Aluminum elicitation improves antioxidant potential and taxol production in hazelnut (*Corylus avellana* L.) cell suspension culture

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Summary

Taxol was originaly found in yew (*Taxus* sp.) but it also ocurrs in other species like hazelnut (*Corylus avellana* L.). We investigated production of taxol in suspension cultures of hazelnut following elicitation by aluminium chloride supplemented at 0, 25, 50 and 100 μM concentration. Aluminium elicitation has a significant effect decreasing cell growth and protein content but increasing production of taxol, total phenolics, anthocyanin and flavonoids. It also increases antioxidant potential and other antioxidant system components such as proline and enzymes like catalases and peroxidases. Additionally, aluminium increased hydrogen peroxide and malondialdehyde content of cells. Apart from taxol production aluminium improved its release into the medium and total yield in comparison to controls. In general, for the increased taxol synthesis application of 50 and 100 μM of aluminium elicitor in cell culture provided acceptable results.

Key words

Taxol, *Corylus avellan*a L., hazelnut, suspension cultures, elicitors, aluminium chloride

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Introduction

Secondary metabolite production is a feature found only in a limited number of plant families. In species that produce them, secondary metabolites have diverse functions (Guerriero et al., 2018) among which trigering defence mechanismas are particulary interesting. Production of secondary metabolites by field cultivation of plants has certain disadvantages including low yields, variability induced by geographical, seasonal, and environmental factors. Therefore, plant cell suspension culture systems were proposed as an attractive alternative for production of secondary metabolites (Ramachandra and Ravishankar, 2002).

Accumulation of secondary compounds in plant cells can be improved in response to addition or aplication of various elicitor types (Ramakrishna and Ravishankar, 2011). They are usualy devided into two cathegories, biotic and abitoic elicitors (Namdeo, 2007). Recently abiotic elicitation with inorganic compounds like minerals and metal ions has been successfully applied in plant suspension cultures (Cai et al., 2013). The stimulation of secondary metabolites accumulation by metal salts has been confirmed in cell cultures of several plants including *Catharanthus roseus* (L.) G.Don (Srivastava and Srivastava, 2010), *Vitis vinifera* L. (Cai et al., 2013) and *Pteris vittata* L. (Pham et al., 2017).

Taxol is a complex diterpene alkaloid well known worldwide as the best-selling anticancer drug with an outstanding activity against various cancer types (Cusido et al., 2014; Ramírez-Estrada et al., 2016; Gallego et al., 2017). Since the resources of *Taxus* sp. tree are limited in nature, attention is focused on finding new taxane sources such as endophytic fungi of *Taxus* and hazelnut (*Corylus avellana* L.) cell cultures, which are promising biological materials for production of taxanes in the future (Rezaei et al., 2011; Miele et al., 2012). Hazelnut (*Corylus avellana*) has recently been considered as an alternative source for taxol production as it is much easier to cultivate it in vitro than yew (Otaggio et al., 2008; Bemani et al., 2013).

Various elicitors have been used to increase the production of taxol in cell suspension cultures. Salicylic acid (Rezaei et al., 2011a; Rezaei et al., 2013), methyl jasmonate (Bestoso et al., 2006; Rezaei et al., 2011b; Gallego et al., 2017), ultrasound waves (Rezaei et al., 2011a; Farrokhzad et al., unpublished), coronatine (Gallego et al., 2017) and silver nanoparticles (Jamshidi et al., 2014) were all successfully used to increase taxol yield in hazelnut (*Corylus avellana*) cell suspension culture. Thus in all of these reports taxol production was increased by elicitation. The search for novel and more effective elicitors for improving the taxoids production is currently one of the most important research tasks (Cusido et al., 2014). Therefore, in this study we have, for the first time, investigated the effects of aluminium elicitation on cell growth, taxol production and other parameters of hazelnut cell suspension cultures. The purpose of our study was therefore to introduce aluminium chloride as a novel elicitor for the production of taxol.

Materials and Methods

Cell Culture Establishment

Immature seeds of hazelnut (*Corylus avellana* 'Gerd Eshkeva') that is cultivated in northern parts of Iran were used in experiments. After removing the shells, kernels were surface sterilized in sodium hypochlorite (containing 5% active chlorine) for 20 min, followed by thorough rinsing in sterile distilled water. Callus induction was done on MS (Murashige and Skoog 1962) medium, supplemented with 3% sucrose, 1 mg/l 2,4-D and 0.5 mg/l benzyladenine, at pH 5.5. Explants consisting of kernel fragments of immature seeds were placed on agar solidified MS medium. Cell suspension cultures were established by placing 2 g of white, friable callus in MS liquid medium supplemented with 1 mg/L 2,4-D and 0.5 mg/L 6-benzylaminopurine (BA) in 250 mL Erlenmeyer flasks without agar. The cultures were incubated at 25°C in the darkness, on the orbital shaker (120 rpm) and were subcultured every two weeks.

Elicitation of Cell Cultures with Aluminium

Fragile, yellowish-white colored callus was trimmed into 2 g explants which were incubated in 30 mL of liquid medium in 200 mL shake flasks. Aluminum chloride ($AlCl_3.6H_2O$) was disolved in sterile water and then sterilized by filtration (0.2 µm) and added to media at final concentrations of 25, 50 and 100 µM on the 7th day of subculture. Controls were supplemented with the same volume of sterilized distilled water. The cells harvested on the 14th day were frozen in liquid N2 and stored at -80°C. until they were used for biochemical analysis.

Growth and Biochemical Analysis

Cell growth was evaluated by measuring the increase of cell fresh and dry weight. The cells were filtered, washed with distilled water and weighed as the fresh weight. Dry weight of the cells was obtained by drying the cells at 70°C in a drying oven for about 48 h until constant weight of samples was achieved. Changes in the medium pH was monitored by the use of an H^+ ion electrode. Electrical conductivity (EC) was measured using an EC-meter, PL-700PC (EZDO, Taiwan), with a precision of 0.01 mS and frequency of 50 Hz. Protein concentration was determined by Bradford (1976) method using bovine serum albumin (BSA, Sigma Aldrich) as standard. Level of damage of membranes was determined by measuring malondialdehyde (MDA) as the end product of peroxidation of membrane lipids according to De Vos et al. (1991). The flavonoid content of cells was determined according to the method as described Djeridane et al. (2006). Phenolic compounds were quantified using Folin-Ciocalteu method (Chua et al., 2007). The anthocyanin and proline content was determined by the method of Wanger (1979) and Bates et al. (1973), respectively.

Antioxidant, Hydrogen Peroxide, Catalase and Peroxidase Activity

Antioxidant activity was evaluated by measuring the scavenging activity of extracts using the 2,2-diphenylhydrazil (DPPH) radical assay as described by Adam et al. (2008). Catalase (CAT, EC 1.11.1.6) activity was measured by following the reduction of H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) at 240 nm according to the method of Dhindsa et al. (1981). The peroxidase activity (POD, EC 1.11.1.7) was assessed using guaiacol as an electron donor (Sahebjamei et al., 2007). Hydrogen peroxide content was assayed according to the method of Velikova et al. (2000).

Taxol Extraction and Assay

Taxol was extracted from medium and powdered dried cells by methods previously described by Rezaei et al. (2011). For extraction of cell-associated (intracellular) taxol, the dried cells were powdered for 5 min at room temperature, and dissolved in 10 mL methanol for 40 min. Homogenized solution was filtered, filtrate was air-dried and re-dissolved in a mixture of methylene chloride and water (1:1), and afterwards centrifuged at 5000 rpm. Methylene chloride phase was collected, air-dried and re-dissolved in 250 µL methanol and filtered with a 0.45-µm syringe filter before high-performance liquid chromatography (HPLC) analysis. Extracellular taxol (in the medium) was extracted with methylene chloride (1:1) in a separator funnel. The methylene chloride phase was collected, air-dried, re-dissolved in 250 µL methanol and filtered with a 0.45-µm syringe filter before HPLC analysis. The HPLC system (Knauer, Berlin, Germany) was equipped with a C-18 column (Perfectsil Target ODS3, (5 µm), (250 3 4.6 mm), MZ-Analysentechnik, Mainz, Germany). Taxol was eluted at a flow rate of 1 mL methanol and water (45:55, v/v) min^{-1} and was detected at 227 nm using an ultraviolet detector (PDA, Berlin, Germany) (Bestoso et al., 2006). Identification of taxol was accomplished by comparison of retention time with authentic standard (Sigma, St. Louis, MO, USA).

Statistical Analysis

Differences between the treatment means were compared using LSD test at 0.05% probability level. This research was performed in a factorial experiment on the basis of a completely randomized design with three replications. Data analysis was performed by SAS 9¬.¬4 software and means comparisons was done using LSD test. All values are shown as mean±SD.

Results

Growth and Biochemical Analysis

The effects of aluminium elicitation are presented in Table 1. It significantly affected biomass accumulation (fresh weight and dry weight), total phenol, anthocyanin, malondialdehyde (MDA), flavonoids, pH, proline and protein content. Aluminium decreased the fresh weight and dry weight of tissue so that the highest values for fresh and dry weight were achieved from controls grown on (Al-free medium). Elicitation significantly affected pH ($P \leq 0.05$). As the elicitor concentration increased, the pH means showed a downward trend so that control samples had higher pH values than Al suplemented media.

As shown in Table 1, the contents of the EC have not been affected by the aluminium treatment, as there was no significant difference between the treated and the control samples. Protein content was significantly decreased with lowest values obtained at highest concentrations of aluminium elicitor whilst the highest protein values was registered in control treatment.

In Al-elicitated cultures the levels of MDA significantly increased compared to those in controls. This finding suggested that the cell membrane has been damaged under aluminium treatment (Table 2). Flavonoids were also increased by Al treatments (Table 2), so that the highest means (11.36 ± 4.14 µM g^{-1} FW) was observed in presence of 100 µM elicitor. The minimal flavonoid values were registered in the control treatment (Table 2). Treatment with Al dramatically increased the production of phenolics compared with the control. The highest Al concentration at 100 µM resulted in high accumulation of phenolics (129.58 ± 31.74 µg ml^{-1}), which was about 3-fold higher than in the control. Aluminium elicitation increased anthocyanin levels, but there was no significant difference between the 25, 50 and 100 µM elicitor application (Table 2). The use of aluminium elicitor increased the proline content in samples so the lowest proline content was found in control treatment.

Antioxidant, Hydrogen Peroxide, Catalase and Peroxidase Activity

Antioxidants, catalase and peroxidase together with hydrogen peroxide content were significantly affected by aluminium elicitor ($P \leq 0.05$, Table 3). The highest amounts of antioxidant activity 70.46 ±14.34% and 53.93 ± 9.27 % were obtained in 25 and 50 µM aluminium treatments, respectively, however antioxidant activity at the 100 µM aluminum concentration was lower. (Table 3). Catalase activity had the highest values with 50 µM aluminium elicitor application. In general, all Al containing treatments improved catalase activity compared to control treatment (Table 3). Peroxidase activity was the highest with 50 and 100 µM Al elicitor and the lowest activity was observed in the untreated cultures (Table 3). As shown in Table 3, application of 100 µM aluminium elicitor caused the highest hydrogen peroxide content, which was the lowest in control treatment .

Taxol Extraction and Assay

Based on results presented in Fig. 1, the effect of aluminium elicitor on extracellular taxol, cell-associated taxol, total taxol, as well as taxol release and specific yield was significant ($P \leq 0.05$). As seen in Fig. 1a, when the elicitor concentration increased, the extracellular taxol content of Al-treated cultures showed an upward trend so that 100 µM of Al elicitor had taxol production of up to 38-fold higher than in Al-free medium (control). Cell associated taxol content in 50 and 100 µM Al elicitation treated cells was 11.4- and 8.3-fold higher, respectively, than that of the control cells (Fig. 1b). There was no significant difference between the control treatment and the concentration of 25 µM in terms of cell associated taxol production (Fig. 1b). Both extracellular and cell-associated taxol contents were affected by Al treatment, although enhancement of extracellular taxol was more pronounced. On the other hand, increasing aluminium elicitor concentration improved the total taxol production in all samples, significantly (Fig. 1c). The highest total taxol amount was in the culture with 100 µM of Al elicitor and it was much more than control treatment (Fig. 1c). The taxol release was also affected significantly by Al treatments so that the highest taxol release amount was in the culture with 25 µM of Al elicitor and followed by the culture with 100 µM of Al elicitor (Fig. 1d). According to the Fig. 1e, as the aluminium concentration increased, the specific yield had a rising trend. Compared with control cells, exposure to 50 and 100 µM Al increased about 10.2 and 15.4-fold the specific yield, respectively.

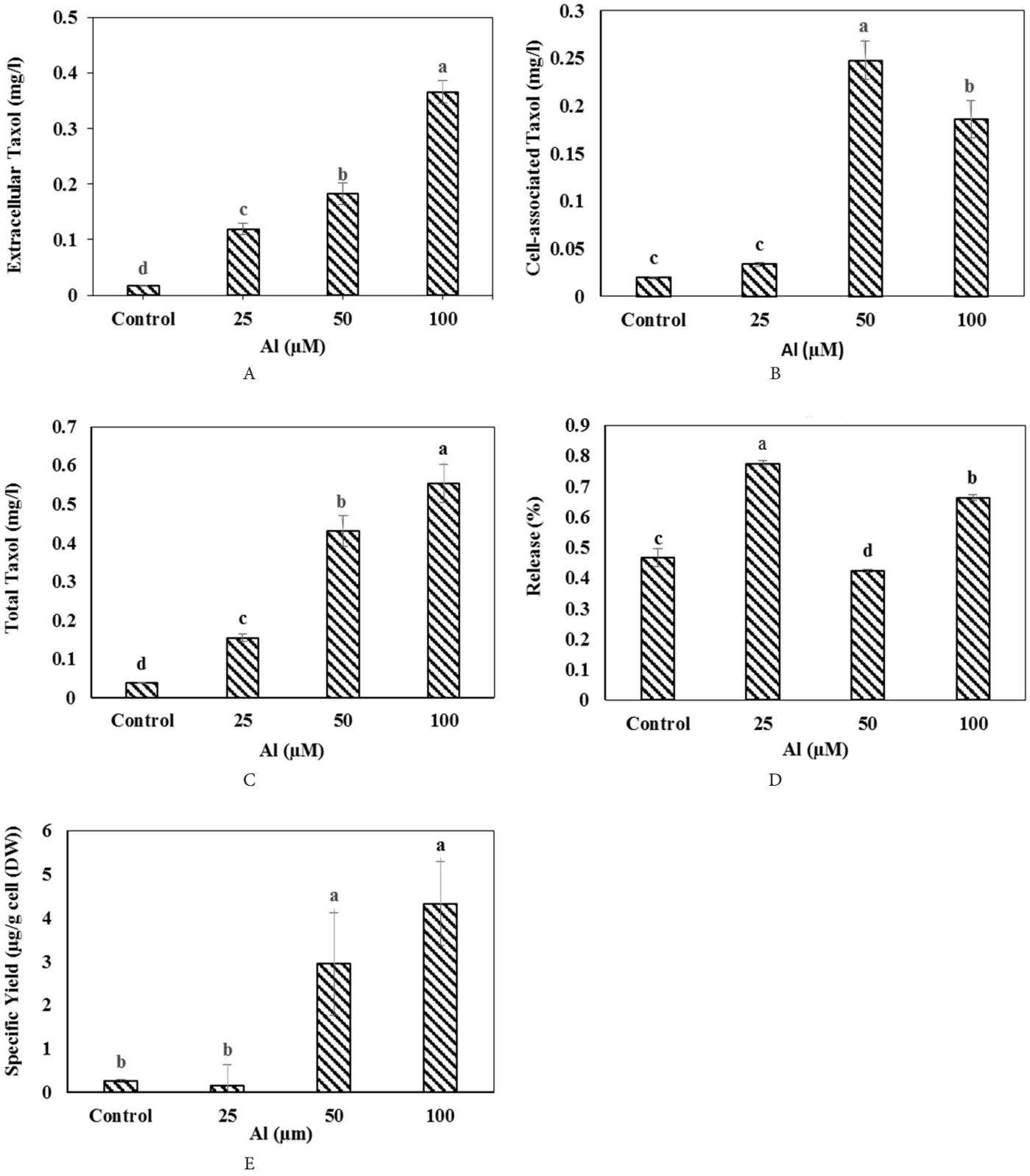

Figure 1. Effect of different levels of Al on taxol production of hazelnut

Note: in each column, means followed by the same letter are not significantly different acoording to LSD test at $P \leq 0.05$

Table 1. Effect of different levels of Al on growth and biochemical traits of hazelnut

Al (µM)	Fresh weight (g)	Dry weight (g)	pH	EC (ds m^{-1})	Protein (mg g^{-1} FW)
Control	4.55±0.39a	0.15±0.00a	8.3±0.36a	84.40±1.65a	1.92±0.56a
25	3.32±0.68b	0.13±0.01ab	5.8±1.41b	89.31±7.16a	1.18±0.26b
50	2.76±0.73b	0.12±0.00b	5.6±1.24b	88.61±7.79 a	0.79±0.12b
100	2.34±0.29b	0.10±0.01c	4.8±0.20c	84.78±1.50a	0.03±0.31c

Note: in each column, means followed by the same letter are not significantly different acoording to LSD test at $P \leq 0.05$

Table 2. Effect of different levels of Al on biochemical traits of hazelnut

Al (µM)	MDA (µM g^{-1} FW)	Flavonoid (µM g^{-1} FW)	Phenolics (µg ml^{-1})	Anthocyanin (µM g^{-1} FW)	Proline (µM g^{-1} FW)
Control	54.19±0.58 b	3.46±0.42b	47.54±5.70b	0.05±0.03b	2.48±0.40b
25	69.48±0.58b	8.32±5.55a	65.42±22.05b	0.20±0.04a	4.63±1.95a
50	76.39±11.62b	10.66±5.04a	82.90±39.00ab	0.21±0.00a	3.77±0.66a
100	124.0±47.90a	11.36±4.14a	129.58±31.74a	0.19±0.00a	3.96±0.11a

Note: in each column, means followed by the same letter are not significantly different acoording to LSD test at $P \leq 0.05$

Table 3. Effect of different levels Al on antioxidant activity traits of hazelnut

Al (µM)	Antioxidant activity (%)	CAT activity (U mg^{-1} FW)	POD activity (U mg^{-1} FW)	H_2O_2 (µMg^{-1} FW)
Control	21.69±8.85b	1.51±0.97b	2.50±0.43b	3.09±0.38c
25	70.46±14.34a	2.34±1.25ab	4.57±2.46ab	5.41±0.84ab
50	53.93±9.27a	4.91±0.97a	5.54±0.69a	4.52±0.73b
100	20.53±5.00b	3.38±2.67ab	7.03±1.80a	5.84±0.02a

Note: in each column, means followed same letter are not significantly different acoording to LSD test at $P \leq 0.05$

Discussion

Aluminium with high ionic charge and small crystalline radius has a level of reactivity which is almost unmatched by any soluble metal (Singh et al., 2017). There is the strong interaction between Al^{3+} as the main Al toxic form and oxygen donor ligands (proteins, nucleic acids, polysaccharides) that results in the inhibition of cell division, cell extension, and transport (Mossor-Pietraszewska, 2001). In the present research, the effect of different concentrations of Al on suspension-cultured hazelnut (*C. avellana*) cells was evaluated. We observed that the application of Al elicitor affected numerous traits of hazelnut, significantly.

The effects of AL on cell growth (fresh and dry weight) in our research were similar to those previously observed in rice under hydroponic culture (Bidhan et al., 2014) and chickpea (*Cicer arietinum* L.) under field condition (Choudhury et al., 2014). Okem et al. (2015) observed that Al in low levels improved the biomass production in *Hypoxis hemerocallidea* Fisch.Mey. & Avé-Lall. up to 2-fold higher than control. It appears therefore that effects of Al treatment varies widely among species and is also related to the organizational level and physiological conditions of plants.

As the intact plant or cell culture are influenced by an elicitor, biochemical responses, such as reactive oxygen species (superoxide and hydrogen peroxide) and cytoplasmic acidification, reduce membrane polarity and increase extracellular pH, NADPH oxidase activation responsible for reactive oxygen production and methyl-jasmonate and salicylic acid as a secondary messenger occur, rapidly (Shilpa et al., 2010). In this experiment increase of aluminium levels, decreased protein content significantly. The reasons for this reduction, based on reports, is decrease of nitrogen content in different plant organs due to increase of aluminium level (Mihailovic et al., 2008; Miguel et al., 2013) and initiation of deleterious events in plant cells induced by generation of free radical chain (Okem et al., 2015). Achary et al. (2008) reported that aluminum treatment increases protein oxidation in the *Allium cepa* L. root cells, which is comparable to our results.

The ROS (reactive oxygen species) contain free radical (such as $O2^{\bullet-}$, superoxide radicals; OH*, hydroxyl radical) and non-radical forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) (Gill et al., 2010). The results from several investigations have revealed that Al stress can develop the generation of ROS, and activates some antioxidant enzymes in plant tissue and cell. There are protective enzymatic and non-enzymatic systems to clean up ROS and mitigate their deleterious effects in plant (Qin et al., 2010; Ahmed et al., 2018). Resistance to aluminium stress in plants relies on antioxidant enzymes activity such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) as well as low molecular weight non-enzymatic molecules such as phenols, flavonoids, carotenoids and so on (Lee et al., 2007; Dabrowska et al., 2007; Inostroza-Blancheteau et al., 2012). In present study, different concentrations of Al elicitor caused increase in the phenolics. This result is in agreement with previous report about promotional effect of Al on phenolic content of *H. hemerocallidea* (Okem et al., 2015). Our results showed that increase of aluminium concentration in medium caused upward trend in recently noted enzyme activity so that the lowest values were observed in Al-free medium. Several studies proved these observations (Yamamoto et al., 2002; Achary et al., 2008; Panda et al., 2009). According to the above, it seems that increased activity of CAT and POD enzymes and non-enzymatic systems (phenolics, flavonoids and anthocyanin) was a response to the stress caused by aluminum. The induction of phenolic compounds production was seen in maize under Al treatment (Díaz et al., 2001). *Phyllantus tenellus* Roxb. leaves possess more phenolics than control plants after the foliar application of copper sulphate as a kind of heavy metal (Díaz et al., 2001). The increase in the content of phenolics in response to heavy metal stress may be related to the improving in activity of enzymes involved in phenolic compounds biosynthesis and conjugate hydrolysis, according to the reports (Parry et al., 1994; Michalak, 2006).

Lipid peroxidation was measured as the amount of malondialdehyde (MDA). MDA is one of the main and common indicators used to assess oxidation and reduction situation in plants. Lower content of MDA means higher antioxidant capacity and reflecting greater resistance to stress condition (Marcinska et al., 2013). Results also showed that increase of antioxidant enzyme activity, such as catalase and peroxidase, decreases MDA means significantly. Proline and soluble sugars are very important organic solvents that accumulate in plants in stress conditions (Changhai et al., 2010). It was shown that elevated aluminium content could increase significantly free proline in plant as aluminium treated samples had more proline content in contrast to control treatment.

Elicitation has been broadly applied to enhance of the biosynthesis or to induce de novo synthesis of secondary metabolites in plant cell suspension cultures (Akula and Ravishankar, 2011). Useful compounds in plant cell culture are often secondary metabolites that accumulate in trace amounts. Accumulation of secondary metabolites in the plant is part of defense response against the pathogen that is induced and activated by elicitors. So plant cell treatment with biotic or abiotic elicitors is one of the beneficial methods to increment of secondary metabolite production in plant cell or organ cultures (Namdeo, 2007). In this study the use of aluminium elicitor in different concentrations had significant effects on taxol production including extracellular taxol, cell-associated taxol and total taxol, release percentage and specific yield in contrast to control treatment. Sivanandhan et al. (2014) tested aluminium chloride on withanolides production in *Withania somnifera* (L.) Dunal cell suspension culture. They observed a dose dependent change in withanolides content under the effect of elicitation. Similarly, elicitation of culture systems such as cell suspensions, hairy roots and adventitious roots by salts (including $AgNO_3$, $AlCl_3$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $HgCl_2$, KCl, $MgSO_4$, $NiSO_4$, $VOSO_4$ and Zn ions) improved secondary metabolites production in a variety of plant species (Verpoorte et al., 2002). Jamshidi et al. (2016) investigated the effect of silver nano particles (AgNPs) on hazelnut cell suspension cultures. They reported that AgNPs elicitor stimulate taxol production at the highest capacity of the cells, meanwhile reserving their viability. Methyl jasmonate (Bestoso et al., 2006; Bonfill et al., 2007; Sabater-Jara, 2014; Gallego et al., 2015) and salicylic acid (Rezaei et al., 2011; Sarmadi et al., 2018) have been widely used as an elicitor to increase the production of taxol in cell cultures of *Taxus* sp. and hazelnut. It has been reported that abiotic stresses, including heavy metals, improve the endogenous production of jasmonate (Dar et al., 2015) and salicylic acid (Khan et al., 2015). It seems that Al elicitor induced taxol production via increase plant phytohormone levels such as jasmonate and salicylic acid. In present research, Al elicitor treatment also improved the H_2O_2 content in hazelnut. According to Jamshidi et al. (2016), silver nanoparticles trigger the defense system by producing hydrogen peroxide as a signaling molecule in cell suspension cultures of *C. avellana*. Increasing levels of hydrogen peroxide may be associated with the taxol production.

Conclusion

Considering to results, aluminium elicitor application (50 and 100 µM concentration) in hazelnut cell culture increased secondary metabolites synthesis, intracellular and cell-associated taxol, total taxol, taxol release and specific yield. Al-elicitor application also increase antioxidant enzyme activity such as catalase and peroxidase as well as proline secondary osmolyte.

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