Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

Optimizing Micropropagation Protocol to Induce Somatic Embryogenesis and Organogenesis from Embryonic Explants of Pinus Gerardiana

Ajmal Khan[1]*, Mudassir Asrar[1]*, Zafran Khan[2]

[1]Botany Department, University of Balochistan, Quetta, Pakistan

[2]Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan

*Corresponding Author: ajmalmandokhail35@gmail.com

Abstract

Pinusgerardiana (chilgoza pine) is animportant ecological and economical pine species. Its forests regenerates certainly through the scattering of seed. The world's largest priest rank of Chilghoza, is on the verge of extinction in the Suleiman range of district Sherani and Zhob, Balochistan, Pakistan. Pinus seeds have low germination percentage due to fungal and bacterial attacks within seeds. These aspects draw attention for in vitro cultivation of *P. gerardiana*. In the current work, mature zygotic embryos (MZE) and juvenile cotyledons were used for in vitro culture. The young plant (Seedling) and the dried seeds of *P. gerardiana* was collected and brought to the lab from Chilghoza forest of District Sherani&Zhob, Balochistan. To achieve bud initiation and embryogenesis, MZEs and embryonic cotyledons were cultivated in MS media with varied concentrations of BAP and 2,4D. The best growth was found at concentration of 0.75mg/l that were recorded of 30%. In WP media the maximum growth was recorded 54% at concentration 0.5mg/l. In IBA treatment the best growth was observed with concentration applied of 0.75mg/l and results produced number of roots as3.0±0.5. AC in MS media showed the superlativeprogression of callus and shoot formation were discovered at 0.50mg/I, in WP media AC showed good effect on callus and shoot formation (40%) at 0. 50mg/l. In current study it was observed that cotyledon erased from the embryos are better option to be induce adventitious buds, here MZE was proved to produce better embryogenic culture in *P. gerardiana.*

Keywords: Pinus, Embryogenesis, Organogenesis,Tissue Culture, Invitro regeneration

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

Introduction:

Pines belongs to the subgroup of the conifers, which is famous for its cone-producing trees(Elferts*et al*., 2007). Chilgoza pine is having broad economic and ecological impacts and is restricted in distributionofBalochistanin the Suleiman Range, famous for containing the world's only absolute standpoint of Chilgoza (*Pinusgerardiana*) forest (Khan *et al*., 2015).

Pines are consumed unprocessed or baked and is also component of cakes, pastries, toffees, pulps, and cakes, along with in green vegetables and red meat meals for their distinct flavor. The nutritional significance of pine nuts is widely established. These are constituted as value of approximately high values in fats of 52%, 32% protein, 12% values for carbohydrate, 4% values of ash and 6% of moisture (Ozguven&Vursavus, 2005). This tree plays a vital role in the livelihood of the people and economic progress (Shalizi and Khurram 2016).Native tribes and wildlife in the area use the tree for food, medicine, and timber. The tree generates local income and revenue, protects the soil surface, maintains an appropriate microclimate, provides shelter, and serves as a sanctuary for wildlife.

*Pinusgerardiana*forests regenerates certainly through the scattering of seed. “The production of seeds is not uniform every year” (Malik and Shamet, 2008). Newly hatched chilgoza seedlings have a slim chance of survival since they are drought susceptible and have least water resistant(Kumar *et al*., 2014).Snow, flash of lightning, wind impairment, insects, and fungus outbreak are some of the natural and manmade threats to the Chilgoza forest. Grazing by animals is degrading the PinusForest at numerous spots where it needs to regrow (Ahmed *et al*., 2009). The species' in vitro research is quite restricted (Gupta *et al*., 1995). Chilgoza pine is being nominated as near threatened (NT) species by IUCN red list” (Anonymous, 1969) (IUCN, 2018).

The Pinusnewly germinating seeds are facing some serious issues like dormancy, seed born fungus and hard seed coat. It is challenging to breakdown the dormancy of seeds.The natural and classical used methods crashed to raise its rate of germination(Akbar *et al*., 2013).In such conditions, pine in vitro culture may be a viable option for conservation and propagation. Large seed size, greater production of cone, and the amount of more seeds per cone are all commercially desired features. Tissue culture techniques provides supplementary means for its propagation. (Murashige, 1974; Start and Cumming, 1976).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

The mature and differentiated tissues of pines are acknowledged to be recalcitrant in response to in vitro propagation. For pine species propagation through means of buds and adventitious somatic embryogenesis have been preferred as method of callus induction (Arya *et al.*, 2000). Because of the tissue's recalcitrance, most researchers used tissues of pines, and applied the invitro propagation mean using immature or matured zygotic embryos. (Bastola*et al*., 2000; Tang &Guo, 2001).

The world's largest priest rank of Chilghoza, is on the verge of extinction in the Suleiman range of district Sherani and Zhob, Balochistan, due to carelessness, overexploitation, and a lack of conservation strategies(Ahmed *et al*., 2011). The Chilghoza forest required immediate interest and must be preserved. Current research work was done to optimize the process for a more fruitful and systematic regeneration of *Pinusgerardiana*. For that purpose, pines samples were collected from district Zhob and Sherani. The outcome of different growth stimulating hormones on the growth of *P. gerardiana* in various media were tested. Many trials to promote in-vitro culturing were performed to optimize the protocol. And this research would help to researchers to promote the invitro propagation of *Pinusgerardiana* in future.

Materials and Methods:

The young plant (Seedling, Sampling) and the dried seeds of *P. gerardiana* was collected from Chilghoza forest of District Sherani&Zhob, Balochistan; and brought to the tissue culture lab, Botany Department,University of Balochistan Quetta. The mature zygotic embryo (MZE) was used as Ex-plant for micro-propagation. The healthy seeds were thoroughly washed with the tap water for period of 10-15 minutes. The seeds were tested for sterilization process and seed coat was removed which uncovered the megagametophyte, afterwards it was subjected to sterilize with 0.1% of mercuric chloride and 2-3 drops of Tween 20 for 4-5 minutes and consequently it was submerged in 70% ethanol for 45 seconds. The megagametophytes were drenched for 15-20 hours at room temperature in a petri dish which was padded with sheets of sanitized filter paper and were treated with 10 ml of double distilled water to make sure the removal of traces of mercuric chloride. The MZE were then aseptically removed from mega gametophytes.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

The removed MZE have beingpositioned for germination in medium of 1 MS (Murashoge&Skoog, 1962) and woody plant medium (WPM) basal medium augmented with different meditations of growth regulators including 2, 4-D, BAP, IBA, PEG, AC, YE and L-Glutamine. After one week, the cotyledons of feasible embryos turned to be opened and were in colour like green. Embryos that were not viable were discarded. Viable MZEs were transplanted to their appropriate media. The horizontally placement in the media were done of MZE. Every third week, the explants were evaluated, and each fourth week, the calluses obtained were then sub cultured. 5 repetitions of 10 cotyledons were placed per petri-dish were utilized in each experiment, while 5 replications of 5 test tubes, each of them was placed with one explant, were used in MZE.

In a plant tissue culture chamber, the culture was kept at 26 ±1 °C with 16 hours of light. For cultures, MS medium with 3.0% Sucrose and it was placed to gell with 0.8 percent agar. The pH level was kept at 5.8. Laminar airflow was used for all treatments, involving washing, cleaning, soaking of seeds, and isolating the MZE.

Following were calculated the mean number of buds for each embryo, standard deviation, and Bud Forming Capacity (BFC) (Capuana and Gianini, 1995). BFC was calculated by the formula:

$$BFC = (\text{Average number of buds per plant} \times \% \text{ explants forming buds}) \div 100$$

Intended for the development of adventitious buds, cotyledons and mature zygotic embryos were used on MS and WP medium for callus induction, treatments were supplemented with 1.0, 0.75, 0.5, and 0.25 mg/liter Benzyl aminopurine (BAP) with 2,4-D. Every third week, the explants were checked for the emergence of embryogenic calluses. The callus was also induced by supplementing with different concentration of IBA and AC in WP and MS medium. The results were recorded after 6 weeks.

Results and Discussion:

In subject to determination of optimizing protocol for *P. gerardiana*different medias were prepared such as MS and WP medias were used to optimize the growth protocol for the of *P. gerardiana*. For callus induction, MZEs and cotyledons were employed as explants. Different

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

amounts of growth hormones were utilized to improve the conditions for rejuvenation and propagation of callus, formation of shoots, and roots development.

Table 1. Responseof BAP and 2,4-D in MS Medium

Media	BAP/2,4-D Conc. (Mg/Liter)	Average Percentage of callus induction	Callus formation
MS	0	0	+
MS	0.25+0.25	10	++
MS	0.50+0.50	15	++
MS	0.75+0.75	30	+++
MS	1.0+1.0	10	++

Callus formation; + = Poor: ++ = Fair; +++ = Good. 30 30 explants were used for each treatment; 5 times replications were done: Results have been expressed as mean ± SD. Growth of callus was recorded after seven weeks.

Table 2. Response of BAP and 2,4-D in WP Medium

Media	BAP/2,4-D Conc. (Mg/Liter)	Average Percentage of Callus induction	Callus formation
WP	0	15	+
WP	0.25+0.25	20	+
WP	0.50+0.50	45	+++
WP	0.75+0.75	35	+++
WP	1.0+1.0	30	+++

Callus formation; + = Poor: ++ = Fair; +++ = Good. 30 30 explants were used for each treatment; 5 times replications were done: Results have been expressed as mean ± SD. Growth of callus was recorded after seven weeks.

Data depicted in figure 1-2 shows the effect of different combinations of Phyto-hormones which were supplemented in MS and WP media. By varying concentration of compounds adversely affected the ratio of callus, shoot and root development. When the propagation material treated with BAP and 2,4-D in MS Medium The frequency of callus and shoot induction were affected. The best growth was found at concentration of 0.75mg/l that were recorded of 30% in MS media.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

While in WP media the maximum growth was recorded 54% at concentration 0.5mg/l. Similar results were found by Zaidi *et al*., (2012).

Table 3. Signifying response of IBA in MS medium

Name of Media	IBA Conc. (Mg/Liter)	Average Percentage of callus induction	Callus formation
MS	0	0.3	-
MS	0.25+0.25	0.5	-
MS	0.50+0.50	2.5	+
MS	0.75+0.75	3±0.5	+++
MS	1.0+1.0	1.5	++

Callus formation; + = Poor: ++ = Fair; +++ =Good Roots formation was recorded afterwards six weeks, and results are stated as mean ± SD.

Table 4. Signifying response of IBA in WP medium

Name of Media	IBA Conc. (Mg/Liter)	Average Percentage of callus induction	Callus formation
WP	0	2.3±0.3	+
WP	0.25+0.25	2.6	+
WP	0.50+0.50	3.5	++
WP	0.75+0.75	4.7	++
WP	1.0+1.0	7.1±0.3	+++

Callus formation; + = Poor: ++ = Fair; +++ =Good. Roots were generated after 6 weeks; results are stated as mean. ± SD.

Table 5. Signifying response of AC in MS medium.

Name of Media	AC Conc. (Mg/Liter)	Average Percentage of callus induction	Callus formation
MS	0	3	+
MS	0.25+0.25	10	+
MS	0.50+0.50	35	+++

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

MS	0.75+0.75	30	+++
MS	1.0+1.0	15	++

Callus formation; + = Poor: ++ = Fair; +++ = Good. Results are stated as in percentage, the formation of callus and shoot were documented after 8 weeks.

Table 6. Signifying response of AC in WP medium.

Name of Media	AC Conc. (Mg/Liter)	Average Percentage of callus induction	Callus formation
WP	0	5	+
WP	0.25+0.25	10	+
WP	0.50+0.50	40	+++
WP	0.75+0.75	30	+++
WP	1.0+1.0	15	++

Callus formation; + = Poor: ++ = Fair; +++ = Good. Results are stated as in percentage, the formation of callus and shoot were documented after 8 weeks.

Data presented in Table 3-6 depicts the role of IBA and AC in callus induction and shoot and root formation in both MS and WP media. In MS media IBA treatment revealed the best growth with treatment of 0.75mg/l, and roots formation was recorded as 3.0±0.5. while IBA in WPM media the greatest formation of roots was observed when treated with 1.0 mg/l and formation of roots number were recorded 7.1±0.3, which was like results achieved by Zaidi *et al.*, (2012). By using AC in MS media depicts the best growth 35% with 0.5 mg/l. The callus induction in WP medium with AC treatment the maximum number of roots were achieved after four to six weeks, and best growth result 40% was recorded at 0.50mg/I in WP media. Which was close to results obtained by the Normah*et al*., (1995).

Conclusion:

We discovered that cotyledons removed from embryos are appropriate parental material to induce adventitious buds. The meditations of BAP and 2,4-D that produced maximum yield were recorded for MS and WP media as 0.75 and 0.5mg/l respectively. The best callus induction in IBA and AC treatment both in MS WP media was recorded as 0.75 mg/l and 0.5 mg/l for shoot and root formation. Yet there is capacity for the improvement in selection of media,

Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 662 - 670
Received 08 November 2021; Accepted 15 December 2021.

optimizing the culturing environments, and use of cytokinin's in best combination. The use of cytokinin was restricted to only BAP as organic oneshaving more affluent to use and due to less viability of *Pinusgerardiana*seeds there is maximum probabilities of dropping the resources which indicates the attention of researchers to utilize more efforts in this section. In *P. gerardiana*, MZE was discovered to yield more embryogenic cultures. However, it has been found that immature zygotic embryos are the optimum material for adventitious bud initiation and somatic embryogenesis in pines nuts (Arya *et al*., 2000). Because the Chilghoza pine breeds in isolated areas and is difficult-to-reach locations in Balochistan, obtaining immature cones at various development stages is problematic, hence MZE was chosen as the preferred element for *in vitro* production.

References

1. Ahmed, M., Palmer, J., Khan, N., Wahab, M., Fenwick, P., Esper, J., & Cook, E. D. (2011). The dendroclimatic potential of conifers from northern Pakistan. Dendrochronologia, 29(2), 77-88.
2. Akbar, M. U. H. A. M. M. A. D., Ahmed, M. O. I. N. U. D. D. I. N., Shaukat, S. S., Hussain, A. L. A. M. D. A. R., Zafar, M. U., Sarangzai, A. M., & Hussain, F. A. I. S. A. L. (2013). Size class structure of some forests from Himalayan range of Gilgit-Baltistan. Sci., Tech. and Dev, 32(1), 56-73.
3. Akbar, M., Khan, H., Hussain, A., Hyder, S., Begum, F., Khan, M., ... & Ali, S. (2014). Present status and future trend of chilghoza forest in Goharabad, District Diamer, Gilgit-Baltistan, Pakistan. Journal of Biodiversity and Environmental Sciences, 5(5), 253-261.
4. Akubugwo, I. E., Obasi, N. A., Chinyere, G. C., &Ugbogu, A. E. (2007). Nutritional and chemical value of Amaranthushybridus L. leaves from Afikpo, Nigeria. African Journal of Biotechnology, 6(24).
5. Anonymous 1969. Wealth of India-raw materials, 8, 65-66
6. Arya, S., Kalia, R. K., & Arya, I. D. (2000). Induction of somatic embryogenesis in PinusroxburghiiSarg.Plant Cell Reports, 19(8), 775-780.
7. Kumar, R., Shamet, G. S., Mehta, H., Alam, N. M., Tomar, J. M. S., Chaturvedi, O. P., &Khajuria, N. (2014). Influence of gibberellic acid and temperature on seed germination

in Chilgoza pine (Pinusgerardiana Wall.). Indian Journal of Plant Physiology, 19(4), 363-367.

8. Malik AR, Shamet GS (2008) Germination and biochemical changes in the seeds of chilgoza pine (Pinusgerardiana Wall.)—by stratification: an endangered conifer species of the Northwest Himalaya. Indian J Plant Physiol 13:278–283.
9. Murashige, 1974; Start and Cumming, 1976
10. Murasnige, T., &Skoog, F. (1962). A revised medium for rapid growth and bio agsays with tohaoco tissue cultures. Physiol. plant, 15(3), 473-497.
11. Normah, M. N., Nor-Azza, A. B., &Aliudin, R. (1995). Factors affecting in vitro shoot proliferation and ex vitro establishment of mangosteen. Plant cell, tissue and organ culture, 43(3), 291-294.
12. Özgüven, F., &Vursavuş, K. (2005). Some physical, mechanical and aerodynamic properties of pine (Pinuspinea) nuts. Journal of food engineering, 68(2), 191-196.
13. Saeed, M., Shahwani, M. N., Ahmed, S. S., Ahmed, N., Shaheen, G., & Raza, A. M. (2013). Somatic Embryogenesis and Organogenesis from Embryonic Explants of Pinusgerardiana. Journal of Applied and Emerging Sciences, 4(1), pp33-36.
14. Shalizi, M. N., &Khurram, S. (2016). Socio-economic importance of chilgoza pine forest of Afghanistan: A survey-based assessment. Asian Journal of Science and Technology, 7, 3556-3559.
15. Tang, W., &Guo, Z. (2001). In vitro propagation of loblolly pine via direct somatic organogenesis from mature cotyledons and hypocotyls. Plant Growth Regulation, 33(1), 25-31.