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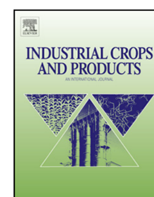

Effects of some elicitors on tanshinone production in adventitious root cultures of *Perovskia abrotanoides* Karel

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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*.
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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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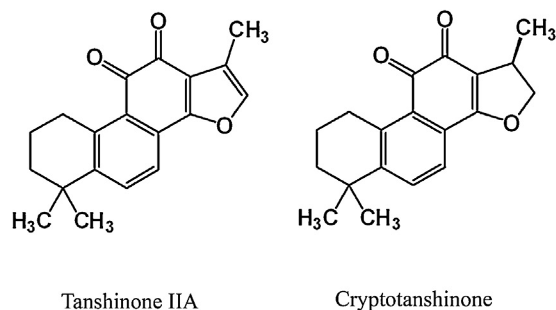

Fig. 1. Molecular structures of two tanshinones in *P. abrotanoides* roots.

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

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