Journal of Medicinal Plants and By-products (2016) 2: 195-204

Original Article

Considerable Azadirachtin Production in Neem Cell Culture under Abiotic Elicitor Induction

Ghasemali Garoosi*, Banafsheh Gholami and Ramin Hosseini

Biotechnology Deptartment, Imam Khomeini International University (IKIU), Qazvin, Iran

Article History: Received: 25 May 2016 /Accepted in revised form: 12 November 2016

Abstract

In this study the effect of different concentrations of some abiotic elicitors (salicylic acid, jasmonic acid, cadmium chloride and sodium chloride) with different incubation times (3, 6, 9 and 12) on azadiractin production and cell growth in the cell culture of the Iranian native Neem were investigated. Cell growth rate was measured by changes in dry cell weight and it was used as a basis for the treatment of elicitor effect. Azadirachtin was extracted with methanol and measured by high performance liquid chromatography (HPLC). The results showed that the AZ content of cells increased significantly in the cells elicited with different types and concentrations of all used elicitors compared to the control. The cell suspension which treated with 0.75 mM salicylic acid showed the highest Azadirachtin content (0.1268 g/g DCW) 6 days after incubation. The highest dry cell weight, with a significant increase, was observed in the suspension cultures treated with 0.1 mM cadmium chloride 12 days after incubation. However, this study highlighted the new valuable Iranian native Neem genotype and salicylic acid as a powerful elicitor for azadirachtin production. In addition, it was approved the effect of exposure time, individual intrinsic and more presumably combined condition of elicitors and their concentration on plant secondary metabolites production.

Keywords: Azadirachtin, Cell suspension culture, Elicitor, Neem

Introduction

Neem tree (*Azadirachta indica* A. Juss.) belongs to the family of Meliaceae, originated from India, is a multi-purpose tree. It is grown in many Asian countries and in tropical regions of the western hemisphere [1] and contains well over 300 secondary compounds, which are responsible for many of its wide-ranging properties [2]. Most of the active principles are terpenoids and are found in Neem fruit, seeds, leaves, stem and root bark. Its products have been used for centuries as insecticidal, antiseptic, contraceptive, antipyretic and antiparasitic purposes [3]. The most important and active ingredient of this plant is Azadirachtin (AZ) ($C_{35}H_{44}O_{16}$), a tetranortriterpenoid which the 90 percent of Neem medicinal and pesticidal feature is referred to it. AZ, a high valuable secondary metabolite initially was isolated from the Neem seeds by Morgan [4]. Because of efficiency, its safety, environmentally benign and insect control [5], such as pests and plasmodium and many issues of safeguarding health of animals and humans [6], its demand is increasing continuously. A considerable variation occurs in AZ content depending upon genetics [7], environment [8], geographical region [7, 9], the developmental stage and different tissues in individual Neem trees [10]. The seeds contain usually higher contents of AZ over other tissues in the Neem tree, but there are numerous limitations in obtaining elite varieties of Neem seeds. Seeds are usually produced only once in a year and cannot be stored for longer durations. However, an alternative solution for producing this specific metabolite is the use of tissue culture techniques, which would ensure the continuous

*Corresponding author: Biotechnology Department, Imam Khomeini International University (IKIU), P.O. Box: 288, Post code: 34149-16818, Qazvin, Iran
Email Address: agaroosi90@yahoo.com

supply of uniform quality, highly specialized, natural components. But many indigenous and exogenous factors can assist with secondary metabolites production in different processes and plants individually. Such processes can easily be optimized during scale up studies for commercial exploitation [2]. Plant secondary metabolites are generally produced in low quantities, and slow growth rates of plant cells. This makes it more difficult to produce the compounds in considerable amounts and in an acceptable time period for commercial purposes, apart from *in vivo* limitation [11]. Exogenous addition of 'elicitor' molecules of biotic and abiotic origin has been reported as one of the most promising strategies for the enhanced production of commercially important plant-derived compounds [2,12-14]. Effect of biotic and abiotic elicitors at a specific time had been reported on *A. indica* suspension culture for AZ synthesis [13], but influence of elicitors may vary from plant-to-plant species/genotypes, season, geographical condition and media content [15] etc. There is no universal effect of a particular elicitor on different plants or cell culture systems. Therefore, selecting the right elicitors and the optimization of their appropriate dose with respect to the plant cells of interest genotype is necessary to ensure the enhancement in product yield. However, the yield of this environment friendly, high valuable plant secondary metabolite is low and efforts are still required to further enhance its production through optimization and improvement of medium content, elicitors, plant genotype, methods and culture conditions etc. Here, we present for the first time noticeable results on AZ production in the cell suspension culture of Iranian native Neem (*Azadirechta indica*). The present study aimed to elucidate the effect of different concentrations of abiotic elicitors (salicylic acid, jasmonic acid, cadmium chloride, sodium chloride) on AZ production and biomass.

Marerial and Methods

Chemicals

Azadirachtin (95% purity) was purchased from Sigma Chemical Co. Methanol (HPLC grade), salicylic acid (SA), $CdCl_2$ (Cd), jasmonic acid (JA) and NaCl were of the highest purity available from Merck AG (Darmstadt, Germany). All other used chemicals were of analytical reagent grade. Stock solution of AZ (100 µg/mL) was prepared by dissolving proper amounts of AZ in 50 mL of double distilled water to prepare standard solutions with 5, 10, 20, 30, 50 and 100 µg/mL. Elicitation studies were carried out with SA (0.25, 0.5 and 0.75 mmol), JA (0, 0.05, 0.25 and 0.5 mM), Cd (0.1, 0.2 and 0.4 mM) and NaCl (50, 75 and 100 mM) with five incubation times (0, 3, 6, 9 and 12 days). Stock solutions of salicylic acid (Sigma, USA) were prepared by dissolving it in distilled water and adjusting the pH to 5.8. Jasmonic acid (Sigma, USA) was dissolved in 96% ethanol solution and filter-sterilized.

Plant Material and Cell Culture Methods

Foliage of Neem tree (*Azadirachta indica*) was collected in spring from Bandar Abbas, Iran. Shoot segments containing lateral buds (1bud/segment) were surface-sterilized as the following: rinsing by tab water for 2 h, soaking in ethanol 95% for 4-5 s, washing with sterilized distilled water. The samples were then submerged in mercuric chloride 0.1% (w/v) for 5 min, rinsed with sterilized distilled water for 3 times, submerged in 10% NaOCl (w/v) for 15 min and washed with sterilized distilled water for 3 times. Sterile shoot segments were placed on a hormone free Murashige & Skoog (MS) basal medium to obtain *in vitro* sterile plantlet and leaves. The leaves of sterilized explants were cultured on MS medium supplemented with 2 mg/L 6-benzylaminopurine (BAP) and 6 mg/L Indole-3-butyric acid (IBA). The explants were stored in dark at 23±2 °C (Fig. 1).

The suitable calli (light-creamy, crispy and crystalline) were used to preparation of cell suspension culture in the liquid MS medium. The medium was the same as the callus induction medium but without agar. The cell suspensions were treated with elicitors after six times of sub-culturing. For elicitation, enough fresh suspension cultures, inoculums, 5 g/L dry cell weight (DCW) basis [13] were transferred into 250 mL Erlenmeyer flasks containing 50 mL of liquid MS medium supplemented with adequate concentrations of elicitors. The suspension cultures were maintained in dark at 100 rpm on a rotary incubator-shaker at 25±2 °C. Control and elicited cultures were harvested 4 times with three-day intervals (3, 6, 9 and 12th days) of cultivation and these samples were used to determine DCW and AZ content.

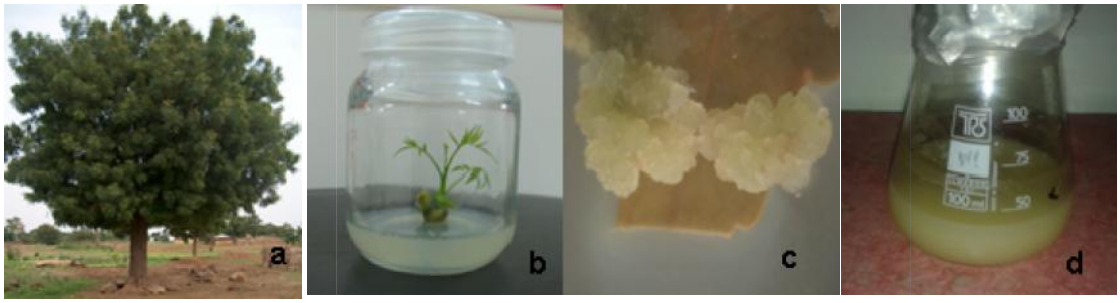

Fig. 1 Plant tissue and cell suspension culture process. (a) Neem tree, (b) *in vitro* sterile plantlet, (c) creamy callus derived from leaf explants, and (d) Neem cell suspension culture.

Dry Cell Weight Estimation

To estimate DCW, cell cultures were harvested and collected by centrifugation at 3000 rpm for 15 min then cells were washed with distilled water. The fresh cells were dried for 72 h at 28±2 °C until dry weight of cells became constant [16].

The Extraction and Quantification of Azadirachtin.

The dried powdered material (20 mg) was homogenized with 1 mL of methanol at room temperature. The samples were transferred to polypropylene mic-rocentrifug tubes and centrifuged at 13000 rpm for 5 min [17]. The supernatant was transferred into clean glass vials and stored at -20 °C until injection to the HPLC.

An Isocratic analytical HPLC was carried out on a KNAUER (Germany) equipped with a K2500 UV-Visible detector. The column was Vertex (Agilent Co.) 250×4.6 mm, 5µm diameter with Eclipse XDB C18, 5µm with integrated pre-column. A B2000 pH meter equipped with a GCFC 11 combination glass electrode was used for pH measurements. Analysis was isocratic at 1.0 mL/min flow rate with binary mixtures of methanol (40%) and ultrapure water as a mobile phase. The mobile phase was premixed, filtered through a 0.45 µm membrane filter to remove any particulate matter and degassed by sonication before use. The mobile phase was prepared freshly each time. Injection volumes of 20 µL were usually employed. The best Rapid resolution liquid chromatography (RRLC) separations were obtained at 25 °C. The absorbance of AZ was higher at 214 nm compared to generally preferred 217 nm and further it was free from any interference. Hence, the eluted peaks were detected at 203 nm.

Statistical Analysis

The experiments were carried out as a factorial experiment based on completely randomized design with three replicates. Data were subjected to one-way ANOVA, using statistical analysis software (SAS) 9.1.3 and reported as means±SE.

Result and Discussion

Effect of Jasmonic Acid

The ANOVA results showed that the AZ content of cells and DCW significantly (p<0.01) affected by JA concentrations, cell incubation time and interactions of them (Table 1). Application of JA increased the AZ content and decreased DCW compared to the control. Among different concentrations of JA the higher AZ content and DCW (0.01224 g/g DCW and 6.556 g/L) were obtained at 0.05 mM JA treatment and control, respectively. Also, with increasing incubation time, AZ content increased and DCW decreased. The highest AZ content and DCW (0.0171 g/g DCW and 6.330 g/L) were observed 9th and first day of culture, respectively (Table 2). Therefore, the highest and lowest AZ content (0.0218±0.0003 and 0.007±0.0003 g/g DCW) were observed when cells were treated with 0.05 m mol of JA on 9th and 12th day of culture, respectively (Fig. 2a). The cell growth index was also demonstrated the same pattern as AZ content, in which the highest and lowest of DCW (7.66±0.17 and 3.93±0.37 g/L) were obtained on 9th and 12th days, respectively (Fig. 2b). Therefore, the highest AZ content of DCW (0.0218±0.0001 g/g DCW and 7.66±0.17 g/L) Over all, results revealed that JA induced significantly the AZ synthesis, but reduced cell growth ultimately at all studied concentrations as compared to the control cultures. It seems that the required time of elicitation by JA for AZ production varies based on its concentration, i. e. each concentration shows its effect at a specific time. JA has been used widely as an abiotic elicitor [11]. JA and SA acid are known as key signaling compounds to mediate plant defense responses to insect herbivores, pathogens and parasites [18].

Involvement of JA as the signal molecule responsible for increased synthesis of hypericin (plant insecticides) is well documented [19]. Balaji et al [20] reported that methyl jasmonate exhibited a great influence on the bioproduction of AZ in cell cultures of Azadirachta indica A. Juss. over some other elicitors (copper sulphate, salicylic acid, pectinase, cellulase, pectolyase, silver nitrate, fungal cell extracts and culture filtrates). Addition of JA into hairy root cultures of *A. indica* induced ~6-fold increase in AZ production, as compared to the control culture [12]. Two fold increases in the production of AZ in cell cultures of *Azadirachta indica* A. Juss. and DCW index by different concentrations of Jasmonate elicitation has also been reported by Prakash and Srivastava [7].

However, the present study exhibited about 4-fold increase in AZ 9 days after induction. These differences may arise from other factors such as genotype [10] media [15,21,22] and culture condition [7].

Effect of Salicylic Scid

According to the ANOVA results, SA concentrations, incubation time and interaction had significant (p<0.01) effect on AZ content of cells and DCW (Table 3). Salicylic acid exhibited itself as a powerful elicitor in AZ stimulation. It was observed that the AZ synthesis was induced significantly upon application of all concentrations of SA as compared to control. Between different concentrations of SA the highest AZ content and DCW (0.06132 g/g DCW and 7.076 g/L) were obtained at 0.75 and 0.5 mM SA treatment, respectively.

Table 1 The ANOVA analysis of the effect of JA and incubation time on AZ content and DCW.

Source of variation	Degree of freedom	Mean of square	
		AZ content	DCW
JA	3	3.88×10^{-5} **	4.214 **
Incubation time	3	2.03×10^{-4} **	4.192 **
JA × Incubation time	9	2.53×10^{-5} **	1.378 **
Error	32	2.8×10^{-6}	0.216

** Significant at 1% probability.

Table 2 The effect of JA and incubation time on AZ content and DCW.

JA (mM)	AZ content (g/g DCW)	DCW (g/L)	Incubation time (days)	AZ content (g/g DCW)	DCW (g/L)
0	0.00924±0.0001	6.556±0.12	0	0.0055±0.0001	6.330±0.06
0.05	0.01224±0.0002	6.022±0.17	3	0.0074±0.0002	5.597±0.20
0.25	0.01220±0.0002	5.424±0.22	6	0.0140±0.0002	5.980±0.25
0.5	0.01070±0.0004	5.798±0.28	9	0.0171±0.0003	6.612±0.22
-	-	-	12	0.0115±0.0002	5.230±0.24

Table 3 The ANOVA analysis of the effect of SA and incubation time on AZ content and DCW.

Source of variation	Degree of freedom	Mean of square	
		AZ content	DCW
SA	3	9.77×10^{-3} **	1.812 **
Incubation time	3	1.98×10^{-3} **	4.656 **
SA × Incubation time	9	1.03×10^{-3} **	0.685 **
Error	32	3.3×10^{-7}	0.0933

** Significant at 1% probability.

Table 4 The effect of SA and incubation time on AZ content and DCW.

SA (mM)	AZ content (g/g DCW)	DCW (g/L)	Incubation time (days)	AZ content (g/g DCW)	DCW (g/L)
0	0.00924±0.0001	6.556±0.12	0	0.00550±0.0001	6.330±0.06
0.25	0.02634±0.0002	6.422±0.10	3	0.01728±0.0003	6.045±0.10
0.50	0.01788±0.0003	7.076±0.16	6	0.04798±0.0003	6.280±0.16
0.75	0.06132±0.0002	6.422±0.17	9	0.03882±0.0002	7.310±0.16
-	-	-	12	0.03390±0.0001	7.130±0.19

Table 5 The ANOVA analysis of the effect of Cd and incubation time on AZ content and DCW.

Source of variation	Degree of freedom	Mean of square	
		AZ content	DCW
Cd	3	0.70×10^{-4} **	18.147 **
Incubation time	3	1.80×10^{-4} **	18.569 **
Cd × Incubation time	9	0.40×10^{-4} **	4.394 **
Error	32	1.5×10^{-7}	0.115

** Significant at 1% probability.

Table 6 The effect of Cd and incubation time on AZ content and DCW.

Cd (mM)	AZ content (g/g DCW)	DCW (g/L)	Incubation time (days)	AZ content (g/g DCW)	DCW (g/L)
0	0.00924±0.0001	6.556±0.12	0	0.0055±0.0001	6.330±0.06
0.1	0.01380±0.0002	7.252±0.17	3	0.0068±0.0002	6.897±0.13
0.2	0.01048±0.0002	6.810±0.13	6	0.0157±0.0002	7.012±0.20
0.4	0.01060±0.0002	7.462±0.11	9	0.0147±0.0001	7.445±0.12
-	-	-	12	0.0123±0.0002	7.415±0.15

Table 7 The ANOVA analysis of the effect of NaCl and incubation time on AZ content and DCW.

Source of variation	Degree of freedom	Mean of square	
		AZ content	DCW
NaCl	3	4.52×10^{-4} **	0.793 **
Incubation time	3	4.75×10^{-4} **	0.0066 ns
NaCl × Incubation time	9	0.70×10^{-4} **	0.485 **
Error	32	1.79×10^{-3}	0.139

**: significant at 1% probability and ns not significant.

Table 8 The effect of NaCl and incubation time on AZ content and DCW.

NaCl (mM)	AZ content (g/g DCW)	DCW (g/L)	Incubation time (days)	AZ content (g/g DCW)	DCW (g/L)
0	0.00924±0.0001	6.556±0.12	0	0.0055±0.0001	6.330±0.06
50	0.01680±0.0003	7.048±0.14	3	0.0105±0.0002	6.977±0.12
75	0.01698±0.0002	6.888±0.12	6	0.0194±0.0003	6.927±0.20
100	0.02136±0.0002	6.850±0.24	9	0.0197±0.0002	6.962±0.26
-	-	-	12	0.0253±0.0002	6.980±0.12

The results indicated that AZ content increases with increasing of inoculation. The maximum AZ content and DCW were 0.047975 g/g DCW and 7.310 g/L observed at 6th and 9th day of culture (Table 4). The highest and lowest contents of AZ (0.1268±0.0003 and 0.0127±0.0009 g/g DCW, > 23fold in camparison to control) were recorded at 0.75 and 0.5 m mol of SA on 6 and 3th day of culture, respectively (Fig. 3a). The highest and lowest DCW accumulation (8.06±0.24 and 5.26±0.06 g/L) occurred on 9 and 12th day of culture, respectively, both in the treatment containing 0.5 m mol SA (Fig. 3b). As it is observed in Fig. 3, SA at concentration of 0.5 m mol not only increased AZ content (over 2-fold), but also it had a positive effect on cell growth, which implies the high potent of SA. However, after 9th day the cell growth rate decreased regardless of the concentration of elicitor.

The considerable increase in AZ content with respect to SA application, could be attributed to this fact that SA triggers a powerful induction signal for this secondary metabolite synthesis in Neem [12, 23], and for other metabolites [24]. SA is also, regarded as a signal molecule, playing an important role in Systemic Acquired Resistance (SAR) and inhibitor of ethylene biosynthesis [25,26]. Therefore, this possibility exists that SA could act in parallel with AZ against insect and pathogens through the inhibition of ethylene biosynthesis [25] or different mechanisms, in the plant defense system. Satdive et al. [12] reported that the addition of SA into the hairy root cultures of *A. indica* induced a ~9-fold increase in AZ production, as compared to the control culture [12]. Enhanced production of AZ by cell cultures of *Azadirachta indica* A. Juss. by elicitation has been previously reported by Prakash and Srivastava [7].

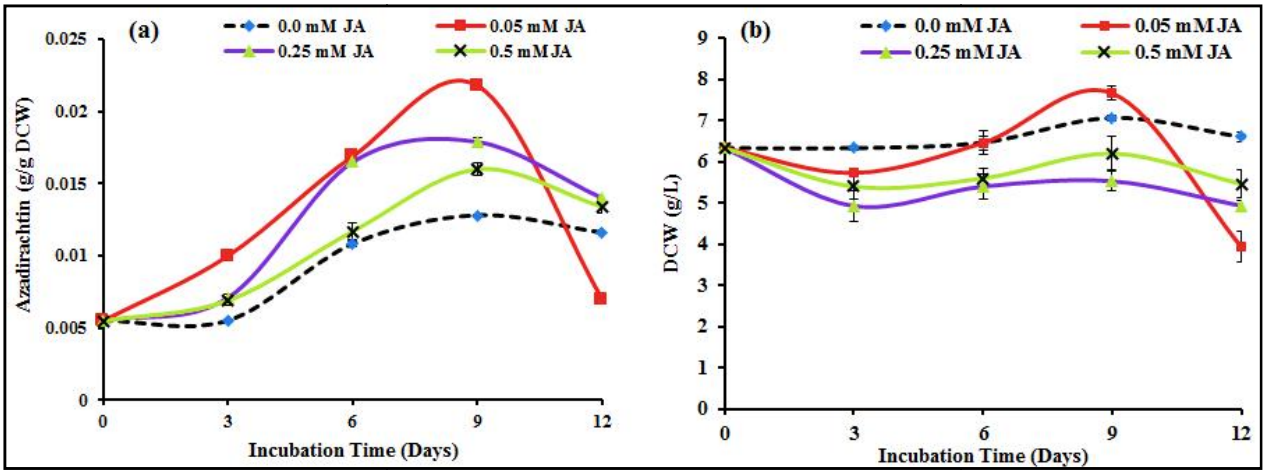

Fig. 2 The interaction effect of JA and incubation time on AZ content and DCW. (a) The AZ content and (b) The dry weight accumulation in the *Azadirachta indica* A. Juss. suspension culture treated with different concentrations of JA (0.0, 0.05, 0.25, 0.5 mM) at different incubation times (0, 3, 6, 9 and 12 days).

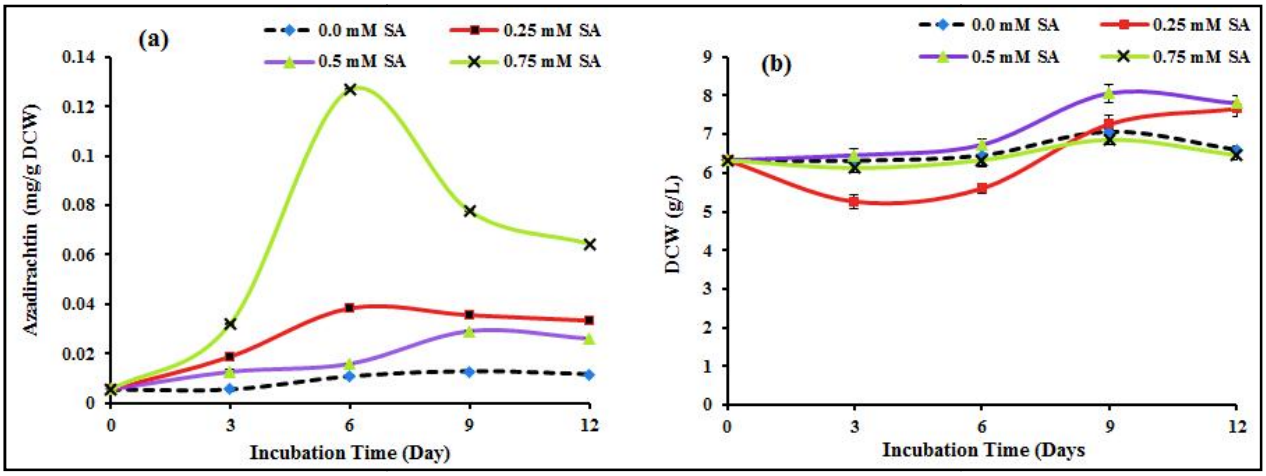

Fig. 3 The interaction effect of SA and incubation time on AZ content and DCW. (a) The AZ content and (b) the dry weight accumulation in the *Azadirachta indica* A. Juss. suspension culture treated with different concentrations of SA (0.0, 0.25, 0.5, 0.75 mM) at different incubation times (0, 3, 6, 9 and 12 days).

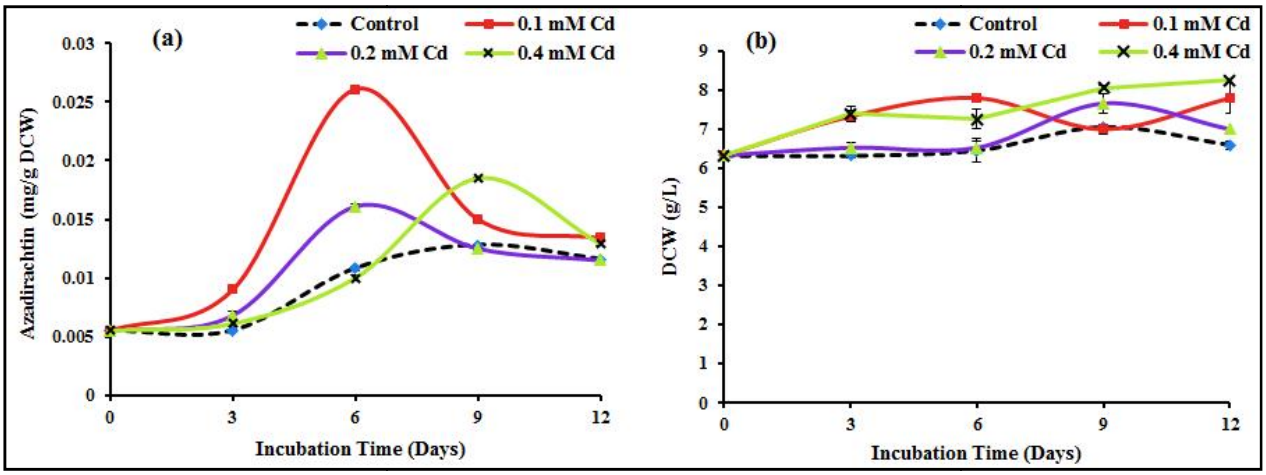

Fig. 4 The interaction effect of Cd and incubation time on AZ content and DCW. (a) AZ content, (b) The DCW accumulation of the *Azadirachta indica* A. Juss. suspension culture treated with different concentrations (0, 0.1, 0.2, 0.4 mM) of Cd at different incubation times (0, 3, 6, 9 and 12 days).

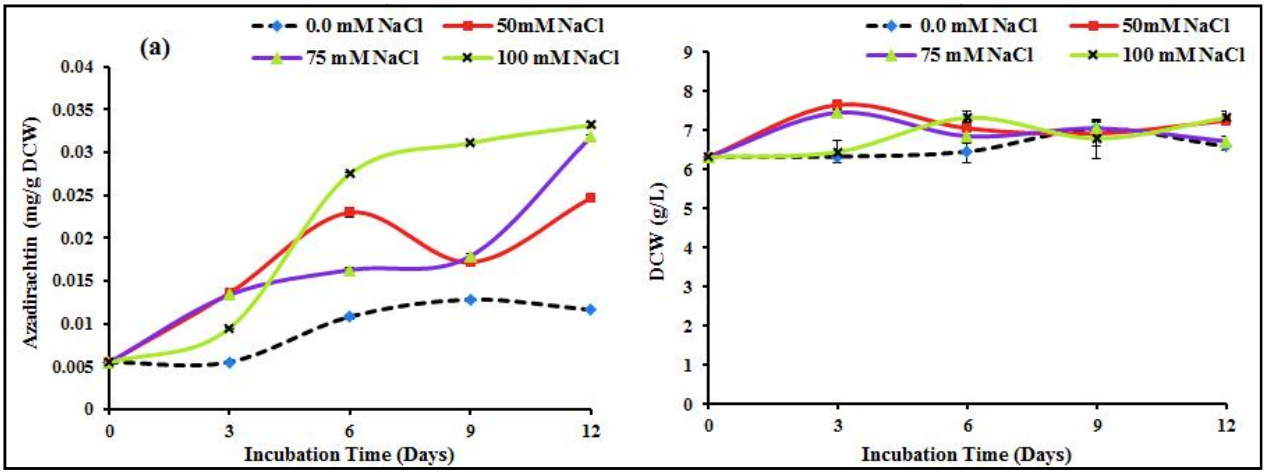

Fig. 5 The interaction effect of NaCl and incubation time on AZ content and DCW. (a) The AZ content, (b) the DCW of the *Azadirachta indica* A. Juss. suspension culture treated with different concentrations of NaCl (0, 50, 75, 100 m mol) at different incubation times (0, 3, 6, 9 and 12 days).

However, our study showed a superior result in which more than 23fold increase of AZ occurred after elicitation by SA at concentration of 0.75 m mol after 6 days of culture over control. As it is observed in Fig. 3a, after 6th day the accumulation of AZ decreased, this may have been caused by several possibilities such as AZ or SA instability, non-proper cell growing and/or the inhibitory effect of AZ accumulation itself on cell viability/growth, deficiency of medium nutrition, or other factors which should be surveyed. The higher elevation of AZ in our case, outlines some hypotheses such as the superior plant genotype and/or combined elicitation conditions [7] (for example, anaerobic + SA) and less importantly cell growth stage. In such condition no significant differences were observed in cell growth at different concentrations of SA (Fig. 3b), which emphases the above discussion and deduction.

Effect of Cadmium Chloride

It was observed that AZ content and cell growth, in term of DCW, significantly (p<0.01) affected by different concentrations of Cd, incubation time and thair interactions (Table 5). Application of different concentrations of Cd increased AZ accumulation in the cells and DCW compared to the control. Among the applied concentrations of Cd, the maximum AZ accumulation and cell growth (0.0138 g/g DCW and 7.462 g/L) were at 0.1 and 0.4 mM treatments. The AZ content and DCW increased by increasing incubation time. The higher AZ content and DCW (0.0157 g/g DCW and 7.445 g/L) were observed at 6th and 9th day of culture (Table 6).

The highest and lowest of DCW values (8.26±0.014 and 6.53±0.13 g/L) were observed in the culture treated with 0.4 mM Cd chloride and control, respectively after 12 days (Fig. 4b). The cell suspension supplemented with 0.1 mM Cd exhibited the highest AZ content (0.0261±0.0002 g/g DCW) on the 6th day of culture. Other concentrations of Cd induced more AZ compared to the control, but on a different day of culture. However, on 12th day of culture AZ content was decreased in all cell cultures including the control (Fig. 4a).

The secondary metabolism is essential for the fitness of plants and many secondary metabolites play important functions as chemical defense compounds against herbivores, microbes or competing plants. At the cellular level, Cd induces changes in the lipid composition, the activity of enzymes associated with membrane, and the distribution of macro and micronutrients in plants [27]. Other studies found that oxidative burst could suppress some polyamines metabolism [28] or induce defense responses and indole alkaloid biosynthesis and accumulation [29]. In the transformed root cultures of *Datura stramonium*, Cd elicited the production of some secondary metabolites, such as the sesquiterpenoids but not the others, such as alkaloids [30]. Lee et al. reported that Cd increased the excretion of tropane alkaloids into the medium from the transformed roots of *Atropa belladonna* [31]. In *Salvia miltiorrhiza* cell cultures also this heavy metal could increase the biosynthesis of tanshinone 5 times and decreased conversely cell growth in comparison to control [13]. In *Vitis vinifera* cv. cell suspension cultures, total phenolics, flavanol, trans-resveratrol and tocopherols contents increased

significantly by cadmium chloride , while dry cell weights reduced [32]. However, our result revealed that Cd can stimulate AZ production and suppress cell growth under prolong cell culture (> 6 days) in Neem.

Effect of Sodium Chloride

The ANOVA results showed that AZ content significantly (p<0.01) affected by NaCl concentrations, incubation time and interaction of them, but DCW significantly (p<0.01) affected by NaCl concentrations and its interaction with incubation time (Table 7). In this study, elicitation of cell suspension with NaCl cause to increases of AZ content and DCW. Among applied different NaCl concentration the maxium AZ content and DCW were 0.02136 g/g DCW and 7.048 g/L observed at 100 and 50 mM NaCl, respectively. Also, the AZ content and DCW increased by increasing incubation time, but this increases was not statisticaly significant. The highest AZ content and DCW (0.0253 g/g DCW and 6.980 g/L) were observed at 12th day of culture (Table 8). In other words, no significant effect was observed after the application of NaCl during the 12 day period on DCW in comparison to control (Fig. 5b). On opposite, NaCl treatments increased significantly AZ content 1.93 to 2.59 fold over control after 12 days with the highest yield (0.0332±0.0003 g/g DCW) at 100 m mol NaCl (Fig. 5a). However, it seems that NaCl can affect positively AZ production at higher concentrations and longer time of the applied treatments. However, this speculation should be examined, further.

In the present study, NaCl improved the production of AZ. Basically, NaCl is an effective molecule responsible for the maintenance of the osmotic balance of the cell. It is also known to generate toxic reactive oxygen species (ROS). Therefore, to reduce the toxicity of ROS and maintain the osmotic balance in the cells synthesizing non-enzymatic antioxidants, such as tocopherols and carotenoids etc, the production of certain enzymatic antioxidants is also inevitable [33]. In response to such conditions, secondary metabolite production has been reported in subjecting the cell cultures of other plants to NaCl stress [11, 18, 34, 35]. Rafiq reported that the production of AZ increased up to 14.88 mg L^{-1} in cell culture of Neem in MS media when supplemented with 0.1 g/L NaCl, but in treatment with 0.2 g/L it was decreased [21]. In comparison to our results, it seems that the genotypes exhibit different sensitivities to NaCl for the production of target metabolites. In this case the Iranian native Neem seems to be more efficient. However, sodium chloride elucidates a potent stimulus to induce AZ in Neem cell culture.

Conclusion

In conclusion, Neem cell suspension culture verified its reliability as a powerful bio-factory to produce AZ, a valuable phytochemical medicine. All abiotic elicitors (SA, JA, Cd and NaCl) enhanced the production of AZ in suspension culture of *Azadirachta indica*. AZ content revealed significant increases in comparison to control at different concentrations of all elicitors. The cell suspension that was supplemented with 0.75 mM salicylic acid, after 6 days of incubation exhibited remarkable AZ production over other elicitors. This is true that not only in this study (> 23fold over control), but also in comparison to other previously reported results. Therefore, it suggests salicylic acid as a very suitable elicitor for the production of AZ compared to other elicitors in *A. indica* cell suspension cultures. NaCl and Cd with 2.17 and 1.7 fold increases in AZ production over control also displayed the next potent elicitors, respectively. The cell dry weight was not affected as expected. However, in the cadmium chloride treated cells a significant increase was observed in dry cell weight. The time length of elicitation was shown to be an important factor in AZ production, so that after 6 to 9 days of elicitation, its production decreased. It seems that combined elicitation condition is a considerable factor which needs to be studied. Overall, this study highlights a high AZ-producing genotype of Neem and SA as a powerful elicitor for AZ production [36]. However, in most elicitors (AS, JA and Cd) at more effective concentrations the cell AZ content was decreased after long time incubation which may be from secretion of AZ from cells into medium [37], which did not studied in this research and needs to be surveyed in next experiments. Research on AZ production by plant cell suspension cultures is still in its initial stages and there is a long way to go towards a commercially viable process. Knowledge of the biosynthetic pathway of AZ in Neem is not yet intricately described and understood. Information is needed at a cellular and molecular level before an efficient alternative for the large-scale commercial production of AZ can be achieved.

Acknowledgment

The authors wish to thank Imam Khomeini International University (IKIU) research council for financial support of this project. We also wish to thank from Dept. of Chemistry, Imam Khomeini International University (IKIU) and Dr. Majid G Afshar for helpful and technical assistance.

Refferences

1. Tinghui X, Wegener MK, O'Shea M, Deling M: World distribution and trade in neem products with reference to their potential in China. 2001 Conference (45th), January 23-25, Adelaide, 2001. Australian Agricultural and Resource Economics Society.
2. Rodrigues M, Festucci-Buselli RA, Silva LC, Otoni WC: Azadirachtin biosynthesis induction in *Azadirachta indica* A. Juss cotyledonary calli with elicitor agents. Braz Arch Biol Technol. 2014;57:155-162.
3. Dasgupta T, Banerjee S, Yadava PK, Rao AR: Chemopreventive potential of *Azadirachta indica* (Neem) leaf extract in murine carcinogenesis model systems. J Ethnopharmacology. 2004;92:23-36.
4. Butterworth JH, Morgan E: Isolation of a substance that suppresses feeding in locusts. Chem Commu (London). 1968;1:23-24.
5. Salehzadeh A, Akhkha A, Cushley W, Adams RLP, Kusel JR, Strang RHC: The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. Insect Biochem Mol Biol. 2003;33:681-689.
6. Tiwari R, Verma AK, Chakraborty S, Dhama K, Singh SV: Neem (*Azadirachta indica*) and its potential for safeguarding health of animals and humans: A review. J Biol Sci. 2014;14:110.
7. Prakash G, Srivastava AK: Statistical elicitor optimization studies for the enhancement of azadirachtin production in bioreactor *Azadirachta indica* cell cultivation. Biochem Eng J. 2008;40:218-226.
8. Sidhu O, Behl H: Seasonal variation in azadirachtins in seeds of *Azadirachta indica*. Curr Sci. 1996;70:1084-1085.
9. Shukor NAA, Seong LK: Azadirachtin variation of six provenances of *Azadimchm excelsa* (Jack) Jacob. Pakistan J Biol Sci. 2006;9:833-836.
10. Sidhu O, Kumar V, Behl HM: Variability in neem (*Azadirachta indica*) with respect to azadirachtin content. J Agric Food Chem. 2003;51:910-915.
11. Baldi A, Dixit V: Enhanced artemisinin production by cell cultures of *Artemisia annua*. Curr Trends Biotechnol Pharm. 2008;2:341-348.
12. Satdive RK, Fulzele DP, Eapen S: Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. J Biotechnol. 2007;128:281-289.
13. Zhao J-L, Zhou L-G, Wu J-Y: Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. App Microbiol Biotechnol. 2010;87:137-144.
14. 14. Zare N, Farjaminezhad R, Asghari-Zakaria R, Farjaminezhad M: Enhanced thebaine production in *Papaver bracteatum* cell suspension culture by combination of elicitation and precursor feeding. Nat Prod Res. 2014;28:711-717.
15. 15. Prakash G, Srivastava AK: Statistical media optimization for cell growth and azadirachtin production in *Azadirachta indica* (A. Juss) suspension cultures. Process Biochem. 2005;40:3795-800.
16. Prakash G, Srivastava AK: Modeling of azadirachtin production by *Azadirachta indica* and its use for feed forward optimization studies. Biochem Eng J. 2006; 29:62-68.
17. Dai J, Yaylayan VA, Vijaya Raghavan G, Pare JR, Liu Z: Multivariate calibration for the determination of total azadirachtin-related limonoids and simple terpenoids in neem extracts using vanillin assay. J Agric Food Chem. 2001;49:1169-1174.
18. Smith JL, De Moraes CM, Mescher MC: Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants. Pest Manag Sci. 2009;65:497-503.
19. Walker TS, Bais HP, Vivanco JM: Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L.(St. John's wort). Phytochemistry. 2002;60:289-293.
20.Balaji K, Veeresham C, Srisilam K, Kokate C: Azadirachtin, a novel biopesticide from cell cultures of *Azadirachta indica*. J Plant Biotechnol. 2003;5:121-130.
21. Muhammad R: Production of azadirachtin contents through *in vitro* culture of neem. University of Sindh, Jamshoro; 2012.
22. Singh M, Chaturvedi R: Statistical optimization of media for enhanced azadirachtin production from redifferentiated zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). In vitro Cell Dev Biol-Plant. 2012;48:92-98.
23. Srivastava S, Srivastava AK: Effect of elicitors and precursors on azadirachtin production in hairy root culture of *Azadirachta indica*. Appl Biochem Biotechnol. 2014;172:2286-2297.
24. Sivanandhan G, Arun M, Mayavan S, Rajesh M, Jeyaraj M, Dev GK, Manickavasagam M, Selvaraj N, Ganapathi A: Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. Appl Biochem Biotechnol. 2012;168:681-696.
25. Kachroo A, Kachroo P: Salicylic acid, jasmonic acid and ethylenemediated regulation of plant defense signaling. Genet Eng: Springer; 2007:55-83.
26. Leslie CA, Romani RJ: Inhibition of ethylene biosynthesis by salicylic acid. Plant Physiol. 1988;88: 833-837.
27. Sandalio L, Dalurzo H, Gomez M, Romero-Puertas M, Del Rio L: Cadmium-induced changes in the growth and

oxidative metabolism of pea plants. J Exp Bot. 2001;52: 2115-2126.
28. Groppa M, Ianuzzo M, Tomaro M, Benavides M: Polyamine metabolism in sunflower plants under long-term cadmium or copper stress. Amino Acids. 2007; 32:265-275.
29. Touiserkani T, Haddad R: Cadmium-induced stress and antioxidative responses in different *Brassica napus* cultivars. J Agri Sci Tech. 2012;14:929-937.
30. Furze JM, Rhodes MJ, Parr AJ, Robins RJ, Withehead IM, Threlfall DR: Abiotic factors elicit sesquiterpenoid phytoalexin production but not alkaloid production in transformed root cultures of *Datura stramonium*. Plant Cell Rep. 1991;10:111-114.
31. Lee K-T, Yamakawa T, Kodama T, Shimomura K: Effects of chemicals on alkaloid production by transformed roots of belladonna. Phytochemistry. 1998; 49:2343-2347.
32. Cetin ES, Babalik Z, Hallac-Turk F, Gokturk-Baydar N: The effects of cadmium chloride on secondary metabolite production in *Vitis vinifera* cv. cell suspension cultures. Biol Res. 2014;47:1.
33. Hojati M, Modarres-Sanavy SAM, Karimi M, Ghanati F: Responses of growth and antioxidant systems in *Carthamus tinctorius* L. under water deficit stress. Acta Physiol Plant. 2011;33:105-112.
34. Ajungla L, Patil P, Barmukh R, Nikam T: Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. Indian J Biotechnol. 2009;8:317-322.
35. Chavan SP, Lokhande VH, Nitnaware KM, Nikam TD: Influence of growth regulators and elicitors on cell growth and α-tocopherol and pigment productions in cell cultures of *Carthamus tinctorius* L. Appl Microbiol Biotechnol. 2011;89:1701-1707.
36. Mahalakshmi R, Eganathan P, Parida AK: Salicylic acid elicitation on production of secondary metabolite by cell cultures of *Jatropha curcas* L. Int J Pharm Sci. 2013;5:655-659.
37. Chawla H: Introduction to plant biotechnology. 3th edition ed: CRC Press Books; 2009.