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Rapid propagation of *Mondia whitei* by embryonic cell suspension culture *in vitro*

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ABSTRACT

We have developed an efficient *in vitro* mass propagation system for *Mondia whitei*, an endangered South African medicinal plant of significant traditional importance, using somatic embryogenesis in cell suspension culture. Leaf explants generated friable embryogenic calli (FEC) when cultured on embryogenic callus induction medium (ECIM), which contained Murashige and Skoog (MS) medium, 8 gL^{-1} agar with 30–50 gL^{-1} sucrose and different plant growth regulators [PGRs: 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram]. Different stages (globular/heart/torpedo/cotyledonary) of somatic embryos (SEs) were produced from FEC using liquid MS medium supplemented with different concentrations and combinations of PGRs. Formation of SEs (torpedo and cotyledonary stages) were significantly higher in liquid MS medium containing 0.5 μM naphthaleneacetic acid (NAA) and 1 μM *meta*-topolin riboside (*m*TR). The highest embryo germination rates achieved in liquid (half-strength MS + 0.5 μM NAA) and solid (MS + 40 gL^{-1} sucrose + 2 μM NAA) media were 98.3% and 97.6%, respectively. The synthetic seeds were prepared with sodium alginate (3% *w*/*v*) and calcium chloride (100 mM) from SEs (torpedo and cotyledonary stages) and stored in darkness at 25 ± 2 °C and 4 °C for 10–60 days. Germination frequency was 68.6% at 25 ± 2 °C after 60 days of storage. All the plantlets from SEs and synthetic seeds were successfully acclimatized (100%) with vermiculite in the greenhouse. These results provide high-frequency, rapid production and germination of SEs and synthetic seeds, and provides further strategies that can be used for overcoming extinction threats, ensuring germplasm conservation, production of bioactive compounds, evaluation of bioactivity and genetic transformation studies.

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1. Introduction

Mondia whitei (Hook.f.) Skeels (Apocynaceae) is a perennial climber, frequently used in South African traditional medicine for treating various diseases including jaundice, bilharzia, diarrhea, gonorrhea, stroke, anemia, asthma, mastitis and allergies (Gerstner, 1941; Kokwaro, 1976; Gelfand et al., 1985; Noumi et al., 1998; SANBI, 2013; Baskaran et al., 2015). Its popularity in traditional medicine has been placed on the biosynthesis of pharmaceutically desirable compounds such as epinephrine, norepinephrine, dopamine, serotonin, ɣ-aminobutyric acid and coumarinolignans (Bunel et al., 2014; Baskaran et al., 2015). Due to its medicinal importance, *M. whitei* is over-exploited and has been reported as an endangered plant (SANBI, 2013). A report of somatic embryogenesis has been published on *in vitro* plant regeneration of *M. whitei* via a solid culture system for conservation purposes (Baskaran et al., 2015). However, the frequency of somatic embryogenesis, number of somatic embryos produced and viability of synthetic seeds was low; therefore, we have attempted to establish another system of somatic embryogenesis using cell suspension culture with a rapid high-frequency and enhanced numbers of viable somatic embryos for synthetic seed production and germination.

Cell suspension culture techniques offer valuable advantages over traditional micropropagation for rapid mass clonal propagation (Shoyama et al., 1997), conservation (Cheruvathur et al., 2013) and research on genetic mechanisms of proembryogenesis (Chung et al., 2016). Synthetic seed technology has been applied for conservation of germplasm and transportation of axenic plant materials of rare and elite genotypes, and transgenic plants (Cheruvathur et al., 2013). Accordingly, the aims of the current studies were (1) to access the influence of different concentrations and combinations of plant growth regulators and sucrose, and MS medium type and strength on higher frequency of somatic embryo production and germination by cell suspension cultures of *M. whitei* and (2) to evaluate the viability of germination of synthetic seeds by analyzing variation in temperature, and duration of storage.

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2. Materials and methods

2.1. Production of embryogenic callus and cell suspension culture

Expanding young leaves of *M. whitei* were collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The decontamination of leaf explants were done as described previously (Baskaran et al., 2015). Leaf explants were inoculated onto embryogenic callus induction medium (ECIM) containing MS (Murashige and Skoog, 1962) solid (8 gL^{-1} agar) medium with 30–50 gL^{-1} sucrose and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram alone for 6 weeks to produce friable embryogenic callus (FEC), as specified in Tables 1 and 2. The FEC from each ECIM were transferred to embryogenic callus proliferation medium (ECPM) with reduced 2,4-D (5 μM) and picloram (5 μM) concentrations alone or in combinations with benzyladenine (BA) and thidiazuron (TDZ) in the respective ECIM (Tables 1 and 2) for 3 weeks. Three-week-old fresh white FEC (approximately 500 mg fresh weight) from each ECPM was placed in 20 mL liquid MS (MS_L) medium containing 30 gL^{-1} sucrose for 3 weeks to optimize the concentration and combination of sucrose and plant growth regulators (PGRs) in ECIM and ECPM for somatic embryo (SE) development. Optimal SEs produced FEC from ECPM (MS medium plus 35 gL^{-1} sucrose, 5 μM 2,4-D and 0.5 μM TDZ; MS medium plus 35 gL^{-1} sucrose, 5 μM picloram and 0.5 μM BA) was placed in embryo development medium (EDM) containing MS_L medium (20 mL) plus 30 gL^{-1} sucrose, 0.5 μM 2,4-D or picloram or naphthaleneacetic acid (NAA) and 1–2 μM BA or TDZ or *meta*-topolin riboside (*m*TR) or 2-isopentenyl adenine (2iP) or kinetin (Kin) in a 100 mL Erlenmeyer flask for 3 weeks to enhance formation of somatic embryos (SEs). The liquid cultures were maintained on an orbital shaker at 180 rpm. After one week in this liquid culture, the supernatant (10 mL) was removed from flasks and then settled cell volume (SCV: 1000 μL) was replaced with an equal volume of fresh medium. The liquid culture cell aggregates with embryos were filtered through sieves (200 μm) after 2 weeks of culture. The filtrates were made up to 30 mL of fresh medium in 250 mL flasks. The SEs (globular, heart, torpedo and cotyledonary) were recorded after 3 weeks from liquid culture initiation. The well-developed plantlets from germinated SEs in liquid cultures were established directly in vermiculite at 25 °C with a 16-h photoperiod. All embryonic stages were photographed using a Leica M Stereo Microscope (JVC-Digital Camera: KY-F 1030U type; 0.5×, Wayne, NJ, USA). In all experiments, medium lacking plant growth regulators served as controls. The chemicals used were analytical grade (Biolab, South Africa; Oxoid, England and Sigma, USA). All media were adjusted to pH 5.8 with 0.1 N NaOH and/or 0.1 N HCl before gelling with 8 gL^{-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. Germination of somatic embryos

Three-week-old SEs (heart, torpedo and cotyledonary stages) were collected from optimal embryo development medium (EDM) (Table 3) and then cultured on different solid or liquid germination media [solid MS medium plus 20–50 gL^{-1} sucrose; solid MS medium plus 40 gL^{-1} sucrose and 2–4 μM NAA; liquid half-strength MS medium plus 15 gL^{-1} sucrose; liquid half-strength MS medium plus 15 gL^{-1} sucrose and 0.5 μM α-naphthaleneacetic acid (NAA)] to improve the frequency of germination of SEs. The solid medium cultures were maintained in darkness for 2 days and then incubated under 40 μmol m^{-2} s^{-1} light intensity and a 16-h photoperiod. The liquid cultures (30 mL in 250 mL flask) were agitated on an orbital shaker at 80 rpm under 40 μmol m^{-2} s^{-1} light intensity and a 6-h photoperiod. All media were adjusted to pH 5.8 with 0.1 N NaOH and/or 0.1 N HCl before gelling with 8 gL^{-1} agar and autoclaved at 121 °C for 20 min. Embryo germination percentage was calculated after 4 weeks (number of germinated SE/total number of SE × 100). The plantlets (approximately, 50–70 mm) were successfully acclimatized with vermiculite in a greenhouse (25 ± 2 °C under natural photoperiod conditions and a midday photosynthetic photon flux density (PPFD) of 950 ± 50 μmol m^{-2} s^{-1}). The plant survival rate experiments included three replicates of 50 plants in each.

2.3. Synthetic seed production, storage and germination

The synthetic seeds of *M. whitei* were prepared from an optimized protocol as described by Baskaran et al. (2015). The synthetic seeds

Table 1
Effects of sucrose concentration and growth regulators on somatic embryo (SE) development from embryogenic callus on cell suspension culture of *M. whitei*.

Sucrose (g L^{-1}) and PGRs (μM) in solid MS medium		Liquid MS medium containing 30 g L^{-1} sucrose			
ECIM for 6 weeks	ECPM for 3 weeks	Number of SEs/SCV (mean ± standard error) Developmental stage			
		Globular	Heart	Torpedo	Cotyledonary
Controls	Controls	0	0	0	0
30 + 5 2,4-D	30 + 5 2,4-D	1.8 e	0	0	0
30 + 15 2,4-D	30 + 5 2,4-D	4.2 de	2.6 de	0.8 fg	0
30 + 25 2,4-D	30 + 5 2,4-D	3.8 de	1.4 fg	0	0
35 + 15 2,4-D	35 + 5 2,4-D	5.2 d	3.4 cd	2.2 de	0
40 + 15 2,4-D	40 + 5 2,4-D	6.0 cd	4.6 bc	2.8 bc	0
50 + 15 2,4-D	50 + 5 2,4-D	6.4 cd	4.8 bc	4.2 a	0
30 + 5 Picloram	30 + 5 Picloram	5.0 d	0	0	0
30 + 15 Picloram	30 + 5 Picloram	6.8 bc	4.2 bc	2.0 de	0
30 + 25 Picloram	30 + 5 Picloram	3.8 de	2.2 ef	1.2 ef	0
35 + 15 Picloram	35 + 5 Picloram	5.4 d	4.8 bc	3.0 ab	0
40 + 15 Picloram	40 + 5 Picloram	5.8 cd	5.2 bc	4.0 a	0
50 + 15 Picloram	50 + 5 Picloram	5.4 d	5.0 bc	2.4 cd	0
30 + 15 2,4-D	30 + 5 2,4-D + 0.5 TDZ	4.6 de	2.0 ef	1.2 ef	0
35 + 15 2,4-D	35 + 5 2,4-D + 0.5 TDZ	10.8 a	7.2 a	3.8 ab	2.6 ab
40 + 15 2,4-D	40 + 5 2,4-D + 0.5 TDZ	8.6 bc	3.2 cd	2.4 cd	1.0 c
30 + 15 Picloram	30 + 5 Picloram + 0.5 BA	7.0 bc	4.2 bc	3.2 ab	0.6 cd
35 + 15 Picloram	35 + 5 Picloram + 0.5 BA	12.0 a	7.6 a	4.8 a	3.8 a
40 + 15 Picloram	40 + 5 Picloram + 0.5 BA	9.2 b	6.2 b	2.6 bc	1.8 b

Controls = MS + 30 or 35 or 40 and 50 g L^{-1} sucrose. PGRs = plant growth regulators. ECIM = embryogenic callus induction medium. ECPM = embryogenic callus proliferation medium. SEs = somatic embryos; SCV = settled cell volume. Mean values derived from 5 replicates (each 500 mg FEC) with 1000 μL settled cell volume of embryonic suspension cells per replicate. Means followed by the same letters in each column are not significantly different at the 5% level as determined by Duncan's multiple range test.

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