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Enhanced phenolic acids production in regenerated shoot cultures of *Salvia virgata* Jacq. after elicitation with Ag^+ ions, methyl jasmonate and yeast extract

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ABSTRACT

Phenolic acids, among the major secondary metabolites of *Salvia* species, are important bioactive phytochemicals, namely for their application in pharmaceutical industries. Biosynthesis of secondary metabolites, including phenolic acids in plant *in vitro* cultures is affected by biotic and abiotic elicitors, leading mostly to higher levels than in non-elicited ones. The present study was initially focused on achievement to a suitable procedure for direct multiple shoot regeneration on multinodal explants from 50 days old seedlings of *Salvia virgata* Jacq. on Murashige and Skoog (MS) solid media supplemented with different concentrations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). Then, the effects of different concentrations of Ag^+ ions, yeast extract (YE) and methyl jasmonate (MeJA) on total phenolic and flavonoid contents, as well as some phenolic acids were studied in the regenerated shoots after 1,3 and 5 days of elicitation in liquid free hormone MS medium. Based on the results, the maximum number of regenerated shoots (4.67) per responsive explant was obtained on MS medium containing 2 ppm BAP and 0.05 ppm IAA. As an effective abiotic elicitor, Ag^+ ions could improve production of phenolic acids in the shoot cultures, while the highest content (26 mg/g DW) of rosmarinic acid (RA) was reported on day 5 after exposure of regenerated shoots to 2.5 ppm of Ag^+ ions. Also, the highest contents of salvianolic acid A (Sal-A) (3.72 mg/g DW) and caffeic acid (CA) (35.5 mg/g DW) were found after elicitation of regenerated shoots with MeJA (11.2 ppm) on day 3 and Ag^+ ions (2.5 ppm) on day 5, respectively. The results suggested that MeJA and Ag^+ ions had the considerable ability to stimulate the production of valuable phenolic acids such as RA in the regenerated shoot cultures of *S. virgata*.

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1. Introduction

Salvia virgata Jacq. (wand sage), belongs to Lamiaceae family and is a perennial medicinal plant native to Asia (northeast of Iran) and southeastern Europe. There are few scientific reports about *S. virgata*. Some traditional uses of the preparations of this little known medicinal plant against skin diseases, wounds and blood cancer (leukemia) have been reported (Baytop, 1999). Recently, modern medical studies have conceived antioxidant ability (Kosar et al., 2008; Tepe, 2008), anti-inflammatory and anti-nociceptive activities of the extracts of this species (Akkol et al., 2008).

It has been identified that the most abundant and important bioactive compounds in the shoots of this species are phenolic acids, especially rosmarinic acid (RA) (Fig. 1) (Kosar et al., 2008).

Some studies have confirmed that RA, as the main bioactive compound in *Salvia* species, especially *S. miltiorrhiza* and *S. officinalis* had some therapeutic effects such as antioxidant (Tepe, 2008), anti-ischemic (Ozturk et al., 2014) anticancer (Sharmila and Manoharan, 2012), anti-inflammatory (Chu et al., 2012), antibacterial (Abedini et al., 2013) and antiviral properties (Dubois et al., 2008). It can also prevent Alzheimer's disease (Airoldi et al., 2013), and acts as a putative inhibitor of HIV-1 integrase (Hooker et al., 2001) and reverse transcriptase (Mazumder et al., 1997).

Abbreviations: Ag^+ ions, silver ions; BAP, 6-benzylaminopurine; CA, caffeic acid; IAA, indole-3-acetic acid; LAB, lithospermic acid B; MeJA, methyl jasmonate; MS, Murashige and Skoog; RA, rosmarinic acid; Sal-A, salvianolic acid A; Sal-B, salvianolic acid B; YE, yeast extract.

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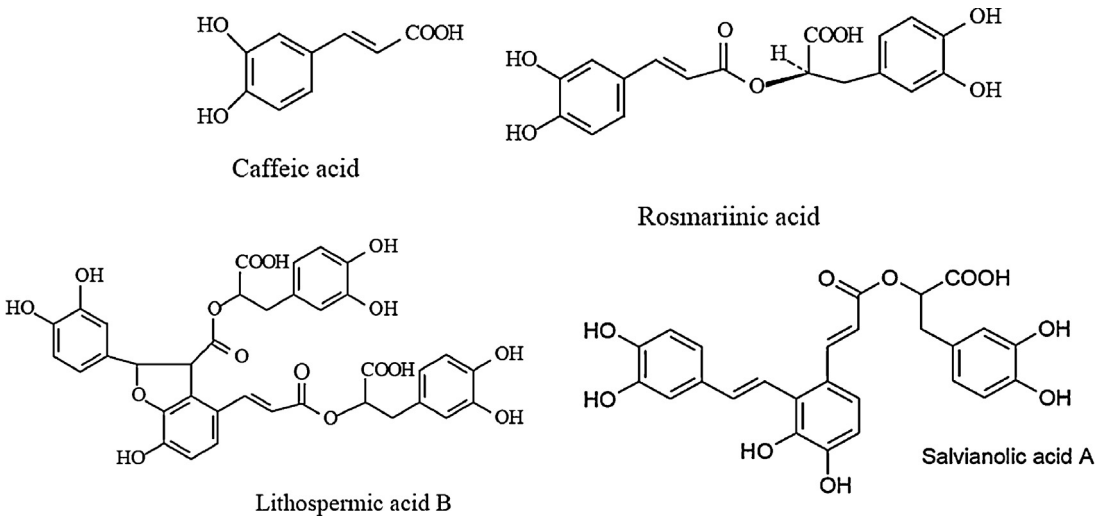

Fig. 1. Molecular structures of four phenolic acids in *Salvia* L. (Xing et al., 2015).

Salvianolic acid B (Sal-B) or lithospermic acid B (LAB), and salvianolic acid A (Sal-A) are dimeric derivatives of RA (Fig. 1), but major details of their biosynthesis pathway from RA have remained unknown (Xiao et al., 2010). Water-soluble phenolic acid, Sal-B, has been successfully isolated and purified from some Chinese *Salvia* plants such as *S. miltiorrhiza, S. bowleyana* Dunn, *S. cavaleriei* var. *simplicifolia* Stib. and *S. dabieshanensis* (Jiang et al., 2005; Min-Hui et al., 2008; Zhao et al., 2010) and Sal-A just has been derived from S.*miltiorrhiza* (Chen et al., 2006; Min-Hui et al., 2008). Among salvianolic acids, Sal-A and Sal-B are the most abundant components in *S. miltiorrhiza* and Sal-A has higher antioxidant activity than Sal-B at the same concentration (Wang et al., 2012).

There are some reports for *in vitro* enhancement of phenolic acids production using shoot cultures of plants (Abraham et al., 2011; Phatak and Heble, 2002; Skrzypczak-Pietraszek et al., 2014). Due to the presence of bioactive phenolic compounds, micropropagation of *S. virgata* through shoot *in vitro* culture could be an effective technique for the large-scale production of these valuable secondary metabolites (Akkol et al., 2008). In the case of RA production, shoot cultures are much better sources than cell suspension cultures (Roy and Mukhopadhyay, 2012). Grzegorczyk and Wysokinska (2009) have reported similar contents of RA for *in vivo* and *in vitro* grown shoots of *S. officinalis*.

Recent studies have shown that plant metabolism and consequently production of some secondary compounds can be affected by a wide range of elicitors, especially metabolites that would not usually be synthesized and accumulated in the parent plants in nature at higher amounts (Baskaran et al., 2012; Cui et al., 2012). Application of various elicitors such as silver ions (Ag^+ ions) and yeast extract (YE) in culture media was widely compatible to promote production and accumulation of secondary metabolites during the culture process (Wang et al., 2012).

Based on the reports of some researchers, YE and Ag^+ ions can stimulate the accumulation of RA and phenolic acids in the hairy root cultures of *S. miltiorrhiza* (Xiao et al., 2010; Xing et al., 2015; Yan et al., 2006). Moreover, it has been proved that production of phenolic acids can be promoted under methyl jasmonate (MeJA) in *S. officinalis* and some other plants (Bauer et al., 2009; Grzegorczyk and Wysokinska, 2009; Skrzypczak-Pietraszek et al., 2014).

Up to this time, there is only one available report (Ejtahed et al., 2015) about the effects of elicitors on phenolic acids production in the regenerated shoot cultures of *S. virgata*. For this reason, the present study was aimed to establish an effective *in vitro* direct shoot regeneration system for *S. virgata* and to investigate the effects of some elicitors on production of RA, CA and Sal-A in the regenerated shoots.

2. Materials and methods

2.1. Plant material

Mature seeds of *S. virgata* were collected from wild grown plants in the August 2013 at Reine village (Bojnoord, North Khorasan province, Iran) with the geographical specifications including latitude: 57° and 2 min North, longitude: 37° and 23 min East and altitude: 1765 m above sea level. The species was identified at the Ferdowsi University of Mashhad herbarium (FUMH), where voucher specimen (no. 38128) of the plant was deposited. Seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 5% sodium hypochlorite (w/v) solution for 5 min. Then, they were rinsed three times in sterile distilled water. For germination, seeds were placed into glass jar containing 25 mL of MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.7% agar. The pH of media was adjusted to 5.6–5.8 before adding agar, and the MS basal medium was autoclaved at 120 °C for 17 min. The glass jars were kept in the dark for 3 days at 25 ± 2 °C and after germination of the seeds, they were placed at 26 ± 2 °C and 16/8 h (light/dark) photoperiod (45 μM photons m^{-2} s^{-1} provided by cool white fluorescent lamps) in a culture room. These conditions were also applied for all the experiments described below.

2.2. Shoot induction and culture conditions

The multinodal explants (1 cm length with 2–3 nodes) were excised from 50-days-old sterile seedlings and were cultured on MS media supplemented with different concentrations of indole-3-acetic acid (IAA) (0, 0.05 and 0.1 ppm) and 6-benzylaminopurine (BAP) (0, 1, 1.5 and 2 ppm) to determine the best culture medium for direct shoot regeneration. Three replications were considered for each treatment and five multinodal explants were cultured in each jar. After 4 weeks, the best treatment for direct shoot regeneration was selected based on the mean shoot number per responsive explant (MSN), the mean shoot length (MSL) and the mean leaves number per regenerated shoot (MLN). Subsequently obtained shoots were moved to free hormone liquid MS media supplemented with 3% sucrose and elicitors (Ejtahed et al., 2015; Grzegorczyk and Wysokinska, 2009; Suarez et al., 2010).

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