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Temperature Pre-treatment of Seeds for Overcoming the Zygotic Embryo Dormancy of *Givotia rottleriformis* Griff. under *in Vitro* Conditions

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ABSTRACT

In vitro zygotic embryo culture provides a means for overcoming the long seed dormancy observed in some forest tree species. Temperature is the major factor that regulates seed dormancy in many plant species. The present study was conducted to evaluate the effect of pre-treatment of seeds at different temperatures in overcoming the zygotic embryo dormancy of *Givotia rottleriformis* Griff. under *in vitro* conditions. The germination frequency and plantlet conversion varied from zygotic embryo axes isolated from seeds pre-treated for 24 h at different temperatures (35-65°C) when cultured on Murashige and Skoog (MS) medium (1/2 macronutrients). High frequency of germination (96.5%) and plantlet conversion (94.5%) was achieved from zygotic embryo axes isolated from seeds that were pre-treated at 55°C for 24 h when cultured on MS (1/2 macronutrients) basal medium. Further, temperature pre-treatment of seeds promoted the development of healthy plantlets with vigorous shoot and root growth from the zygotic embryos cultured with endosperm on MS (1/2 macronutrients) medium. The plantlets developed from zygotic embryos with endosperm of temperature-treated (55°C for 24 h) seeds exhibited superior growth with increased height (37.8 cm), number of leaves (11.8), leaf length (19.6 cm) and leaf width (18.7 cm) per plants. These results demonstrated the effectiveness of temperature pre-treatment of seeds in overcoming the zygotic embryo dormancy *in vitro* and inducing rapid development of plantlets which can be exploited for mass propagation and conservation of *G. rottleriformis*.

Keywords: Euphorbiaceae, endosperm, germination, plantlet conversion, zygotic embryo axes

INTRODUCTION

Givotia rottleriformis Griff (family Euphorbiaceae) is a softwood tree species which is of high economic importance with wide applications (Rao *et al*. 1999). The wood of this species is light and soft and used for carving figures, toys and fancy articles in toy making industry. It is also used for making boxes, catamarans, and for purposes where lightness is required (Reddy *et al*. 2001). The seeds and bark powder of this tree species are known to have medicinal properties and used for the treatment of rheumatism, psoriasis and dandruff (Thammanna and Narayana Rao 1990). In addition, seeds possess high oil content which is valuable and used for lubricating fine machinery (Somasundaram 1980; Nayar *et al*. 1989). The tree has limited distribution and confined to small regions in forests of Andhra Pradesh, Karnataka, Tamil Nadu and West Bengal states of India. Natural regeneration of this species is constrained due to long seed dormancy for 1-2 years, vertebrate predation and poor seed germination in soil (Rao *et al*. 1999). The problem is further compounded by the over-exploitation of this species for softwood and habitat destruction which is endangering the population of this tree species. Propagation of this species through stem cuttings has been achieved by treatment with auxins such as IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) and NAA (α-naphthaleneacetic acid) which varied with the seasons. Reddy *et al*. (2001) observed that the treatment of the stem cuttings with 4000 mg/l NAA induced higher percentage of rooting (40%) than 4000 mg/l IBA (30%) or IAA (28%) in summer (June-July) as compared to winter (November-December) season. Babitha *et al*. (2002) reported highest rooting percentage (77.8%) from stem cuttings of *G. rottleriformis* treated with 3000 mg/l IBA which differed significantly from those treated with NAA (73.3%) and IAA (40.0%) at 3000 mg/l. Although propagation has been achieved from stem cuttings of *Givotia*, the lack of availability of sufficient number of stem cuttings imposes practical constraints in raising plantations on large scale. There has been growing interest to establish plantations of *Givotia* particularly to meet the demand of softwood which has multiple uses. However, the major problem in growing these trees is the lengthy seed dormancy and lack of methods for breaking seed dormancy in soil.

Seed dormancy can be caused by the maternally derived seed covering structures or by embryonic factors, acting individually or in combination (Koornneef *et al*. 2002; Black *et al*. 2008). It is controlled by several environmental factors, such as light, temperature and the duration of seed storage. The most prevalent form of seed dormancy is physiological dormancy (Baskin and Baskin 2004) which decreases with time under dry storage conditions through a process called after-ripening (Holdsworth *et al*. 2008). Temperature is a major environmental factor responsible for changes in dormancy status of seeds, and dormancy can be broken by fluctuating temperature or warm/cold stratification (Baskin and Baskin 1998). In most species, dormancy is alleviated faster with increasing after-ripening temperature (Bazin *et al*. 2011). Various methods are used for breaking seed dormancy, which depends on the plant species and type of dormancy. Treatments such as sulphuric acid, hot water and feeding to goats were used to break seed dormancy in *Givotia* which were unsuccessful (Naidu *et al*. 2001). The plant hormones gibberellins (GAs) and abscisic acid (ABA) are involved in seed dormancy and germination. It has been shown that ABA plays a role in the induction and maintenance of seed dormancy, whereas gibberellic acid (GA_3) is associated with dormancy breaking and ger-

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mination (Kucera *et al*. 2005). Attempts have been made to break the seed dormancy in *Givotia* by treatment with GA_3 which proved to be ineffective in inducing seedling emergence (Kiran *et al*. 2001). Rambabu *et al*. (2005) showed that pre-treatment with 86.6 mM GA_3 results in an enhancement of *in vivo* seed germination in *Givotia*.

Embryo culture *in vitro* is an increasingly applied method for overcoming seed dormancy and reduces the generation cycle of plant species (Collins and Grosser 1984). The first studies on zygotic embryo culture in *Givotia* were performed by Rambabu *et al*. (2005) who reported 100% germination of zygotic embryo axes on MS full-strength basal medium with 3% sucrose and promotion of seedling growth by addition of 100 mg/l tyrosine in the medium. In our previous report (Samuel *et al*. 2009), we showed that acid scarification of fresh seeds has resulted in high frequency of germination from zygotic embryo axes when cultured on MS (Murashige and Skoog 1962) medium (1/2 macronutrients) with 28.9 µM GA_3 whereas zygotic embryo axes of non-acid scarified one-year-old seeds germinated and converted into plantlets on MS basal (1/2 macronutrients) medium. The present investigation aimed to evaluate the effect of temperature pre-treatment of seeds in overcoming the zygotic embryo dormancy under *in vitro* conditions.

MATERIALS AND METHODS

Plant material, pre-treatments and surface sterilization of seeds

The mature seeds of *G. rottleriformis* were collected during February 2009 from trees growing at the Regional Forest Research Centre, Rajahmundry, Andhra Pradesh. Embryo culture studies were conducted from seeds stored for 8-9 months at room temperature. The epicarp and mesocarp of the seeds is manually removed with a cutter and the seeds with stony endocarp were subjected to pre-treatments at different temperatures (35, 45, 55 and 65°C) for 24 h in a hot air oven (Cassia Siamia Technologies Pvt. Ltd., Hyderabad, India) followed by soaking in tap water for 24 h. The seeds were washed in a solution of 0.1% (v/v) Tween-20 (Sigma-Aldrich, St. Louis, USA) for 10 min and then rinsed 2-3 times in sterile double distilled water. The seeds were treated with 1% bavistin for 10 min followed by 2-3 washes with sterile double distilled water. The seeds were surface sterilized with 70% ethanol for 3 min followed by treatment with 0.1% $HgCl_2$ (mercuric chloride) for 10 min and then rinsed 4-5 times with sterile double distilled water.

In vitro culture of zygotic embryo axes (with or without endosperm) of temperature pre-treated and untreated seeds

The surface-sterilized seeds were cut gently and the mature zygotic embryo axes from temperature pre-treated and untreated seeds were cultured on MS (Murashige and Skoog 1962) medium (1/2 macronutrients) containing 3% sucrose (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), 0.8% (w/v) agar (Qualigens, Mumbai, India). In another experiment, the zygotic embryos with endosperm isolated from temperature-treated (55°C for 24 h) or untreated seeds were cultured on MS medium (1/2 macronutrients). The explants were inoculated into test tubes (25 × 150 mm) containing 20 ml of medium and closed with steristoppers (Riviera, Mumbai, India). A minimum of 20 explants was used for each treatment and the experiments were repeated three times. The pH of the media were adjusted to 5.8, before adding 0.8% (w/v) agar (Qualigens) and autoclaved at 121°C for 15 min. The cultures were incubated in the dark for one week and then moved to culture racks under light provided by white fluorescent tubes (65 µE m^{-2} s^{-2}) for a 16 h photoperiod at 25 ± 2°C. The data on germination percentage and plantlet conversion was determined after 45 days of culture. The zygotic embryos that showed emergence of hypocotyls, epicotyls or both were scored as germinated. The development of shoots with leaves and roots from the zygotic embryos was used as the criteria for calculating the plantlet conversion frequency. The data was analyzed statistically using Sigmaplot 12 software (Systat Software Inc.) by one-way analysis of variance (ANOVA) and the pair-wise means were compared by Newman-Keul's multiple comparison test at the 5% probability level.

Acclimatization of plantlets and establishment in soil

The *in vitro* developed plantlets of 2-months-old produced from zygotic embryo axes or zygotic embryos with endosperm on different media were carefully removed from the culture tubes and washed under running tap water until the agar sticking to the roots was completely removed. The plantlets were placed in plastic pots containing autoclaved mixture of soil: manure: vermiculite (3:1:1) and acclimatized in culture room for 4 weeks by covering with polythene covers and then transferred to glasshouse. The polythene covers were gradually removed and after 4 weeks of acclimatization in glasshouse, the plantlets were transplanted into earthen pots containing soil and farmyard manure in 3:1 (v/v) and irrigated with tap water every 2 d. For each treatment, a minimum of 20 plantlets were transferred to soil, and the data on the percentage of survival of plants, height of the plants (cm), number of leaves, leaf length (cm) and leaf width (cm) was taken after 5 months of transfer to soil. The measurements of leaf length and leaf width were taken from the third fully expanded leaves of the plantlets obtained from different treatments. The data represents the means of 10 plantlets which was subjected to one-way analysis of variance and the pair-wise means were compared using Newman-Keul's multiple comparisons test at the 5% probability level. After acclimatization in glasshouse, the plantlets were transplanted to soil and farmyard manure (3:1, v/v) in open field.

RESULTS

Effects of temperature pre-treatment of seeds on zygotic embryo germination and plantlet conversion

Zygotic embryo axes isolated from untreated seeds exhibited 51.6% germination on MS (1/2 macronutrients) medium but the germinated embryo axes failed to convert into plantlets. Experiments were conducted to investigate the effects of pre-treatment of seeds at different temperatures on zygotic embryo germination and plantlet conversion after culture on MS (1/2 macronutrients) medium. The optimal response in terms of germination frequency (96.5%) and plantlet conversion (94.5%) was achieved from zygotic embryo axes isolated from seeds that were pre-treated at 55°C for 24 h as compared to other temperature treatments tested (**Table 1; Fig. 1A**). The frequency of germination (71.3%) as well as plantlet conversion (37.7%) from zygotic embryo axes decreased with further increase in temperature to 65°C. *In vitro* cultured zygotic embryo axes of temperature-treated seeds started to germinate after 3-4 days of culture compared to those isolated from untreated seeds which showed signs of germination after 7 to 8 days of culture.

Combined effect of temperature pre-treatment of seeds and endosperm on germination and plantlet conversion

In the present study, pre-treatment of seeds at 55°C for 24 h followed by culture of zygotic embryos along with endosperm induced high frequency of germination (95.9%) along with subsequent plantlet conversion (94.8%) with development of vigorous shoots and well expanded leaves and profuse root growth (**Table 1; Fig. 1B**). In contrast, no germination was observed from zygotic embryos with endosperm of untreated seeds when cultured on MS (1/2 macronutrients) medium. Temperature-treated seeds did not respond for germination when cultured wholly on MS (1/2 macronutrients) medium indicating the necessity to isolate the zygotic embryos for recovering plants.

Table 1 Effect of temperature pre-treatment of seeds on germination frequency and plantlet conversion from zygotic embryo axes (with or without endosperm), plant survival and growth after 5 months of transfer to soil.

Explant type	Temperature used for pre-treatment	% germination *	% plantlet development *	% survival in soil	Average height of the plants (cm)**	Average No. of leaves**	Average leaf length** (cm)	Average leaf width** (cm)
Zygotic embryo axes	Without temperature treatment	51.6 ± 0.5 d	0.0	0.0	0.0	0.0	0.0	0.0
Zygotic embryo axes	35°C	52.9 ± 1.2 d	0.0	0.0	0.0	0.0	0.0	0.0
Zygotic embryo axes	45°C	62.2 ± 0.3 c	24.1 ± 2.2 c	62.0	10.5 ± 0.3 c	6.4 ± 0.2 c	10.6 ± 0.2c	9.8 ± 0.2 c
Zygotic embryo axes	55°C	96.5 ± 0.6 a	94.5 ± 0.2 a	80.0	26.2 ± 1.3 b	9.6 ± 0.5 b	14.7 ± 0.6b	13.5 ± 0.2 b
Zygotic embryo axes	65°C	71.3 ± 2.9 b	37.7 ± 0.8 b	60.0	12.2 ± 1.0 c	7.2 ± 0.3 c	10.8 ± 0.1c	10.2 ± 0.1 c
Zygotic embryo axes with endosperm	55°C	95.9 ± 0.5 a	94.8 ± 0.6 a	96.0	37.8 ± 1.0 a	11.8 ± 0.4 a	19.6 ± 0.4a	18.7 ± 0.6 a
Zygotic embryo axes with endosperm	Without temperature treatment	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*The percentage of germination and plantlet development was determined after 6 weeks of culture of the explants on MS (1/2 macronutrients) medium. Values are means ± SE of three experiments with each experiment consisting of a minimum of 20 explants.

** The data represents means ± SE of 10 plantlets.

Means followed by the same letter in a column are not significantly different at 5% probability level by Newman-Keul's multiple comparison test.

Acclimatization and survival of the plantlets obtained from different treatments

The plantlets developed from zygotic embryo axes of temperature-treated seeds were transferred to soil and initially acclimatized in culture room and then in glasshouse. The plantlets recovered from zygotic embryos with endosperm of temperature-treated seeds established at high frequency (96.0%) upon transfer to soil in comparison to those raised from zygotic embryo axes obtained from temperature-treated seeds which survived at a frequency of 80%. Further, it was observed that the growth of the plantlets from zygotic embryos with endosperm was superior in terms of plant height, number of leaves, leaf length and leaf width as compared to plantlets obtained from zygotic embryo axes of temperature-treated seeds (**Table 1; Fig. 1C, 1D**). The average height of the plantlets obtained from zygotic embryos with endosperm was found to be 37.8 cm whereas it ranged from 10.5 to 26.2 cm for plantlets obtained from other treatments. The average number of leaves (11.8), average leaf length (19.6 cm) and average leaf width (18.7 cm) also remained higher for plantlets obtained from zygotic embryos with endosperm of temperature-treated seeds as compared to plantlets recovered from zygotic embryo axes alone (**Fig. 1D**). The acclimatized plantlets exhibited normal growth in glasshouse (**Fig. 1E**) and continued their further development upon transplantation to soil in the field (**Fig. 1F**).

DISCUSSION

Zygotic embryo germination and plantlet conversion is stimulated by temperature pre-treatment of seeds

Zygotic embryo culture has been used to overcome seed dormancy in several plant species (Ho 1987) although the optimal conditions for germination vary with the species. Various studies involving embryo culture have shown the importance of pre-treatment of seeds, the stage of embryo development, type and concentration of the basal medium, the presence of carbohydrates, growth regulators, physical conditions such as temperature and light regime on germination and seedling development (Bridgen 1994; Raghavan 2003). Recently, Tafreshi *et al*. (2011) reported that leaching the seeds in water prior to embryo isolation removed germination inhibitors and led to a proper germination of the embryos producing seedlings with extended roots and shoots in *Taxus baccata* L. In our previous study, we observed that the zygotic embryo dormancy in *Givotia* was released after one year upon dry storage of seeds at room temperature (Samuel *et al*. 2009). This prompted us to evaluate the effect of temperature pre-treatment of seeds on *in vitro* zygotic embryo germination and plantlet development. To our knowledge, this is the first report concerning the effect of temperature pre-treatment of seeds on *in vitro* zygotic embryo germination and plantlet conversion in *G. rottleriformis*. The results showed that temperature-treatment of seeds at 55°C for 24 h was found to be optimum in terms of obtaining high percentage of zygotic embryo germination (96.5%) and plantlet conversion (94.5%) compared with other temperature treatments. The zygotic embryo axes of temperature-treated seeds germinated within 3-4 days whereas as the zygotic embryo axes of untreated seeds exhibited germination after 7-8 days. The promotive effect of high temperature treatment of seeds on germination has been demonstrated in different species (Sarker *et al*. 2000; Carneiro *et al*. 2010). Huarte and Benech-Arnold (2010) found that fluctuating temperature decreased ABA concentration prior to radicle emergence, and exogenous GA_3 enhanced seed germination at constant temperature in *Cynara cardunculus* L. In the present study, the zygotic embryo axes of seeds that were pre-treated at 35°C for 24 h exhibited low frequency of germination (52.9%) and lacked the potential for plantlet conversion suggesting that the exposure to 35°C was not sufficient to release embryo dormancy. In the present study, germination percentage (71.3%) and plantlet conversion (37.7%) from zygotic embryo axes of seeds decreased with further increase in temperature to 65°C which could be due to embryo weakening caused by higher temperature affecting their potential for germination as well as plantlet conversion. Sanyang *et al*. (2008) observed that pre-treatment of *Acacia* seeds with simmering hot water for longer than 10 min resulted in embryo weakening and delayed seed emergence.

Effect of endosperm in zygotic embryo germination and plantlet conversion

The characteristics of the endosperm affect the embryo during germination and subsequent seedling growth. The structure and chemical composition of the endosperm may alter the quality and quantity of the metabolites available for the embryo, seed osmotic potential, and germination (Parera *et al*. 1996). The *in vitro* culture studies with isolated mature zygotic embryos of conifers have indicated the importance of the megagametophyte to the embryo during early seedling growth (David *et al*. 1995). In the present study, we observed that growth of the zygotic embryo axes of temperature-treated seeds was promoted when cultured along with endosperm on MS (1/2 macronutrients) basal medium. These shoots were robust in nature with well expanded leaves and profuse root induction as compared to plantlets recovered from zygotic embryo axes without endosperm of temperature-treated seeds. The superior growth observed in these cultures could be due to the presence of growth promoting substances present in the endosperm of temperature-treated seeds. Lin and Leung (2002) noted that

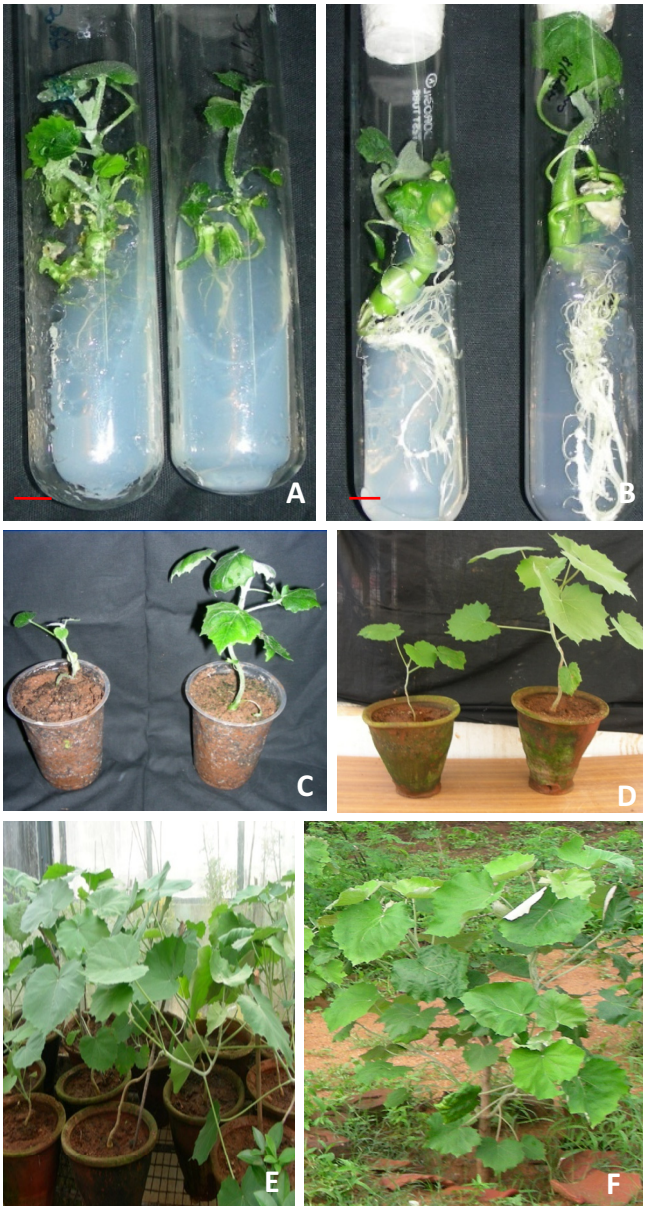

Fig. 1 Plantlet development from zygotic embryos of temperature-treated seeds of *Givotia rottleriformis* on MS medium (1/2 macronutrients) and their acclimatization and establishment in the field. (**A**) Development of plantlets from zygotic embryo axes of temperature-treated (55°C for 24 h) seeds after 45 days of culture on MS basal medium. (**B**) Development of plantlets from zygotic embryos with endosperm of temperature-treated (55°C for 24 h) seeds after 45 days of culture on MS medium. (Scale bar = 10 mm). (**C**) Comparison of growth of plantlets developed from zygotic embryo axes (left) and zygotic embryos with endosperm (right) after 2 months of transfer to glasshouse. (**D**) Comparison of growth from plantlets developed from zygotic embryo axes (left) and zygotic embryos with endosperm (right) after 5 months of transfer to glasshouse. (**E**) Establishment of plantlets developed from zygotic embryos with endosperm in glasshouse. (**F**) Establishment of plantlet developed from zygotic embryos with endosperm in soil in the field.

the emblings (plantlets developed from the culture of isolated zygotic embryos) of *Pinus radiata* were smaller than the seedlings, inspite of the fact that the former were grown in a nutrient-supplemented culture medium. A similar observation was made in a study on alfalfa emblings and seedlings (Lai *et al*. 1995).

The present experiments showed lack of germination from zygotic embryos with endosperm of untreated seeds on MS basal medium even after 30 days of culture. This could be due to the growth inhibitory substance(s) present in the endosperm of untreated seeds which was transported to the embryo during imbibition and suppressed its growth. Further research needs to be undertaken for identifying the growth promoting or inhibitory substances present in the endosperm of non-dormant and dormant seeds, respectively. Tzec-Sima *et al*. (2006) found that the presence of the endosperm, whether still attached to the embryos or separated from them but in direct contact with the nutrient medium, greatly reduced germination in isolated embryos of *Bactris major* Jacq. and *Desmoncus orthacanthos* Mart. Liao *et al*. (2006) achieved rapid and efficient *in vitro* germination and seedling development from mature embryos of *Taxus* by removal of endosperms followed by culture on half-strength MS media supplemented with 1.0 mg/l BAP (6-benzylaminopurine) and 0.1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). Zarck (2007) observed negative effect when the embryo was placed on the culture medium together with some residues of the endosperm independently from medium composition.

Our previous study showed that the dormancy present in the fresh mature seeds of *Givotia* was physiological and physical in nature (Samuel *et al*. 2009) and the intact fresh seeds failed to germinate on MS (1/2 macronutrients) medium containing GA_3 which supported the germination from isolated zygotic embryo axes. Embryo dormancy is characterized by inhibition of extension growth, while coat dormancy manifested as mechanical resistance from testa and endosperm to embryo growth (Finch-Savage and Leubener-Metzger 2006), or as chemical dormancy due to presence of inhibitor compounds in the covering layers of the seeds (Baskin and Baskin 2004). The present observations showed lack of germination from temperature treated (55°C for 24 h) seeds when cultured on MS (1/2 macronutrients) medium. This could be possibly due to the hard endocarp which acts as a mechanical barrier for germination since the isolated embryos of temperature-treated seeds germinated and converted into plantlets on MS medium.

Acclimatization and growth performance of plantlets

Acclimatization of *in vitro* produced plantlets is the most critical and difficult stage for tree species. Most studies related to *in vitro* zygotic embryo culture of tree species have focussed on identification of media and growth regulators to overcome the seed dormancy and there is limited information on acclimatization and performance of the plantlets derived from embryo cultures in the soil. Kaur *et al*. (2006) reported that plantlets produced from embryo cultures of walnut had roots that were robust and more developed than the leaves and these plantlets were successfully hardened and performed well in the field. In *Boswellia serrata*, the survival of seedlings produced from zygotic embryos on media lacking PVP (polyvinylpyrrolidone) and growth regulator remained in the range of 31 to 67% whereas the seedlings produced on growth regulator (auxin, cytokinin and GA_3) containing media were susceptible to environmental stress in the field (Ghorpade *et al*. 2010). Pech y Ake *et al*. (2007) studied the effect of GA_3 on plant conversion of coconut zygotic embryos and *ex vitro* survival. They observed that the survival was not dependent on the treatment but rather on the stage of development that had to be attained *in vitro* before the transfer to *ex vitro*. The present study revealed differences with respect to survival frequency and growth of the plantlets depending on the source of the explant and the temperature treatment given to the seeds. The study resulted in high rates of survival along with enhanced growth from plantlets recovered from zygotic embryos with endosperm of temperature-treated seeds (55°C for 24 h) as these plants had thicker shoots, expanded leaves and well developed roots. The procedure reported here represents an improvement to our previous published work (Samuel *et al*. 2009) in terms of achieving higher survival rate of plants in soil along with enhanced growth and can be used for reforestation and conservation of this species.

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