Somatic Embryogenesis and Organogenesis from Embryonic Explants of *Pinus gerardiana*

Mohammad Saeed[1], Mohammad Naeem Shahwani[1], Shahjahan Shabbir Ahmed[1], Nazeer Ahmed[1], Ghazala Shaheen[2], Agha Mohammad Raza[1]

[1]Faculty of Life Sciences and Informatics, Balochistan University of Information Technology, Engineering and Management Sciences, Quetta, [2]Department of Botany, University of Balochistan, Quetta.

Abstract

Pinus gerardiana (chilgoza pine) is an ecologically and economically important pine species. It is a gourmet non timber product harvested in natural stands in Pakistan. These forests are confined to the eastern Afghanistan parts of Pakistan and India scattered in dry inner valleys of northern Himalayas. Extensive destruction through logging of trees is leading to fast deforestation. Its seeds have low rate of germination and lose their viability relatively faster due to seed borne fungi and bacteria. These factors call attention to the in vitro culture of chilgoza pine nut. The pines are known to have inherent recalcitrancy of the mature and differentiated tissues. Therefore mature zygotic embryos (MZE) and juvenile cotyledons have been used for the in vitro culture in present study. MZEs and embryonic cotyledons were cultured in MS medium containing various concentrations of BAP and 2,4D, so as to achieve bud initiation and embryogenesis. The frequency of bud initiation is affected by BAP concentration. Buds were induced on MZEs in MS medium containing 7.5µM and 10 µM concentration of BAP. While at 2.5µM and 5.0 µM concentration of BAP only large calluses were observed on both MZEs and embryonic cotyledons. Embryogenic calluses were induced on MZEs in MS medium containing 2.5µM and 5.0 µM concentration of 2,4D. While at 7.5µM and 10 µM concentrations of 2,4D profuse callusing was observed in both MZEs and embryonic cotyledons. In present study, it has been observed that cotyledons excised from the embryos are appropriate material for the induction of adventitious buds. MZE were found to produce embryogenic cultures in P.gerardiana

Keywords: Pinus, Forests, MZEs, BAP, P.gerardiana

Corresponding author's email: msaeed1963@gmail.com

INTRODUCTION

P. Gerardiana Wall. Ex Lamb (*chilgoza* pine) is a small to moderate sized tree. It is confined to the eastern Afghanistan parts of Pakistan and India, scattered in dry inner valleys of northern Himalayas (Critchfeild & Little, 1966). It grows on the elevation from 1600 m to 3400 m above the sea level.

Chilgoza pine carries ecological and economic importance. The pine is valued for its large edible seeds which are relished as a dry fruit. *Chilgoza* nut are harvested in natural stands. The pine earns cash for local inhabitants. It is among very few pine species that have been considered as important pine nut producers (Siberian pine, *Pinus sibirica*; Korean pine, *Pinus koraiensis*; Italian stone pine, *Pinus pinea*; pinyon pines, including, *Pinus monophylla* and Colorado pinyon, *Pinus edulis*).

The *chilgoza* pine forest faces many threats which include indiscriminate felling of trees for timber and clearing of forest for orchards. Due to seed borne fungi and bacteria *P. gerardiana* seeds have low rate of germination and a brief period of viability (Saeed, 2000).

In such circumstances *in vitro* culture of the pine can be a potential tool for the conservation and propagation. *In vitro* culture can be used to propagate economically desirable characters in *P. gerardiana* such

as large seed size, increased cone production and more seeds per cone. Since pines have long life cycles and are open pollinated plants, the need for unconventional propagation particularly increases to propagate the plants with desirable traits.

The pines are known to have inherent recalcitrancy of the mature and differentiated tissues in response to *in vitro* propagation. Adventitious bud initiation and somatic embryogenesis have been considered the methods of choice for pine species for propagation. (Arya *et al*., 2000). Most of the studies on pine tissue culture have used immature or mature zygotic embryos due to the recalcitrant nature of the tissue (Dimantoglou *et al*., 1990; Bastola *et al*., 2000; Tang & Guo, 2001). The present study also uses mature zygotic embryos (MZE) and juvenile cotyledons for *in vitro* culture.

The economic significance of chilgoza pine is not limited to nut and timber production, they also perform ecological functions like water shed protection and food for wildlife. Even then scientific research work performed on *P. gerardiana* is very scarce. *In vitro* research on the species is yet limited; Gupta *et al.,* (1995) have reported shoot buds initiation from cotyledonary leaves of *P. gerardiana*. The present work presents the results of effect of MZEs and embryonic cotyledons cultured on MS medium containing various concentrations of BAP and 2,4D, so as to achieve bud initiation and embryogenesis.

MATERIALS AND METHODS

The seeds of *P. gerardiana* were provided by Department of Forest and Wildlife Balochistan, which were obtained from open pollinated trees growing in State forest District Zhob; Balochistan. The seeds were washed with distilled water. Seed coat was removed from the seeds and megagametophyte was exposed. The megagametophytes were washed in distilled water with 2-3 drops of Tween® 20 and subsequently immersed in 0.1%solution of $HgCl_2$ for 5 min or alternatively treated with 20 % solution of commercial bleach for 20 min and afterward immersed in 70 % ethanol for 30 sec. The megagametophytes were soaked for 12-18 hr at 24 °C in a petri dish which was lined with two layers of sterilized filter paper and moistened with 10 mL of distilled water. MZEs were then aseptically excised from megagametophytes.

The excised embryos were placed for germination on 1× MS (Murashoge & Skoog, 1962) basal medium with their radicle ends submerged in medium. The cotyledons of viable embryos open after one week and turn green (figure. 1). Non-viable embryos were discarded. Viable MZEs and excised juvenile cotyledons from MZEs were then transferred to their respective media. Both MZE and excised cotyledons were horizontally placed on the media. The explants were examined every third week, the calluses were sub cultured every fourth week. For each experiment, 5 replicates of 10 cotyledons per petri-dish were used, while in case of MZE 5 replicates of 5 test tubes, each containing one explant were used.

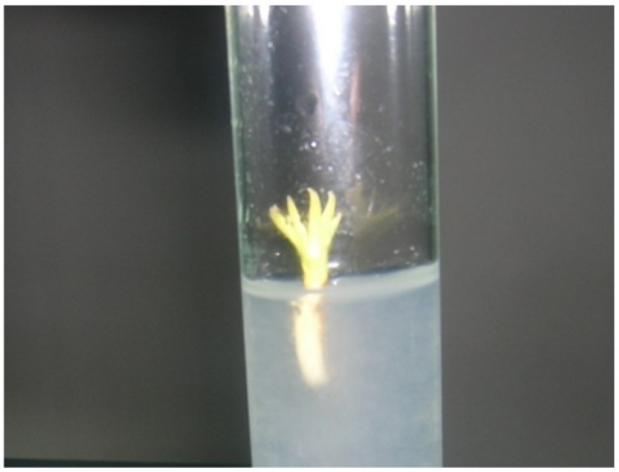

Figure 1: Mature zygotic embryo of *P. gerardiana* placed cultured on basal MS medium.

The culture was maintained at 24 ± 2 °C and 16h of light in plant tissue culture chamber. 1× MS media was used for all cultures. Media contained 2.0% Sucrose and was gelled with 0.8 % agar. pH was maintained at 5.8. All manipulations including washing, disinfecting, soaking of seeds and isolation of MZE were accomplished under laminar airflow.

The mean number of buds per embryo, standard deviation and Bud Forming Capacity (BFC) was determined following (Capuana and Giannini, 1995). BFC was determined as following:

BFC = (Average number of buds per plant × % explants forming buds) ÷100

Mature zygotic embryos and cotyledons were cultured on MS medium supplemented with 10.0, 7.5, 5.0, and 2.5 μM Benzylaminopurine (BAP) for the induction of adventitious buds. Mature zygotic embryos and cotyledons were cultured on MS medium supplemented with 10.0, 7.5, 5.0, and 2.5 μM 2, 4-dichlorophenoxyacetic acid (2,4D). The explants were examined every 3rd week for initiation of embryogenic calluses.

RESULTS AND DISCUSSION

Four concentrations of BAP *i.e*. 10.0, 7.5, 5.0, and 2.5 μM were used to induce adventitious buds on MZEs and cotyledons. The frequency of bud formation was affected by the concentration of BAP on cotyledons. Almost similar BFC was observed at 7.5 and 10.0 μM concentrations (figure. 2); though mean number of buds was slightly higher in 7.5 μM (Table. 1). On MZE no buds were initiated and only callusing was observed in all concentrations of BAP applied. Cotyledons have been used previously to initiate buds in *P. gerardiana* (Gupta *et al.,* 1995) and some other pines like *Pinus taeda* (Tang & Guo, 2001).

Figure 2: Buds formed on cotyledons of P. gerardiana in response to BAP on cotyledons.

Table 1. Effect of BAP concentration on Bud Forming Capacity (BFC) on juvenile cotyledons.

Conc. of BAP	Mean No. of Buds formed on explants	% explants forming buds ± SE	BFC
2.5	0	Only callus formed	0
5	0	Only callus formed	0
7.5	6	54± 0.54	3.24
10	6.5	52± 1.3	3.38

White and shiny calluses were produced in a large number of MZE and cotyledons. However they did not produce embryogenic calluses. The number of embryogenic culture formed on cotyledons was negligible in media supplemented with 2, 4D. The embryogenic calluses were observed in MZE cultured in media supplemented with 2.5, 5.0, 7.5, and 10.0 μM concentrations of 2, 4D, however at 2.5 and 5.0 μM the percentage of such calluses was higher as compared to 7.5 and 10.0 μM (figure. 3, 4). For maturation the cultures were transferred to MS basal media with 0.5 % activated charcoal (unpublished data). MZE have been successfully used to initiate embryonic cultures in some pine species (Klimaszweska *et al.,* 2000). However immature zygotic embryos have reported to be best explant for adventitious bud initiation as well as for somatic embryogenesis (Arya *et al*., 2000).

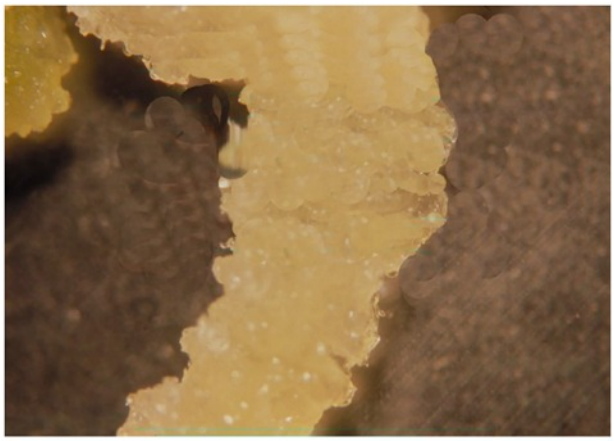

Figure 3: Embryogenic culture of *P. gerardiana* on MZE.

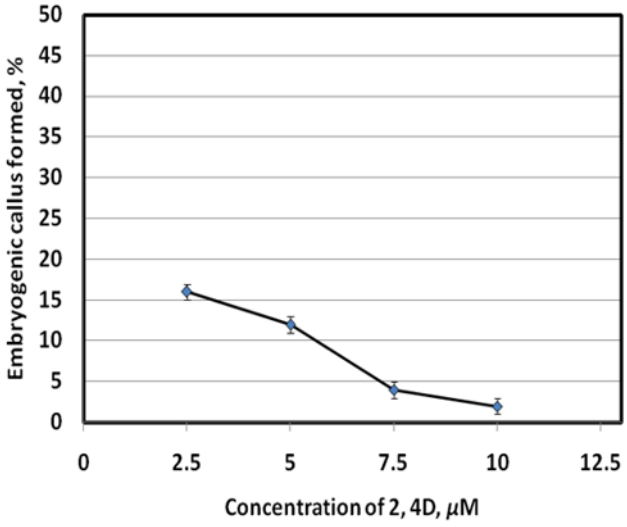

Figure 4: MZE of P. gerardiana forming embryogenic culture in 2, 4D.

CONCLUSION

In present study, we have observed that cotyledons excised from the embryos are appropriate material for the induction of adventitious buds. The concentrations of BAP that yielded optimum results for bud initiation in our study was 7.5 and 10.0 uM, however there is a room for the improvement in selection of media, optimization of culture conditions, and selection of right combination of cytokinins. The selection of cytokinin for the present study was restricted only to BAP as natural ones are more expensive, and due to short viability of *P*. *gerardiana* seeds there were always chances of losing the resources. This warrants further studies in this area.
MZE were found to produce embryogenic cultures in *P. gerardiana* However immature zygotic embryos have reported to be best material for adventitious bud initiation as well as for somatic embryogenesis in pines (Arya *et al*., 2000). The chilgoza pine grows in remote and hard to access areas in Balochistan it is very difficult to obtained immature cones at different developmental stages therefore MZE was adopted as choice material for *in vitro* culture.

REFERENCES

- Arya S, Kalia RK and Arya ID. (2000). Induction of somatic embryogenesis in *Pinus roxburghii* Sarg. Plant Cell Reports 19(8):775-780.

- Bastola DR, Agrawal VP and Joshee N. (2000). *In vitro* propagation of a Himalayan pine- *P. wallichiana* A. B. Jacks. Curr. Sci. 78(3):338-341.

- Capuana M and Giannini R. (1995). *In vitro* plantlet regeneration from embryogenic explants of *Pinus pinea* L. *In Vitro* Cell. Dev. Biol. Plant. 31: 202–206.

- Critchfield WB, Little EL. (1966). Geographic Distribution of the Pines of the world. US Department of Agriculture Forest service Miscellaneous Publication. Washington, DC.

- Diamantoglou S, Panagopoulos I, Munoz-Ferriz A, Rhizopolou S. (1990). *In vitro* studies of embryo growth callus formation and multiple bud induction of *Pinus pinea* L. Journal of Plant Physiology. 137: 58-63.

- Gupta D, Purohit M, Srivastava PS. (1995). Adventitious buds from cotyledonary leaves of *Pinus gerardiana* Wall., the chilgoza pine. Beitraege zur Biologie der Pflanzen. 68: 291-296.

- Klimaszweska K, Bernier-Cardou M, Cyr DR, Sutton BCS. (2000). Influence of gelling agents on medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. *In Vitro* Cell Dev. Biol.-Plant 36: 279-286.

- Murashige T, Skog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 15: 473-493.

- Saeed M. (2000). Comparative physiology and ecophysiology of regeneration (germination, reproduction, reproductive potential) of genus *Pinus*. PhD thesis, National & Kapodestrian University of Athens, Athens Greece.

- Tang W and Guo Z. (2001). *In vitro* propagation of loblolly pine via direct somatic organogenesis from mature cotyledons and hypocotyls. Plant Growth Regulation. 33: 25–31.