

First In vitro micropropagation of Vitis vinifera cultivars for production of virus free germplasm by apical meristem tip culture in Pakistan

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Abstract

The study aimed to develop a procedure for virus-free grapevine plant production through in vitro micropropagation. Collected explants from three grapevine cultivars were sterilized and grown on Murashige and Skoog medium with varying concentrations of cytokinins (BAP) and auxins (NAA and IBA). Different combinations of BAP and NAA were tested for shoot induction, while IBA and NAA combinations were used for root induction. This study demonstrates the successful production of virus-free grapevine cultivars using apical meristem tip culture, enabling the preservation of clean planting material and large-scale production of plantlets regardless of season.

INTRODUCTION

Grape (*Vitis vinifera* L.) is one of the most valuable and economically significant crop of world (Myles et al., 2011). It is valued for its use for many purposes as it is used as fresh fruit, raisins, jams, jelly, juices and many others. The crop is mostly grown in tropical and temperate regions of the world (Mullins et al., 1992). Allah has blessed Pakistan with best agro climatic conditions suitable for the growth of 21 different kinds of fruit (Aujla et al., 2011). Grapes rank on 10th position in terms of fruit production in Pakistan and mainly European grapes are cultivated (Jaskani et al., 2008; Ali et al., 2017). In Pakistan 66,036 tons grapes were produced during year 2017 using total area of 15,360 ha (FAO, 2019). Hilly regions of the northern Punjab and NWFP, and highlands of Baluchistan are limited areas for cultivation of grapes in Pakistan (Khair et al., 2009). According to Government of Pakistan, Baluchistan produces 98% of total grape production of country. Grapes are produced in all districts of Baluchistan and some important local and imported cultivars includes Kishmish, Shundo Khanni, Haitha, Askari, Hussaini, Alphanso, Cardinal, Flame Seedless, Kings Ruby and Regenia. These varieties are mainly grown in Pishin, Kalat, Killa Abdullah, Quetta, Mastung, Zhob and Loralai districts of Baluchistan (Pakistan., 2007). According to FAO, Pakistan stands on 57th position in world in terms of grape production (FAO, 2019). Generally, In Pakistan the production of grapes is not as good as compare to other countries of the world. This directs a necessity to classify various factors which are the reason for low production of grape.

MATERIALS AND METHODS

Plant material

Three commercial grapevine cultivars namely Flame Seedless, Crimson Seedless and Thomson Seedless were collected as a source plant material from Agricultural Research Institute, Quetta.

Pre-indexing of explants

The RNA extraction from the collected cultivars was done by commercial kit RNeasy Plant Mini kit (Qiagen Inc. Valencia, CA); after DNase treatment and synthesis of cDNA, RT-PCR based molecular virus

diagnosis was done for pre indexing of targeted viruses as described by (Faggioli et al., 2013).

Surface disinfection of explant

The shoots (1–2 mm) were washed with 70% ethanol for 60 seconds. The ethanol was discarded and shoots were dipped for 10 minutes in 10% Sodium Hypochlorite solution (NaClO) followed by three to four times washing with sterile water.

Initiation of shoots

Sterile excised apical meristems were cultured on MS medium provided with 0.0–2.0 (mg/l) BAP and 0.1 (mg/l) NAA.

Multiplication of shoots

The initiated shoots were shifted to fresh MS medium with different concentrations of BAP (1.0, 2.0, 3.0 and 4.0 (mg/l) and NAA (0.0, 0.1, 0.2 and 0.3).

Root Induction

Plantlets with 2–3 cm length of *in vitro* grown microshoots were excised and transferred to MS medium accompanied with different concentrations and combinations of NAA (0.0, 0.1 and 0.5 mg/l) along with various concentrations of IBA (0.0, 0.5, 1.0 and 1.5).

Acclimatization

The roots of newly formed plantlets were rinsed thoroughly with double distilled and autoclaved water to remove any residue of MS medium and the plantlets were shifted to plastic pots containing jiffy 7 pellets and then covered with transparent plastic bags, to retain high level of humidity for 3 to 4 weeks in growth chamber.

Hardening in green house

Plantlets with 12 inches length were shifted to the 10 inches plastic pots to undertake the necessity of higher nutrients essential for their further development providing large surface area. After four weeks they were shifted to one gallon pots for hardening in green house.

Post indexing of grapevine plantlets

The success of virus elimination was analyzed by the RT-PCR amplification. Positive controls for all viruses were run to check the proficiency of amplification.

RESULTS AND DISCUSSIONS

Surface disinfection of explant

The successful micropropagation is dependent on the choice of the explants and time duration of sterilization (Garg et al., 2014). Therefore, NaClO was used as a conventional bleaching agent that acts as a wonderful disinfectant reported by (Block, 2001; Panattoni et al., 2013). 10% NaClO along with three different time duration was experimented. Treatment with 10% NaClO for 5 minutes produced only 30% clean cultures. However, insignificant differences were observed with 10% NaClO for 10 and 15 minutes which resulted in 100% plants free from contamination (Table 1). Our results resembled with (Jaskani et al., 2008) who also studied disinfection with 10% NaClO.

Table 1
Influence of time duration on surface sterilization with 10% Sodium Hypochlorite (NaClO)

Time Duration (minutes)	No. of explants	No. of explants of contaminated	% of contamination	No. of clean cultures	% of clean cultures
5	20	14	70	6	30
10	20	0	0	20	100
15	20	0	0	20	100

Effect of various concentration of growth hormone for initiation of shoot

BAP is considered to be most efficient cytokinin for the establishment of shoots *in vitro* (Banilas and Korkas, 2007; Mukherjee et al., 2010). In current study, MS medium supplemented with various concentration of PGRs [0.0–2.0 (mg/l) BAP and 0.0-0.1 (mg/l) NAA for development of shoots. The three grapevine cultivars showed different growth patterns with respect to various parameters including survival rate, percentage of explants showing growth, number of shoots formed, shoot length and number of days required for initiation of shoots. It was observed that in the case of Crimson seedless, the survival rate and number of explant produced was good as compare to Thomson Seedless and ranged between 80–95 and 70–95 respectively. However, late shoot initiation was observed in low concentrations of BAP and in the absence of NAA. Maximum number of shoots was produced in MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l of NAA. Best results in terms of survival rate, number of explant produced and other related factors was observed in Flame Seedless. The shoot initiation started within 25 days after inoculation. Shoot length is also dependent on BAP concentration.

2.90 cm of shoot length was achieved with 1.0 mg/l of BAP however, decrease in shoot length was observed with the increase concentration of BAP. Our results were similar to study performed by (Ali et al., 2017) who reported that the use of higher concentrations could suppress development and is sometimes toxic for the plant. Greater decrease in shoot growth and shoot length was observed in the cultivar Thomson Seedless with the increase concentration of BAP. Maximum shoot length was obtained using 0.5 mg/l of BAP in this case. Our results were similar to the studies conducted previously which reported the role of BAP in combination of NAA for the development of shoots (Abido et al., 2013). Table No. 2 and Fig. 1.

Effect of various concentration of growth hormone on multiplication of shoots

Effect of various concentrations of BAP and NAA on multiplication rate of grapevine shoots provided significant role of BAP regarding mean no. of shoots developed. Maximum mean value was observed at 2.00 mg/l BAP and minimum mean value was recorded in its absence. Amount of NAA used was also produced significant results. The maximum value was observed at 0.3 mg/l of NAA, but, the minimum response was observed in the absence of BAP. Amount of NAA used was also produced significant results. However, maximum value was noticed when BAP was used in combination of maximum NAA. Both PGRs play significant role with respect to shoot length. Additionally, the medium supplemented with higher BAP concentrations provided maximum results, in contrary, NAA showed proportional relation with mean value showing maximum results at 0.1 (mg/l). Use of auxins with cytokinins produced effective shoot multiplication. Moreover, Butiuc Keul *et al.* (2008) reported that multiplication rate can be improved using cytokines in the culture medium. Sub culturing in fresh medium was done after every two weeks to remove any brownish material from the developed shoots.

Effect of various concentrations of IBA and NAA on root induction

IBA provides greatest response with reference to number of roots formed but late rooting was noticed in the absence of NAA. In the meantime, efficient root initiation was observed by cv. Flame Seedless using 0.5 (mg/l) of NAA and 1.5 (mg/l) of IBA. The results showed maximum root length (4.8 cm), however, Crimson Seedless and Thomson Seedless produce 4.2 cm and 4.6 cm in 18 and 15 days respectively as shown in Table 4. Our results are similar to (Munir et al., 2015) who reported that both NAA and IBA were the most suitable plant growth regulators for rooting of plants.

Acclimatization

The plants were gradually acclimatized in two steps, first in the growth chamber providing suitable conditions and then acclimatized in green house. For this purpose, 12 inches tall plantlets were shifted to the 10 inches plastic pots to undertake the necessity of higher nutrients essential for their further development providing large surface area. (Pathirana and McKenzie, 2005) also practiced the same strategy for producing pathogen free grapevine. The pots were covered with the plastic tent to ensure the travel of light to the newly developed plantlets, Furthermore, the survival of plantlets was achieved as the humidity and CO₂ level was easy to maintain in plastic tent. Our findings were similar to (Abido et al., 2013). After successful survival of plants in growth chamber, they were shifted to green house for further acclimatization and hardening by exposing to environment.

Post indexing

The newly grown plantlets were subjected to post indexation, 80% of the plantlets were found free of grapevine viruses detected by RT-PCR. Positive controls and ladder was also run in gel electrophoresis to confirm the authenticity of the PCR reaction (Fig. 6).

Declarations

Author Contribution

Dr. Madiha Mahmood Gillani. Write up of manuscript. Dr. Shagufta Naz. Idea and Assistance in Research. Francesco Faggioli, Andrea Gentili and Anna Taglienti . Assistance in research work. Dr. Sunniya Rasool Figures and Tables.

References

1. Abido, A., Aly, M., Hassanen, S.A. and Rayan, G. 2013. In vitro propagation of grapevine (*Vitis vinifera* L.) Muscat of Alexandria cv. For conservation of endangerment. *Middle-East Journal of Scientific Research*, 13(3): 328–337.
2. Ali, N., Afrasiab, H. and Anwar, S. 2017. Vinefera L.
3. Aujla, K., Shah, N., Ishaq, M. and Fraooq, A. 2011. Postharvest losses and marketing of grapes in Pakistan. *Sarhad Journal*, 27(3): 485–490.
4. Banilas, G. and Korkas, E. 2007. Rapid micropropagation of grapevine cv. Agiorgitiko through lateral bud development. *Journal of Science and Technology*, 2: 31–38.
5. Block, S.S. 2001. *Disinfection, sterilization, and preservation*, ed. Lippincott Williams & Wilkins, Place Published.
6. Faggioli, F., Anaclerio, F., Angelini, E., Antonelli, M., Bertazzon, E., Bianchi, G., Bianchedi, P., Bianco, P., Botti, S. and Bragagna, P. 2013. Harmonization and validation of diagnostic protocols for the

- detection of grapevine viruses covered by phytosanitary rules. *Advances in Horticultural Science*, 27(3): 107–108.
7. Garg, R.K., Srivastava, V., Kaur, K. and Gosal, S. 2014. Effect of sterilization treatments on culture establishment in *Jatropha curcas* L. *Karnataka J Agric Sci*, 27: 190–192.
 8. Jaskani, M.J., Abbas, H., Khan, M., Qasim, M. and Khan, I. 2008. Effect of growth hormones on micropropagation of *Vitis vinifera* L. cv. Perlette. *Pakistan Journal of Botany*, 40(1): 105.
 9. Khair, S., Maqsood, A. and Ehsanullah, K. 2009. Profitability analysis of grapes orchards in Pishin: an ex-post analysis. *Sarhad Journal of Agriculture*, 25(1): 103–111.
 10. Mukherjee, P., Husain, N., Misra, S. and Rao, V. 2010. In vitro propagation of a grape rootstock, deGrasset (*Vitis champinii* Planch.): Effects of medium compositions and plant growth regulators. *Scientia horticulturae*, 126(1): 13–19.
 11. Mullins, M.G., Bouquet, A. and Williams, L.E. 1992. *Biology of the grapevine*, ed. Cambridge University Press, Place Published.
 12. Munir, N., Safdar, I. and Naz, S. 2015. Effect of growth regulators on callus induction and micropropagation of three grape varieties. *J. Agric. Res*, 53(2).
 13. Myles, S., Boyko, A.R., Owens, C.L., Brown, P.J., Grassi, F., Aradhya, M.K., Prins, B., Reynolds, A., Chia, J.-M. and Ware, D. 2011. Genetic structure and domestication history of the grape. *Proceedings of the National Academy of Sciences*, 108(9): 3530–3535.
 14. Pakistan., G.o. (2007). Fruit, vegetables and condiments statistics of Pakistan, Ministry of Food, Agriculture and Livestock, Food and Agriculture Division ...
 15. Panattoni, A., Luvisi, A. and Triolo, E. 2013. Elimination of viruses in plants: twenty years of progress. *Spanish Journal of Agricultural Research*, (1): 173–188.
 16. Pathirana, R. and McKenzie, M.J. 2005. Early detection of grapevine leafroll virus in *Vitis vinifera* using in vitro micrografting. *Plant cell, tissue and organ culture*, 81(1): 11–18.

Tables 2 and 3

Tables 2 and 3 are not available with this version.

Figures



Figure 1

Initiation of shoots.



Figure 2

Development of internode segments.

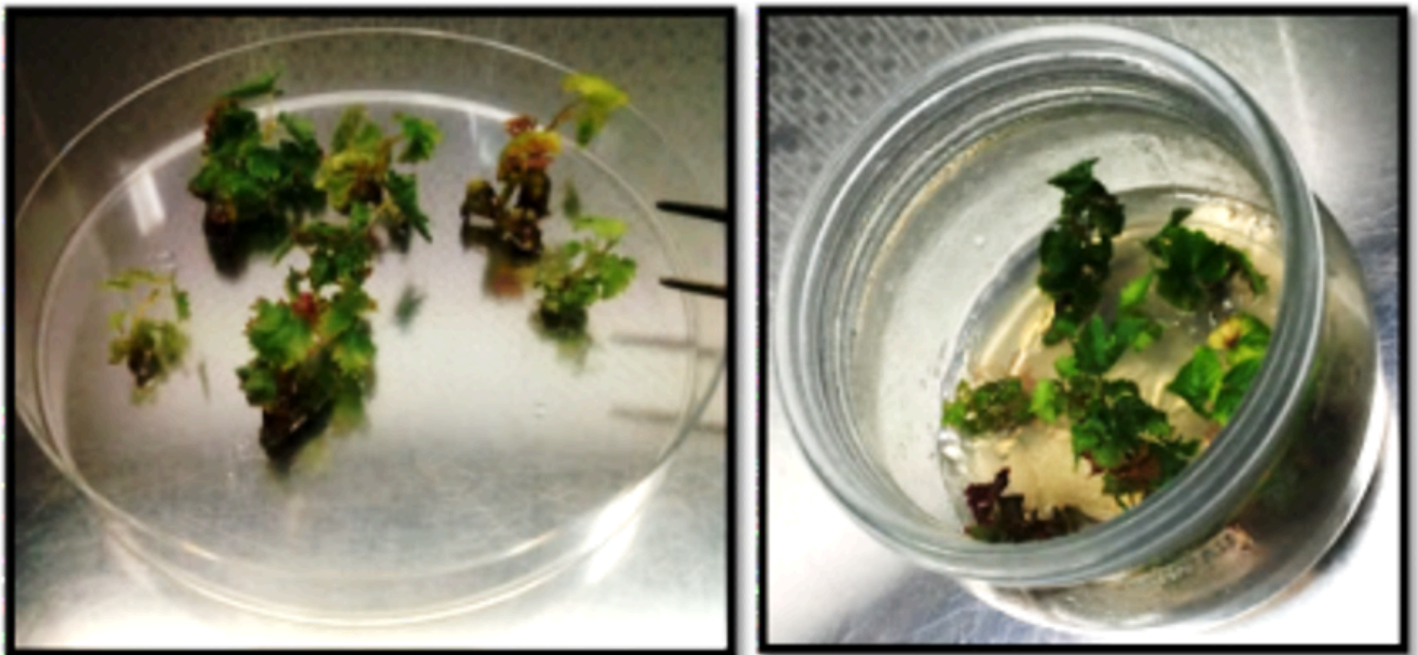


Figure 3

Multiplication of shoots



Figure 4

Induction of roots in media provided with IBA (1.0 mg/l) and NAA (0.5mg/l)



Figure 5

(A) Acclimatization of grapevine plantlets in growth chamber, (B) Acclimatization of grapevine plantlets in green house.

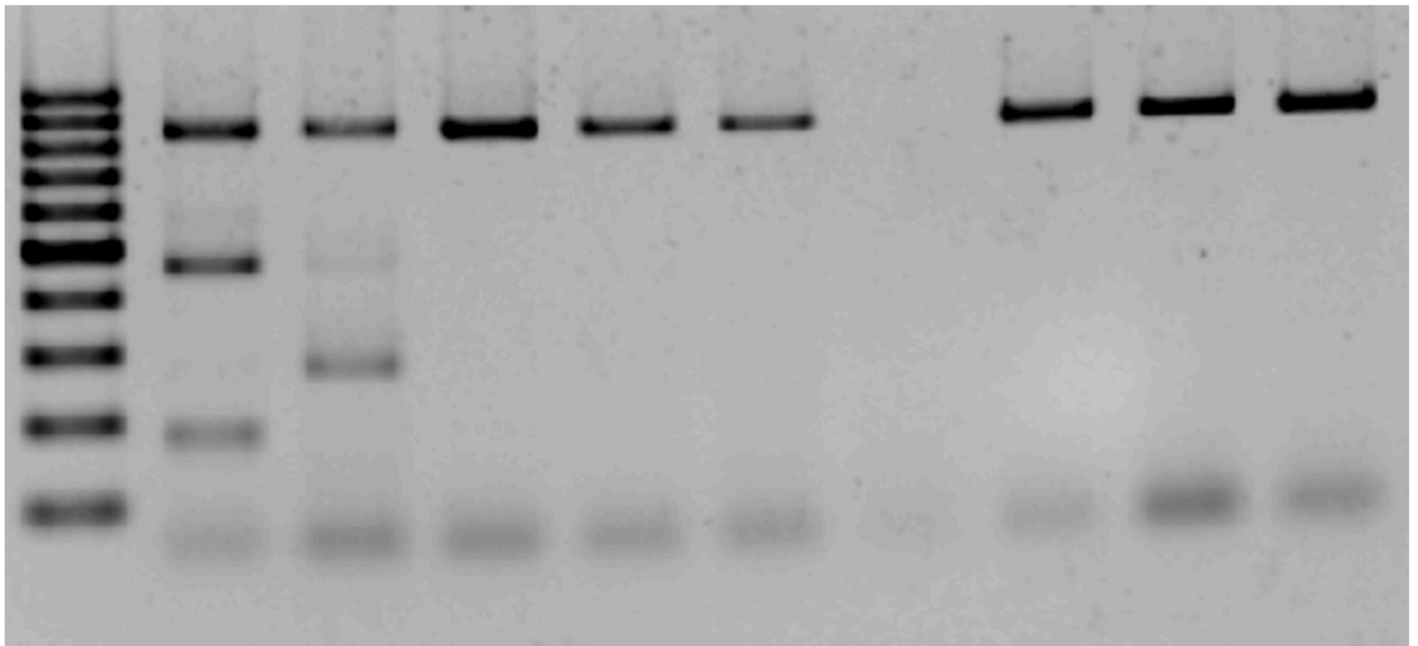


Figure 6

Gel electrophoresis of RT-PCR of new plantlets achieved after meristem tip culture. (M = marker 100 bp, K = Water. Samples 1, 2= Positive controls for targeted viruses 3-8 showing amplification of only 18S ribosomal RNA (844bp) as an internal control, therefore they are virus free plantlets).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables2and3.docx](#)