Improvement in androgenic response of borage (*Borago officinalis* L.) cultured anthers using antibrowning agents and picloram

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Abstract: Given the importance of borage (*Borago officinalis* L.) as a medicinal herb, androgenic studies are necessary in order to speed up the breeding programs of this plant species. In this work, we evaluated the effect of silver nitrate, activated charcoal, ascorbic acid, and polyvinylpyrrolidone (PVP), previously described as antibrowning agents and promoters of androgenesis. Adding silver nitrate at a concentration of 5 mg L⁻¹ significantly enhanced the androgenic responses of cultured anthers up to ~2-fold for total calli production (53.33%) and up to ~2.5-fold for embryonic calli and embryo formation (30% and 0.3 embryos/anther, respectively) compared to control culture medium. Culture media containing 5 and 10 mg L⁻¹ silver nitrate and 2 mg L⁻¹ PVP significantly reduced the callus browning and led to increased callus viability in borage cultured anthers. We also evaluated the effect of various combinations of picloram with 2-isopentyl adenine and 2,4-dichlorophenoxyacetic acid (2,4-D) as growth regulators in the standard protocol. Significantly better results were obtained by adding 2 mg L⁻¹ picloram in combination with 2 mg L⁻¹ 2,4-D in culture medium. This medium produced the highest frequencies of callogenesis (48.33%), embryogenic calli (20%), and embryo formation (0.2 embryos/anther) compared to control and other growth regulator combinations.

Key words: Anther culture, callogenesis, embryogenic calli, plant growth regulators

1. Introduction

Homozygous plants are valuable materials for plant breeding and genetic studies (Jia et al., 2014). The production of homozygous plants by conventional plant breeding methods is lengthy and laborious, requiring 7 or 8 recurrent cycles of inbreeding. On the other hand, these approaches are impractical for self-incompatible and male sterile plant species (Forster et al., 2007). These homozygous plants can be obtained in a single generation by in vitro culture methods. Androgenesis, as the process of haploid induction from male gametic cells, is often the method of choice for doubled haploid (DH) production in many crop plants (Sopory and Munshi, 1997). Anther culture is generally a simple method of androgenesis and has been effectively used for production of haploid and DH lines in many crops and horticultural species such as rice, barley, wheat, maize, watermelon, and cucumber (Chen et al., 2001; Jacquard et al., 2006; Broughton, 2008; Abdollahi et al., 2015, 2016; Ismaili and Pour Mohammadi, 2016), but its use is still limited in medicinal plants compared to agronomical important crops (Ferrie, 2013).

The efficiency of embryogenesis in anther cultures is influenced by many factors such as genotype, developmental stage of microspores, stress pretreatment, growth regulators, and chemical compounds in the culture medium (Dunwell, 2010; Germanà, 2011; Asif et al., 2014; Sinha and Eudes, 2015). One of the most important is the induction medium and its various components. Antioxidants and antibrowning agents are other components that have been previously used in induction media and may help improve the efficiency of embryogenesis in anther cultures. These chemical agents have often been reported to prevent accumulation of phenolic compounds and their deleterious effects on plant tissue culture phenomena such as callus and somatic embryo induction (Malabadi and van Staden, 2005). Among the antibrowning agents, silver nitrate, activated charcoal, ascorbic acid, and polyvinylpyrrolidone (PVP) have previously been employed in androgenesis studies (Johansson, 1983; Ockendon and McClenaghan, 1993; Zhong et al., 1995; Pinto et al., 2008; Prem et al., 2008; Shahvali-Kohshour et al., 2013).

One of the most critical factors controlling the androgenic response in plant species is growth regulator (Dieu and Dunwell, 1988; Peixe et al., 2004). The type and
concentration of the growth regulators, particularly the auxins and cytokinins, can be the deciding medium factor that would influence pollen embryogenesis in microspore or anther cultures (Sood and Dwivedi, 2015). Various types and concentrations of auxins are required to obtain efficient yields of embryogenic structures in crop species (Zhou and Konzak, 1989; Ishizaka, 1998; Han et al., 2000). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most widely used auxin in anther culture studies (Ball et al., 1993) and, depending on the plant species, its optimum concentration for induction of androgenesis is different (Custodio et al., 2005). Picloram (4-amino-3,5,6-trichloropicolinic acid), another auxin acid, has been reported to induce embryogenesis from anther and ovary cultures in grapevines (Nakano et al., 1997), and it was also a favorable auxin for inducing calli from anthers of ‘Connecticut King’ (Han et al., 1997, 2000).

Borage is a self-incompatible annual medicinal plant that is cultivated for medicinal and culinary uses and has different usages in pharmaceutical, industrial, and forage fields (Peiretti et al., 2004). It has oil seed that contains a high amount of γ-linolenic acid (30%–40%), a volatile fatty acid synthesized by just a few plant varieties such as borage and primrose (Yang et al., 2002). In borage Chardoli Eshaghi et al. (2015) first reported androgenesis by anther culture method. These researchers evaluated the effect of various androgenic factors including developmental stage of microspores, carbon source, temperature pretreatments, and growth regulators on the anther culture efficiency of borage. Their results were limited to the production of mostly calli that turned brown and eventually died. The callus browning led to the production of a small number of haploid embryos. Thus, in order to increase the callus viability and efficiency of embryo formation in cultured anthers, the present study was undertaken to test the effect of different antibrowning agents and some other plant growth regulators (PGRs) such as picloram in anther culture medium.

2. Materials and methods

2.1. Plant material and donor plant growth condition

Donor plants used to obtain anthers were derived from a local Iranian variety of borage (Borago officinalis L.). Seeds of this local variety were obtained from Pakan Bazr Company (Isfahan, Iran). Plants were grown in 25-cm pots in a greenhouse under 28–32 °C during the day and 18–24 °C at night with a natural photoperiod from May 2015 to September 2015.

Donor plants were watered two or three times a week with the addition of a nutrient solution (N/P/K = 15/12/24) every 3 weeks. Treatments against pathogens were undertaken as necessary.

2.2. Determination of microspore developmental stage

The developmental stages of the microspores were determined by staining in aceticarmine (1 g of carmine in 100 mL of 45% glacial acetic acid). Anthers from harvested flower buds with different lengths were used to prepare slides. The slides were observed under a microscope to determine the suitable microspore stage.

2.3. Anther culture

Anther culture conditions were provided according to Chardoli Eshaghi et al. (2015). Borage buds (5–7 mm) containing anthers with microspores at the mid-to-late uninucleate stage were collected from donor plants of 50–60 days old and disinfected as follows: surface sterilization with 70% (v/v) ethanol for 30 s, followed by 2.5% (v/v) cold (4 °C) sodium hypochlorite for 10 min, and then rinses of 5 min each in cold (4 °C) sterile distilled water. Anthers with microspores at the mid-to-late uninucleate stage (Chardoli Eshaghi et al., 2015) were dissected and plated with 20 anthers per petri dish (90–100 mm in diameter) on 20 mL of induction medium comprising the macroelements and microelements of B5 (Gamborg et al., 1968), vitamins of NLN medium (Lichter, 1982), 0.8% agar, 3% maltose, 2 mg L⁻¹ 2,4-D, and 1 mg L⁻¹ BAP (Chardoli Eshaghi et al., 2015). Dishes were placed at 4 °C in the dark for 5 days and then transferred to 25 °C in a growth room under a 16/8 photoperiod with a light intensity of 40 µmol m⁻² s⁻¹ for 2 weeks. Callogenetic and embryogenic anthers were recorded 3 weeks after anther inoculation. One week later, embryogenic calli or embryos were transferred to B5 regeneration medium supplemented with 2 mg L⁻¹ BA and 0.5 mg L⁻¹ naphthalene acetic acid, 3% sucrose, and 0.8% agar with pH 5.7 under the growth room conditions described above. These anther culture conditions were kept unchanged in all experiments, except for altered parameters. In order to evaluate the effect of antibrowning agents and PGR combinations on calli and embryo production from borage anther cultures, we designed two separate experiments, described below.

2.4. Effect of different concentrations of antibrowning agents

In this experiment, we assessed the influence of antibrowning agents on the androgenic response of borage anthers and on the viability of induced calli 3–4 weeks after culture initiation. Borage anthers were cultured on the basal medium (B5 medium salts and NLN medium vitamins) supplemented with various concentrations of the following antibrowning compounds: silver nitrate (5, 10, and 15 mg L⁻¹), activated charcoal (5, 10, and 15 mg L⁻¹), ascorbic acid (10, 25, and 50 mg L⁻¹), and PVP (0.5, 1.0, and 2.0 mg L⁻¹), separately.

2.5. Effects of picloram, 2-isopentyl adenine, 2,4-D, and their combinations

In this experiment, the basal medium (as described in the first experiment) supplemented by various combinations
of picloram (0, 1.0, and 2.0 mg L\(^{-1}\)) with 2-isopentyl adenine (2ip) (0, 0.3, and 0.6 mg L\(^{-1}\)) and also by different combinations of picloram (0 and 2 mg L\(^{-1}\)) with 2 and 5 mg L\(^{-1}\) 2,4-D was used to stimulate callus and induction during anther culture of borage. The combination of 2 mg L\(^{-1}\) 2,4-D with 1 mg L\(^{-1}\) BAP, the best PGR treatment in the previous study by Chardoli Eshaghi et al. (2015), was used as a further control treatment. Observations on callus and embryo production in anther culture were made 3 weeks after culture initiation. In both experiments, 60 anthers (20 anthers per petri dish and 3 dishes per treatment) were cultured for each treatment.

2.6. Determination of chromosome number and ploidy level

Cytological analyses of the diploid root of borage seedling and anther-derived calli and embryos were carried out by the method described by Chardoli Eshaghi et al. (2015). In order to determine the ploidy level of anther-derived calli and embryos and also the borage seedlings, flow cytometry was carried out with a ploidy analyzer (Partec GmbH, Germany). Using a sharp blade, a sample anther-derived embryo or small callus or a leaf segment of borage seedling (approximately 1 cm) was cut into small pieces in 2 mL of cold (8 °C) DAPI staining solution (5 µg mL\(^{-1}\), Partec GmbH) and passed through nylon gauze (mesh size: 50 µm). The filtrate was used for flow cytometric analysis, at a gain FL1 of 400–415 (relative fluorescence). Leaf tissue from plants derived from seeds of tomato was used as a standard. In chromosome counting experiments, the plant materials were first treated by alpha-bromonaphthalene for 3 h, followed by fixation in acetic acid and absolute alcohol (1:3 v/v) for 24 h. After fixation, the plant materials were washed in distilled water and hydrolyzed in 1 M HCl for 6 min at 60 °C. The treated samples were squashed in 1% acetocarmine and 45% acetic acid and then the prepared samples were observed under light microscope magnified by 40 × 10 to determine the chromosome numbers of the cells. Chromosome numbers were counted and photos were taken using a 100 × 10 objective and eyepiece combination, respectively.

2.7. Statistical analyses

Three weeks after culture initiation, the number of total calli (the number of calli produced on anthers in each petri dish), number of embryogenic calli (the calli capable of forming embryos) per dish, and the number of embryos per anther were recorded and were calculated as the percentage of anthers producing calli (C %), the percentage of anthers producing embryogenic calli (EC %), and the mean number of embryos per anther (ME/A), respectively. In first experiment, the mean number of days from callus induction until the time of 50% callus browning was also recorded for each antioxidant treatment. Both experiments were designed as complete randomized designs and were repeated three times. Each treatment consisted of 3 replications (one petri dish containing 20 anthers was regarded as one replicate). The data were analyzed by SPSS 16. Mean comparisons were done by Duncan’s multiple range test (Duncan, 1955) following ANOVA for the studied parameters. The percentage data that were not normally distributed in residual plot analysis were transformed by the square root function before the analyses of variance.

3. Result

Immature borage anthers were inoculated on standard anther culture medium. Some of cultured anthers did not form calli, turned brown in color, and died. The anthers that stayed fresh and did not show browning symptoms usually had a high chance of producing calli (Figure 1a). These anthers became swollen and callus-like structures formed approximately 2 weeks after anther inoculation (Figure 1b), and globular embryos gradually formed in some embryogenic calli (arrowheads in Figure 1c). After 2 more weeks, embryos at the torpedo stage developed (arrowheads in Figures 1d–1f). These torpedo embryos were characterized by an elongated hypocotyl and two primordial cotyledons with an embryo-typical texture (arrowheads in Figure 1f). Some anther-derived embryos did not show normal shoot apices and had no identifiable embryo structure (arrowhead in Figure 1g). In this study, we also observed some embryo-like structures, originating directly from anthers without callus formation and considered as direct embryogenesis (arrowheads in Figures 1h and 1i). Embryogenic calli and embryo-like structures failed to germinate when placed on B5 germination medium and turned brown and eventually died. Likewise, the chromosome numbers in root tips of diploid seedling plants and anther-derived calli or embryos were counted.

3.1. Effect of the exogenous addition of antibrowning agents

The results of this experiment indicated that application of 5 mg L\(^{-1}\) silver nitrate to the anther culture medium produced the highest C % (53.33%, Figure 2a), EC % (30%, Figure 2b), and ME/A (0.3, Figure 2c) when compared with media containing other antibrowning agents and without antibrowning agents. Use of culture media supplemented with silver nitrate (5 and 10 mg L\(^{-1}\)) and PVP (2 mg L\(^{-1}\)) also significantly (P < 0.05) decreased callus browning compared with the control and other antibrowning agents and enhanced the mean time of callus viability up to 12 days after induction (Figure 2d). The addition of different concentrations of activated charcoal repressed EC and embryo formation and only led to little callus production (Figure 2a). This compound did not reduce browning, and calli eventually died in these culture media (3–5 days after induction, Figure 2d).
3.2. Effects of different combinations of PGRs

Different combination of PGRs significantly (P < 0.05) influenced the androgenic response of borage cultured anthers. Addition of picloram to the culture medium either alone or in combination with different concentrations of 2,4-D and 2ip was effective in enhancing the C %, EC %, and ME/A (Figures 3a–3c). However, 2ip alone (at 0.3 or 0.6 mg L\(^{-1}\)) was ineffective in calli/embryo formation and reduced all androgenic traits compared to control medium without any PGR. The greatest values of C %, EC %, and ME/A (48.33%, 20%, and 0.2, respectively, Figures 3a–3c) were observed in culture medium supplemented with 2 mg L\(^{-1}\) picloram and 2 mg L\(^{-1}\) 2,4-D compared to the control and all PGR treatments (Figures 3a–3c). Among the 13 culture media tested in this experiment, only the culture medium supplemented with 2 mg L\(^{-1}\) picloram and 2 mg

**Figure 1.** Callus and embryo induction from anther cultures of *Borago officinalis*: (a) suitable anthers (2.5–3.5 mm) inoculated in a standard culture medium; (b) an embryogenic green callus emerged from a cultured anther 3 weeks after anther inoculation; (c) an embryo-like structure at globular stage (white arrowhead) induced on the surface of an embryogenic callus; (d and e) torpedo embryos (white arrowheads) arose from green embryogenic calli 2 weeks after callus induction; (f) a developed embryo with two primordial cotyledons (white arrowheads), embryogenic callus turned brown in color 3 weeks after callogenesis initiation (black arrowhead); (g) an elongated callus-derived embryo showing malformation of absent shoot apex (white arrowhead); (h and i) elongated embryos (white arrowheads) directly originated from anthers without a callus-mediated phase. Bars: 1 mm.
L-1 2,4-D significantly improved the C %, EC %, and ME/A compared to this further control medium (Figures 3a–3c).

3.3. Determination of ploidy level in anther-derived calli and callus-derived embryos
Using flow cytometry, the ploidy levels of a total of 5 anther-derived calli, 5 callus-derived embryos, and 3 direct anther-derived embryos were determined. The fluorescence G1 and G2 peak values of the control tissue (diploid borage seedling) were ~120 and ~240, respectively (Figure 4a), so G1 and G2 peaks of ~60 and ~120 were interpreted as indicating the presence of haploid plant tissue (Figure 4b). A diploid tomato leaf used as a standard produced G1 and G2 peaks at gains of ~75 and ~150, respectively (Figure 4c). The results of flow cytometric analysis showed two different ploidy levels. Among the five anther-derived calli tested, three calli were haploid (60%) and two calli were diploid (40%), while four callus-derived embryos (80%) presented haploid ploidy levels. Among the three embryos that originated directly from anthers, one embryo showed haploid ploidy level and two embryos (67.67%) were diploid, suggesting that they may have developed from anther walls (somatic origin) or from the microspores (spontaneous diploids). Chromosome number was also determined in the anther culture-derived tissues by microscopy. The root tips of borage diploid seedlings and anther-derived tissues (callus/embryo) were cytologically examined under a microscope by counting the number of chromosomes of metaphase-stage mitotic cells. Diploid root tips of *B. officinalis* had a chromosome number of 2n = 2x = 16 (Figure 4d), while the chromosome number in the haploid tissues tested by flow cytometry was 8 (Figure 4e).

4. Discussion
Explant browning is a major unsolved problem in the initiation of tissue cultures and is generally attributed to the accumulation of phenolic compounds in plant tissues (Thomas and Ravindra, 1997). This phenomenon leads to a decrease in culture competence (Benson, 2000). In our previous report (Chardoli Eshaghi et al., 2015), we first reported an anther culture protocol for borage under...
Figure 3. Effect of different combinations of picloram with 2ip and 2,4-D on: (a) the percentages of anthers producing calli, (b) the percentages of anthers producing embryogenic calli, and (c) the mean number of embryos obtained per anther in borage anther cultures. Different letters show significant differences (Duncan's multiple range test, P < 0.05).
Figure 4. Determination of the ploidy level in borage seedlings and anther-derived embryos by flow cytometry analysis and chromosome counting: (a) flow cytometry histogram of a diploid borage seedling that produced G1 and G2 peaks at gains of *120 and *240, respectively; (b) flow cytometry histogram of a haploid embryo showed G1 and G2 peaks at gains of *60 and *120, respectively; (c) flow cytometry histogram of a diploid tomato leaf was used as a standard; (d) chromosome number of a root tip cell of a borage seedling (2n = 2x = 16); (e) an anther-derived haploid embryo cell with eight chromosomes confirmed the presence of a chromosome set half that of the diploid seedlings. Bars: 10 µm.
the influence of temperature pretreatments and some hormone treatments. We successfully obtained a limited number of gametic embryos in that study. However, the most important bottleneck in borage anther culture was that the eventual browning and dying of induced calli led to a decline in the androgenic potential of the cultured anthers. Therefore, in first part of this report, we have critically analyzed the effect of different antibrowning agents, which interfere with callus browning in borage anther culture. Among the different antibrowning agents tested in this work, the most effective to promote callus and embryo induction was silver nitrate at the concentration of 5 mg L⁻¹. In accordance with this result, Lashermes (1992) also reported a significant enhancement in the androgenic potential of wheat anthers when they were cultured on media containing 5 mg L⁻¹ silver nitrate. The positive effect of silver nitrate on callogenesis and gametic embryogenesis in the present study is further supported by similar studies on the androgenesis of rice (Niroula and Bimb, 2009), wheat (Lashermes, 1992), maize (Bhojwani and Razdan, 1996), tobacco (Dunwell et al., 1987), broccoli (Na et al., 2011), and strawberry (Shahvali-Kohshour et al., 2013).

Silver ion is known as an inhibitor of ethylene action in classical plant responses such as abscission, senescence, and growth retardation (Beyer, 1976; Lashermes, 1992), and silver nitrate has been shown to have a positive effect on embryogenesis by blocking the inhibitory effect of endogenously produced ethylene in culture vessels (Niroula and Bimb, 2009).

In this study, we observed that the addition of PVP to the culture medium had no positive effect on androgenic responses of borage cultured anthers, but it was efficient in decreasing the browning reaction in anther-derived calli. According to these results, Zhong et al. (1995) alleviated anther and medium browning in anther culture of sunflower by the addition of 0.1% PVP in culture medium. PVP is insoluble with high molecular weight and has also shown promotive effects on anther culture of Datura (Tyagi et al., 1981).

The addition of different concentrations of activated charcoal and ascorbic acid to culture medium negatively affected the androgenic traits of borage cultured anthers or had no positive effects on these traits compared to control culture medium. These results are in accordance with previous studies in Eucalyptus somatic embryogenesis (Pinto et al., 2008). Those researchers added several antibrowning compounds (ascorbic acid, charcoal, DTE, DTT, PVP, PVPP, and silver nitrate) to the expression medium of Eucalyptus somatic embryogenesis but all decreased somatic embryogenesis potential. These negative effects were confirmed in other species for charcoal (Von Arnold, 1982; Pan and van Staden, 1998; Von Aderkas et al., 2002). However, application of charcoal in culture medium has resulted in the improvement of androgenic responses in a majority of anther culture studies (Anagnostakis, 1974; Horner et al., 1977; Johansson et al., 1982; Johansson, 1983; Calic et al., 2005, 2010).

Because of the essential role of growth regulators in most in vitro proliferation and differentiation processes such as plant androgenesis, we previously studied the role of different combinations of 2,4-D and BAP on callogenesis and embryo formation from borage anthers (Chardoli Eshaghi et al., 2015). In this work, we also evaluated the role of other growth regulators, picloram, 2ip, and 2,4-D, either alone or in combination with each other in the process of microspore embryogenesis from borage cultured anthers. We found that the androgenic response of borage anthers could be increased by adding picloram to culture medium. However, the highest increases in callogenesis percentage were achieved by using picloram alone or in combination with 2 mg L⁻¹ 2,4-D and 0.6 mg L⁻¹ 2ip, whereas the combined addition of 2 mg L⁻¹ picloram and 2 mg L⁻¹ 2,4-D produced the maximum number of embryos compared to the control and the best hormone treatment in the previous study (Chardoli Eshaghi et al., 2015). This result is consistent with the results obtained by Han et al. (1997, 2000), whereby picloram was found to be essential for induction and maintenance of calli in cultured anthers of Lilium species. Those researchers showed that picloram was a favorable auxin for inducing yellowish nodular calli in anther cultures of the cultivar ‘Connecticut King’ (Han et al., 1997) and it also was suitable for maintaining haploid calli in this cultivar (Han et al., 2000). One of the principal conclusions of this study is that silver nitrate (at a concentration of 5 mg L⁻¹) should be routinely used in the anther culture protocol of borage in order to delay callus browning and to increase the androgenic responses.

Our results showed that including 2 mg L⁻¹ picloram in combination with 2 mg L⁻¹ 2,4-D in anther culture medium can efficiently enhance the callus/embryo formation in cultured anthers of borage. In this study, we improved the borage anther culture protocol in order to increase embryo yield, but application of DH technology in breeding programs of borage needs to obtain borage haploid plants. Therefore, in future studies, it will be necessary to define more new conditions favoring the androgenic potential of borage in order to obtain borage DHs.
References


