evaluation of PSY and PDS genes expression and carotenoid content (μg g^{-1} dry weight). Results expressed as mean ±SD (n=3) in different plasma and CNT treated groups of *M. charantia**.

	Control	**CNT50**	**CNT100**	**CNT200**	**P60**	**CNT50+P60**	**CNT100+P60**	**CNT200+P60**	**P120**	**CNT50+P120**	**CNT100+P120**	**CNT200+P120**
PSY gene	1 ± 0.001^{f}	6.69 ± 0.19^{b}	0.07 ± 0.03^{g}	3.95 ± 0.14^{c}	4 ± 0.38^{c}	26.62 ± 0.05^{a}	1.12 ± 0.04^{f}	2.21 ± 0.01^{e}	0.36 ± 0.02^{g}	3.03 ± 0.03^{d}	0.14 ± 0.02^{g}	$1.1\pm<0.001^{f}$
PDS gene	1 ± 0.001^{g}	58.06 ± 0.16^{a}	0.43 ± 0.11^{gh}	5.69 ± 0.02^{d}	0.33 ± 0.02^{gh}	37.45 ± 0.81^{b}	0.28 ± 0.09^{gh}	27.09 ± 0.02^{c}	0.73 ± 0.01^{gh}	3.81 ± 0.005^{e}	0.12 ± 0.03^{h}	2.88 ± 0.16^{f}
β-carotene	104.8 ± 8.08^{d}	249.3 ± 20.2^{a}	171.6 ± 12.8^{bc}	121.9 ± 8.14^{cd}	155.5 ± 19.3^{bcd}	245 ± 16.14^{a}	202 ± 12.29^{ab}	152.5 ± 20.9^{cd}	99.6 ± 14.42^{d}	209.6 ± 37.1^{bc}	141.3 ± 17.4^{cd}	113.6 ± 4.1^{cd}
zeaxanthin	2.31 ± 0.71^{bc}	8.43 ± 1.72^{a}	2.9 ± 0.86^{bc}	3.97 ± 0.32^{b}	3.87 ± 1.17^{bc}	7.44 ± 1.09^{a}	7.32 ± 0.56^{a}	3.02 ± 0.58^{bc}	1.07 ± 0.25^{c}	4.34 ± 1.12^{bc}	3.98 ± 0.87^{bc}	3 ± 0.43^{bc}
α-carotene	3.28 ± 0.48^{e}	11.53 ± 1.74^{abc}	10.3 ± 1.9^{abcd}	4.44 ± 0.87^{de}	5.89 ± 0.95^{cde}	14.34 ± 1.93^{a}	13.18 ± 2.28^{a}	10.6 ± 1.32^{abc}	6.3 ± 1.14^{cde}	8.34 ± 1.5^{bcde}	12.45 ± 1.8^{ab}	7.16 ± 1.61^{cde}
Lutein	39.23 ± 6.42^{a}	123.5 ± 19.3^{ab}	73.6 ± 10.1^{bcd}	69.4 ± 10.38^{cd}	45.67 ± 8.51^{d}	171.6 ± 21.5^{a}	118.6 ± 15.6^{abc}	98.37 ± 9.89^{bc}	64.8 ± 7.31^{cd}	108.5 ± 4.98^{abc}	85.2 ± 5.3^{bcd}	98.3 ± 35^{bcd}

*Statistical significance of the differences between treated groups was determined using ANOVA followed by paired-group comparisons. The different letters (a, b, c and d) indicate significance at $P< 0.05$.

in CNT-treated groups similar to α-carotene, the CNT50 group had a significantly higher lutein level (p<0.001) than the CNT100, CNT200, and control groups (Tab. 4). However, there were no significant differences in lutein levels between plasma-treated groups. Meanwhile, the Plasma 60 group contained the lowest level of lutein (Tab. 4).

The correlation between carotenoid contents and the expression of McPSY and McPDS

Correlation analysis, *inter se McPSY* and carotenoid contents in all groups revealed significant association between the *McPSY* with all forms of carotenoid [β-carotene (r=0.602, *P< 0.001*), zeaxanthin (r=0.539, *P< 0.001*), α-carotene (r=0.405, *P< 0.014*) and lutein (r=0.646, *P< 0.001*)]. Also, correlation analysis, *inter se* the *McPDS* and carotenoid contents in all groups revealed significant association between this gene with all forms of carotenoid [β-carotene (R=0.643, *P< 0.001*), zeaxanthin (r=0.568, *P< 0.001*), α-carotene (r=0.442, *P< 0.007*), and lutein (r=0.587, *P<0.001*)]. Regarding the coefficients, correlation between the expression of two genes and other variables is significant (p <0.001), showing a direct relationship between them (Tab. 5).

Tab. 5: Correlation coefficients (R) exhibiting relationship between the expression of two evaluated genes (PSY and PDS) and the measured related carotenoids (lutein, α-carotene, zeaxanthin, and β-carotene).

Genes	lutein	α-carotene	zeaxanthin	β-carotene
McPSY	0.646**	0.405*	0.539**	0.602**
McPDS	0.587**	0.442**	0.568**	0.643**

*: p≤0.05
**: p≤0.01

Discussion

In the present study, the relationship between carotenoid accumulations and the gene expression of *McPSY* and *McPDS,* under individual and combined treatments of cold plasma and carbon nanotube, were investigated in *M. charantia,* which could potentially be used as a source of carotenoid in human nutrition. The seed priming with cold plasma and supplementation of nutrient solution with MWCNT in both individual and combined treatments, not only improved plant early growth but also modified the secondary metabolism through changes in expression patterns of two key genes, *McPSY* and *McPDS*, contributed to the synthesis of important terpenoid metabolites. In our previous report, we provided comprehensive evidence on how CNT and cold plasma treatments were associated with significant modifications in plant growth, biomass accumulation, yield, and differentiation of tissues (especially xylem conducting tissue) (Seddighinia, 2020).

The individual or combined treatments of cold plasma and MWCNTs, especially CNT50+plasma60, were capable of upregulating genes involved in carotenoid metabolism. As it is well known, carotenoids contribute to protecting the photosynthesis apparatus through the Xanthophyll cycle. There is limited molecular evidence on transcriptional responses to CNTs. In this regard, earlier reports showed that CNT application was associated with changes in growth and development-related genes, including *SLR1*, *RTCS*, *RTH1*, and *RTH3* genes in maize (Yan et al., 2013) and *CycB*, *NtLRX1*, and *NtPIP1* in tomato (Khodakovskaya et al., 2012). Moreover, several reports indicated that CNTs were associated with upregulation in genes involved in secondary metabolism which our results are consistent with their findings. In line with our results, the CNT treatments mediated changes in secondary metabolism through upregulating several genes, like *DAT* in *Catharanthus roseus* (Ghasempour, 2019), *PAL*, *TAT*, and *RAS* in *Salvia verticillate* (Rahmani, 2020), and *HPPR*, *RAS*, *PAL*, *TAT* genes in *Satureja khuzistanica* (Fatemi, 2019). LC-MS analysis revealed that MWCNT supplementation altered total fruit metabolome in tomato crops (McGehee, 2017). These results manifested that similar to other nanomaterials, CNTs can trigger signalling thereby influencing transcriptions of genes and metabolism. Apart from CNTs, cold plasma is also capable of affecting the transcription of genes. Seed priming with cold plasma affected the expression of several genes in diverse plant species, like *PAL* and *USP* in *Astragalus fridae* (Moghanloo, 2019), defense-related genes in tomato (Adhikari, 2020), and *WRKY1* transcription factor, *THCAS*, *OAC*, *CBDAS*, and *OLS* in Hemp (Iranbakhsh et al., 2020). In sunflower, the cold plasma priming was also associated with transcriptional responses and substantial variation in phytohormones (Mildaziene et al., 2019). The plasma-mediated changes in the transcription of genes can be explained by bioactive signalling agents generated during the plasma generation (Babajani, 2018; Iranbakhsh, 2017; Iranbakhsh, 2018; Nasrin Safari, 2017; Sheteiwy et al., 2018). Our results along with these recent reports underline this hypothesis that cold plasma perception and signal transduction can modify transcription of genes, thereby improving plant growth and metabolism. However, more molecular studies are required to fill knowledge gaps and elucidate the potentially involved mechanisms in plant responses to cold plasma and CNTs.

In the present study, it was shown that these two treatments could have synergistic effects. In this connection, the results of this study also demonstrated that CNT50 + plasma 60 can stimulate the higher expression of *McPSY* and *McPDS*, compared to the other treated groups; however, the other had acceptable expression. Thus, it can be concluded that plasma 60 can increase the efficiency of the CNT50. Furthermore, the CNT treatments (long-term), especially CNT50, were more efficient than the individual plasma priming (short-term treatment) in affecting the gene expression or carotenoid levels. It should be noted that the significant correlations were found between the *McPSY* and *McPDS* expressions and carotenoids' concentrations. However, the similar regular linear relationship between the CNT concentrations, gene expression levels, carotenoid contents, and growth performance was not found and the individual or combined treatments of the CNT50 provoked the highest expression of *McPSY* and *McPDS*, as well as terpenoid derivatives. Interestingly, the CNT200 treatment led to the best plant growth performance, while CNT50 provoked the highest expression. Hence, it can be proposed that CNT can affect diverse signalling pathways, thereby altering growth and metabolism. Therefore, it seems that the individual or combined treatments of the CNT and plasma, differentially affect plant cell at diverse growth, physiological, and molecular levels in a dose-dependent manner.

To the best of our knowledge, this experiment provides the first molecular evidence on how cold plasma and CNT treatment may synergistically improve carotenoid metabolism which is an important mechanism in terms of photosynthesis performance and protection. The observed molecular variations at the transcriptional level may be explained by modulation in redox homeostasis in response to CNT (Fatemi, 2019; Rahmani, 2020) and cold plasma (Ghasempour, 2019; Iranbakhsh et al., 2020; Mildaziene et al., 2019; Moghanloo, 2019). Monitoring the potential variation in cellular redox status following cold plasma and CNTs is, therefore, recommended for designing future studies.

Conclusions

In the present article, we addressed the efficacy of CNT and cold plasma treatments to affect the metabolism of carotenoids. According to our results, the CNT and plasma-mediated modification in the me-

tabolism of carotenoids can be considered as an important mechanism by which these treatments may protect the photosynthesis apparatus against photoinhibition. Herein, a pilot experiment is presented for introducing new effective methods, which could be exploited in the food, medicinal, and agricultural industries.

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Conflict of interest
The authors declare no conflict of interest.

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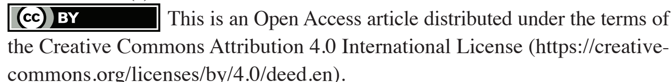
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