Research in Plant Biology, 2(5): 32-40, 2012 ISSN : 2231-5101
www.resplantbiol.com

Regular Article

Smoke saturated water promoted *in vitro* seed germination of an epiphytic orchid *Oberonia ensiformis* (Rees) Lindl.

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This study highlights for the first time an *in vitro* seed germination of an epiphytic orchid *Oberonia ensiformis* (Rees) Lindl, from the Western Ghat Forest of Karnataka state, India promoted by smoke saturated water (SSW). High percentage germination (85%) and high percentage of plantlet recovery (73%) was achieved by culturing seeds on Mitra *et al.* (1976) basal medium supplemented with 10% (v/v) SSW. Well-rooted shoots were transferred to pots containing charcoal chips, coconut husk and broken tiles (2:2:1) and 90% survived. Therefore, the role of SSW as a natural growth regulator at different stages of development from seed germination to plant regeneration has been confirmed.

Key words: Belgaum, Karnataka state, India, micropropagation, semi-dry grasses, Western Ghat Forests

Abbreviations: butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one, **PLB**, protocorm-like body; **TRIA**, triacontanol, **SSW**, smoke saturated water

Smoke influences seed germination in many plant species, and acts as a catalyst during germination process (Malabadi and Vijayakumar, 2006, 2008; Malabadi *et al.* 2009c; Baxter and van Staden, 1994; Baxter *et al.* 1994; Drews *et al.* 1995; Dixon *et al.* 1995; Keeley, 1993; Keeley and Fotheringham, 1998; Brown, 1993; Brown and van Staden 1997; van Staden *et al*. 2000; Brown *et al*. 2003; Light and van Staden 2004; Daws *et al*. 2008; Dixon *et al*. 1995; Pierce *et al*. 1995; Roche *et al*. 1997; Brown and Botha, 2004; Blank and Young, 1998; Jain *et al.* 2008; Jain and van Staden, 2006; Abdollahi *et al.* 2011). Smoke water has also induced somatic embryogenesis in plants (Senaratna *et al.* 1999; Malabadi and Nataraja, 2007c; Malabadi *et al.* 2009c; Abdollahi *et al.* 2012; Ghazanfari *et al*. 2012). The ability of plant-derived smoke to break dormancy and stimulate germination was first reported by de Lange and Boucher (1990) for *Audouinia capitata*, a fynbos species growing in a fire-prone habitat. The potentiality of smoke promoting seed germination has been

Received: 21.8.2012; Revised and accepted: 8.10.2012

extensively discussed in a number of reviews and research articles (Daws et al. 2008; Kulkarni et al. 2006a, 2006b; Light and van Staden, 2004; Light et al. 2002; Ma et al. 2006; Pierce et al. 1995; Roche et al. 1997; Sparg et al. 2005a, 2005b; Strydom et al. 1996; Taylor and van Staden, 1996; Thomas and van Staden, 1995; Brown and van Staden, 1997; van Staden *et al*. 2000; Light and van Staden 2004). Smoke contains several thousand compounds (Maga, 1988). A highly active germination promoting compound has recently been identified as a water soluble butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one, from the smoke of burnt fynbos *Passerina vulgaris* Thoday and the grass *Themeda triandra* L. (van Staden *et al*. 2004) as well as from the combustion of cellulose (Flematti *et al*. 2004). This compound, which is water soluble and heat stable, can stimulate seed germination at very low concentrations (10^{-9} M; Flematti *et al*. 2004; van Staden *et al*. 2004) and can be stored as an aqueous solution for long periods while retaining its activity after autoclaving (van Staden *et al*. 2000, 2004, 2006).

One of the major problems in orchid production is the difficulty in seed germination. Generally orchids are propagated through seed germination, but seedling development can be a long process and flowering plants are often produced only after 3-5 years of growth (Arditti, 1968; Arditti *et al*. 1981). The greatest threat to orchid diversity is habitat loss; for orchids this may occur on a very small scale because a single tropical tree may bear hundreds of epiphytic orchid species. Orchids are badly affected by habitat destruction and their unabated collection. If orchids are present in an ecosystem, this is a good indicator of a healthy, functioning ecosystem. *Oberonia ensiformis* (Rees) Lindl., is one of the native epiphytic orchids from the Western Ghat forests of Karnataka, and this epiphytic orchid is commercially important as potted floriculture crop. There is also a growing perception that *Oberonia ensiformis* (Rees) Lindl., could be endangered in the near future. Tissue culture techniques have been widely used for the *in vitro* mass multiplication of several commercially important orchids (Arditti, 1968; Arditti *et al.* 1981; Johnson and Kane, 2007; Kauth *et al.* 2006, 2008; Morel 1964; Rao 1977; Sharma *et al*. 1991; Lakshmanan *et al*. 1995; Ichihashi 1997, 1998; Kanjilal *et al*. 1999; Malabadi *et al*. 2004, 2005; Teixeira da Silva *et al*. 2006; Das *et al*. 2007; Malabadi and Nataraja 2007a, 2007b; Malabadi *et al*. 2008a, 2008b, 2008c; Malabadi *et al*. 2009a, 2009b; Malabadi *et al.* 2011; Mulgund *et al.* 2011, 2012). This paper highlights for the first time an efficient propagation method for *Oberonia ensiformis* (Rees) Lindl, through *in vitro* seed germination culture by incorporation of smoke-saturated water (SSW) in the nutrient medium, and extend this low cost technology for the conservation of native orchids.

Materials and methods

Preparation of smoke-saturated-water

SSW was prepared according to the procedure described by Thomas and van Staden (1995) and Dixon *et al*. (1995). This was achieved by slow burning of a mixture of two local (Indian) semi-dry grasses *Aristida setacea* and *Cymbopogon martini* (Graminiaceae) (Malabadi and Vijaykumar 2006, 2007d; Malabadi and Nataraja 2007c). The resulting smoke was first passed into a metal drum connected to a flask containing 500 ml of distilled water through a pipe. The smoke was forced to pass through the water by blowing air using a fan or compressed air for 1 to 2 h at the rate of 50 to 60 psi continuously. The SSW was collected and stored at 2°C until further use. Different concentrations of SSW (5, 10, 15 and 20%) were used in the following *in vitro* seed germination of an epiphytic orchid *Oberonia ensiformis* experiments.

In vitro seed germination using smoke-saturated water

Green capsules (approx. 2 to 6 cm in length) of *Oberonia ensiformis* were collected from the Western Ghat Forests of Karnataka near Belgaum–Panaji road India. These capsules were carefully washed in sterilized double distilled water. They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma, USA), and thoroughly rinsed with sterilized double distilled water. After sterilization, the capsules were dried and dissected longitudinally with a surgical blade under aseptic conditions. The seeds were scooped out from sterilized capsules and sown by spreading as thinly as possible over the surface of Mitra *et al*. (1976) basal medium with 3.0% sucrose, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin (all reagents Sigma), the control medium, in 250-ml wide mouth tissue culture bottles (3 tissue culture bottles per capsule and one capsule for each treatment, and experiments were repeated 3 times). The effect of SSW was also studied on the germination of the orchid under study by incorporating different concentrations (5, 10, 15 and 20%) into the control medium. The pH of the medium was adjusted to 5.7 with 1 N NaOH or HCl before agar was added. The medium was then sterilized by autoclaving at 121°C and 1.05 kg/cm^2 for 15 min. L-glutamine and casein hydrolysate were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm), and added to the medium after it had cooled to below 50°C. All the cultures were maintained in the dark at 25 ± 2°C. Percentage germination was calculated by dividing the number of germinating seeds by total number of seeds in the sample under the microscope. Various developmental stages of seed germination of an epiphytic orchid *Oberonia ensiformis* were adopted from Kauth *et al*. (2006) and Johnson and Kane (2007). The cultures were maintained for 6-10 weeks to initiate protocorm-like bodies (PLBs) or proliferating shoot buds. The freshly initiated individual PLBs were transferred (~5-10 PLBs per conical flask) to basal medium containing 10% SSW (this is the optimum concentration for growth and development) (Senaratna *et al*. 1999; Malabadi and Nataraja 2007c). Healthy shoots with 2-3 leaves developed within 10-12 weeks. They were subcultured on the same medium for another 2 weeks for further shoot development.

Plantlet hardening and acclimatization

The well-developed shoots were further transferred to fresh basal medium supplemented with or without (control) 2.0 µM triacontanol (TRIA) for improving rooting. The shoots with well developed roots on TRIA-supplemented basal medium were washed thoroughly under running tap water and transplanted into 15-cm diameter pots containing a potting mixture of charcoal chips, coconut husks and broken tiles (2: 2: 1). Three to four plantlets were planted in each pot, watered daily and fertilized weekly with a foliar spray of a mixture of commercial DAP (di-ammonium phosphate) and NPK (nitrogen 20: phosphorous 10: potassium 10) (Malabadi *et al*. 2004, 2005; Malabadi and Nataraja 2007a; Malabadi *et al*. 2008).

Statistical analyses

All experiments contained 10 cultures per replicate, with five replicates (50 cultures) per experimental treatment, and each treatment was repeated three times (50 × 3 = 150). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA, and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at the 5% level using the SPSS (Microsoft Windows v. 13.0.1.1) statistical software package.

Results and discussion

In the present study, the cultured immature embryo of *Oberonia ensiformis* onto the media slowly turned green and formed swollen structures called protocorms within 10 weeks (**Fig. 1A**). In this study an increase in percentage germination as well as early differentiation of protocorms into seedlings was observed on 10% (v/v) SSW-supplemented Mitra *et al*. (1976) basal medium compared to control (**Table 1**). Maximum percentage germination (95%) was observed on 10% (v/v) and seed germination percentage was greatly inhibited at higher concentrations of SSW (15 and 20%) compared to the control and most seeds turned brown without germinating (**Table 1**). This higher percentage of seed germination also corresponds to the highest percentage recovery of seedlings (93%) with well developed roots (**Fig. 1B**) (**Table 1**). In this study, the presence of SSW at 10% (v/v) in basal medium resulted in faster differentiation of protocorms to form plantlets (i.e. leaves and roots) than the control (**Table 1**). By 50 days of culture, a significantly high percentage of propagules formed on SSW-supplemented basal medium regardless of the concentration compared to the control. The effect of quickening differentiation of protocorms to form plantlets (i.e. leaves and roots) by the presence of SSW in basal medium indicated that SSW has some growth-promotive substance(s). Finally, the well-rooted shoots that regenerated on 10% (v/v) SSW-supplemented basal medium formed plants during hardening that were normal and showed healthy growth with a 90% survival rate, i.e. SSW at 10% (v/v) aids in rapid regeneration of *Oberonia ensiformis*. In one of our previous studies of the effect of smoke and SSW on seed germination of four medicinal plants (*Acacia pennata* (Mimosaceae), *Basella alba* (Basellaceae), *Celastrus asiatica* (Celastraceae), and *Cleome gynandra* (Cleomaceae) (Malabadi and Vijay**a**kumar 2007), it was noticed that all plants showed a higher rate of germination under 16:8 h light/dark in the control and smoke treatments (Malabadi and Vijaykumar 2007; Malabadi *et al.* 2008a). Smoke influences seed germination and post-germination processes. SSW may protect seeds and seedlings against microbial attack and thus result in higher seedling survival (Light and van Staden 2004; Malabadi *et al.* 2008a).

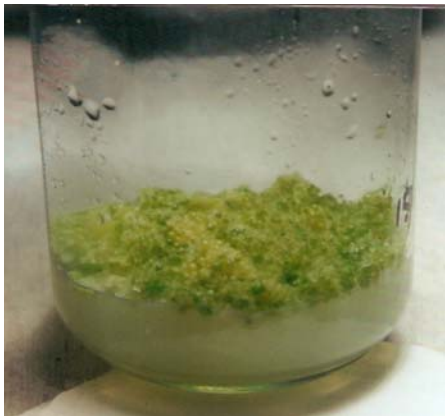

Fig. 1A. Influence of 10% SSW on seed germination of *Oberonia ensiformis* (Rees) Lindl. *In vitro* seed germination on 10% SSW-incorporated Mitra *et al*. (1976) basal medium showing protocorm development.

Fig. 1B. Seedlings with well developed roots after 16 weeks and ready for hardening.

Table 1 Effect of different concentrations of SSW-supplemented Mitra *et al*. (1976) basal medium on seed germination of an epiphytic orchid, *Oberonia ensiformis* (Rees) Lindl.

SSW concentrations (%, v/v)	No. of protocorms	Time taken for germination (weeks)	No. of protocorms with 2-3 leaves (%)	No. of seedlings with roots (%)
*control	4.0 ± 0.1 b	13-16	0.0 ± 0.0 c	0.0 ± 0.0 c
5	18.0 ± 0.2 b	8-10	10.0 ± 1.0 b	7.0 ± 0.5 b
*control	0.0 ± 0.0 c	14-16	0.0 ± 0.0 c	0.0 ± 0.0c
10	85.0 ± 1.6 a	8-10	80.0 ± 1.8 a	73.0 ± 1.4 a
*control	0.0 ± 0.0c	13-16	0.0 ± 0.0c	0.0 ± 0.0 c
15	12.0 ± 1.0 b	8-10	16.0 ± 1.2 b	10.0 ± 0.3 b
*control	0.0 ± 0.0 c	13-16	0.0 ± 0.0c	0.0 ± 0.0 c
20	5.0 ± 0.3 b	8-10	0.0 ± 0.0 c	0.0 ± 0.0 c

*Control = Mitra *et al*. (1976) basal medium without SSW. Data scored after 16 weeks and represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different (P<0.05) according to DMRT (Duncan's multiple range test). SSW-smoke saturated water.

Depending on the plant species of different geographical locations smoke treatments and butenolide applications are able to improve seedling vigour, and survival rates in some South African indigenous medicinal plants (Sparg *et al*. 2005), a commercial maize cultivar (Sparg *et al*. 2006), rice (Kulkarni *et al*. 2006), vegetables such as tomatoes, okra and beans (Jain and van Staden 2006; van Staden *et al*. 2006), grasses (Baxter and van Staden 1994; Blank and Young 1998) and woody *Acacia* species (Kulkarni *et al*. 2007). SSW was also able to stimulate somatic embryogenesis geranium (Senaratna *et al*. 1999) specifically at 10% using vegetative shoot apices of mature trees of *Pinus wallichiana* (Himalayan blue or Bhutan pine) (Malabadi and Nataraja 2007c) and, flowering in fire-lily *Cyrtanthus ventricosus* (Keeley 1993) and rooting in *Vigna radiata* (L.) Wilczek hypocotyl cuttings (Taylor and van Staden 1996; Malabadi *et al.* 2008a). SSW and aerosol smoke by slow burning of a mixture of semi-dry grasses *Aristida setacea* and *Cymbopogon martini* (Graminaceae) improved the seed germination and seedling vigour of four Indian indigenous medicinal plants (*Terminalia chebula*, *Holorrhina antidysentrica*, *Clitoria ternatea* and *Gymnema sylvestre*) (Malabadi and Vijay Kumar 2006). Therefore, from the above results it is clear that active compound(s) within SSW play a regulatory role in plant development (Malabadi *et al.* 2008a). As all these physiological effects are in part controlled by plant growth regulators (PGRs), indications are that the smoke extracts interact in same way with endogenous PGRs (van Staden *et al*. 2000; Jain *et al*. 2008). SSW does not have any significant effect on the germination period of somatic embryos in all the three genotypes of *P. wallichiana* although it did affect the total number of somatic embryos that germinated (Malabadi and Nataraja 2007c; Malabadi *et al.* 2008a). In geranium (*Pelargonium hortorum* Bailey cv. 'Elite'), SSW treatment (10% v/v) of the explant prior to induction, or together with the inductive signal (TDZ) produced the highest number of somatic embryos (Senaratna *et al*. 1999; Malabadi *et al.* 2008a). However, the mode of action of SSW is still unknown even after the identification of butenolide (Malabadi *et al.* 2008a). Therefore, on the basis of this study it was concluded that smoke saturated

water influences orchid seed germination and hence played an important role in the conservation of orchid species. The use of smoke saturated water for *in vitro* cultures is very cost effective and it will definitely help to save the endangered plant species.

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