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EVALUATION OF VARIOUS LEVELS OF MINERAL NUTRIENTS AND PLANT GROWTH REGULATORS FOR *IN VITRO* CULTURE OF GRAPE

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Abstract

In order to circumvent the constraints in the existing methods for *In vitro* culture growth, germplasm conservation and large-scale propagation of grape, a study was undertaken to devise and formulate an appropriate growth media for micropropagation of new grape genotypes. Explants of two germplasm accessions viz., Sunder Khani (SK) and 019972 from a recently established field gene bank were used. In total, 12 different media formulations involving macro- and micro-nutrients (MS media), Fe-EDTA levels employed in Murashige-Skoog (MS) media were tested for their effects on *In vitro* growth performance of the two grape germplasm accessions. It was found that 75% levels of macronutrients worked best for the genotypes tested. The protocol fine tuned in this study for *In vitro* growth of grape cultures and micropropagation process achieved as many as 72 and 62 plants in each cycle of subculturing every 4-6 weeks, in case of Sundar Khani and 019972 accession, respectively. Use of coconut husk produced 80% rooting frequency when double node cuttings were used, without any application of rooting hormone within a period of 6 weeks, whereas, single node cuttings failed to root. On the other hand, *In vitro* plants showed maximum rooting incidence (1.26gm per explant) when grown on media containing adenine sulfate (543 uM) in combination with IAA (1.1 uM) and kinetin (4.6 uM). The protocol developed in this study has paved the way to establish *In vitro* conservation of all grape germplasm accessions of our gene bank as a back up for sustainable plant genetic resources (PGRs) conservation and their utilization.

Introduction

The grape belongs to the genus *Vitis*, which comprises of about 60 species. However, the principal species from which the cultivated grape has been derived is Vitis vinifera. They are consumed fresh, as juices and wine, as raisins, jams and jellies and as frozen products. In Pakistan, only European grapes are cultivated for eating. Over 70% of grapes are grown in Baluchistan, while there is some acreage in NWFP. A survey of northern areas of Pakistan, NWFP and Baluchistan shows that there is a considerable amount of biodiversity available in the grape germplasm for exploitation in genetic improvement and characterization for profitable cultivation of grapes. Germplasm is valuable as it contains the diversity of genotypes needed to develop new and improved lines (Khawale & Singh, 2005). Invasive species threaten native biodiversity, agricultural and other production systems (Pimentel 2002). More stringent restrictions on germplasm exchanges are helpful in curbing the rate of spread (Chapin *et al*., 2000).

Both the cross border germplasm exchange and conventional propagation of grapes for commercial cultivation involves long shoot cuttings derived from dormant hardwood as a source. This method is economical, simple and farmer friendly. But it does not allow materializing a rapid growth of planting material, which is mostly a limiting factor in

clonal selection programs, which are based on the hard work of breeders and virologists, who identify and develop new varieties that are virus free and genetically unique. Also germplasm exchange *via* shoot cuttings often has inadvertently led to detrimental spread of fungal, viral and bacterial diseases within and between countries (Khawale & Singh 2005). Many countries have restrictions on import of bud wood because of the hazards of introducing new and virulent diseases and it is confined only through seeds. Viral and fungal diseases are rarely seed transmitted (Clay, 2004). However, when seed is used as genetic material for germplasm exchange and propagation, germinability loss, desiccation and heterogeneity are the major problems. Therefore, an alternate system for their conservation and rapid propagation is essential for international germplasm exchange and to cultivate these materials on commercial scale in the shortest possible time. Elsewhere, a novel approach adopted by nurserymen involves rooting of softwood cuttings, growth forcing and then re-propagating them by repeated root induction in the cuttings thereof. This method is moderately speedy but still has drawbacks, as it does not ensure virus free status of the clonal material resulting from the process. A more scientific and fool proof approach is the *In vitro* method of vegetative multiplication or micropropagation, more commonly referred to as tissue culture technology. It ensures large scale propagation of planting material in an industrial setup around the year without being affected by the natural climatic fluctuations and above all, genetic constitution of the resulting planting material remains unaltered, thus preserving the agronomic, horticultural, biological and physiological characters of the clone which are inculcated into the genotypes after long term development efforts of breeder and his collaborators of diverse disciplines. Moreover, health status of the resulting plants is also superior (Barbara, 2004), ensuring better fruit yield at the farms, thus fetching premium prices for the growers as well. We had previously reported that a large collection of grape germplasm has been established as field gene bank at our institute, where more than 150 accessions collected from different agro-ecological zones of Pakistan and also exotic accessions are being maintained (Sajid & Kashif, 2006). The field gene bank serves as a precious resource for breeders, agronomists and horticulturists for evolving new cultivars and selecting unique and superior genotypes suitable for cultivation on commercial scale in specific ecologies of Pakistan. An *In vitro* gene bank is also being established as a backup facility for conservation and protection from epidemics and other natural and man made disasters, which threaten biodiversity. We have earlier reported establishment of *In vitro* cultures but found culture mortality of different accessions due to their sensitivity to high salt levels which are inherent in the commonly prescribed media formulations (Kashif & Sajid, 2005). Therefore, it was speculated that more elaborate study on the micronutrients and macronutrient requirements of grape cultures, needs to be carried out for perfecting the process of *In vitro* culture conservation, multiplication and rooting of grape plants to meet the future challenges of supplies of grape germplasm for dissemination of planting material, conservation studies and quarantine requirements for international exchanges of plant germplasm (Sajid *et al*., 2003).

Materials and Methods

Plant material: Plants used in this study were two germplasm accessions, of which one (Sunder Khani) was collected from Baluchistan and the other 019972 was acquired from Upland Crop Research Station, Japan. Both these accessions have been established in the clonal repository of the field gene bank to serve as explant sources for subsequent culture

establishment. In the present study, these accessions were retrieved from our *In vitro* gene bank established earlier as a part of our long-term strategy for biodiversity conservation.

Growth media formulations: In one of the experiments, as many as 12 different combinations of macronutrients, micronutrients and iron chelate were employed to test the growth responses of grape explants transferred and cultured on them for a period of six weeks. These formulations included 10% to 100% MS levels of macronutrients, 50 to 100% levels of micronutrients and 50 to 100% levels of iron chelate. All the nutrients used in this study were of Sigma Chemical Company, USA. All the media formulations were supplemented with myo-Inositol (100mg-1), sucrose (3% w/v), vitamins (as in MS), and media were gelled with the help of agar (0.8% w/v). pH was adjusted to 5.8 before addition of agar. In one set of experiments, media formulations were devoid of any growth regulator but in the other set of experiments, they were also supplemented with varying ranges of BAP (0 to 606 uM), adenine sulfate (326 to 869 uM), kinetin (2.3 to 6.9 uM) and auxins (1.1 to 19 uM). Aliquots of media were dispensed into glass jars at the rate of 50 ml per jar and capped before autoclaving at 121^{o}C for 15 minutes. Culture vessels were stored in the cold room at 15^{o}C until they were used for transferring the explants.

Culture incubation conditions: Explants were derived from *In vitro* plant material maintained in the *In vitro* gene bank and prepared by aseptically sectioning the plants to obtain double node cuttings which were then cultured in the glass jars and were incubated in the growth room under fluorescent light of 1500 lux, at a temperature of 25^{o}C and a photoperiod of 16 hours daily. The data on growth parameters namely number of shoots, shoot length, shoot mass, number of nodes per stem, root mass and mortality were recorded and analyzed for *In vitro* culture growth responses of the two grape accessions used in this study.

Root induction: Shoot cuttings from hardwood winter hardened dormant plants were sliced in to either double node or single node cuttings with the help of sharp scalpels and cut surfaces were treated with 19M IBA prepared in chalk powder before immersing them into the potting mix for root induction. The potting substrate consisted of 100% coconut husk that was moistened and drained thoroughly with tap water for eluting away any toxic materials in the husk. Mini-pots trays containing the cuttings were placed under high humidity conditions maintained by enclosing them in an air-tight polyethylene cover sealed from all sides to preserve vapors emerging from the surface of the pots. The cuttings were watered with plain tap water on daily basis for 6 weeks after which data on rooting was recorded on both types of cuttings used in this set of experiments. Root induction was also studied using plant growth regulators in the MS media on which stem slices of *In vitro* grown plants were cultured.

Results and Discussion

Culture viability and mortality: The *In vitro* plantlets of two grape germplasm accessions namely Sundar Khani (SK) and 019972 were aseptically sliced into double-node stem pieces, which were then cultured on twelve different media formulations to test the growth performance of these explants against the specified levels of macronutrients and micronutrients including iron chelate as depicted in Table 1. It was

observed that cultures underwent a maximum mortality (60%) when grown on media containing 100% MS levels of each of macronutrient, micronutrients and iron chelate in case of the 019972 accession whereas Sunder Khani (SK) accession did not show any mortality when grown on this media formulation, suggesting the culture viability to be genotype dependent. This may be attributed to the differential tolerance of genotypes to osmotic potentials of the growth media. When grown on media containing any level of nutrients equal to or less than 50%, a 100% survival was recorded among the cultures of 019972 accession, whereas the response of Sunder Khani accession was also good at these levels. Similar findings were reported by Dalal *et al*., (1991) when they observed that culture browning and media browning were reduced and culture survival was improved by growing the explants on media containing 50% strength of the macronutrients of MS media. Since the explants were derived from already established aseptic cultures, the contamination was found to be fully controlled and none of the cultures encountered any contamination. Strict maintenance of asepsis during the culture transfer operations is also crucial in reducing or eliminating the incidence of contamination. Some of the healthy and well-grown *In vitro* cultures are shown in Fig. 1.

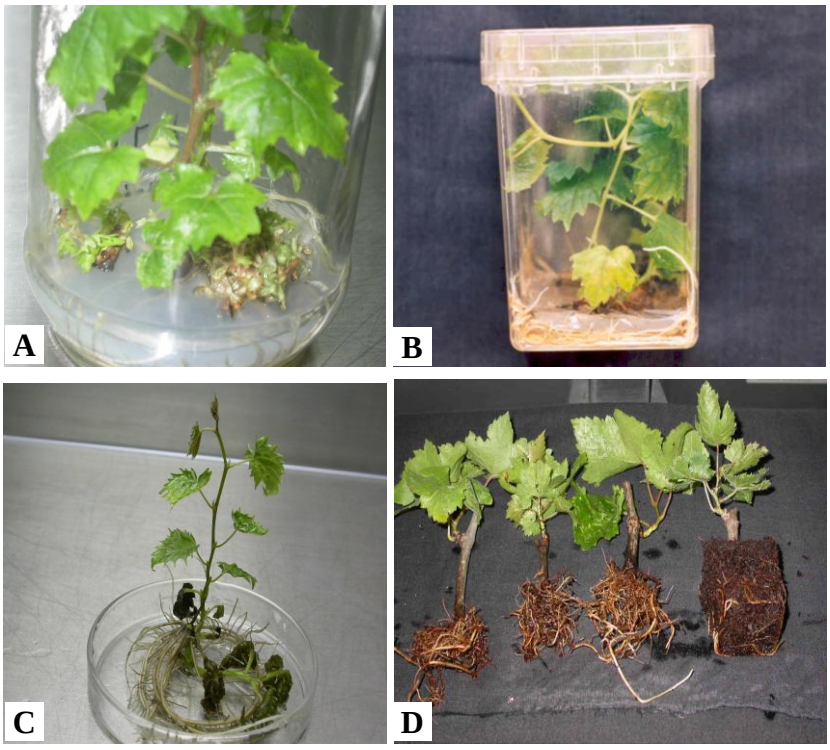

Fig. 1. *In vitro* grown grape plant showing a well established healthy shoot growth (A). A well rooted *In vitro* grown grape plant ready for transplantation in the field conditions for hardening (B and C). Well rooted *ex vitro* plants of grape obtained from winter woodcuttings of double nodes each after 6 weeks of immersion in well moistened coconut husk (D).

Shoot elongation and shoot mass: Since none of 12 media formulations tested in this part of study employed any plant growth regulator, therefore shoot proliferation was not expected, and rather a vertical shoot growth was observed to be variably influenced by media formulation. Shoot number in all the cultures was mostly limited to one shoot per explant at the end of the incubation for 6 weeks except in a few cases where it was recorded to vary from 1 to 2 shoots per culture in either of the two accessions tested, which is not a multiplying factor for the purpose of shoot proliferation. Mean shoot length however, ranged from 1.50cm to 6.5 cm per shoot in case of the 019972 accession, depending upon the media formulation on which the explants were grown. On the other hand, shoot length varied from 1.5 cm to 5.12 cm per shoot in case of Sunder Khani accession when the explants were grown on the described media formulations. Maximum shoot length was recorded on the media containing 100% levels of each category of the nutrients used, whereas moderate shoot lengths were recorded on media containing either 75% or 50% levels of each of the macronutrients and micronutrients, despite the fact that mortality was also among cultures grown on high strength media formulations. Largest shoot mass, however, was gathered by 019972 accession when cultured on the media containing 50% levels of each category of the nutrients used. These findings suggest that as a compromise among shoot length, shoot mass and culture mortality, the levels of nutrient salts employed for *In vitro* culture of all grape accessions be adjusted at 50% of MS levels rather than 100% MS salts as a guiding principle for *In vitro* gene bank management.

Shoot proliferation: After assessing the effects of macronutrient and micronutrient levels in hormone free regimes on culture growth and viability, it was envisaged to exploit several plant growth regulators used at varying strengths in obtaining higher shoot multiplication rates (Table 2). Therefore, BAP, kinetin, adenine sulfate and auxins were used at the prescribed levels in the 50% levels of salts optimized in the aforementioned studies. When grape explants were transferred on media containing various levels of BAP, it was observed shoot mass per explant showed an increment in a linear fashion along with the increase in BAP concentration from 0 to 6.6uM. Shoot mass of 019972 accession increased from 0.66 gm per explant to 11 gm per explant when BAP was raised from 0 to 6.6uM. This increase in shoot mass was paralleled by the increase in shoot number per explant as the latter also showed an upward trend from 1 shoot per culture to 62 shoots per culture in case of this accession. The second accession namely Sunder Khani followed the same pattern (increase from 2.79 to 10.80 gm per explant) when its explants were grown on media containing same levels of BAP. However, shoot proliferation rate of this accession was even higher (1:70 per subculture) than the 019972 accession (1:62 per subculture) at the highest BAP concentration used in this study (Table 2). Thus, shoot proliferation rate is dependent on the concentration of BAP in the media and also the genotype in question. In contrast, shoot length showed an almost linear drop in value (11.33 cm to 2.50cm per explant in case of 019972 accession and 4.5cm to 1.37 cm in case of Sundar Khani accession)) when BAP was enhanced from 0 to 6.6uM. Thus, BAP was found to promote shoot proliferation at high concentrations but shoot height is shortened in both the accessions used in this study. Extent of the shortening effect is genotype and doze dependent in a relatively linear fashion. Kinetin and adenine sulfate when employed alone or in combinations with IAA or with each other at the levels described have not shown any visible impact on shoot proliferation in either of the two accessions as compared to a consistent impact of BAP in both the accessions used.

Root induction: When *In vitro* cultured plants of grapes were grown on media containing various levels of the nutrients, the highest root mass was obtained on the media containing full strength nutrients of each category in case of the accession 019972. But in the other accession, Sunder Khani, a greater root mass was gathered on the media containing full strength each of macronutrients and iron chelate but half strength micronutrients (Table 1). Thus, root growth response is also linked with the genotype and nutrient composition of the growth media. Choi *et al*., (1992) recommended the use of NAA at a concentration of 2.6uM for root induction in grapes although they did not compare other auxins or a hormone free media for their effectiveness in root induction. Highest root mass was obtained on media containing either low level of BAP (2.2uM) in case of 019972 accession or no BAP in case of Sundar Khani (Table 2). Adenine sulfate when used in combination with kinetin and IAA at the prescribed levels also generated high root mass (1.26 gm per explant) in case of Wild grape accession but none in the other accession. Basinger *et al*., (2000) have compared rooting ability of many species of native grape vine by culturing them on half strength MS media and noticed a relatively high rooting ability except in one of the species used. They also reported high contamination incidence among their cultures. Contamination incidence was negligible in our cultures and rooting ability depended on the genotype used.

In the experiments on *ex vitro* root induction, it was observed that shoot cuttings which consisted of 2 nodes were more suitable for root induction as almost all the other shoot cuttings which were consisted of single nodes, failed to produce roots when embedded in the coconut husk used as a growth substrate (Fig. 1-D). More than 80% of the double- node shoot cuttings produced well grown root mass and were successfully established into healthy plantations at the site for future plantation.

Conclusion

The technique developed and described here for *In vitro* culture growth of grape germplasm can be effectively used for germplasm conservation for protecting the grape biodiversity available in the country from severe losses arising from epidemics. Besides facilitating the germplasm exchange, the technique by virtue of maximizing shoot mass and subsequent root induction both in the *In vitro* cultures and *ex vitro* plant cuttings ensure that the plants are produced on large scale without losses and in a minimum possible time. This makes it possible to speedily raise plantations of new varieties, which are developed after a long-term research and development exercise of scientists. These findings will also prove helpful in managing the in vitro gene bank for more than 150 germplasm accessions collected from across the country and elsewhere.

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