Journal of Nuts

Journal homepage: ijnrs.damghaniau.ac.ir

Priming with CuO Nanoparticles and Ultrasound Enhanced Antioxidant Potential and Total Taxol in the Cell Suspension Culture of *Corylus avellana* L.

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A R T I C L E I N F O

Keywords:

Antioxidant responses;

Elicitors;

Sonication;

Total taxol

A B S T R A C T

The main superiority of taxol production in hazelnuts compared to the yew tree is that hazelnut is broadly accessible, grows much faster in nature, and is easier to establish in the *in vitro* systems. The aim of this experiment was to improve taxol production and evaluate other characteristics of cell suspension culture treated with 4 concentrations (0, 2, 4, 6 mg L^{-1}) of copper oxide nanoparticles (CuO NPs), ultrasonication (30 and 60 seconds) in two sampling times (9 and 18 days after treatment). Interaction of all three factors was significant on fresh weight (FW), protein content, H_2O_2 generation, and taxol content. The highest fresh and dry weights (DW) and protein content were obtained from non-application treatments of CuO NPs. The content of anthocyanins, proline, antioxidant activity, and catalase and peroxidase enzymes increased with the application of silver nitrate nanoparticles. Comparing means for interaction effects showed that H_2O_2 and taxol content have nearly similar trend, indicating the stimulation of taxol production pathway by reactive oxygen species (ROS). Ultrasonication (US) enhanced most of the traits studied in this experiment and stimulated the antioxidant system without negatively affecting on cellular growth. The antioxidant response and taxol content evoked by stress treatments i.e. US and CuO NPs is partially relieved by increasing the duration after treatment (the 18th day after treatment).

Introduction

Taxol is one of the most effective anti-cancer drugs on the market. The easiest way for taxol production is to elicit it from hundred-year-old yew trees, but this elicitation leads to the destruction of these natural resources because the amount of taxol metabolite is very low and this tree has very slow growth (Itokawa, 2003). This metabolite was also found in small amounts in different parts of hazelnut (Itokawa, 2003) as well as callus and its cell culture (Bestoso *et al*., 2006, Rahpeyma *et al*., 2015). The growing demand for this compound due to its use in the cure of many types of cancer, as well as its high cost, has prompted efforts to find alternative ways to obtain this compound. Hazelnut (*Corylus avellana* L.), formerly grown for its nutritional aspects, is now gaining attention due to its phytochemical content (Gallego *et al*., 2017).

The elicitation of secondary metabolites can be enhanced through cell cultures by selecting a proper cell line, and controlling the culture conditions (Fett-neto *et al*, 1993), using different eliminators (Tabata, 2004; Farrokhzad and Rezaie, 2020), and optimizing the extraction procedures (Zhao *et al*., 2005). The search for

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Received: 23 March 2021; Received in revised form:5 May 2021; Accepted: 31 July 2021

DOI: 10.22034/jon.2021.1925927.1109

new and more potent elicitors for developing taxol biosynthesis has become one of the most important scientific issues (Cusido *et al*., 2014). In cell suspension culture, elicitor is an additive compound capable of improving the production of a specific metabolite. Elicitors are usually divided into biotic (i.e. yeast extract, chitosan) and abiotic (i.e. methyl jasmonate, salicylic acid, vanadyl sulfate) categories depending on their chemical nature and endo- or exogenous origins (Gatahi *et al*., 2016; Ramirez-Estrada *et al*., 2016).

Heavy metals are among the abiotic metabolites that have found interesting applications. Farrokhzad and Rezaie (2020) reported the positive effects of the aluminum elements on taxol production in hazelnut cell suspension cultures. Chung *et al.* (2019) investigated the role of copper oxide nanoparticles on cell suspension culture (*Gymnema sylvestre*). They concluded that these nanoparticles significantly increase total phenol, gymnemic acid II, and flavonoids. Low-energy sonication can promote the plant defense mechanisms and increase membrane permeability; giving rise to enhance extraction of metabolites (Chung *et al*, 2019). Rezaei *et al.* (2011) showed that sonication at a low frequency can stimulate the accumulation of taxol in cell suspension culture.

The key purpose of this research is to assess the possibility of priming with two abiotic elicitors (CuO NPs and US) in order to stimulate taxol production and antioxidant responses. To this end, in addition to measuring different physiological indices, the yield of hazelnut cells in suspension culture medium was also evaluated in terms of taxol production at two-time intervals of 9 and 18 days.

Materials and Methods

Establishment of cell cultures

After eliminating the shells, seed kernels were disinfected in NaOCl solutions containing 0.5% chlorine concentration for 20 min, followed by three times rinsing with sterile deionized water. Callus was developed on MS basal salt solution (Murashige and Skoog, 1962), augmented with 3% sucrose, 1 mg L^{-1} 2,4-D, and 0.5 mg L^{-1} N6-benzyladenine (BA), at a pH of 5.5. Kernel fragments of immature hazelnut seeds were kept on a solid MS salt medium (Fig. 1 a). Cell suspension cultures were initiated by adding 2/5 g callus to the 25 mL liquid MS medium complemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA in the agar-free culture containers culture containers (Fig. 1 b). The cultures were nurtured at 24±2 °C in the darkness using a shaking incubator (110 rpm) and were subcultured every three weeks. Next, the cultures were analyzed in terms of fresh weight (FW), dry weight (DW), and biochemical and taxol contents 9 and 18 days after treatment.

Elicitation of copper oxide nanoparticles (CuO NPs) and ultrasound (US) in cell suspension culture

CuO NPs (copper (II) oxide nanoparticles) less than 100 nm in size were bought from ARMINANO Company (Iran, Tehran). After 4 subcultures, sonication was done at 40 kHz for 30, and 60s and then the cell suspension cultures were completed with 0, 2, 4, or 6 mg L^{-1} CuO NPs. After 48 h, the explants were subcultured on a new liquid medium without CuO NPs. The cell mass was harvested from the medium 9 and 18 days after treatment to quantify FW, DW, and other indices. Cell mass gathered by filtering followed by raising with sterile distilled water and blotting on the sterile Whatman filter to eliminate moisture before FW quantification. The gathered cell mass was frozen in liquid N_2 and kept at -80°C until further analyses.

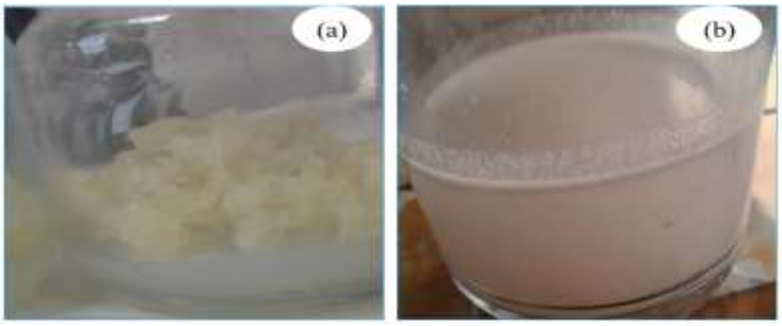

Fig. 1. Callus development from immature seed fragments (a) and suspension cultures (b) of *C. avellana*

Growth and biochemical analysis

The tests were carried out in Simin Sepehr Mehrandish lab, Tehran-Karaj branch. Mitotic cell growth progression was assessed by quantifying FW and DW. The weight of filtered and distilled water-washed cells was recorded as FW, while the DW of the cell mass was achieved by desiccating the cells at 70 °C in the oven for about 48 h until reaching a constant weight. The method of Bradford (1976), BSA (bovine serum albumin, Sigma Aldrich) was used as a standard to calculate the soluble protein content in the cell samples. The proline level was assessed by the Bates *et al.* (1973) technique as already stated by the other researchers (Lotfi *et al.*, 2010; Aslani Aslamarz *et al.*, 2011; Jariteh *et al.*, 2015). The samples (0.5 g) were grounded with mortar and pestle for homogenization with 3 % (w/v) sulphosalicylic acid followed by filtering with filter papers. After adding ninhydrin acid and glacial acetic acid, the homogenate was incubated at 100 ° C for 60 min in a water bath. An ice bath was used to stop the reaction. The homogenate extraction was conducted using toluene, and the absorbance of the fraction in toluene was recorded at 520 nm.

For determining the anthocyanin content of the samples, 1 g of collected cells was ground and homogenized in 10 ml methanol, and the extract was stored at 4°C in the dark for 24h. The homogenate was centrifuged at 4000 rpm for 10 min. The soluble anthocyanin in the supernatant was quantified at 520 nm based on the Wagner (1979) method.

Antioxidant, hydrogen peroxide, catalase and peroxidase activity

Peroxidase activity was evaluated using a solution containing 5.8 ml of phosphate buffer (0.1 M, pH 6.8), 0.2 ml enzyme extract, and 2 ml of 20 mM hydrogen peroxide. After the addition of 2 mL of 20 mM pyrogallol, the increase in the absorbance of the pyrogallol was determined using a spectrophotometer for the 60s at 470 nm. Following the addition of 2 ml of 20 mM pyrogallol, the increase in pyrogallol absorbance was measured at 470 nm for 60s using a spectrophotometer (Kar *et al.*, 1976). Catalase activity was calculated according to the method explained by previous researchers (Aebi and Bergmeyer, 1983; Jariteh *et al.*, 2011). The reaction mixture at a final volume of 10 ml containing 9.96 mL H_2O_2 phosphate buffer (0.1 M, pH 6.8) was added to the reaction mixture to reach a final volume of 10 mL containing 40 µL enzyme extract. CAT activity was spectrophotometrically monitored considering the variation rate of hydrogen peroxide absorbance at 250 nm over 60 s.

The radical scavenging assay of cell suspension culture extracts was carried out using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) test (Chung *et al.*, 2018).

Extracts (100 µL) were added to 1.4 mL DPPH solution and kept in the darkness at 25°C for 45 min. The absorbance (A) was spectrophotometrically recorded at 517 nm. Inhibition (%) was calculated as [(Ablank − Asample)/Ablank] × 100.

Total taxol quantification

The total content was evaluated based on the technique proposed by Rezaei *et al.* (2011). To quantify intracellular taxol, the desiccated cell mass was ground for 5 min at 25 °C, homogenized in 10 ml methanol, and sonicated for 40 min followed by filtering. The filtered cells were then dehydrated at 25°C and reliquefied in the methylene chloride and water solution (1:1), followed by centrifugation at 5000 rpm. The methylene chloride phase was harvested, dehydrated at 25°C, and reliquefied in 250 ml of methanol, and filtered through a 0.45 mm syringe filter before analysis by high-performance liquid chromatography (HPLC). Moreover, extracellular taxol (in medium) was elicited with methylene chloride (1:1) in a separating funnel. The collected methylene chloride phase was dissolved in 250 ml of methanol, dried at 25°C, and filtered with a 0.45 mm syringe filter before the HPLC test. The HPLC assay was equipped with a C-18 column. Taxol was eluted at a flow rate of 1 mL/min (in the methanol-to-water ratio of 45:55, v/v) and characterized at 227 nm using an ultraviolet detector (Bestoso *et al.*, 2006). Taxol detection was performed by comparing the retention time and peak area with the original standard (Sigma). The total taxol refers to the sum of intra- and extracellular taxol contents.

Statistical analysis

Differences between the treatment means were compared using the least significant difference (LSD) test at the significance level of 0.05%. This study was conducted in a three-factor factorial test on the basis of a completely randomized design with three replications. Factors included 4 CuO NPs concentrations (0, 2, 4, 6 mg L^{-1}), two sonication times (30 and 60 s), and two sampling times (9 and 18th day after treatment) (Table 1). Data analysis was done by SAS 9.1 software and mean of interactions was done using MSTATC software.

Table 1. Treatments used during the present investigation for cell suspension culture of *C. avellena*

Treatment codes	CuO NPs (mg L^{-1})	Time (days)	US (s)
T1	0	9	30
T2	0	9	60
T3	0	18	30
T4	0	18	60
T5	2	9	30
T6	2	9	60
T7	2	18	30
T8	2	18	60
T9	4	9	30
T10	4	9	60
T11	4	18	30
T12	4	18	60
T13	6	9	30
T14	6	9	60
T15	6	18	30
T16	6	18	60

Results

The interactive effect of CuO NPs, US, and sampling time was significant on fresh weight, H_2O_2 amount, protein content, and total taxol at a significance level of 5%. Sampling time significantly influenced all traits evaluated in this study at the level of 1 %. The US also substantially affected most of the traits evaluated in this study.

The highest and lowest FW values were obtained in T4 (0 CuO + 60 s sonication+ sampling on the 18th day) and T13 (6 CuO + sampling on 9th day + 30 s sonication), respectively. Increasing the concentration of CuO NPs decreased cell growth, however, the 60 s of sonication and 18th day of sampling increased FW level (Fig. 2). The interaction of different treatments on DW was not significant. The highest DW was achieved in the treatment free of CuO NPs. Dry weight decreased with prolonging the sampling time, while longer sonication durations enhanced the dry weight (Fig. 3).

Maximum total protein content was recorded in T1 (0 CuO + 9^{th} day sampling + 30 s sonication). The lowest total protein content was recorded in T14, T15, and T12 (Fig. 4). This finding indicates that increasing the concentration of CuO NPs and duration of sonication reduced the total protein of hazelnut cell samples. Due to the insignificance of interactions on anthocyanin content, our results showed that the content of this plant pigment in all culture flasks containing CuO NPs first increased and then decreased so that the highest content of anthocyanin detected in samples treated with 2 mg L^{-1} CuO NPs and the lowest anthocyanin content was observed at a concentration of 0 mg L^{-1} CuO NPs. Increasing the duration of sonication and 18 days of sampling also increased the anthocyanin content in hazelnut cell samples (Fig. 5).The content of proline as a molecule that is a marker of stress was also affected. Maximum proline content was observed in cell samples containing CuO NPs, especially in concentration of 4 mg L^{-1} CuO NPs and the lowest in flask without CuO NPs (0 concentration). The results of this study showed that proline also increased with increasing duration of sonication so that the 18^{th} day after treatment was more than the 9^{th} day. US as a stressful treatment enhanced proline concentration. Increasing sonication time also increased proline content (Fig. 6).

The main effects of different treatments on antioxidant activity were significant. The highest antioxidant activity was recorded under 2 mg L^{-1} CuO NPs. The lowest level of antioxidant activity was obtained in flasks without CuO NPs. As the duration of sonication (60 s) increased, the antioxidant activity improved, but with increasing the duration after treatment (18^{th} day after treatment), the antioxidant activity reduced (Fig. 7).

The activity of two enzymes, catalase, and peroxidase, showed a similar trend so that the activity of these two enzymes had the highest level at 4 mg L^{-1} CuO NPs and the lowest in untreated flasks (concentration 0 mg L^{-1} CuO NPs). The results showed that the enzyme activity decreased with increasing duration after treatment (18^{th} days of sampling). However, the activity of these two enzymes improved with increasing sonication time (60 s) (Fig. 8, 9).

H_2O_2, which is a ROS species, was affected by the interaction of three treatments, so that its highest content was recorded in T14 (6 mg L^{-1} CuO NPs, 60 s sonication and 9^{th} day of sampling) and T16 (6 mg L^{-1} CuO NPs, 60 s sonication and 18^{th} day of sampling) treatments, however, its lowest in T3 (0 mg L^{-1} CuO NPs, 30 s sonication and 18^{th} day of sampling) and T1 (0 mg L^{-1} CuO NPs, 30 s sonication and 9^{th} day of sampling) treatments (Fig. 10).

Taxol production, which was the most important aim of this experiment, was also affected by the interaction of all three treatments, so that the highest total taxol content was seen in T14 (0 mg L^{-1} CuO NPs, 30 s sonication, and 9^{th} day of sampling) and then T16 (6 mg

L^{-1} CuO NPs, 60 s sonication and 18th day of sampling). The lowest was observed in T1-T4 (non-CuO NPs treatments) (Fig. 11).

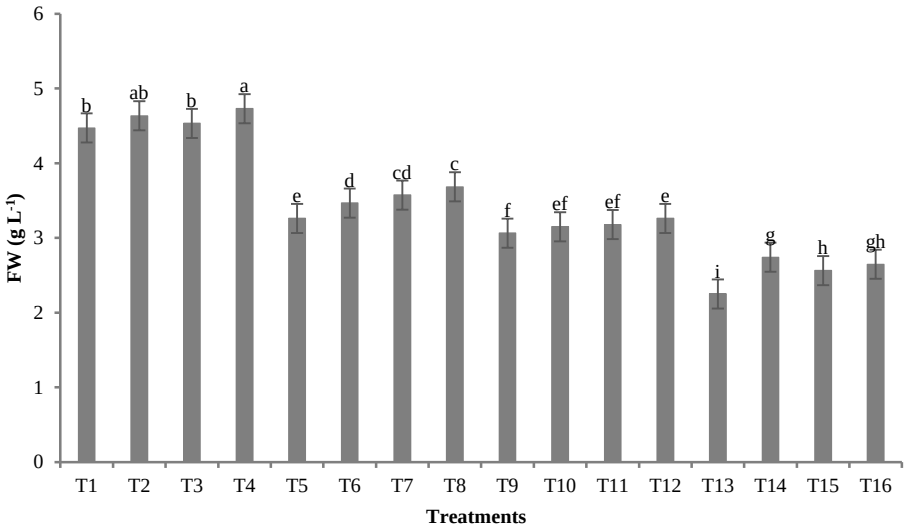

Fig. 2. Effect of different treatments on fresh weight (FW) of *C.avellana* in cell suspension cultures (T1-T16 presented in Table 1)

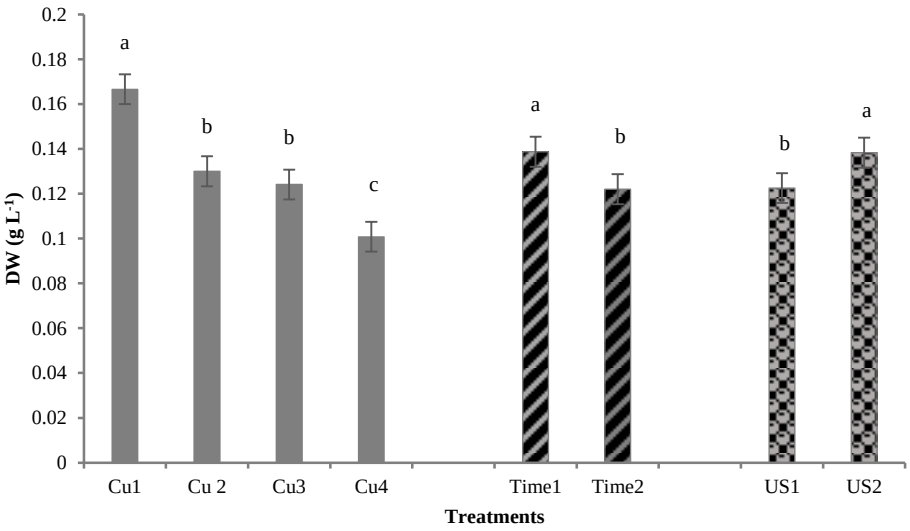

Fig. 3. Effect of different treatments on dry weight (FW) of *C. avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

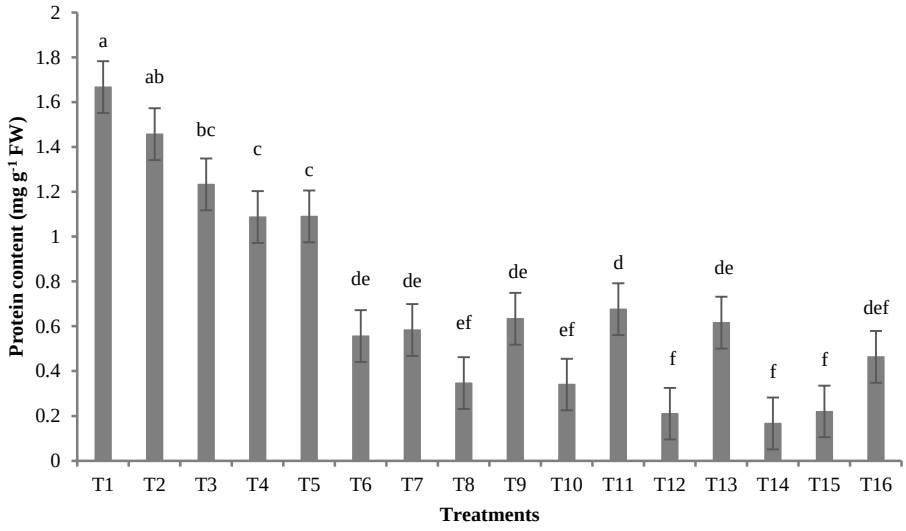

Fig. 4. Effect of different treatments on protein of *C. avellana* in cell suspension cultures (T1-T16 presented in Table 1)

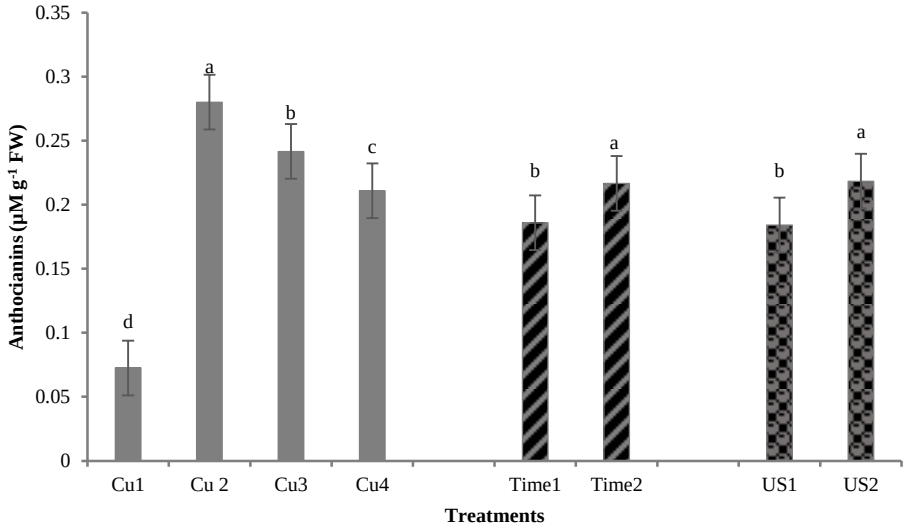

Fig. 5. Effect of different treatments on antioxidant content of *C. avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

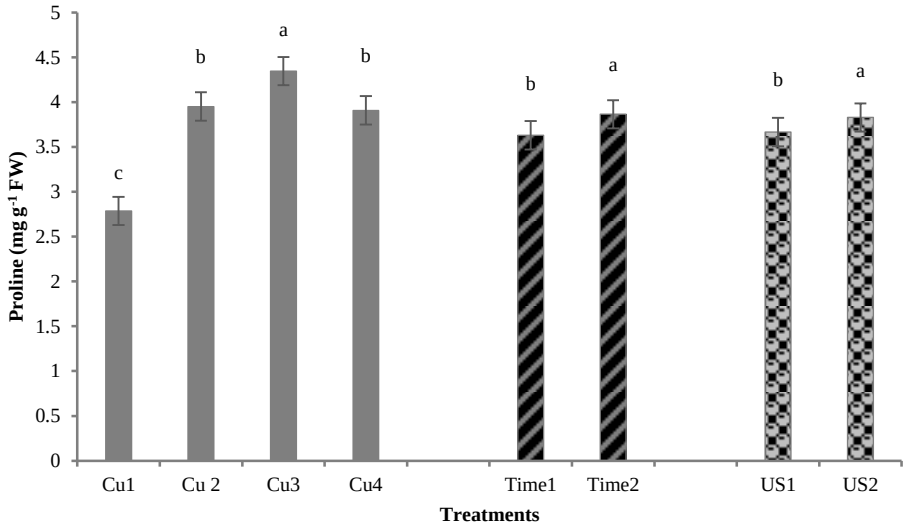

Fig. 6. Effect of different treatments on proline of *C. avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

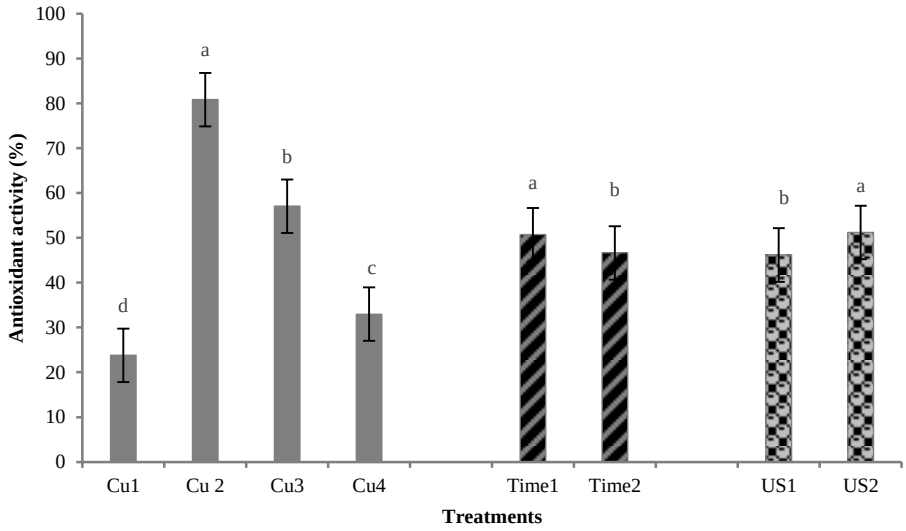

Fig. 7. Effect of different treatments on antioxidant activity of *C.avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

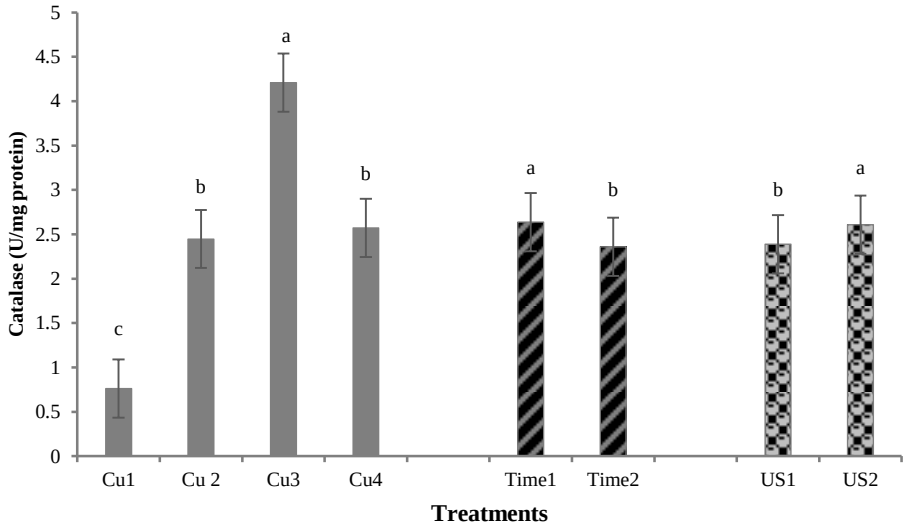

Fig. 8. Effect of different treatments on Catalase activity of *C.avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

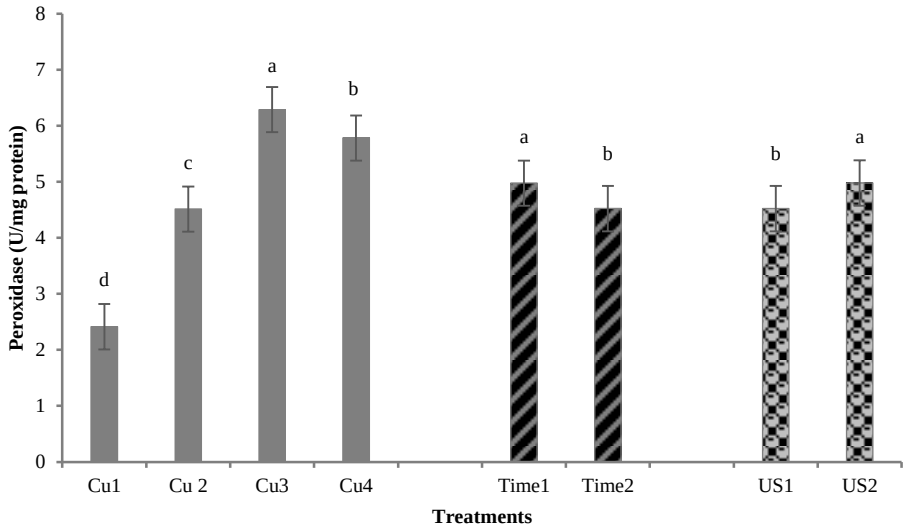

Fig. 9. Effect of different treatments on Peroxidase activity of *C.avellana* in cell suspension cultures (Cu1-Cu4: 0, 2, 4, 6 mg L^{-1} CuO NPs, respectively; sampling time1 and 2: 9 and 18th day after treatments; Sonication time of US1 and US2: 30 and 60s, respectively)

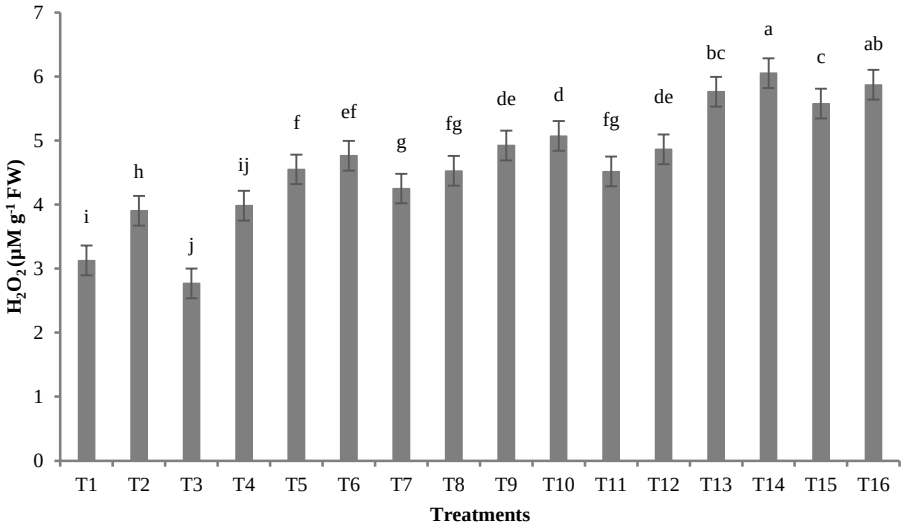

Fig. 10. Effect of different treatments on H_2O_2 generation of *C.avellana* in cell suspension cultures (T1-T16 presented in Table 1).

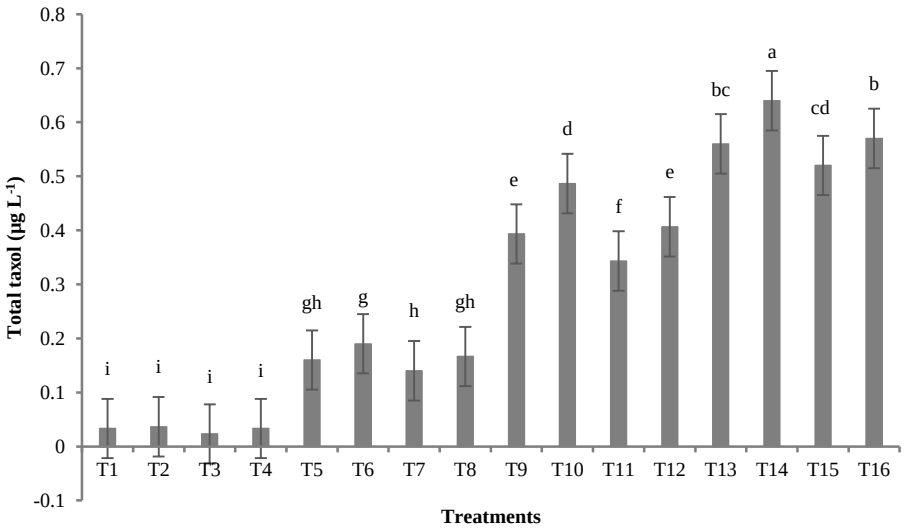

Fig. 11. Effect of different treatments on total taxol (FW) of *C. avellana* in cell suspension cultures (T1-T16 presented in Table 1).

Discussion

The effects of nanoparticle stimulants in the promotion of stress responses in the main plants have found economic and medicinal importance. NPs can be used as non-living elicitors in plant biotechnology to promote the biosynthesis of valuable bioactive compounds (Fakruddin *et al*., 2012).

Our results presented that the total protein content reduced with the increasing concentration of CuO NPs (Fig. 4). Anthocyanin pigment increased under low concentrations of CuO NPs (2 mg L^{-1}) but decrease somewhat at higher levels (4 and 6 mg L^{-1} CuO NPs). However, in general, the anthocyanin content at all CuO NPs treated flasks was higher than non-CuO NPs treated (0 mg L^{-1} CuO NPs) (Fig. 5). Antioxidant activity showed a similar trend as anthocyanin content (Fig. 7). Flasks treated with CuO NPs have a higher proline

content than untreated flasks (0 mg L^{-1} CuO NPs). The activity of catalase and peroxidase elevated with increasing concentration of CuO NPs but decreased to some extent at a concentration of 6 mg L^{-1} (Fig. 8 and 9). It can be concluded that the increase in H_2O_2 concentration may be due to a decrease in the activity of enzymes in the high level of CuO NPs (6 mg L^{-1}) because the catalase and peroxidase enzymes play a key role in suppressing ROS species. All of these effects indicate the fact that the increase in the level of CuO NPs causes the cell cultures exposed to stress conditions and stress responses are seen in the flasks treated with high levels of CuO NPs. Although cell growth continued under all treatments, it seems that the main reason for fresh and dry weight loss is the stressful effects of high levels of CuO NPs.

The generation of H_2O_2 as a reactive oxygen species (ROS) increased under high levels of CuO NPs, which was destructive to plant tissues and raised oxidative stress, leading to cell death. ROS damaged biological molecules such as DNA, proteins, and lipids and served as a secondary signal in low/medium concentrations in intracellular signaling cascades, thus intervening in various responses of plant cells. Plants can eliminate ROS by activating their enzymatic and non-enzymatic antioxidant systems (Nasibi *et al.*, 2020; Jariteh *et al.*, 2011). This mechanism plays a key role in reducing copper toxicity in plants such as *Zea mays* L. (Murakami et al., 2009). Furthermore, a high level of heavy metals can suppress the antioxidant defense of a plant (Bertini *et al*., 2019).

The results of this study showed that increasing the total taxol content has a similar process to H_2O_2. According to Jamshidi *et al*. (2016), the generation of efficient amounts of H_2O_2 can stimulate the antioxidant defense system and affect taxol production in hazelnut cell suspension cultures. Farrokhzad and Rezaei (2020) investigated the effect of aluminum as a heavy metal on hazelnut cell culture. Its effect on reducing cell growth, augmenting the antioxidant system, and taxol production were similar to the present study. Our results are in line with the results of Jamshidi *et al*. (2016) who examined the effect of silver nitrate nanoparticles on hazelnut cell suspension culture.

In this study, the influence of two sampling times (9 and 18th days after treatment) on physiological and biochemical characteristics of hazelnut cell culture was investigated. The results showed that with increasing time after treatments (18th day after treatment), dry weight, antioxidant activity, enzymatic activity (catalase and peroxidase) decreased, however, proline and anthocyanin content increased, also, the taxol yield in the samples found decreased on the 18th day after treatment. After exposing cells to treatments, the taxol production of cells was increased , but after a while, cultures adapted to the stress conditions, and the stress responses quenched to some extent. This should be considered in the production of valuable metabolites such as taxol because various studies have shown that the use of eliminators temporarily stimulates cellular pathways and production of secondary metabolites and decreases in subsequent passages (Gallego *et al*., 2017).

The effect of ultrasound on the production of taxol has already been investigated by Rezaei *et al*. (2011). However, the effect of sonication time in combination with CuO NPs is new and according to reports, a synergistic effect is often observed between several elicitors in the biosynthesis of secondary metabolites (Zhao *et al*., 2005). In the present study, ultrasound increased the majority of the studied traits, and 60s sonication stimulated the major traits related to the antioxidant response. Ultrasound (60s sonication) in combination with CuO NPs on the 9th day after treatment (T14) was the best treatment combination in terms of taxol production. Farrokhzad *et al*. (2016) reported that ultrasound treatment stimulates callus growth in *Vitis vinifera* cv.Kondori. One of the reasons that may be the cause of the decrease in taxol production in the next passages is the decrease in cell growth. The use of elicitors, which are mainly stressful, reduces cell growth

in cell suspension cultures. However, it seems that under ultrasound treatment, while maintaining cell growth, the taxol content also increases. However, in addition to the sonication time, US frequency (kHz) must also be optimized.

Conclusions

Considering the obtained results, CuO NPs application (6 and 4 mg L^{-1} concentration) in combination with 60s sonication on the 9^{th} day after treatment enhanced total taxol content. CuO NPs-elicitor application combined with sonication (60 s) also increased enzymatic and non-enzymatic antioxidant systems. However, antioxidant and oxidant responses quenched after a while (18^{th} day after treatment). Therefore, our procedure could be valuable for further research around the biosynthesis of taxol in the cell suspension cultures of *C. avellana*.

Acknowledgments

The authors acknowledge Simin Sepehr Mehrandish Laboratory, Tehran-Karaj Branch for laboratory services and facilities.

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