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# Putrescine Influences Somatic Embryogenesis and Plant Regeneration in *Pinus gerardiana* Wall

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**Abstract:** This study for the first time highlights the influence of putrescine on somatic embryogenesis in a commercially important Indian pine *Pinus gerardiana*. Mature zygotic embryos produced the highest percentage of embryogenic tissue on half strength MSG basal medium supplemented with 9.0 μM 2, 4-D and 2.0 mg $L^{-1}$ putrescine. However, the percentage of somatic embryogenesis was not similar in all the three genotypes of Chilgoza pine. The highest percentage of somatic embryogenesis (81.2±2.8a) was recorded in PG321 genotype. The lowest percentage of somatic embryogenesis (69.0±2.1a) was obtained in PG11 genotype. Incorporation of polyamine biosynthesis inhibitors in the initiation medium has inhibited somatic embryogenesis. Explants regained the embryogenic potential after the addition of the putrescine in the basal medium. These results support earlier findings of the role of polyamines in the process of somatic embryogenesis in several plant species including conifers.

**Key words:** Chilgoza pine, edible nut, micropropagation, polyamines

## INTRODUCTION

Plant regeneration *via* somatic embryogenesis is a complex and little understood process. It is the most promising technology for the large-scale production of elite genotypes. Factors limiting commercialization of somatic embryos for conifers include low initiation percentages, low culture survival, culture decline causing low or no embryo production and the inability of somatic embryos to fully mature, resulting in low germination and slow growth of somatic seedlings (Malabadi *et al*., 2002; Pullman *et al*., 2003). Another major problem for the commercialization of somatic embryogenesis is the recalcitrant nature of pines, which has resulted in the formation of very lower percentage of embryogenesis. *P. gerardiana* (Chilgoza pine) Walls is well known for fruit trade and its fruit is highly valuable as an edible nut. In India it is very much restricted in dry temperate regions of North-Western Himalayas between altitudes of 1600 to 3000 m above mean sea level. It is mainly distributed in district Kinnaur of Himachal Pradesh state which is situated between 77° 45' and 79° 35' E longitudes and between 31° 50' and 32°15' N latitudes. The larger proportion of nut production (190 tones per year) comes from Kinnaur alone and remaining requirement of this nut is met through import from Afghanistan. There is a lot of scope to domesticate and improve this crop through establishment of clonal seed orchard and control breeding for the enhancement of nut production both in quality and quantity (Singh, 1990). The seeds are locally marketed as chilgoza which are rich in carbohydrates and proteins. It is one of the most important cash crops of tribal people residing in the Kinnaur district of Himachal Pradesh state, India.

Polyamines, which include putrescine, cadaverine, spermidine and spermine, are naturally occurring low molecular weight, polycationic, aliphatic nitrogenous compounds that have been

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implicated in many important cellular processes such as cell division, protein synthesis, DNA replication and response to abiotic stress (Kakkar and Sawhney, 2002). Recent reports have indicated that polyamines could enhance somatic embryogenesis in several plant species including conifers (Fowler *et al*., 1996; Kakkar and Sawhney, 2002; Kevers *et al*., 2002; Sakhanokho *et al*., 2005; Pullman *et al*., 2003; Minocha *et al*., 1999; Amarsinghe *et al*., 1996; Santanen and Simola, 1997). *In vitro* clonal propagation of Chilgoza pine was reported by Singh (1990). Till today, no reports of somatic embryogenesis of Chilgoza pine are available in the literature. The objective of the present study was to determine the effect of putrescine on the initiation and establishment of embryogenic tissue using mature zygotic embryos of *Pinus gerardiana*. Our results demonstrated that putrescine can be used as a growth regulator for the initiation of embryogenic system in other recalcitrant genotypes of Chilgoza pine for the commercial forestry.

## MATERIALS AND METHODS

### Plant Material

*Pinus gerardiana* Walls seeds of a genotype (PG11 PG321 and PG100) of open pollinated trees were procured from the Himachal Pradesh Forest Department, Shimla, India. Seeds were surface cleansed with 1% citramide for 2 min and washed thoroughly with sterilized distilled water for three times. Seeds were further treated with sodium hypochlorite solution (4-5% available chlorine) for 2 min, rinsed 5 times with sterile double distilled water and treated with 6% hydrogen peroxide for 24 h. Prior to dissection of embryos, seeds were surface decontaminated sequentially with 0.1% $HgCl_2$ for 2 min, immersed in 70% ethanol for 3 min and finally rinsed thoroughly five times with sterile distilled water (Malabadi *et al*., 2002, 2005).

### Culture Medium and Initiation of Embryogenic Tissue

Mature zygotic embryos (Fig. 1A) of 3 genotypes (PG11 PG321 and PG100) were cultured individually on half-strength inorganic salts MSG medium (Becwar *et al*., 1990) containing 2.0 g $L^{-1}$ Gellan gum (Sigma), 90 mM maltose (Hi-media, Mumbai), 1 g $L^{-1}$ L-glutamine, 1 g $L^{-1}$ casein hydrosylate, 0.5 g $L^{-1}$ meso-inositol, 0.2 g $L^{-1}$ p-aminobenzoic acid and 0.1 g $L^{-1}$ folic acid. The medium was supplemented with a range putrescine concentrations (0.5, 1, 2, 3, 5, 7, 10, 12 and 15 mg $L^{-1}$) and 9.0 μM 2, 4-D. The cultures were raised in 25×145 mm glass culture tubes (Borosil) containing 15 mL of the medium and maintained in dark for 4-6 weeks at 27±3°C. Nutrient medium without putrescine (Sigma Co. St Louis, USA) is served as a control. The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium was then sterilized by autoclaving at 121°C and 1.08 kg $cm^{-2}$ for 15 min. L-glutamine, p-aminobenzoic acid and putrescine were filter sterilized and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonal suspensor masses by morphological and cytological observations of callus. The cultures showing white mucilaginous embryogenic tissue were identified and subcultured on the initiation medium (Fig. 1B) for further three weeks for the better development of embryonal suspensor masses. The half-strength (inorganic salts) MSG medium (Becwar *et al*., 1990) supplemented with 9.0 μM 2, 4-D and 2.0 mg $L^{-1}$. Putrescine was used as an initiation medium for this purpose.

### Effect of Polyamine Biosynthesis Inhibitors

In order to check the role or effect of putrescine, the initiation and following maintenance medium were modified by the addition of polyamine biosynthesis inhibitors DFMO (difluromethylornithine) and DFMA (difluromethylarginine) at various concentrations ($10^{-5}$ to $10^{-3}$ M) after autoclaving. These additives did not appear affect the general health of the cultures, except that there was some

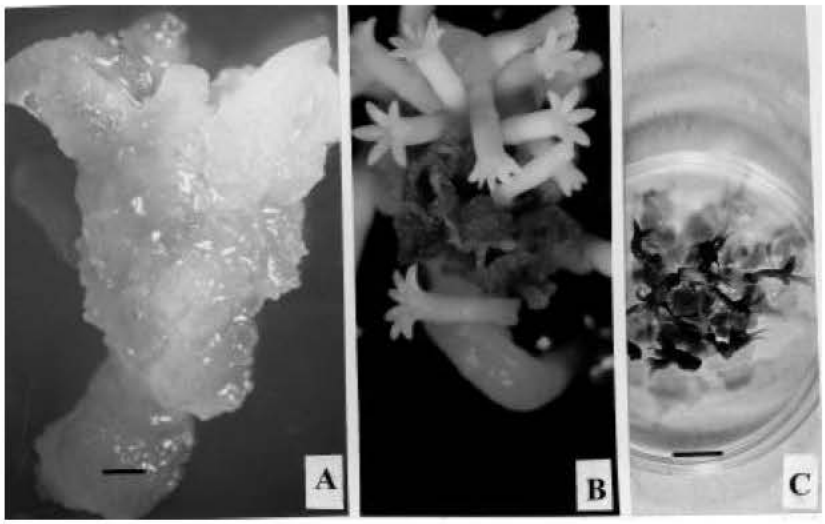


Fig. 1: Effect of putrescine on somatic embryogenesis of *P. gerardiana*. (A) Zygotic embryo showing white mucilaginous embryogenic callus on initiation medium (I) (scale bar 1 = 1.3 cm). (B) Development of advanced cotyledonary somatic embryos on maturation medium (scale bar 1 = 0.9 cm). (C) Somatic seedlings on germination medium (scale bar 1 = 1.2 cm)

delayed cleavage polyembryogenesis at the highest concentration ($10^{-3}$M). Putrescine was obtained as its hydrochloride from Sigma Co (St. Louis, MO, USA). DFMO and DFMA were obtained from Marion Merrell Research Co. Cincinnati, OH and were incorporated into the media after filter sterilization using a 0.22 μM filter (Sartorius Ltd.) to obtain the desired concentration range of $10^{-5}$ to $10^{-3}$ M.

**Maintenance of Embryogenic Tissue**

The white mucilaginous embryogenic tissue developed on the above initiation medium (I) was subcultured on maintenance medium (II). The half-strength (inorganic salts) MSG medium containing 130 mM maltose, 4 g $L^{-1}$ Gellan gum and supplemented with 2 μM 2, 4-D and 0.5 mg $L^{-1}$ putrescine (maintenance medium) was used for this purpose. On the maintenance medium, the embryogenic tissue containing embryonal suspensor masses was maintained for 3 weeks with two subcultures. All the cultures were maintained in dark and microscopic observation of cultures was conducted to ensure the development of pro-embryo.

**Maturation of Somatic Embryos**

After partial desiccation of 24 h (Malabadi and van Staden, 2005a-c; Malabadi *et al*., 2004; Malabadi and Nataraja, 2006; Malabadi *et al*., 2006), the embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development (Fig. 1C). The half strength (inorganic salts) MSG medium supplemented with 180 mM Maltose, 60 μM ABA and 8 g $L^{-1}$ Gellan gum (maturation medium) was tested for this purpose (Malabadi *et al*., 2005). All the cultures were again maintained in the dark for 12 to 14 weeks.

**Germination and Plantlet Recovery**

After 12 to 14 weeks of maturation, advanced cotyledonary somatic embryos were picked from the cultures for germination. The germination medium used was half strength (inorganic salts) MSG

medium with 2 g $L^{-1}$ Gellan gum. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in growth room under a 16 h photoperiod (50 µmol $m^{-2}$ $sec^{-1}$) for hardening.

**Statistical Analysis**

In all the above experiments each culture tube received a single explant. Each replicate contained 50 cultures and one set of experiment is made up of 2 replicates (100 zygotic embryos were cultured for each genotype for one set of experiment). All the experiments were repeated 3 times (total 900 cultures for 3 independent experiments of three genotypes). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's Multiple Range test (a = 0.05) following ANOVA. All statistical analysis was performed using the SPSS statistical software package.

## RESULTS AND DISCUSSION

This study demonstrated that putrescine, a natural member of polyamine, enhances embryogenic tissue formation in all the three genotypes of *P. gerardiana*. In the present study, mature zygotic embryos produced the highest percentage of embryogenic tissue on half strength MSG medium supplemented with 9.0 µM 2, 4-D and 2.0 mg $L^{-1}$ putrescine (Initiation medium) in all three genotypes tested (Fig. 1A and Table 1). However, the percentage of somatic embryogenesis was not similar in all the three genotypes of Chilgoza pine (Table 1 and 2). The highest percentage of somatic embryogenesis (81.2±2.8a) was recorded in PG321 genotype. On the other hand the lowest percentage of somatic embryogenesis (69.0±2.1a) was obtained in PG11 genotype. In case of PG100 (70.0±1.6a) of somatic embryogenesis was noticed (Table 1 and 2). On the other hand mature zygotic embryos cultured on half-strength MSG basal medium containing 9.0 µM 2, 4-D without putrescine (control) and with higher concentrations of putrescine (7, 10, 12 and 15 mg $L^{-1}$) produced white glossy non-embryogenic tissue in all three genotypes of *P. gerardiana*. The cultures were failed to produce embryonal suspensor masses and ultimately resulted in the browning of tissue and discarded. On the other hand mature zygotic embryo explants produced white mucilaginous embryogenic tissue on MSG containing 9.0 µM 2, 4-D with putrescine (0.5-5.0 mg $L^{-1}$) (Table 1). The embryogenic tissue showed elongated cells with a few undergoing cleavage polyembryony. In all the three genotypes, explants failed to produce embryogenic tissue after the addition of polyamine biosynthesis inhibitors in the

Table 1: The effect of various concentrations of putrescine on the initiation of embryogenic cultures in three genotypes of *P. gerardiana* cultured on half-strength MSG medium containing 9.0 µM 2,4-D

| | Embryogenic cultures in 3 genotypes (%) | | |
|---|---|---|---|
| Putrescine (mg $L^{-1}$) | PG11 | PG321 | PG100 |
| Control | 0 | 0 | 0 |
| 0.5 | 3.0±0.1c | 1.0±0.1c | 6.0±0.1c |
| 1.0 | 15.0±0.1b | 1.0±0.3 c | 14.0±1.2b |
| 2.0 | 69.0±2.1a | 81.2±2.8a | 70.0±1.6a |
| 3.0 | 27.4±2.3b | 20.8±4.0 b | 11.5±3.0b |
| 5.0 | 2.0±0.1c | 4.0±0.1c | 1.0±0.1c |
| 7.0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 |

Data scored after 6 weeks and represents the mean±E of at least 3 different experiments. In each column the values with different letters are significantly different ($p<0.05$); Control = MSG medium without putrescine

Table 2: Somatic embryogenesis and seedling recovery in three genotypes of *P. wallichiana*. *Explants produced maximum number of embryogenic cultures on half-strength MSG medium supplemented with 9.0 μM 2,4-D and 2.0 mg $L^{-1}$ putrescine

| Genotype | Somatic embryogenesis (%) | Total No. of somatic embryos recovered $g^{-1}$ fresh wt. of embryogenic tissue | Total No. of seedlings recovered $g^{-1}$ fresh wt. of embryogenic tissue |
|---|---|---|---|
| PG11 | 69.0±2.1a* | 41.0±2.5b | 20.0±2.6c |
| PG321 | 81.2±2.8a* | 32.0±1.8b | 17.0±1.1c |
| PG100 | 70.0±1.6a* | 30.6±2.6b | 19.0±1.3c |

Data scored after 14 weeks and represents the means (±SE) followed by the same letter in each column were not significantly different at $p<0.05$

basal medium. Cultures regained the embryogenic efficiency by the addition of external putrescine in the basal medium (data not shown). This paper presents only optimum results.

The white mucilaginous embryogenic callus was subcultured on maintenance medium for the further development of embryonal suspensor masses. The pro-embryos developed on the maintenance medium could not grow further, until they were transferred on a medium with enhanced maltose, ABA and Gellan gum, respectively. The callus developed somatic embryos on maturation medium after 12 to 14 weeks in all the three genotypes of *P. gerardiana* (Fig. 1B and Table 2). The total number of somatic embryos recovered per gram fresh weight of embryogenic tissue and somatic seedlings is shown in Table 2. After maturation, the advanced cotyledonary somatic embryos were picked up for the germination (Fig. 1C). The half-strength MSG medium without growth regulators was used as a germination medium. After 6 weeks, the somatic seedlings were recovered and hardened in all three genotypes of Chilgoza pine.

In our present study the highest percentage of embryogenic cultures was recorded at 2.0 mg $L^{-1}$ putrescine in all the three genotypes tested (Table 1). This clearly indicates the positive role of putrescine on induction of embryogenesis in *P. gerardiana*. The addition of putrescine in the basal medium did not significantly affect callus growth but promoted the formation of somatic embryos. The stimulation of somatic embryogenesis by exogenous putrescine has already been reported in a number of other plant tissue and cell cultures (Yadav and Rajam, 1997; Sakhanokho *et al*., 2005; Kakkar and Sawhney, 2002; Kevers *et al*., 2002). In case of cotton which was essentially recalcitrant without putrescine treatment, inclusion of 0.5 mg $L^{-1}$ putrescine improved somatic embryo induction for most treatments and lines tested (Sakhanokho *et al*., 2005). Putrescine also enhanced somatic embryo formation and plant regeneration in elite oat species (*Avena sativa* and *Avena nuda*) (Kelley *et al*., 2002). Exogenous administration of putrescine at a concentration of 40 mM resulted in maximum tissue response in terms of *in vitro* shoot numbers and shoot lengths in *Cichorium intybus* cv. Lucknow local (Bais *et al*., 2000). In *Melia azedarach*, somatic embryos without putrescine developed abnormal cotyledons and the addition of putrescine in the culture medium avoided this problem (Sharry *et al*., 2006). In addition, the addition of putrescine also induced secondary somatic embryogenesis in *Melia azedarach* (Sharry *et al*., 2006). This promoting action of putrescine on somatic embryogenesis may be due to an efficient conversion of competent cells into embryos (Kumar *et al*., 2002; Ponce *et al*., 2006; Kumar *et al*., 1996). The stimulatory effect of putrescine in somatic embryogenesis of *Solanum melongena* has been shown not to be due to the added nitrogen (Yadav and Rajam, 1997). The present counter-experiments using inhibitors of polyamine biosynthesis indirectly confirm this fact. The endogenous polyamine level of the tissue and the stage at which polyamine was applied influenced the action of exogenous putrescine on differentiation of somatic embryos (Bouchereau *et al*., 1999; Roy and Ghosh, 1996; Roy *et al*., 1996; Kelley *et al*., 2002; Kevers *et al*., 2002; Yadav and Rajam, 1997; Cvikrova *et al*., 1999). In case of ginseng cell suspensions, it was showed that addition of polyamine biosynthesis inhibitors (DFMA and DFMO) to either induction or regeneration medium significantly reduced the number of regenerated structures (Kevers *et al*., 2000).

In our present study also explants in all the three genotypes failed to produce embryogenic tissue after the addition of polyamine biosynthesis inhibitors in the basal medium. Cultures regained the embryogenic efficiency by the addition of external putrescine in the basal medium. These observations suggest that putrescine is involved in the induction and regeneration of somatic embryos (Pullman *et al*., 2003). In carrot, the expression of mouse ornithine decarboxylase cDNA promoted somatic embryogenesis (Andersen *et al*., 1998).

Further, in conifers the active polyamine metabolism during development of somatic embryos and maturation of zygotic embryos of *Picea abies* suggest that polyamines regulate embryo development (Santanen and Simola, 1997). Until, now the role of polyamines in embryogenesis has mainly been studied as variations in levels of free polyamine and by inhibiting polyamine synthesis, which usually blocks embryo development. Polyamines are however, ubiquitous molecules and also participate in so-called house-keeping reactions in cells (Amarasinghe *et al*., 1996). Therefore, it is important to compare their metabolism in cells entering embryogenesis with their metabolism in cells only dividing and ageing on nutrient medium (Walden *et al*., 1997). Polyamines bind covalently to enzymes and structural proteins, modifying them post-transnationally and changing properties which may exert a regulatory function in embryogenesis and other developmental manifestations (Santanen and Simola, 1997; Amarsinghe *et al*., 1996). A characteristic pattern of changes in polyamine metabolism during the development of somatic as well as zygotic embryos in *Pinus radiata* is evident (Minocha *et al*., 1999). Furthermore, the ratios of polyamines are significantly different in the developing embryos and the non-differentiating residual tissue. This has resulted in higher yields of somatic embryos per gram fresh weight of cultured tissue as well as improved maintenance of the embryogenic potential of these cell lines through serial transfers in *Pinus radiata* (Minocha *et al*., 1999).

Polyamines are small polycations found in most organisms and are essential for cellular proliferation and normal cellular function (Kevers *et al*., 2002; Kakkar *et al*., 2000). In higher plants, putrescine can be derived through two different pathways from ornithine *via* ornithine decarboxylase or from *via* arginine decarboxylase, both of which are specific, rate-limiting enzymes (Malmberg *et al*., 1998; Bais *et al*., 2000; Berta *et al*., 1997). Many of the studies demonstrating enzyme and putrescine functions have been made possible due to use of inhibitors, such as Difluromethylarginine (DFMA, a specific and irreversible inhibitor or arginine decarboxylase), Difluromethylornithine (DFMO, an inhibitor of ornithine decarboxylase) (Martin-Tanguy, 1997). It has been demonstrated in various plant tissues that ornithine decarboxylase pathway is particularly active in cell proliferation and that the arginine decarboxylase pathway is involved in embryo and organ differentiation and stress responses (Gemperlova *et al*., 2005; Ponce *et al*., 2006; Bais *et al*., 2000). Inhibition of polyamine biosynthesis blocks differentiation in plants, but the effect can be removed by addition of polyamines (Walden *et al*., 1997; Kumar *et al*., 1996, 1997, 2002). However, the role of polyamines in plant morphogenesis is not yet clear (Minocha *et al*., 1999; Amarsinghe *et al*., 1996; Santanen and Simola, 1997). Our results are in agreement with the available literature.

A low conversion of somatic embryos to plantlets can constitute a serious hurdle as it reduces the likelihood of recovering transformation events. Therefore, a good regeneration protocol should not only induce a high frequency of somatic embryos but also subsequently convert those somatic embryos into plantlets. A lower conversion frequency of somatic embryos into plantlets was also noticed in our present study (Table 2). In conclusion the addition of putrescine in culture media resulted in the formation of higher percentage of embryogenic cultures in a recalcitrant commercially important Indian pine *P. gerardiana*. The data presented also confirm that production of somatic embryos can be regulated by the manipulations of polyamine levels and metabolism either by using exogenous polyamines or their specific metabolic inhibitors. Under appropriate conditions, the embryogenic tissue obtained can further develop into somatic embryos and plantlets, which opens up the possibility of mass production of somatic embryos of *P. gerardiana* for the commercial forestry.

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