Research in Plant Biology, 2(2): 24-29, 2012 ISSN : 2231-5101
www.resplantbiol.com

Research Article

Smoke promoted *in vitro* seed germination of *Pholidota pallida* Lindl.

Gangadhar S. Mulgund[1], Neelambika T. Meti[2], Ravindra B. Malabadi[1]*, K. Nataraja[1] and S. Vijaya Kumar[3]

[1]Department of Botany, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka, India
[2]Department of Agricultural Biotechnology, Bharati Vidyapeeth University, Pune-Satara Road, Katraj, Pune - 411046, Maharashtra, India
[3]Department of Biotechnology, Madanapalle Institute of Technology and Science, Madanpalle-517325, Chitoor District, Andhra Pradesh, India
*Corresponding author e-mail: mlbd712@rediffmail.com

This study reports the influence of smoke saturated water (SSW) on asymbiotic seed germination and an early differentiation of protocorms and plant regeneration of *Pholidota pallida* Lindl. High percentage germination (85%), and percentage of plantlet recovery (71%) 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, smoke saturated water is an efficient, cheap and easy way to improve the seed germination, *in vitro* development and *ex vitro* establishment of orchids.

Key words: Belgaum, 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

The ability of plant-derived smoke to break dormancy and stimulate germination was first reported for *Audouinia capitata*, a fynbos species growing in a fire-prone habitat (Daws *et al.* 2008; Flematt *et al.* 2004). Since this discovery, the application of smoke and aqueous smoke extracts to improve seed germination has been shown in a wide range of plants from many families, irrespective of their fire sensitivity (Daws *et al.* 2008; Flematt *et al.* 2004). 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. as well as from the combustion of cellulose (Daws *et al.* 2008; Flematt *et al.* 2004).

Orchids are produced through seed germination, but seedling development can be a long process and flowering plants are often produced only after 3-5 years of growth (Ichihashi, 1998; Johnson and Kane, 2007; Kanjilal *et al.* 1999). *Pholidota pallida* Lindl. is one of the native epiphytic orchids from the Western Ghat forests of Karnataka. Tissue

culture techniques have been widely used for the *in vitro* mass multiplication of several commercially important orchids (Ichihashi, 1998; Johnson and Kane, 2007; Kanjilal *et al.* 1999). The orchid seeds contain a small embryo and lack enzymes to metabolize polysaccharides, but utilize lipids as a major nutrient source. Symbiotic relationship with a mycorrhizal fungus is very much required to germinate under natural conditions (Ichihashi, 1998; Johnson and Kane, 2007; Kanjilal *et al.* 1999). The *in vitro* seed germination of orchids and culture of apical meristematic tissue has been reported in many orchid species (Kulkarni *et al.* 2006; Lakshmanan *et al*. 1995; Malabadi *et al.* 2004, 2005a; Malabadi and Nataraja, 2007a, 2007b, 2007c; Malabadi *et al.* 2008a, 2008b, 2008c; Malabadi and Vijaykumar, 2006, 2007; Senaratna *et al*. 1999; van Staden *et al*. 2004). This paper reports the multiplication method for *P. pallida Lindl* through *in vitro* seed germination culture by incorporation of smoke-saturated water (SSW) in the nutrient medium.

Materials and methods

Preparation of smoke-saturated-water

SSW was prepared and was achieved by slow burning of a mixture of two local (Indian) semi-dry grasses *Aristida setacea* and *Cymbopogon martini* (Graminiaceae) (Malabadi *et al*. 2008c; 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 (Malabadi *et al*. 2008c; Malabadi and Nataraja, 2007c). 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 experiments (Malabadi *et al*. 2008c; Malabadi and Nataraja, 2007c).

In vitro seed germination using smoke-saturated water

Green capsules (approx. 3 to 5 cm in length) of *P. pallida* were collected from the Western Ghat Forests of Karnataka near Khanapur, Belgaum, 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 and co-workers basal medium (Mitra *et al.* 1976) 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 conical flasks (3 conical flasks per capsule and one capsule for each treatment, and experiments were repeated 3 times). The effect of SSW was also studied on the initiation of embryogenic tissue by incorporating different concentrations (5, 10, 15 and 20%) into the control medium. The pH of the medium was adjusted to 5.8 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 *P. pallida* were adopted from our previous work. These stages are (stage 0 = ungerminated seed with embryo; stage 1 = enlarged embryo, testa ruptured (= germination); stage 2 = appearance of protomeristem or rhizoids; stage 3 = emergence and elongation of first leaf; stage 4 = protocorm with developing leaves and rhizoids; stage 5 = two leaves and one or more roots present; stage 6 = presence of two or more leaves, roots present (= seedling). The protocorms (60-70) in various stages of development were subcultured on fresh medium for 30 days. The percentage of propagules in each stage was calculated by dividing the number of propagules in that stage by the total number of propagules × 100. 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). 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).

Statistical analyses

All experiments contained 25 cultures per replicate, with four replicates (100 cultures) per experimental treatment, and each treatment was repeated three times (100 × 3 = 300). 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 an increase in percentage germination as well as early differentiation of protocorms into seedlings (**Fig. 1A, B, C**) was observed on 10% (v/v) SSW-supplemented Mitra *et al*. (1976) basal medium compared to control (**Table 1**). Germination was marked by swelling and emergence of the embryo from the testa (Fig. 1B). Maximum percentage germination (85%) 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**). 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. In our previous study related to orchid seed germination, 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 *V. parviflora* (Malabadi *et al*. 2008c).

SSW was also able to stimulate somatic embryogenesis geranium specifically at 10% using vegetative shoot apices of mature trees of *Pinus wallichiana* (Himalayan blue or Bhutan pine), and flowering in fire-lily *Cyrtanthus ventricosus*, and rooting in *Vigna radiata* (L.) Wilczek hypocotyl

cuttings (Malabadi and Nataraja, 2007c; Malabadi *et al.* 2008c; Malabadi and Vijaykumar, 2006, 2007; Senaratna *et al*. 1999; van Staden *et al*. 2004). 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*) (Kulkarni *et al.* 2006; Malabadi and Nataraja, 2007c; Malabadi *et al.* 2008c; Malabadi and Vijaykumar, 2006, 2007; Senaratna *et al*. 1999; van Staden *et al*. 2004). However, the mode of action of SSW is still unknown even after the identification of butenolide.

Table 1. Effect of different concentrations of SSW-supplemented Mitra *et al.* (1976) basal medium on seed germination of *P. pallida* Lindl.

SSW concentrations (%, v/v)	**№ of protocorms**	**Time taken for germination (weeks)**	**№ of protocorms with 2-3 leaves (%)**	**№ of seedlings with roots (%)**
*control	4.0 ± 0.1 b	12-16	2.5 ± 0.1 b	2.0 ± 0.1 b
5	17.0 ± 0.3 b	6-8	11.0 ± 1.0 b	8.0 ± 0.2 b
control	3.0 ± 0.1 b	12-16	2.3 ± 0.1 b	2.0 ± 0.1 b
10	85.0 ± 2.1 a	7-8	80.0 ± 2.1 a	71.0 ± 1.4 a
control	3.0 ± 0.1 b	12-16	2.1 ± 0.2 b	2.0 ± 0.1 b
15	12.0 ± 1.0 b	7-8	9.0 ± 1.0 b	8.0 ± 1.0 b
control	4.0 ± 0.1 b	12-16	2.5 ± 0.1 b	2.0 ± 0.1 b
20	3.0 ± 0.2 b	7-8	2.0 ± 0.1 b	1.0 ± 0.1 b

*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).

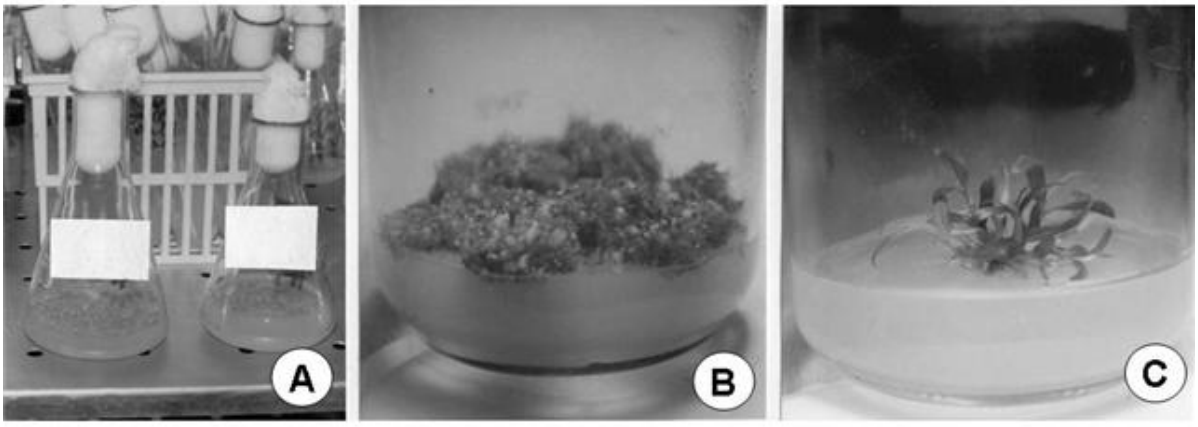

Fig. 1 Influence of 10% SSW on seed germination of *Pholidota pallida* Lindl. (**A**) *In vitro* seed germination on 10% SSW-incorporated Mitra *et al*. (1976) basal medium (bar = 1.0 cm). (**B**) Protocorm formation and growth of shoot buds with leaf primordia within 7-9 weeks (bar = 1.2 cm). (**C**) Seedlings with well developed roots after 16 weeks and ready for hardening (bar = 0.8 cm).

It has been suggested that the smoke compound acts either by modulating the sensitivity of the tissue to PGRs, activation of enzymes or by modifying the receptor molecules (Malabadi and Vijaykumar, 2006, 2007; Senaratna *et al*. 1999; van Staden *et al*. 2004). Therefore, from the above results it is clear that active compound(s) within SSW

play a regulatory role in plant development (Kulkarni *et al.* 2006; Malabadi and Nataraja, 2007c; Malabadi *et al.* 2008c; Malabadi and Vijaykumar, 2006, 2007; Senaratna *et al*. 1999; van Staden *et al*. 2004). 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.

References

Daws MI, Pritchard HW, van Staden J **(**2008) Butenolide from plant-derived smoke functions as a strigolactone analogue: Evidence from parasitic weed seed germination. *South African Journal of Botany* **74**, 116-120

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD (2004) A compound from smoke that promotes seed germination. *Science* **305**, 977

Ichihashi S (1998) Control of growth and development of protocorm-like bodies derived from callus by carbon sources. *Plant Biotechnology* **15**, 183-187

Johnson TE, Kane ME (2007) Asymbiotic germination of ornamental *Vanda*: *in vitro* germination and development of three hybrids. *Plant Cell Tissue and Organ Culture* **91**, 251-261

Kanjilal B, Sarkar DD, Mitra J, Datta KB (1999) Stem disc culture: development of a rapid mass propagation method for *Dendrobium moschatum*, an endangered orchid. *Current Science* **77**, 497-500

Kulkarni MG, Sparg SG, Light ME, van Staden J (2006) Stimulation of rice (*Oryza sativa* L.) seedling vigour by smoke-water and butenolide. *Journal of Agronomy and Crop Sciences* **192**, 395-398

Lakshmanan P, Lee CL, Loh CS, Goh CJ (1995) *In vitro* propagation of commercial orchids. An assessment of current methodology and development of a novel approach of thin cross section culture. In: Islam AS (Ed) *Plant Tissue Culture*, Oxford IBH New Delhi, India, pp 42-49

Malabadi RB Mulgund GS, Nataraja K (2004) Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. *Plant Cell, Tissue and Organ Culture* **76**, 289-293

Malabadi RB, Mulgund GS, Nataraja K (2005a) Micropropagation of *Dendrobium nobile* from shoot tip sections. *Journal of Plant Physiology* **162**, 473-478

Malabadi RB, Nataraja K (2007a) Brassinosteroids influences *in vitro* regeneration using shoot tip sections of *Cymbidium elegans* Lindl. *Asian Journal of Plant Sciences* **6 (2)**, 308-313

Malabadi RB, Nataraja K (2007b) Genetic transformation of *Vanilla planifolia* by *Agrobacterium tumefaciens* using shoot tip sections. *Research Journal of Botany* **2 (2)**, 86-94

Malabadi RB, Teixeira da Silva JA, Mulgund GS (2008a) Micropropagation of *Eria dalzelli* (Dalz.) Lindl. through TCL *in vitro* culture. Floriculture and Ornamental Biotechnology 2(2):77-80.

Malabadi RB, Teixeira da Silva JA, Nataraja K, Mulgund GS (2008b) Shoot tip transverse thin cell layers and 24-epibrassinolide in the micropropagation of *Cymbidium bicolor* Lindl. Floriculture and Ornamental Biotechnology 2(2): 44-48.

Malabadi RB, Teixeira da Silva JA, Mulgund GS (2008c) Smoke-saturated water influences *in vitro* seed germination of *Vanda parviflora* Lindl. Seed Science and Biotechnology 2(2):65-69.

Malabadi RB, Nataraja K (2007c) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana*

A. B. Jacks. *Journal of Plant Sciences* **2**, 45-53

Malabadi RB, Vijaya Kumar S (2006) Smoke induced germination of some important medicinal plants. *Journal of Phytological Research* **19**, 221-226

Malabadi RB, Vijaya Kumar S (2007) Effect of smoke on seed vigour response of selected medicinal plants. *Journal of Phytological Research* **21**(1):71-75

Mitra GC, Prasad RN, Choudhury RA (1976) Inorganic salts and differentiation of protocorms in seed callus of orchid correlative changes in its free amino acid content. *Indian Journal of Experimental Biology* **14**, 350-351

Senaratna T, Dixon K, Bunn E, Touchell D (1999) Smoke-saturated water promotes somatic embryogenesis in geranium. *Plant Growth Regulation* **28**, 95-99

van Staden J, Ja¨ger AK, Light ME, Burger BV (2004) Isolation of the major germination cue from plant-derived smoke. *South African Journal of Botany* **70**, 654-657