Industrial Crops and Products 77 (2015) 51–58

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

In vitro propagation and biochemical changes in *Aloe pruinosa*

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ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form 13 August 2015
Accepted 15 August 2015
Available online 3 September 2015

Keywords:
Aloe pruinosa
Medicinal plant
In vitro propagation
Somatic embryogenesis
Polyphenolic enzyme activity
Phenolic content

ABSTRACT

Aloe pruinosa is a vulnerable traditional medicinal plant extensively used in South Africa. Efficient *in vitro* mass propagation and analysis of biochemical changes of the species was established to counter extinction threats, for commercial cultivation and to understand the effect of oxidative enzymes and phenolic metabolism on oxidative browning *in vitro* from shoot-tip and leaf explants using various concentrations and combinations of plant growth regulators (PGRs), sucrose and phloroglucinol (PG). The highest number of regenerated shoots per shoot-tip explant (15.4 ± 0.9) was obtained on solid Murashige and Skoog (MS_S) medium supplemented with a combination of 5 μM benzyladenine (BA) and 4 μM indole-3-acetic acid (IAA). Shoots developed significantly more roots (8.6 ± 0.7 per shoot) on ½-MS_S medium containing 2 μM indole-3-butyric acid (IBA) and 40 μM PG. The plantlets were successfully acclimatized (100%) *ex vitro* in a vermiculite-soil mixture (1:1 v/v) in the greenhouse. Somatic embryogenesis with all developmental stages of somatic embryos (SEs: 38–26) were obtained from friable embryogenic callus (FEC) of leaf explants in liquid MS (MS_L) containing 0.5 μM picloram (Pic), 2 μM BA or 1 μM thidiazuron (TDZ) and 10 μM PG. However, germination and conversion of plantlets from SEs were inhibited as a result of oxidative browning. Polyphenolic enzymes (polyphenol oxidase (PPO) and peroxidase (POD)) activity and total phenolics of different *in vitro* growth phases (adventitious shoots, regenerated plantlets and calli) with different PGR treatments were determined. The application of PGRs and PG resulted in the control of morphogenic properties, oxidative exudates and browning in *A. pruinosa*. The study highlights the role of natural PGR (*meta*-topolin riboside) and PG in reducing the impact of polyphenolic exudation and can be used to improve future *in vitro* programmes. In addition, micropropagation protocols facilitate commercial and rapid propagation of *A. pruinosa* for conservation and could satisfy pharmaceutical, cosmetic and food industry demands.

1. Introduction

The genus *Aloe* (Asphodelaceae) has a long history in the ornamental and horticultural trades worldwide. In addition, the genus is also used medicinally in the treatment of a wide variety of medical disorders and conditions. *Aloe* species in general are used in traditional medicine for therapeutic benefits, production of bioactive compounds and in commercial food products (Grace et al., 2008; Grace, 2011; Mukherjee et al., 2014). *Aloe* species are harvested in the wild in South Africa and exported globally (Smith et al., 2008). Currently, the wild populations of *Aloe* are threatened due to indiscriminate, illegal collections and habitat destruction (Malda et al., 1999). *Aloe* species are protected by the Convention on International Trade in Endangered Species (CITES) as a conservation measure (Smith et al., 2008). *Aloe pruinosa* is an important evergreen perennial succulent medicinal plant. It is commonly used in South African traditional medicine (SANBI, 2014) and contains the medicinally important compound isovitexin (apigenin 6-C-glucoside) (Grace et al., 2010). Wild populations of *A. pruinosa* have declined mainly due to urban expansion and harvesting for medicinal purposes (SANBI, 2014). Accordingly, it is reported as vulnerable in the Red Data List of South African plants (SANBI, 2014). An efficient *in vitro* regeneration system via micropropagation and somatic embryogenesis by embryonic cell suspension culture would alleviate harvesting pressure and can then be used for clonal propagation, germplasm conservation, large-scale com-

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; ECPM, embryogenic callus proliferation medium; FEC, friable embryogenic callus; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MSL, liquid MS medium; MS, Murashige and Skoog medium; mTR, 6-(-3-hydroxybenzylamino)-9-β-D-ribofuranosylpurine; NAA, α-naphthaleneacetic acid; POD, peroxidase; PPO, polyphenol oxidase; PG, phloroglucinol; PGRs, plant growth regulators; Pic, picloram; PPF, photosynthetic photon flux; SCV, settled cell volume; MS_S, solid MS medium; SEs, somatic embryos; TDZ, thidiazuron; Zea, zeatin.

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http://dx.doi.org/10.1016/j.indcrop.2015.08.036
0926-6690/

mercial production, genetic improvement, evaluation of bioactivity and to satisfy the pharmaceutical demands.

Micropropagation and embryonic cell suspension culture offers a viable alternative method for plant propagation and are suitable techniques for clonal propagation and industrial applications (Sanchez et al., 1988; Shoyama et al., 1997; Haque and Ghosh, 2013), production of artificial seeds (Maruyama et al., 2003), cryopreservation (Kong and von Aderkas, 2011), genetic manipulations (Parimalan et al., 2010), the production of bioactive compounds (Jeong et al., 2005) and analysis of bioactivity (Baskaran et al., 2014). The technique is also a promising tool for research into the basic physiology and biochemistry of plant cells (Georgiev et al., 2011). However, the *in vitro* establishment of *Aloe* is challenged by severe phenolic exudation and oxidative browning. Phenolic exudation, oxidation and quantity of total phenols are deleterious during *in vitro* culture and cause browning and necrosis of the plant tissues (Ozyigit, 2008; Reis et al., 2008). Antioxidant enzymes such as peroxidases (POD) and polyphenol oxidase (PPO) are essential for converting H_2O_2 to H_2O in the plant cells which neutralizes the toxic effects of H_2O_2. However, PPO is a Cu^{2+}-containing enzyme localized on the thylakoids of chloroplasts and is involved in catalyzing the aerobic oxidation of different phenolic compounds to quinines which are autooxidised to dark brown pigments (Jain et al., 2014). The enzymatic actions of monophenol oxidase and PPO promotes oxidation leading to the production of quinines, which inhibit plant cellular growth and destroy enzymatic activities (Loomis and Battaile, 1966). Chromone, anthraquinone or anthrone derivatives have been identified in *Aloe* species (Mukherjee et al., 2014). These may impede growth and development *in vitro*. To the contrary, in several cases, phenolic compounds seem to be essential for the control of some morphogenic processes (stimulation of organogenesis and somatic embryogenesis) occurring *in vitro*, indicating that their role is far from being resolved (Teixeira da Silva et al., 2013). Phloroglucinol (PG: benzenetriol, occurs in nature in the A-ring of flavonoid compounds and many other plant phenolic compounds) is used as a plant growth regulator (PGR) in *in vitro* culture for plant growth processes and development. In *Aloe in vitro* culture programmes PG may be beneficial. Accordingly, *in vitro* propagation technique could play important roles in conservation, genetic improvement and pharmaceutical, cosmetic and food industries. The present investigation aimed to develop a simple and effective, rapid *in vitro* plant regeneration system for mass propagation through micropropagation and somatic embryogenesis of *A. pruinosa* from shoot-tip and leaf explants. In addition, the metabolism of phenolic compounds through the analysis of enzyme (PPO and POD) activity and phenolic contents (total phenolic and flavonoids) accumulation in *in vitro* regenerated tissues in comparison with different PGR treatments and plant growth phases were also determined.

2. Material and methods

2.1. Plant material, micropropagation and somatic embryogenesis

Mature seeds of *Aloe pruinosa* were collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Seeds were washed thoroughly with 0.1% (v/v) of Tween® 20 for 5 min, and decontaminated with 70% ethanol for 30 s followed by 2% aqueous $HgCl_2$ for 10 min. The seeds were then rinsed five times with sterile distilled water for *in vitro* germination. Shoot-tip (approximately, 10 mm) and leaf (approximately, 10 × 3.0 mm) explants of *A. pruinosa* were excised from twenty-day-old *in vitro* germinated seedlings grown on solid Murashige and Skoog (1962) (MS_S) medium containing 8 g L^{-1} agar. Shoot-tip explants were inoculated onto MS_S medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) and phloroglucinol (PG) for shoot proliferation. Microshoots (□2.0 cm in length) were cultured in MS_S or ½-MS_S medium alone and ½-MS_S medium in combination with IBA (indole-3-butyric acid) and PG for root induction. The PGR and PG treatments are indicated in Tables 1 and 2.

Leaf explants were inoculated onto MS_S medium containing 8 g L^{-1} agar, 30–50 g L^{-1} sucrose and 20 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram (Pic) alone or in combinations with 20 μM Pic, 5 μM benzyladenine (BA) or *meta*-topolin riboside (*m*TR) or zeatin (Zea) or thidiazuron (TDZ) and PG for induction of friable embryogenic callus (FEC) for 6 weeks, as specified in Table 3. Callus containing FEC from each treatment were transferred to embryogenic callus proliferation medium (ECPM) containing MS_S medium with respective sucrose concentration and reduced 2,4-D (5 μM) or Pic (5 μM) concentrations alone or in combinations of Pic (2–5 μM) and 5–15 μM BA or *m*TR or Zea or TDZ and with or without 25 μM PG for 4 weeks for shoot regeneration and FEC proliferation (Table 3). Three week-old fresh FEC (approximately 500 mg fresh weight) from each treatment of ECPM was placed in 20 mL of liquid MS (MS_L) medium containing 30 g L^{-1} sucrose or MS_L medium plus 30 g L^{-1} sucrose, 0.5 μM 2,4-D or Pic and 1-2 μM BA or 1 μM Zea or *m*TR or TDZ and with or without 10 μM PG in a 100 mL Erlenmeyer flask. The PGRs treatments are indicated in Table 3. Suspension culture of *A. pruinosa* was performed as previously described (Baskaran and Van Staden, 2012) and modified with settled cell volume (SCV: the volume of cells in 250 mL flasks was maintained as 1000 μL). Developmental stage somatic embryos (SEs: globular, club-shaped, torpedo-shaped and cotyledonary) were recorded after 4 weeks from suspension culture initiation. The SEs germinated on the same medium. All the stages of embryo were inoculated onto ½-MS_L or ½-MS_S medium alone or in combination with 10–40 μM PG for germination and plantlet formation. All embryonic stages were photographed using a Leica M Stereo Microscope (JVC-Digital Camera: KY-F 1030U type; 0.5X, Wayne, NJ, USA). In all experiments, medium lacking plant growth regulators served as controls. The chemicals used were of analytical grade (Biolab, South Africa; Oxoid, England and Sigma, USA). All media were adjusted to pH 5.8 with 0.1 N NaOH before gelling with 8 g L^{-1} agar and autoclaved at 121 °C for 20 min. The cultures were maintained at a temperature of 25 ± 2 °C and light intensity of 40 μmol m^{-2} s^{-1} provided by cool white fluorescent light (OSRAM L 58 W/740, South Africa) with a 16 h photoperiod.

2.2. *Acclimatization* ex vitro

All rooted shoots were removed gently from the rooting medium after 6 weeks, and transferred to terracotta pots (95 × 120 mm, 500 mL) containing a 1:1 (v/v) vermiculite:sand mixture and irrigated with tap water every third day. These plantlets were maintained in the greenhouse (25 ± 2 °C under natural photoperiod conditions and a midday PPF of 950 ± 50 μmol m^{-2} s^{-1}) for acclimatization *ex vitro*.

2.3. Polyphenol oxidase and peroxidase assays

One gram of fresh leaves and calli were collected from different treatments of shooting (10 weeks-old), rooting (6 weeks-old) and callus (10 weeks-old) induction media, as specified in Fig. 3, and polyphenol oxidase (PPO) and peroxidase (POD) enzyme activities were assayed as described (Jain et al., 2014). The enzyme activities were recorded as units of activity (UA) which corresponds to the change in absorbance of 0.001 per second per gram fresh weight. PPO and POD activities corresponds to the level of enzyme activity