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ORIGINAL PAPER

# Embryogenesis and synthetic seed production in *Mondia whitei*

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**Abstract** An effective plant regeneration system via somatic embryogenesis and synthetic seeds was developed for *Mondia whitei*, an endangered medicinal plant. Friable embryogenic callus was induced by culturing leaf explants on solid Murashige and Skoog (MS) medium containing various concentrations and combinations of sucrose and plant growth regulators. The highest frequency of somatic embryogenesis (100 %) and production of all developmental stages of somatic embryos were obtained on MS medium with 40 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, 20 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 µM thidiazuron. This was followed by establishment in MS medium with 20 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, 0.5 µM *meta*-topolin riboside (*m*TR) and 0.25 µM indole-3-acetic acid (IAA). All the embryos germinated and produced healthy plantlets on the same medium. Somatic embryos at the heart, torpedo and cotyledonary-stages were collected from media (EDM) containing MS medium plus 20 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, 0.5 µM *m*TR and 0.25 µM IAA. The embryos were encapsulated with liquid MS medium plus different concentrations of sodium alginate (SA) and calcium chloride ($CaCl_2{\cdot}2H_2O$) with a 10 min exposure. A combination of 3 % SA and 100 mM $CaCl_2{\cdot}2H_2O$ provided higher survival (95.7 %) and germination (73 %) frequencies of synthetic seeds. Germination frequency of synthetic seeds was 51.6 % after 50 days of storage at 4 °C. Somatic embryos and synthetic seed-developed plantlets were successfully acclimatized in the greenhouse with 90 % survival ex vitro. Application of the protocol provides a relatively simple and rapid system for conservation of natural populations for germplasm conservation. Analysis of bioactive compounds and genetic transformation studies can also be performed.

**Keywords** Cell suspension · Medicinal plant · *Mondia whitei* · Somatic embryogenesis · Synthetic seeds

**Abbreviations**

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| BA | 6-Benzyladenine |
| EDM | Embryo development medium |
| FEC | Friable embryogenic callus |
| IAA | Indole-3-acetic acid |
| Kin | Kinetin |
| MS | Murashige and Skoog medium |
| *m*TR | 6-(-3-Hydroxybenzylamino)-9-*β*-D-ribofuranosylpurine |
| NAA | α-Naphthaleneacetic acid |
| PGRs | Plant growth regulators |
| PPF | Photosynthetic photon flux |
| SA | Sodium alginate |
| SEs | Somatic embryos |
| TDZ | Thidiazuron |

## Introduction

*Mondia whitei* (Hook.f.) Skeels (Apocynaceae) is a woody climber with a tuberous root stock. It is a truly versatile South African medicinal plant and is reported as endangered in the Red Data List of South African plants (SANBI 2013). It is sold in Traditional Medicinal markets in South Africa

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(McCartan and Crouch 1998; SANBI 2013). It produces various bioactive compounds such as epinephrine, norepinephrine, dopamine, serotonin, γ-aminobutyric acid and coumarinolignans (Bunel et al. 2014). The roots are used as spices, aphrodisiacs and in the treatment of urinary tract infections, jaundice, headache, easing of flatulence, abdominal pains, constipation, bilharzia, diarrhoea and gonorrhoea (Gerstner 1941; Kokwaro 1976; Gelfand et al. 1985; Noumi et al. 1998) and a plethora of other ailments including hypertension, stroke, anemia, asthma, hangover, mastitis and allergies, to improve sleep, ease birth pains, mouth freshener, antacid, indigestion, and to stimulate appetite (Oketch-Rabah 2012). It also contains androgenic properties (Watcho et al. 2004). In addition, the plant is used to treat fits in children and for stress and tension in adults. The leaves serve as animal fodder, human vegetables and food supplement (Oketch-Rabah 2012). Accordingly, wild populations of *M. whitei* are over-exploited and are becoming threatened with extinction. In this context, mass propagation of *M. whitei* through in vitro regeneration is essential for conservation and commercial production. Although a micropropagation protocol has been developed for *M. whitei* (McCartan and Crouch 1998), high frequency rapid in vitro plant regeneration for mass propagation and conservation remains a major challenge. An efficient in vitro plant regeneration through somatic embryogenesis and production of synthetic seeds could play an important role in *M. whitei* including fulfilling the rapid mass propagation, conservation and traditional medicine demands.

Plant somatic cells are able to produce somatic embryos (bipolar structures) through somatic embryogenesis (Bhojwani and Razdan 1996). The somatic embryo develops into characteristic morphological stages (globular, heart, torpedo and cotyledonary) leading to plantlet formation. Various novel and innovative ideas like optimization of plant growth regulators and adjustment of osmotic conditions have been used to improve somatic embryogenesis frequency in plants (Karami et al. 2006; Deo et al. 2010; Baskaran and Van Staden 2014). Somatic embryogenesis holds great promise for *en masse* propagation, bioactive compound production, the improvement of the species by genetic engineering, germplasm cryopreservation and production of artificial or synthetic seeds (Fowler 1983; Mathews et al. 1992; Wilde et al. 1992; Blakesley et al. 1995; Manjkhola et al. 2005). Artificial or synthetic seeds are considered as analogous to natural seeds. Synthetic seeds consist of somatic embryos surrounded by an artificial layer creating a capsule (Murashige 1977). The first successful report of synthetic seed production was reported for carrot (Kitto and Janick 1982). The technology has been extended to a wide variety of agricultural, ornamental and medicinal plants (Redenbaugh 1993; Ghosh and Sen 1994; Janeiro et al. 1997; Pattnaik and Chand 2000; Utomo et al. 2008; Cheruvathur et al. 2013). Synthetic seed technology provides an opportunity for easy handling, storage, shipping, ex situ conservation of the germplasm of elite and endangered plant species, exchange of axenic plant materials between laboratories and pharmaceutical industries (Rai et al. 2009; Cheruvathur et al. 2013) and propagation of rare hybrids, elite genotypes, and genetically engineered plants for which the seeds are either very expensive or not available. In addition, the technology is an alternative system for in situ conservation and is also useful to avoid environmental disasters. Therefore, the aim of the current study was to develop a simple and effective, rapid in vitro plant regeneration system through somatic embryogenesis, synthetic seed production and synthetic seed storage for the conservation and large-scale commercial production of *M. whitei* from leaf explants.

## Materials and methods

### Plant material and somatic embryogenesis

Expanding young leaves of *Mondia whitei* were collected from the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Leaves were washed under running tap water for 15 min to remove loose dirt, washed with Tween® 20 for 1 min and then decontaminated with 0.2 % aqueous $HgCl_2$ for 7 min. Leaves were then rinsed five times with sterile distilled water. Leaf explants (approximately, 10 × 5.0 mm) of *M. whitei* were excised and cultured on MS (Murashige and Skoog 1962) solid (8 g $l^{-1}$ agar) medium with 30–50 g $l^{-1}$ sucrose and different concentrations of plant growth regulators [PGRs: 2,4-dichlorophenoxyacetic acid (2,4-D), picloram, benzyladenine (BA), *meta*-topolin riboside (*m*TR), kinetin (Kin) and thidiazuron (TDZ)] alone or in combination for induction of somatic embryos (SEs) for 8 weeks. This was followed by establishment of individual treatments in MS medium with 20 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar for SE maturation and plantlet formation. The PGR treatments are indicated in Tables 1 and 2. Friable embryogenic callus (FEC) with somatic embryoids produced from an optimized somatic embryogenesis medium (MS medium plus 40 g $l^{-1}$ sucrose, 20 μM 2,4-D and 1 μM TDZ) were transferred to MS medium containing 20 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar and different concentrations and combinations of PGRs [BA, *m*TR, TDZ, Kin, indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA)] to enhance somatic embryogenesis and plantlet development. The PGR treatments are as indicated in Table 3. The SEs (globular, heart, torpedo and cotyledonary) and degree of plantlet development were recorded after 12 weeks from culture initiation. All embryo stages were photographed under a Leica M Stereo Microscope (JVC-

**Table 1** Effect of sucrose concentration and growth regulators on somatic embryo (SE) and plantlet development from embryogenic callus in *M. whitei* leaf explants

| Sucrose (g $l^{-1}$) + PGR (μM) in MS medium | Frequency (%) of embryogenesis | No. of SEs developmental stage/explant (mean ± SEM) | | | | |
|---|---|---|---|---|---|---|
| | | Globular | Heart | Torpedo | Cotyledonary | Plantlets |
| Controls | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 + 10 2,4-D | 36.0 ± 1.43 l | 7.2 ± 1.06 de | 4.6 ± 1.36 ef | 1.4 ± 0.74 fg | 0.4 ± 0.24 g | 0.4 ± 0.24 i |
| 30 + 15 2,4-D | 44.0 ± 0.51 k | 11.4 ± 2.24 cd | 6.0 ± 1.28 bc | 2.4 ± 0.62 ef | 0.8 ± 0.37 g | 0.6 ± 0.40 hi |
| 30 + 20 2,4-D | 56.0 ± 0.51 i | 12.8 ± 2.62 cd | 7.6 ± 1.50 b | 4.0 ± 0.70 de | 1.4 ± 0.53 fg | 0.8 ± 0.42 hi |
| 30 + 25 2,4-D | 70.0 ± 0.70 g | 11.6 ± 1.86 cd | 6.2 ± 1.93 bc | 2.6 ± 0.92 ef | 1.2 ± 0.58 g | 1.0 ± 0.44 hi |
| 35 + 20 2,4-D | 64.0 ± 0.67 h | 18.0 ± 2.58 bc | 6.8 ± 0.86 bc | 6.0 ± 0.70 bc | 1.6 ± 0.51 ef | 1.6 ± 0.67 gh |
| 35 + 25 2,4-D | 52.0 ± 1.02 j | 15.0 ± 1.16 bc | 5.4 ± 1.63 cd | 3.8 ± 1.65 de | 0.8 ± 0.37 g | 0.6 ± 0.24 hi |
| 40 + 20 2,4-D | 80.0 ± 0.31 de | 21.0 ± 3.09 ab | 7.8 ± 0.86 b | 8.6 ± 0.51 ab | 2.0 ± 0.70 ef | 9.0 ± 1.18 b |
| 40 + 25 2,4-D | 54.0 ± 1.03 i | 16.6 ± 2.61 bc | 5.6 ± 0.74 cd | 6.0 ± 0.70 bc | 1.2 ± 0.49 g | 5.4 ± 1.03 de |
| 50 + 20 2,4-D | 78.0 ± 0.58 e | 24.0 ± 2.55 a | 9.4 ± 1.07 b | 9.2 ± 1.20 a | 3.6 ± 0.92 de | 12.0 ± 0.94 a |
| 50 + 25 2,4-D | 88.0 ± 0.37 c | 25.4 ± 5.02 a | 8.6 ± 2.20 b | 5.2 ± 0.73 cd | 2.0 ± 0.72 ef | 7.6 ± 0.98 bc |
| 30 + 10 picloram | 78.0 ± 0.49 e | 2.2 ± 0.58 g | 1.4 ± 0.51 i | 1.2 ± 0.58 gh | 1.0 ± 0.54 g | 0.4 ± 0.24 i |
| 30 + 15 picloram | 82.0 ± 0.86 d | 3.0 ± 1.44 fg | 1.8 ± 0.91 hi | 1.0 ± 0.44 hi | 1.4 ± 0.51 fg | 1.2 ± 0.58 gh |
| 30 + 20 picloram | 84.0 ± 0.73 d | 3.8 ± 0.86 ef | 2.4 ± 0.92 gh | 0.8 ± 0.37 i | 2.2 ± 0.70 ef | 1.4 ± 0.37 gh |
| 30 + 25 picloram | 74.0 ± 1.12 f | 6.8 ± 0.86 ef | 4.2 ± 0.58 fg | 1.6 ± 0.40 fg | 1.2 ± 0.58 g | 1.8 ± 0.37 fg |
| 35 + 20 picloram | 92.0 ± 0.37 b | 7.0 ± 1.64 ef | 4.8 ± 1.06 de | 4.2 ± 0.86 cd | 3.8 ± 0.86 de | 2.8 ± 0.97 fg |
| 35 + 25 picloram | 94.0 ± 0.24 b | 10.0 ± 1.94 cd | 6.0 ± 1.04 bc | 4.8 ± 0.82 cd | 4.4 ± 1.03 cd | 3.4 ± 0.81 ef |
| 40 + 20 picloram | 98.0 ± 0.20 a | 13.4 ± 1.91 bc | 13.6 ± 1.20 a | 7.0 ± 0.83 ab | 6.8 ± 0.73 b | 4.0 ± 0.70 de |
| 40 + 25 picloram | 86.0 ± 0.51 c | 9.8 ± 1.06 cd | 9.0 ± 1.22 b | 6.2 ± 0.86 bc | 5.0 ± 0.70 bc | 3.0 ± 0.71 fg |
| 50 + 20 picloram | 78.0 ± 0.58 e | 7.2 ± 1.15 de | 10.0 ± 1.78 b | 9.2 ± 1.06 a | 10.0 ± 1.30 a | 6.0 ± 1.00 cd |
| 50 + 25 picloram | 72.0 ± 0.58 f | 6.6 ± 0.92 ef | 8.4 ± 1.24 b | 6.4 ± 1.32 bc | 6.0 ± 0.74 bc | 3.2 ± 0.85 ef |

Controls = MS + 30 or 35 or 40 and 50 g $l^{-1}$ sucrose. Results are mean ± SEM of % of embryo from 25 replicate per treatment. Mean values followed by same letters in each column are not significantly different according to the Duncan's multiple range test at 5 % level

*PGR* plant growth regulator, *SEs* somatic embryos

Digital Camera: KY-F 1030U type; 0.5×, Wayne, NJ, USA). In all experiments, the medium lacking PGRs served as control. 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 irradiance of 40 μmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent tubes (OSRAM L 58 W/740, South Africa) under a 16 h photoperiod. All the plantlets (approximately, 70–90 mm) from somatic embryos were transferred to a plastic box containing a 1:1 (v/v) vermiculite and soil mixture and placed 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 and established in the greenhouse.

## Synthetic seed production, storage and germination

Somatic embryos (heart, torpedo and cotyledonary) were collected equally from an optimized embryo development medium (EDM: MS medium plus 40 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, 20 μM 2,4-D and 1 μM TDZ followed by establishment in MS medium plus 20 g $l^{-1}$ sucrose, 8 g $l^{-1}$ agar, 0.5 μM *m*TR and 0.25 IAA) and were suspended in a capsule matrix of liquid MS medium containing sodium alginate (SA: 1–4 % w/v) dropped into a complexing agent solution of calcium chloride ($CaCl_2 \cdot 2H_2O$: 75–125 mM). Each drop-containing embryo was maintained and shaken gently by hand in $CaCl_2 \cdot 2H_2O$ for 10 min for proper bead formation. The beads were washed in sterile distilled water 3 times to remove traces of $CaCl_2$ and then placed on sterilized filter paper to remove excess water. After optimization of the capsule matrix (3 % SA) and complexing agent (100 mM $CaCl_2$), experiments were conducted further for synthetic seeds production from SEs (heart, torpedo and cotyledonary stages) derived from different EDM to study their ability to germinate. The EDM is indicated in Table 4. The synthetic seeds stored at 4 °C were evaluated for viability over 10–50 days through survival and germination tests for germplasm conservation. Non-stored synthetic seeds were used as a control. Synthetic seeds were incubated in MS medium containing 30 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar at 25 ± 2 °C with a 16-h photoperiod for

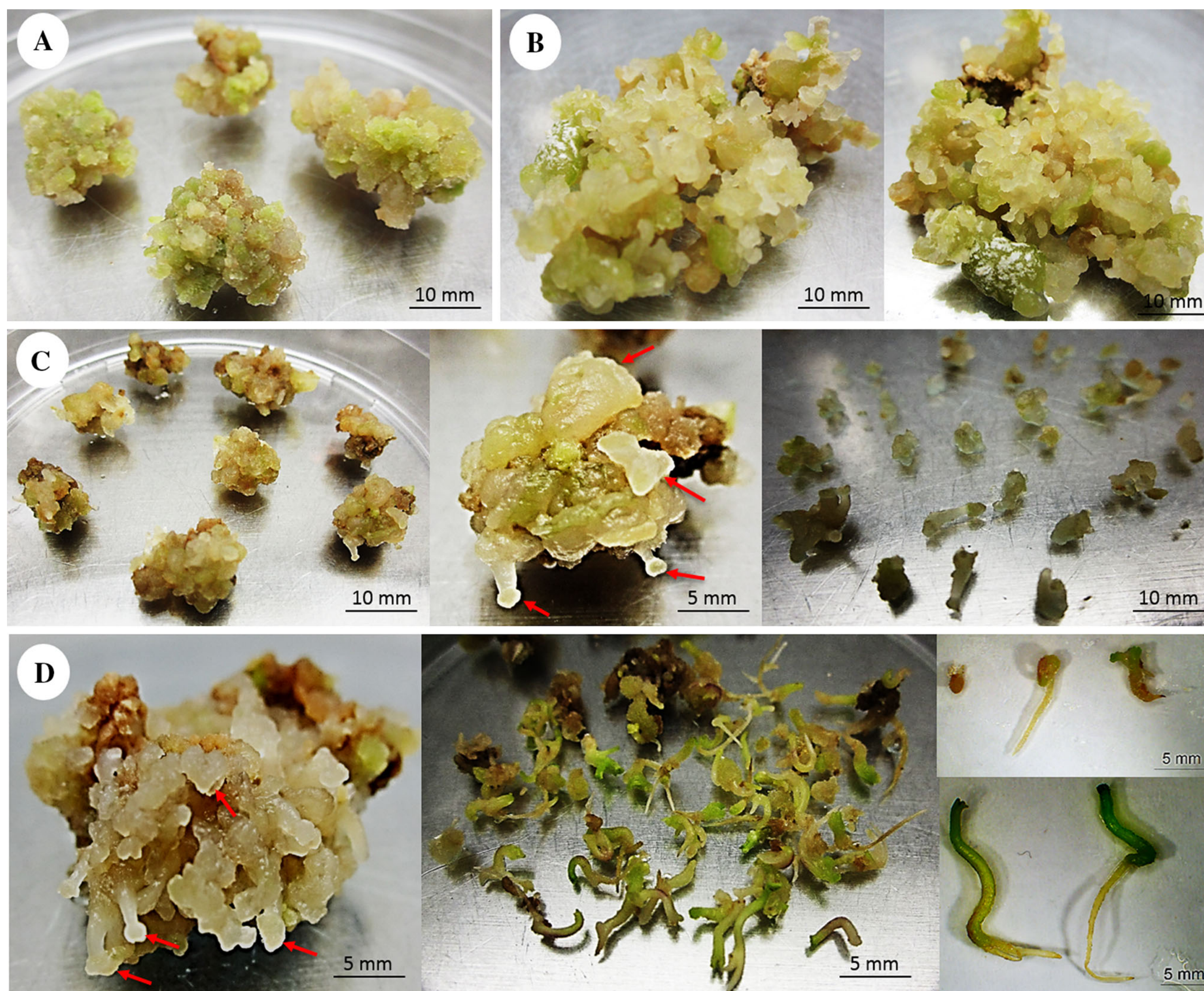


**Fig. 1** Somatic embryogenesis from leaf explants of *M. whitei*: **a** Induction of whitish green FEC on MS medium containing 40 g $l^{-1}$ sucrose, 20 µM 2,4-D and 1 µM TDZ. **b** Formation of embryoids in MS medium containing 40 g $l^{-1}$ sucrose, 20 µM 2,4-D and 1 µM TDZ. **c** Induction of globular, heart, torpedo and cotyledonary stage embryos on MS medium containing 40 g $l^{-1}$ sucrose, 20 µM 2,4-D and 1 µM TDZ. **d** Photograph of developmental stages of SEs and plantlets (×5). In **c** and **d**, *arrows* indicate the heart and torpedo stage embryos

germination. The survival and germination of synthetic seeds were recorded after 6 weeks of culture. The experiments were conducted in aseptic conditions. The plantlets (approximately, 70–90 mm) were successfully acclimatized in 1:1 (v/v) vermiculite:soil mixture and established in the greenhouse.

### Statistical analysis

All experiments were repeated at least three times with 25 replicates for somatic embryogenesis and 50 replicates for synthetic seed germination per treatment. Data were statistically analyzed using analysis of variance (ANOVA), and are presented as mean ± standard error of three independent experiments. Treatment means were separated using Duncan's multiple range test at the 5 % probability level and analyzed using SPSS Windows version 21 (SPSS Inc., Chicago, IL, USA).

## Results and discussion

### Development of somatic embryos and plantlets

Somatic embryogenesis and plantlet development was achieved from leaf explants on MS medium containing different concentrations of sucrose with various PGRs alone or in combination. The leaf explants enlarged and

**Table 2** Effect of sucrose concentration in combination with growth regulators on somatic embryo (SE) and plantlet development from embryogenic callus in *M. whitei* leaf explants

| Sucrose (g $l^{-1}$) + PGR (μM) in MS medium | Frequency (%) of embryogenesis | No. of SEs developmental stage/explant (mean ± SEM) | | | | |
|---|---|---|---|---|---|---|
| | | Globular | Heart | Torpedo | Cotyledonary | Plantlets |
| Controls | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 + 20 2,4-D + 1 Kin | 84.0 ± 0.40 ef | 15.0 ± 3.50 b | 10.2 ± 1.88 bc | 6.4 ± 0.81 cd | 2.2 ± 0.58 e | 1.8 ± 0.80 c |
| 35 + 20 2,4-D + 1 TDZ | 96.0 ± 0.24 ab | 17.4 ± 2.22 b | 14.0 ± 2.08 b | 9.0 ± 1.52 bc | 4.8 ± 1.06 de | 5.4 ± 1.50 bc |
| 35 + 20 2,4-D + 1 *m*TR | 92.0 ± 0.37 c | 13.0 ± 2.16 bc | 10.8 ± 1.46 bc | 7.0 ± 1.05 cd | 4.4 ± 1.32 de | 4.2 ± 1.06 c |
| 35 + 20 2,4-D + 1 BA | 86.0 ± 0.51 de | 13.2 ± 2.87 bc | 9.8 ± 1.74 bc | 7.6 ± 1.74 bc | 4.0 ± 0.86 de | 4.0 ± 1.14 c |
| 40 + 20 2,4-D + 1 Kin | 90.0 ± 0.37 cd | 19.6 ± 3.81 b | 10.0 ± 1.09 bc | 10.2 ± 1.15 bc | 4.2 ± 0.86 de | 9.0 ± 1.41 b |
| 40 + 20 2,4-D + 1 TDZ | 96.0 ± 0.24 ab | 25.2 ± 3.38 a | 12.0 ± 1.30 bc | 15.0 ± 1.09 a | 6.6 ± 1.20 cd | 14.0 ± 1.87 a |
| 40 + 20 2,4-D + 1 *m*TR | 92.0 ± 0.27 c | 15.6 ± 2.54 b | 8.6 ± 1.50 cd | 8.2 ± 1.39 bc | 4.0 ± 1.34 de | 8.6 ± 0.92 b |
| 40 + 20 2,4-D + 1 BA | 88.0 ± 0.58 d | 15.2 ± 2.17 b | 9.4 ± 2.37 bc | 8.0 ± 2.34 bc | 3.2 ± 0.86 de | 7.8 ± 0.66 b |
| 35 + 20 picloram + 1 Kin | 70.0 ± 1.22 h | 5.8 ± 1.06 e | 5.0 ± 0.70 e | 4.8 ± 1.24 d | 6.8 ± 0.86 cd | 2.6 ± 0.51 c |
| 35 + 20 picloram + 1 TDZ | 78.0 ± 0.69 g | 7.6 ± 1.21 de | 7.6 ± 1.07 de | 7.4 ± 1.96 bc | 8.2 ± 0.86 bc | 4.8 ± 0.80 bc |
| 35 + 20 picloram + 1 *m*TR | 90.0 ± 0.44 cd | 12.2 ± 1.06 bc | 11.0 ± 1.46 bc | 10.6 ± 1.20 bc | 9.2 ± 1.39 bc | 8.0 ± 1.00 b |
| 35 + 20 picloram + 1 BA | 86.0 ± 0.24 de | 11.4 ± 1.86 cd | 11.4 ± 1.91 bc | 10.4 ± 1.86 bc | 8.4 ± 1.20 bc | 8.4 ± 1.20 b |
| 40 + 20 picloram + 1 Kin | 94.0 ± 0.20 c | 16.0 ± 2.34 b | 13.2 ± 2.08 b | 8.4 ± 0.87 bc | 9.2 ± 0.66 bc | 5.2 ± 0.86 bc |
| 40 + 20 picloram + 1 TDZ | 84.0 ± 0.40 ef | 13.4 ± 1.28 b | 10.4 ± 1.50 bc | 5.8 ± 1.11 cd | 6.4 ± 1.03 cd | 3.6 ± 0.67c |
| 40 + 20 picloram + 1 *m*TR | 98.0 ± 0.20 a | 18.4 ± 1.91 b | 15.0 ± 1.70 b | 10.0 ± 1.51 bc | 13.2 ± 1.85 a | 10.2 ± 0.86 b |
| 40 + 20 picloram + 1 BA | 82.0 ± 0.51 f | 17.6 ± 2.21 b | 19.0 ± 2.61 a | 11.2 ± 1.35 bc | 9.2 ± 1.31 bc | 9.6 ± 0.92 b |

Controls = MS + 35 or 40 g $l^{-1}$ sucrose. Results are mean ± SEM of % of embryo from 25 replicate per treatment. Mean values followed by same letters in each column are not significantly different according to the Duncan's multiple range test at 5 % level

*PGR* plant growth regulator, *SEs* somatic embryos

initiated profuse friable embryogenic calli (FEC) at wounding sites during 2 weeks of culture. Later calli (whitish or whitish green) covered the explants on different media treatments (Fig. 1a). The FEC were differentiated into white globular embryoids in all treatments except the control after 6 weeks (Fig. 1b). Similarly, embryoids were achieved from callus in *Rhinacanthus nasutus* (Cheruvathur et al. 2013). In this study, FEC promoted different stages of somatic embryos (SEs: globular, heart and torpedo) in all treatments after 8 weeks of culture (Fig. 1c). Increasing sucrose concentrations enhanced induction of FEC as well as SEs. This indicates that sucrose at higher concentrations may create both nutritional and excess osmotic stress that helps to improve somatic embryogenesis. Therefore, it is suggested that, nutrient and osmotic effects of sucrose may cause normal development of somatic embryos. The positive effect of high osmolarity may mimic the osmolarity alterations that occur surrounding the embryo in nature (Merkle et al. 1995). Different developmental stages (globular, heart, torpedo and cotyledonary) of SEs and plantlet formation from all treatments improved when transferred to MS medium with 20 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar. The frequency of embryogenesis and mean number of SEs and plantlets varied with treatment after 12 weeks from culture initiation (Table 1). The frequency (98 %) and number of heart-stage embryos (13.6) increased significantly on MS medium containing 40 g $l^{-1}$ sucrose and 20 μM picloram, while the globular, torpedo and cotyledonary-stage embryos and plantlet number were increased on media containing 50 g $l^{-1}$ sucrose and 20 μM 2,4-D or picloram (Table 1; Fig. 1d). However, no significant difference were observed between the two treatments (20 μM 2,4-D or picloram) in formation of torpedo-stage embryos (Table 1). Therefore, higher concentrations of 2,4-D or picloram and sucrose were essential in enhancement of *M. whitei* somatic embryogenesis.

Combination of 2,4-D or picloram and Kin or TDZ or *m*TR or BA was more effective in production of SEs and plantlet development (Table 2). The combined favorable influence of auxin and cytokinin observed in the present system were in accordance with the culture response of other Apocynaceae species (Sudha and Seeni 2006; Siddiqui et al. 2011; Yuan et al. 2011; Dipti et al. 2014). Whitish and whitish-green SEs and plantlets were produced varying in number with each treatment (Table 2; Fig. 2a). The SEs and plantlet development were increased by 40 g $l^{-1}$ sucrose, 20 μM 2,4-D or picloram and 1 μM TDZ or *m*TR or BA (Table 2; Fig. 2b). However, among the different combinations, the medium containing 40 g $l^{-1}$

**Fig. 2** In vitro plant regeneration by somatic embryogenesis from leaf explants of *M. whitei*. **a** Proliferation of whitish green somatic embryos and plantlets on MS medium containing 40 g $l^{-1}$ sucrose, 20 μM 2,4-D and 1 μM TDZ. **b** Development of somatic embryos to plantlet formation (×5). **c** Proliferation of plantlets from somatic embryos on MS medium containing 40 g $l^{-1}$ sucrose, 20 μM 2,4-D and 1 μM TDZ

sucrose, 20 μM 2,4-D and 1 μM TDZ markedly increased the number of globular and torpedo-stage embryos and plantlet numbers (Table 2; Fig. 2c). Induction of heart and cotyledonary stage embryos was enhanced in 40 g $l^{-1}$ sucrose, 20 μM picloram and 1 μM BA or *m*TR (Table 2). These results reveal that sucrose at higher concentration and in combination with 2,4-D and TDZ or picloram and BA or *m*TR were important for significant SEs and plantlet production in *M. whitei.*

Enhancement of somatic embryos and plantlets

The prolific SEs and plantlets from optimized somatic embryogenesis medium were significantly multiplied on MS media plus 20 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar containing various treatments of PGRs singly and/or in combination (Table 3). Somatic embryos were induced rapidly in all the treatments after one week of culture compared to control. Different stages of SEs were observed simultaneously on the same medium treatments indicating that somatic embryo production in *M. whitei* is an asynchronous phenomenon. Similar findings have been reported for other plant species (Karami et al. 2006; Baskaran and Van Staden 2014). Clearly, globular-shaped embryos were further developed into heart, torpedo and cotyledonary-stage embryos and plantlet development were observed after 10 weeks of culture (Fig. 3a). However, SEs improved on medium containing 0.5 μM BA or *m*TR (Table 3). Similarly, addition of cytokinin in the medium has been reported to improve somatic embryogenesis in other plant species (Karami et al. 2006; Siddiqui et al. 2011; Dipti et al. 2014). In this study, addition of IAA or NAA with cytokinin was even more effective in inducing SEs and plantlet formation (Table 3; Fig. 3b). Medium containing

**Table 3** Enhancement of somatic embryos (SEs) and plantlets from embryogenic callus of leaf explants of *M. whitei* incubated in solid MS medium with 40 g $l^{-1}$ sucrose, 20 μM 2,4-D and 1 μM TDZ

| Sucrose (20 g $l^{-1}$) + PGR (μM) in MS medium | Frequency (%) of embryogenesis | No. of SEs developmental stage/explant (mean ± SEM) | | | | |
|---|---|---|---|---|---|---|
| | | Globular | Heart | Torpedo | Cotyledonary | Plantlets |
| 0 (Control) | 96.0 ± 0.24 bc | 21.2 ± 3.38 bc | 11.6 ± 1.56 c | 12.0 ± 1.09 bc | 6.8 ± 1.11 c | 11.0 ± 2.34 bc |
| 0.5 BA | 98.0 ± 0.20 ab | 23.8 ± 2.22 bc | 13.2 ± 0.66 bc | 13.2 ± 0.94 bc | 9.0 ± 1.22 bc | 12.6 ± 1.32 bc |
| 0.5 *m*TR | 96.0 ± 0.60 bc | 24.4 ± 2.97 bc | 14.6 ± 1.32 bc | 14.6 ± 1.65 bc | 10.2 ± 2.22 bc | 13.8 ± 1.50 bc |
| 0.5 TDZ | 94.0 ± 0.24 cd | 16.6 ± 1.80 bc | 16.0 ± 1.96 bc | 12.8 ± 2.16 bc | 10.8 ± 1.49 bc | 14.0 ± 1.56 bc |
| 0.5 Kin | 92.0 ± 0.58 d | 17.8 ± 2.95 bc | 13.8 ± 1.88 bc | 14.2 ± 0.66 bc | 8.8 ± 1.56 bc | 12.4 ± 1.65 bc |
| 0.5 BA + 0.25 IAA | 98.0 ± 0.20 ab | 27.2 ± 3.38 a | 15.8 ± 2.05 bc | 15.0 ± 1.78 bc | 10.2 ± 1.59 bc | 14.8 ± 1.68 bc |
| 0.5 *m*TR + 0.25 IAA | 100 ± 0.00 a | 23.2 ± 5.00 bc | 20.2 ± 2.13 a | 18.6 ± 2.15 a | 16.0 ± 2.70 a | 20.4 ± 2.65 a |
| 0.5 TDZ + 0.25 IAA | 96.0 ± 0.24 bc | 17.6 ± 1.93 bc | 18.4 ± 1.16 bc | 14.2 ± 1.56 bc | 13.2 ± 1.24 bc | 13.4 ± 2.27 bc |
| 0.5 Kin + 0.25 IAA | 92.0 ± 0.58 d | 19.0 ± 1.43 bc | 16.2 ± 1.98 bc | 13.6 ± 1.69 bc | 10.6 ± 1.20 bc | 12.6 ± 1.20 bc |
| 0.5 BA + 0.25 NAA | 90.0 ± 0.31 d | 19.8 ± 2.45 bc | 13.0 ± 1.77 bc | 11.2 ± 1.24 c | 8.2 ± 1.62 bc | 10.2 ± 0.97 bc |
| 0.5 *m*TR + 0.25 NAA | 98.0 ± 0.24 ab | 20.8 ± 3.56 bc | 16.8 ± 2.05 bc | 14.0 ± 1.91 bc | 12.6 ± 2.65 bc | 15.2 ± 2.59 bc |
| 0.5 TDZ + 0.25 NAA | 84.0 ± 0.51 e | 13.4 ± 1.32 c | 14.4 ± 1.43 bc | 10.4 ± 1.50 c | 9.4 ± 1.74 bc | 13.2 ± 1.35bc |
| 0.5 Kin + 0.25 NAA | 80.0 ± 0.63 f | 15.0 ± 1.24 bc | 11.2 ± 0.58 c | 12.0 ± 1.14 bc | 6.2 ± 0.86 c | 9.0 ± 2.34 c |

Control = MS + 20 g $l^{-1}$ sucrose. Results are mean ± SEM of % of embryo from 25 replicate per treatment. Mean values followed by same letters in each column are not significantly different according to the Duncan's multiple range test at 5 % level

*PGR* plant growth regulator, *SEs* somatic embryos

0.5 μM BA and 0.25 μM IAA produced the highest number of globular-stage embryos (27.2), while the heart (20.2), torpedo (18.6) and cotyledonary-shaped embryos (16.0) and plantlets (20.4) were higher from medium containing 0.5 μM *m*TR and 0.25 μM IAA (Table 2). In addition, combination of 0.5 μM *m*TR and 0.25 μM IAA induced longer plantlet (approximately, 2–3 cm shoot and 5–6 cm radicle) compared to other treatments (Fig. 3c). Accordingly, *meta*-topolin riboside (*m*TR) in combination with tested auxins was effective in inducing somatic embryogenesis in *M. whitei*. The aromatic cytokinin, *meta*-Topolin is well documented for in vitro cultures in many plant species (Aremu et al. 2012; Baskaran et al. 2012). The present study indicated that SE production in *M. whitei* is dependent on the type and combination of PGRs. Similar phenomena have also been reported for other plant species (Sudha and Seeni 2006; Baskaran and Van Staden 2014; Dipti et al. 2014). Well-developed plantlets (approximately, 70–90 mm) (Fig. 3d) were separated and then transferred to a plastic box containing a 1:1 (v/v) vermiculite:soil mixture. The plantlets were successfully acclimatized and established in the greenhouse with 90 % survival rate (Fig. 3e, f).

## Synthetic seed production, storage and germination

Somatic embryos (SEs: heart, torpedo and cotyledonary) from different embryo development media (EDM) were used for synthetic seed production and viability testing in various concentrations of sodium alginate (SA: 1, 2, 3 and 4 %) and calcium chloride ($CaCl_2 \cdot 2H_2O$: 75, 100 and 125 mM for 10 min exposure) (Table 4). Ideal concentrations and combinations of SA (3 %) and $CaCl_2 \cdot 2H_2O$ (100 mM) were optimized (Fig. 3g) from EDM (0.5 μM *m*TR and 0.25 μM IAA)-derived embryos. The standardized concentrations of SA and $CaCl_2 \cdot 2H_2O$ were essential to obtain the most uniform beads with a hard enough coat for survival as well as viability of synthetic seeds. Germination of synthetic seeds was initiated after 1 week culture (Fig. 3h). The survival and germination frequencies varied with concentration combination of SA and $CaCl_2 \cdot 2H_2O$ after 6 weeks (Table 4). Among the different concentrations of SA and $CaCl_2 \cdot 2H_2O$ used, 3 % SA and 100 mM $CaCl_2 \cdot 2H_2O$ proved to be the best for production of most uniform beads, and supported significantly higher survival (95.7) and germination (73 %) frequencies (Table 4; Fig. 3i). Increasing concentrations of SA (>3 %) and $CaCl_2 \cdot 2H_2O$ (>100 mM) with 10 min exposure resulted in hard beads with lower survival and germination frequencies (Table 4). These results are in agreement with other reports and suggest the direct relation between higher frequency of germination of synthetic seed with lower concentration and exposure time to $CaCl_2 \cdot 2H_2O$ (Malabadi and Van Staden 2005; Cheruvathur et al. 2013). High concentration or excessive exposure time of $CaCl_2 \cdot 2H_2O$ leads to more absorption and penetration to the embryos which generates inhibition of germination, growth and development in the field (Redenbaugh et al. 1986; Malabadi and Van Staden 2005). In this study, germination of synthetic seed from heart,

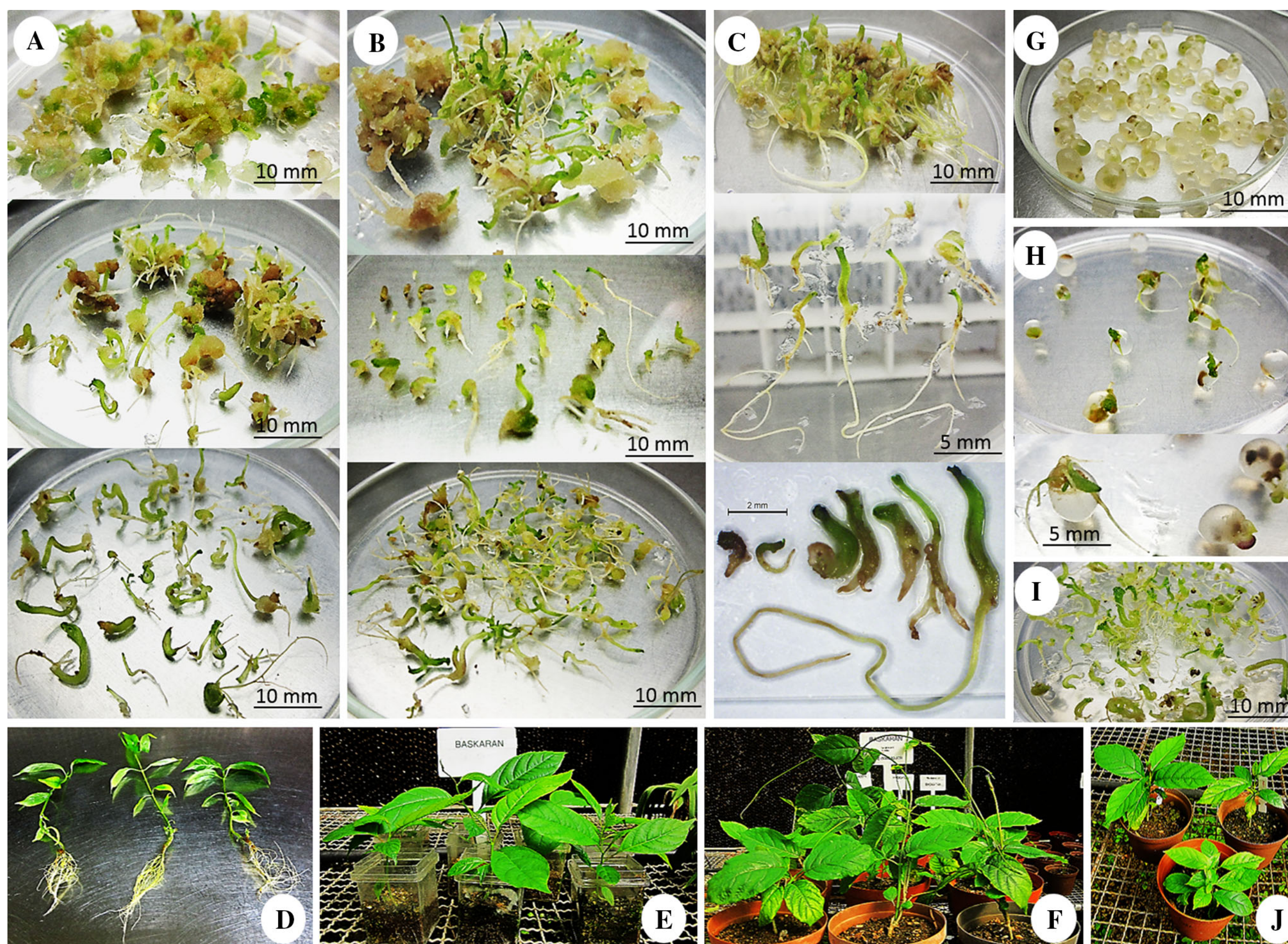


**Fig. 3** In vitro plant regeneration from somatic embryos (SEs) and synthetic seeds from leaf explants of *M. whitei*. **a** Conversion of somatic embryos to plantlet formation on MS medium containing 20 g $l^{-1}$ sucrose and 0.5 µM *m*TR. **b** Proliferation of plantlets from SEs on MS medium containing 20 g $l^{-1}$ sucrose, 0.5 µM *m*TR and 0.25 µM IAA. **c** Development of longer plantlets on MS medium containing 20 g $l^{-1}$ sucrose, 0.5 µM *m*TR and 0.25 µM IAA. **d** Well developed plants from somatic embryo. **e** Acclimatized plants of *M. whitei* in the greenhouse after 3 months. **f** Ex vitro plants of *M. whitei* after 6 months. **g** Production of synthetic seeds from 3 % SA and 100 mM $CaCl_2{\cdot}2H_2O$. **h** Germination of synthetic seed after 1 week. **i** Development of plantlets from synthetic seeds. **j** Acclimatized plants from synthetic seeds after 4 months

torpedo and cotyledonary stage embryos was rapid, with survival and germination frequencies significantly higher in embryos derived from EDM containing 0.5 µM *m*TR and 0.25 µM IAA (Table 4). Therefore, the medium for production of embryos is essential for healthy and viable synthetic seeds.

The synthetic seeds were stored at 4 °C for different time periods to test viability. Thereafter, synthetic seeds were transferred to MS medium containing 30 g $l^{-1}$ sucrose and 8 g $l^{-1}$ agar for testing competence of germination. The present study provided promising results for survival and germination frequencies of synthetic seeds; however, it was significantly lower than control (non-stored synthetic seed) (Table 4). Similar results were also reported for other plant species (Ipekci and Gozukirmizi 2003; Krishna Kumar and Thomas 2012). Poor viability of stored synthetic seeds may be related to both oxygen deficiency in the gel bead and rapid drying (Redenbaugh et al. 1991). All the plantlets (approximately, 70–90 mm) produced from synthetic seeds were successfully acclimatized in 1:1 (v/v) vermiculite:soil mixture and established in the greenhouse with a survival rate of 90 % (Fig. 3j).

In conclusion, this is the first report on somatic embryogenesis and synthetic seed production in *M. whitei* using leaf explant-derived FEC. Promising plant regeneration from somatic embryogenesis was influenced markedly by sucrose at higher concentrations and combinations of PGRs. The synthetic seeds of *M. whitei* could be stored at low temperature (4 °C) for 50 days. This facilitates

**Table 4** Growth response from stored (4 °C) and non-stored synthetic seeds of heart-, torpedo- and cotyledonary-embryo from leaf explants of *M. whitei*

| Plant growth regulators (μM) in EDM | Capsule matrix (%) | Complexing agent (mM) | Storage (days) | Survival (%) | Germination (%) |
|---|---|---|---|---|---|
| 0.5 *m*TR + 0.25 IAA | 1 | 75 | 0 | 46.7 ± 1.76 fg | 28.0 ± 1.52 f |
| | | 100 | 0 | 52.0 ± 2.08 ef | 36.0 ± 2.08 ef |
| | | 125 | 0 | 33.3 ± 1.45 h | 21.3 ± 1.20 g |
| | 2 | 75 | 0 | 68.0 ± 1.52 bc | 48.0 ± 1.15 cd |
| | | 100 | 0 | 69.3 ± 2.33 bc | 52.0 ± 2.08 c |
| | | 125 | 0 | 37.3 ± 1.20 gh | 22.7 ± 0.33 g |
| | 3 | 75 | 0 | 74.6 ± 1.20 bc | 61.3 ± 0.33 bc |
| | | 100 | 0 | 95.7 ± 0.90 a | 73.0 ± 1.15 a |
| | | 125 | 0 | 62.7 ± 2.18 cd | 48.0 ± 1.52 cd |
| | 4 | 75 | 0 | 89.3 ± 1.26 bc | 69.3 ± 0.88 bc |
| | | 100 | 0 | 63.0 ± 1.45 cd | 52.0 ± 1.15 c |
| | | 125 | 0 | 56.0 ± 0.58 de | 44.0 ± 0.58 cd |
| 0.5 BA + 0.25 IAA | 3 | 100 | 0 | 76.0 ± 0.58 bc | 54.3 ± 0.90 c |
| 0.5 BA + 0.25 NAA | 3 | 100 | 0 | 72.6 ± 0.90 bc | 57.3 ± 1.45 bc |
| 0.5 *m*TR + 0.25 NAA | 3 | 100 | 0 | 78.0 ± 0.90 bc | 64.6 ± 0.90 bc |
| 0.5 *m*TR + 0.25 IAA | 3 | 100 | 10 | 70.6 ± 0.90 bc | 67.3 ± 1.20 bc |
| 0.5 *m*TR + 0.25 IAA | 3 | 100 | 30 | 63.3 ± 1.76 cd | 58.3 ± 1.20 bc |
| 0.5 *m*TR + 0.25 IAA | 3 | 100 | 50 | 58.0 ± 1.76 de | 51.6 ± 1.20 c |

Embryo development medium (EDM) = solid MS + 20 g $l^{-1}$ sucrose + growth regulators. Capsule matrix = Liquid MS medium + sodium alginate (%). Complexing agent = $CaCl_2 \cdot 2H_2O$ for 10 min. Results are mean ± SEM derived from 50 replicate per treatment after 6 weeks. Mean values followed by same letters in each column are not significantly different according to the Duncan's multiple range test at 5 % level

transportation and exchange of germplasm across international borders. The system can be used for conservation, production of bioactive compounds and genetic transformation.

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