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Effects of some elicitors on tanshinone production in adventitious root cultures of *Perovskia abrotanoides* Karel

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ABSTRACT

In this study, we investigated the effects of different biotic and abiotic elicitors including yeast extract (YE), methyl jasmonate (MJ), $AgNO_3$ and sorbitol on biomass and production of cryptotanshinone and tanshinone IIA in adventitious roots cultures of *Perovskia abrotanoides* Karel. Elicitors had no significant effect on root dry weight. The highest cryptotanshinone and tanshinone IIA production was achieved with 200 mg/l YE and 25 μM $AgNO_3$, respectively. YE and $AgNO_3$ were the most effective elicitors to stimulate the tanshinones production while the lowest concentration of MJ, only moderately promoted tanshinone accumulation. Sorbitol was almost ineffective in enhancing tanshinone content. Cryptotanshinone formation was stimulated more significantly by elicitation than tanshinone IIA. The results suggested that elicitors have the ability to stimulate tanshinone content in adventitious roots culture of *P. abrotanoides*.

1. Introduction

Perovskia abrotanoides Karel., a member of the Lamiaceae family, is a traditional medicinal plant, growing in various regions of Iran. The roots of this little known medicinal plant are mainly used for the treatment of leishmaniasis in Iranian folk medicine (Jaafari et al., 2007). There are only few scientific reports about *P. abrotanoides*. Some of them are implicated to the pharmacological effects such as leishmanicidal, antiplasmodial, antinociceptive, anti-inflammatory, antibacterial, and cytotoxic effects (Hosseinzadeh and Amel, 2001; Nassiri Asl et al., 2002). It has been reported that tanshinones are the most abundant and important bioactive compounds in the roots of this species (Sairafianpour et al., 2001).

Tanshinones, abietane-type norditerpenoid quinones, have been shown to exhibit diverse pharmacological activities, including antibacterial (Lee et al., 1999), antioxidant (Cao et al., 1996), anti-diabetes (Kim et al., 2007), anti-cancer (Wang et al., 2005; Liu et al., 2009; Chiu and Su, 2010; Pan et al., 2010) and anti-inflammatory (Jang et al., 2003) activity.

Since the roots of *P. abrotanoides* contain therapeutically applicable tanshinones, the mass cultivation of roots *in vitro* could be an effective technique for the large-scale production of these valuable secondary metabolites. For the commercial production of some secondary metabolites, field-grown plant material has generally been used but the quality of these products can be highly affected by various biotic and abiotic factors (Sivanandhan et al., 2012). Besides, field cultivation is a time consuming and labor intensive process, so the use of plant cell, tissue, and organ cultures has been acknowledged as a potential alternative source for the more efficient production of valuable secondary metabolites (Zhang et al., 2012; Jeong et al., 2006; Yu et al., 2005). Among these, root cultures are percieved as an effective means of biomass production because they grow fast, are easy to handle and show stable metabolite productivity (Subotic et al., 2009; Kang et al., 2004; Yu et al., 2002). Furthermore, the large-scale harvesting of roots of naturally growing medicinal plants to meet the demand for secondary metabolites has in turn exerted great pressure on the existence of many plant species (Martin et al., 2008). So, the development of a fast growing root culture system would offer unique opportunities for the production of root drugs *in vitro*, independent of field cultivation (Wasnik et al., 2009).

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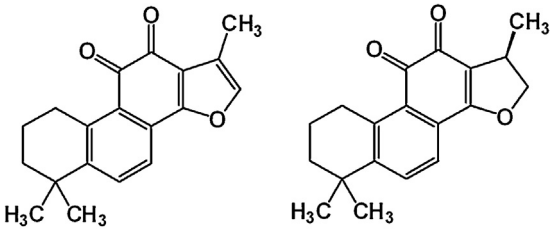

Fig. 1. Molecular structures of two tanshinones in *P. abrotanoides* roots.

Adventitious roots are post-embryonic roots that can arise from the stem and leaves and from non-pericyclic tissues in old roots (Li et al., 2009). These roots are natural, grow fast in phytohormone supplemented medium with a high rate of proliferation and show remarkable potentialities of secondary metabolites accumulation (Wasnik et al., 2009; Martin et al., 2008; Murthy et al., 2008).

There are different approaches to improve the yield of secondary products in *in vitro* cultures, some of which, using biotic and abiotic elicitors, have been well established for the stimulation of a diverse range of secondary metabolite production (Baskaran et al., 2012; Yu et al., 2005). Treatment with elicitors is one of the most effective strategies for improving secondary metabolite production in plant tissue cultures, and has recently found commercial application (Sivanandhan et al., 2012; Zhao et al., 2010; Smetanska 2008; Wu and Shi, 2008; Baskaran et al., 2012). Recent studies have shown that a wide range of elicitors can modify plant metabolism and result in elevated production of some secondary compounds, as well as accumulation of chemicals that would not usually be synthesized in the source plant (Cui et al., 2012; Baskaran et al., 2012).

To the best of our knowledge, no reports are available about the effects of elicitors on growth and tanshinone production in *in vitro* cultures of *P. abrotanoides*. In this study, we have established for the first time adventitious root culture of *P. abrotanoides* and investigated the effects of some elicitors on root growth and production of tanshinone IIA and cryptotanshinone (Fig. 1). Moreover, this is the first report on the quantitative determination of these metabolites in whole plant roots.

2. Materials and methods

2.1. Plant material

Mature seeds of *P. abrotanoides* were collected from wild grown plants in Khorasan-e Razavi Province, Iran. The plants were identified at the Research Center of Plant Science, Ferdowsi University of Mashhad, Mashhad, Iran (Voucher Sample Number: 39299). The seeds were surface sterilized with 70% (V/V) ethanol for 30 s and 20% sodium hypochlorite (V/V) for 5 min. Then, they were washed 4 times with sterilized water. Seed germination was carried out in distilled water. The seeds germinated after 4–6 days. Subsequently, one week old seedlings were transferred to Hoagland medium (Hoagland and Arnon, 1950). The plants were grown in a culture room under a 16 h photoperiod, (45 μmol m^{-2} s^{-2} irradiance level) and 25 ± 1 °C.

2.2. Adventitious root induction

Young leaves from 3-month-old hydroponically cultured plants were used as explants. The explants were surface sterilized with 70% (V/V) ethanol for 30 s and 20% sodium hypochlorite (V/V) for 5 min. Then, they were washed 4 times with sterilized water. Explants were inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 1-Naphthaleneacetic acid (NAA), 3% sucrose and 7 g/l agar (Zaker et al., 2013). The pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving at 1.2 kg/cm^2 and 121 °C for 20 min. The cultures were maintained at 25 ± 1 °C under dark conditions for 3 weeks. Adventitious root formation started after 2 weeks. Two grams of adventitious roots were subsequently transferred to 250 ml flasks with 75 ml liquid MS medium supplemented with 2 mg/l NAA, and kept in the dark on a rotary shaker (80 rpm) at 25 ± 1 °C with regular subcultures every 3 weeks into the same medium for multiplication. Five months old roots were used for the elicitation experiments.

2.3. Elicitor preparation

The elicitor types and concentrations were selected with respect to the references about tanshinone production in *in vitro* cultures (cell suspension and hairy root cultures) of *Salvia miltiorrhiza*, a well-known Chinese medicinal plant (Zhao et al., 2010; Wu and Shi, 2008; Shi et al., 2007; Ge and Wu, 2005; Yan et al., 2005). Four elicitors were tested at 3 concentrations each, including $AgNO_3$ (5, 25, and 50 μM), sorbitol (5, 25, and 50 g/l), yeast extract (YE; 50, 100, and 200 mg/l) and methyl jasmonate (MJ; 10, 50, and 100 μM). Stock solutions of $AgNO_3$ and sorbitol were prepared by dissolving them in deionized water. A YE carbohydrate fraction was prepared from yeast extract (Sigma) by ethanol precipitation as described by Hahn and Albersheim (1978). Briefly, YE was dissolved in distilled water (50 g/250 ml) and ethanol was added to a final concentration of 80% (V/V). The mixture was allowed to precipitate for 4 days at 4 °C in a refrigerator. The precipitate was then redissolved in 250 ml distilled water and subjected to another round of ethanol precipitation. The final gummy precipitate was dissolved in distilled water, stored at 4 °C and used as stock solution. MJ was dissolved in 96% ethanol. All elicitors were sterilized by filtering through a microfilter (0.2 μm).

2.4. Elicitor treatment

Two grams of adventitious roots were subcultured into a 250 ml flasks containing 75 ml liquid MS medium supplemented with 2 mg/l NAA and grown on a rotary shaker at 80 rpm and 25 ± 1 °C in dark. For elicitation, various concentrations of elicitors were added to 21-day-old root cultures. The same amount of water or ethanol was added to the control cultures. Adventitious roots were harvested 7 days after elicitor treatment.

2.5. Determination of root growth

Controls and elicited cultures were harvested after four weeks of cultivation. The roots were separated from the culture medium and the fresh weight was recorded after rinsing with distilled water and blotting dry with tissue paper. The adventitious roots dry weight was determined after drying in a freeze dryer.

2.6. Extraction and HPLC analysis of tanshinones

Freeze dried adventitious roots were ground into fine powder. Roots of wild growing plants were dried at room temperature under dark conditions and also crushed to powder. Tanshinones were extracted with methanol (500 mg root material/30 ml solvent) by sonication for 60 min at room temperature. The extract was then filtered through Whatman No.1 filter paper. After evaporating the solvent, the remaining residue was redissolved in 1 ml methanol and centrifuged at 5000 rpm for 5 min. The supernatant was used for HPLC analysis. Tanshinone content in the methanolic

Table 1
Effects of different elicitors on adventitious root growth of *P. abrotanoides* Karel.

Treatment	Dry weight (g)	Fresh weight (g)
Control 1	0.944 ± 0.037^{a}	13.898 ± 0.176406^{ab}
Control 2	0.888 ± 0.218^{a}	12.976 ± 1.562404^{ab}
MJ 10 μM	0.845 ± 0.068^{a}	14.207 ± 0.923474^{ab}
MJ 50 μM	0.564 ± 0.284^{a}	12.865 ± 2.743450^{ab}
MJ 100 μM	0.787 ± 0.237^{a}	16.064 ± 3.136722^{ab}
Ag^{+} 5 μM	1.377 ± 0.602^{a}	17.085 ± 2.236462^{a}
Ag^{+} 25 μM	0.707 ± 0.064^{a}	12.179 ± 0.842439^{ab}
Ag^{+} 50 μM	0.576 ± 0.028^{a}	10.655 ± 0.192905^{b}
YE 50 mg/l	0.759 ± 0.052^{a}	16.887 ± 2.619101^{a}
YE 100 mg/l	0.938 ± 0.179^{a}	13.486 ± 1.382542^{ab}
YE 200 mg/l	0.960 ± 0.288^{a}	12.014 ± 1.034016^{ab}
Sorbitol 5 g/l	1.050 ± 0.248^{a}	15.298 ± 1.451444^{ab}
Sorbitol 25 g/l	0.965 ± 0.057^{a}	13.928 ± 0.406589^{ab}
Sorbitol 50 g/l	1.291 ± 0.272^{a}	15.574 ± 2.255194^{ab}

Each value represents mean ± S.E. of four replicates. Within a column, means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's Multiple Range Test. Control 1, untreated roots; control 2, ethanol-treated roots.

Table 2
Effect of different concentrations of various elicitors on CT and Tan IIA accumulation in *P. abrotanoides* adventitious root culture.

Treatment	CT content (μg/g DW)	Tan IIA content (μg/g DW)
Control 1	122.06 ± 0.4^{g}	6.79 ± 0.18^{de}
Control 2	192.99 ± 0.33$^{d–g}$	6.73 ± 0.14^{de}
Ag^{+} 5 μM	283.79 ± 0.91$^{c–e}$	11.38 ± 0.24^{b}
Ag^{+} 25 μM	363.93 ± 3.59^{bc}	12.98 ± 0.38^{bc}
Ag^{+} 50 μM	361.20 ± 0.84^{bc}	9.99 ± 0.24^{bcd}
Sorbitol 5 g/l	114.08 ± 1.02^{g}	5.10 ± 0.026^{e}
Sorbitol 25 g/l	178.14 ± 0.10^{fg}	5.54 ± 0.097^{e}
Sorbitol 50 g/l	226.18 ± 1.67$^{d–f}$	4.87 ± 0.11^{e}
YE 50 mg/l	198.50 ± 0.75$^{d–g}$	5.84 ± 0.15^{e}
YE 100 mg/l	330.43 ± 1.29^{c}	8.29 ± 0.188^{cde}
YE 200 mg/l	443.62 ± 16.33^{b}	8.76 ± 0.739^{cde}
MJ 10 μM	283.65 ± 0.181$^{c–e}$	7.25 ± 0.16^{de}
MJ 50 μM	182.82 ± 0.60$^{d–g}$	5.72 ± 0.45^{e}
MJ 100 μM	181.46 ± 2.63$^{d–g}$	5.44 ± 0.14^{e}
Wild growing plants	801.20 ± 120.65^{a}	30.67 ± 4.57^{a}

Each value represents mean ± S.E. of three replicates. Within a column, means followed by the same letter are not significantly different ($p = 0.05$) according to Duncan's Multiple Range Test. Control 1, untreated roots; control 2, ethanol-treated roots.

extract solution was determined with an analytical HPLC system (Shimadzu, SIL-10AD VP Auto Injector), equipped with an auto sampler, 250 × 4 mm C18 column (5 μm particle size) and a PDA detector operating at a wavelength of 254 nm. The mobile phase was a mixture of acetonitrile and water eluted gradiantly from 20:80 V/V to 95:5 V/V, at a flow rate of 1 ml min^{-1}. The injection volume was 10 μl. Tanshinone IIA and Cryptotanshinone (Fig. 1) were detected and quantified with authentic standards obtained from Sigma. The tanshinones concentrations were calculated using a calibration curve compared with the standards.

2.7. Statistical analysis

All experiments were carried out in a completely randomized design. Statistical analyses were performed using Statistica software version 10. Data were subjected to Duncan's Multiple Range Test and reported as means ± standard error (S.E.). A probability of $p \leq 0.05$ was considered to be significant.

3. Results

3.1. Effects of various elicitors on adventitious root growth

Table 1 shows the effect of elicitor treatments on dry and fresh weight of adventitious roots of *P. abrotanoides*. As seen from the table, elicitor treatments did not affect root dry weight. Adventitious root fresh weight significantly increased at 5 μM Ag^{+} and 50 mg/l YE concentrations. The highest root dry and fresh weights were found in the medium supplemented with 5 μM Ag^{+} (1.377 and 17.085 g, respectively). All roots produced in culture media supplemented with different amounts of various elicitors were normal, healthy and similar in appearance.

3.2. Effects of elicitor treatments on cryptotanshinone (CT) content

Adventitious roots of *P. abrotanoides* could be easily induced and showed fast growth in liquid culture medium. Our results indicate that elicitation increased CT content in adventitious roots, as demonstrated in Table 2. Maximum CT accumulation (443.62 μg/g DW) was achieved with the addition of 200 mg/l YE. The content of CT in this treatment was 3.6 fold higher than that of the controls. However, no significant difference in CT concentration was observed between this treatments and adventitious roots treated with 25 or 50 μM Ag^{+}. The application of Ag^{+}, led to a significant increase of CT accumulation at all concentrations tested, as compared to the control culture.

The roots treated with 5 g/l sorbitol had the lowest CT content (114.08 μg/g DW), which was not significantly different to the CT content in the control. Among the different MJ concentrations tested, only the lowest concentration (10 μM MJ), resulted in enhanced production of CT when compared to the control. The effect of MJ at this concentration was stronger than sorbitol treatments, but weaker than high concentrations of Ag^{+} and YE.

Sorbitol at 5 and 25 g/l, MJ at 50 and 100 μM and YE at 50 mg/l concentrations failed to increase the CT content. In summary, the higher concentrations of YE (100 and 200 mg/l) and Ag^{+} (25 and 50 μM) were the most effective elicitors to stimulate CT production in *P. abrotanoides* root cultures. Fig. 2 shows HPLC chromatograms of the extracts from adventitious roots (a–c) and roots of wild grown plants (d).

3.3. Effects of elicitor treatments on tanshinone IIA (Tan IIA) content

Ag^{+} at all tested concentrations enhanced the Tan IIA content significantly. This elicitor had a dose-dependent stimulating effect on tanshinone production (Table 2). The highest concentration of Tan IIA (12.98 μg/g DW) was measured in roots treated with 25 μM Ag^{+}, about two fold higher than that of the control (6.79 μg/g DW).

Yeast extract at higher concentrations (100 and 200 mg/l) increased Tan IIA accumulation up to 1.3 times compared to the control.

The results indicate that Tan IIA content of adventitious roots treated with 50 or 100 μM MJ was considerably decreased. At a concentration of 10 μM MJ, Tan IIA production was similar to that in the control roots. Sorbitol treatments reduced Tan IIA accumulation significantly. Briefly, among the elicitors tested, Ag^{+} was the most effective elicitor for Tan IIA production in *P. abrotanoides* root culture.

In summary, Ag^{+} and YE elicitors stimulated tanshinone production in adventitious root culture of *P. abrotanoides* and CT formation was stimulated more significantly than that of Tan IIA.

4. Discussion

Roots play vital roles in whole plant growth and crop production. They also are sources of secondary metabolites, especially in plants producing pharmaceutical substances in their roots. In these

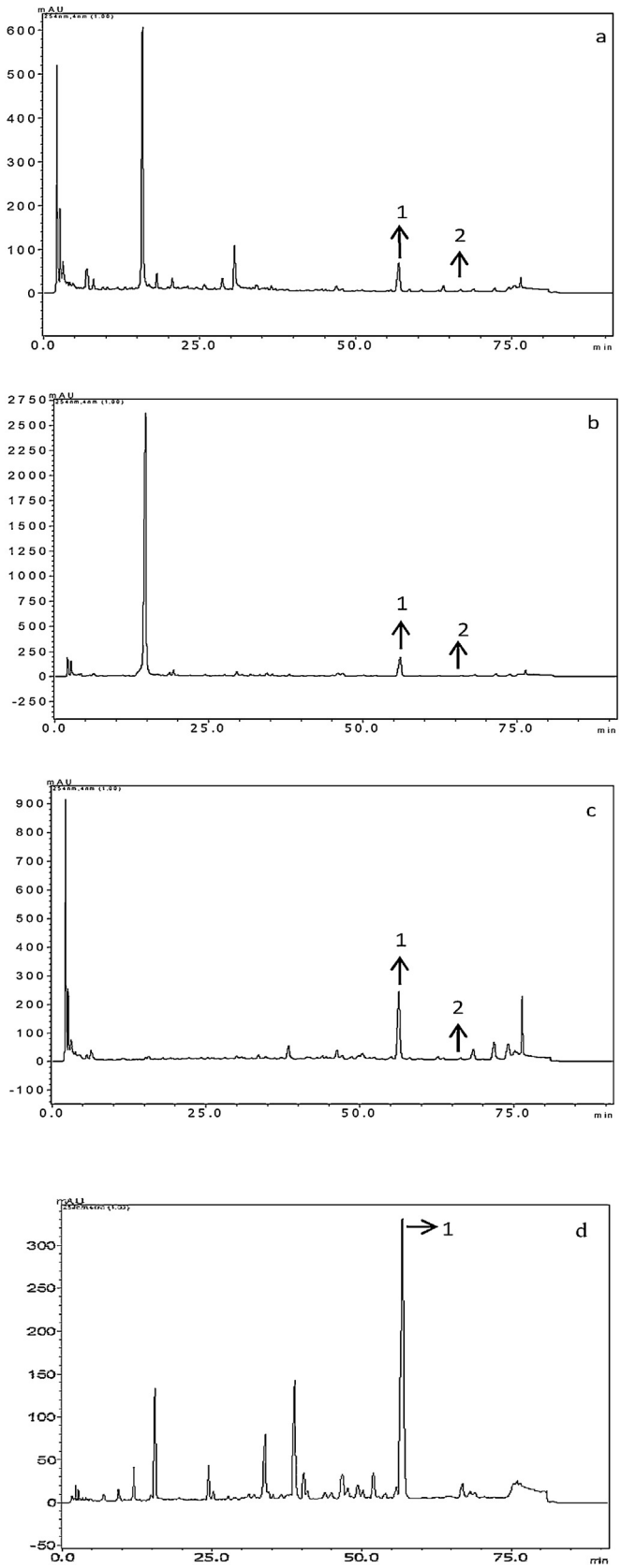

Fig. 2. HPLC profiles of cryptotanshinone and tanshinone IIA in *P. abrotanoides* adventitious roots and roots of wild grown plants. Control culture (a), culture treated with 25 μM Ag^+(b), culture treated with 200 mg/l YE (c) and root extract of wild grown plants (d). Peaks 1 and 2 represent cryptotanshinone and tanshinone IIA, respectively.

cases, mass production of roots including adventitious roots can be an alternative way to produce secondary metabolites (Martin et al., 2008). In the present study, adventitious root cultures in liquid medium supplemented with different elicitors were examined for root growth and tanshinone production.

Our experimental results showed that tanshinone accumulation in *P. abrotanoides* adventitious roots can be increased by both biotic and abiotic elicitors. In our study, elicitation did not significantly affect root dry weight. Other investigations also reported that various elicitors did not influence biomass production in root cultures of some plant species such as *Brugmansia candida* (Pitta-Alvarez et al., 2000), *Coleus forkohlii* (Li et al., 2005), *Gloriosa superba* (Ghosh et al., 2006), *Hypericum niger* (Hong et al., 2012) or *Angelica gigas* (Rhee et al., 2010).

Sorbitol is widely used as osmoticum in plant cell and tissue cultures and can have a positive effect on secondary metabolite production (Hong et al., 2012). Our results indicate that high osmotic pressure in the medium, as induced by sorbitol, had a slight enhancing effect on root growth. Similar results indicating a positive effect of sorbitol on biomass production and tanshinone accumulation were reported by Shi et al. (2007) and Wu and Shi (2008). However, sorbitol as an osmotic agent did not significantly stimulate tanshinone production in cell cultures of *S. miltiorrhiza*. This indicates that elicitor functions for a given metabolite can vary with the type of culture system (Zhao et al., 2010).

In this investigation, YE treatment resulted in enhanced biomass and tanshinone accumulation in adventitious roots of *P. abrotanoides*, particularly at the highest concentration of 200 mg/l. YE had a dose-dependent increasing effect on biomass and tanshinone production, stronger at 200 mg/l than at other concentrations. Similarly, tanshinone production in hairy root and cell cultures of *S. miltiorrhiza* was also stimulated significantly by YE (Zhao et al., 2010; Chen et al., 2001; Wu and Shi, 2008; Shi et al., 2007; Yan et al., 2005). Besides, YE elicitation promoted CT accumulation more than the formation of Tan IIA. YE is one of the most commonly used biotic elicitors to stimulate production of various secondary metabolites such as plumbagin in *Drosera indica* (Thaweesak et al., 2011), scopolamine and hyoscyamine in *Atropa belladonna* hairy root culture (Eskandari et al., 2012) and decursinol angelate in *A. gigas* Nakai root culture (Rhee et al., 2010). YE is composed of various substances including amino acids, vitamins and minerals. It is possible that the stimulating effect of this elicitor on secondary metabolites production may be due to the content of cations including Ca, Zn, and Co (Eskandari et al., 2012). YE-induced accumulation of tanshinones is mainly derived from a non-mevalonate pathway, involving 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Ge and Wu 2005).

Metal ions also influence secondary metabolite production. The present study demonstrated that addition of Ag^+ greatly improved the accumulation of CT and Tan IIA in *P. abrotanoides* adventitious root cultures. This observation is in agreement with results of Zhao et al. (2010) and Ge and Wu (2005). Ge and Wu (2005), found that Ag-stimulated production of tanshinones in hairy root culture of *S. miltiorrhiza* can be dependent on both mevalonate and non-mevalonate pathways, due to induction of key enzymes. Pitta-Alvarez and co-workers reported similar results about the overproduction of two tropane alkaloids, scopolamine and hyoscyamine, in hairy root culture of *B. candida* (Pitta-Alvarez et al., 2000). Similar results were also achieved for the production of lettucenin A in *Lactuca sativa* (Ong, 2009).

MJ showed only a moderate and insignificant stimulating effect on tanshinone accumulation in adventitious root culture of *P. abrotanoides*. Except for 10 μM MJ, different concentrations of MJ did not improve the production of CT. However, addition of 50 and 100 μM MJ exhibited a negative effect on root growth and Tan IIA production. These results are in accordance with Zhao et al. (2010) about *S. miltiorrhiza* cell cultures. Jasmonates are signal molecules

and plant regulators that mediate various developmental processes and induce plant defense responses to pathogens and insects (Chen et al., 2007; Yu et al., 2002; Rhee et al., 2010). In particular, exogenous application of MJ as an elicitor can be effective to stimulate the production of various secondary metabolites belonging to different structural groups in plant cell and tissue cultures (Yoo et al., 2011; Zhao et al., 2010; Rhee et al., 2010), including the formation of decursinol angelate in *A. gigas* root culture (Rhee et al., 2010), ginsenosides in *Panax ginseng* hairy root and adventitious root culture (Yu et al., 2002; Hong et al., 2012), podophyllotoxin in *Podophyllum peltatum* adventitious root and cell cultures (Anbazhagan et al., 2008), saikosaponins in *Bupleurum kaoi* adventitious root culture (Chen et al., 2007) and hyoscyamine and scopolamine in *A. belladonna* hairy root culture (Eskandari et al., 2012).

5. Conclusion

In conclusion, we established an adventitious root culture system for *P. abrotanoides*. The present study demonstrated that different elicitors had different effects on tanshinone accumulation in *P. abrotanoides* adventitious root cultures. YE and Ag^+ have been shown to be the most effective elicitors for stimulating tanshinone production. Cryptotanshinone was more responsive to elicitors and increased more significantly in content than Tan IIA. Therefore this in vitro system can be an efficient tool and alternative source for the production of these pharmacologically important metabolites.

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