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In Vitro Establishment, Conservation and its Implications for Grape Germplasm Biodiversity

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Abstract

A series of experiments were carried out to establish the in vitro cultures from the explants taken from the field gene bank after surface sterilization of seven different grape accessions. Seven accessions of grape germplasm were tested on 75% Murashige-Skoog media containing varying levels of inorganic salts. Among all the accessions, maximum viability (90%) and shoot length (4.18 cm) and highest no. of nodes (3.6) were achieved in 4472 (18). Moreover, response of cultures to different treatments was found to be dependent on the accession and the duration of the treatment. In another set of experiments two different kinds of osmotics, namely, sorbitol and mannitol were used to study the growth retardation of in vitro grown grape cultures. Each of the two was separately supplemented in the 75% MS media at different concentrations i.e. 0, 0.01, 0.05, 0.2, 1.0 or 1.5 g/l under standard light and temperature. Observations on eight different parameters viz. Viability, Mortality, Contamination, Shoot number, Shoot length, Number of nodes , Shoot mass, Root mass and Chlorophyll content were performed. The results revealed that there was a decline in culture growth along with the increase in the concentration of either sorbitol or the mannitol. The retardation effect was found to be maximal at the highest concentration of either sorbitol or mannitol. The latter proved slightly better than the former in inducing growth retardation to the accession under study.

Keywords: Grape, *In vitro* conservation, Germplasm biodiversity, *In vitro* establishment.

Introduction

Grape (*Vitis vinifera* L.) is one of the extensively grown fruit crops in the world [1].*Vitis Vinifera* L. has given rise to over 1400 cultivars grown today in most of the grape growing areas of the world [2]. Grape is a perennial crop and its flower bud formation generally occurs during late summer or autumn in the subtopics and temperate climate. These buds open during next year. Based on use and commerce, the grapes are broadly classified as: table grapes, raisins grapes, wine grapes, juice grape and canning grapes [3]. Pakistan has been bestowed with varying agro-climatic conditions by nature making it suitable for growing a wide variety of fruits including grapes. In Pakistan, grapes are grown over an area of 13,000 ha with annual production of 49.0 thousand tones [4]. Cultivation of fruits not only helps in improving biological productivity but also assists in maintaining ecological sustainability, earning of foreign exchange and providing direct and indirect employment to the poor. After apple, grape is the second major fruit in Balochistan. Within fruits, it contributed 15.22 per cent and 10.17 per cent both in area and production respectively in Balochistan while grapes contribute 98.42 per cent and 95.37 per cent in overall area and production of Pakistan. A number of varieties of grapes are grown in highland areas of the province. The districts, Pishin, Killa Abdullah and Mastung contributed more than 70 per cent to area and production of grapes in the province. Grape production during the last decade increased from 31,010 to 73,787 tonnes with an increase of 138 per cent mainly due to rural electrification, which resulted in extensive installation of tubewells.

For any plant breeding programme, plant germplasm preservation is an integral part but some of grape varieties are being vanished owing to genetic drift, bad agronomic practices and short of conservation strategies. The most efficient and economical way of germplasm storage is in the form of seeds. However, this kind of storage is not always feasible because: 1) some plants do not produce seeds and only require vegetative propagation, 2) seeds remain viable only for a limited duration, 3) some seeds are heterozygous as these plants lose their unique characteristics such as growth, behavior, yield and quality, consequently not appropriate for maintaining true-to-type genotypes and, 4) seeds of certain species deteriorate rapidly because of seed-borne pathogens [5].The vegetative propagation also faces challenge due to high rate of mortality and low rate of rooting in soil planted cuttings [6]. In such crops like grape, clonal propagation is imperative as each cell is totipotent, containing compulsory genetic information to generate true to type plants. Propagation by axillary shooting has proved to be the most applicable and reliable method of *in vitro* propagation of *Vitis vinifera* [7, 8]. Commercial tissue culture laboratories are now able to propagate a large number of herbaceous and horticultural species and several woody plants [9]. Tissue culture techniques in conjunction with careful manipulation of cryobiological methods could be advantageously used for the preservation of recalcitrant plant germplasm. Preservation of plant cells, meristems and somatic embryos has become an important tool for the long-term storage of plant species using lowest space and maintenance [10].

To maintain the genetic diversity of the endangered plant species, *in-vitro* conservation is a technique employed specifically for vegetatively propagated crops such as sugarcane, potato, sweet potato, banana and grape. Furthermore *in-vitro* conservation involves the maintenance of explants in sterile and pathogen free environments. Keeping in view the problems related to grape propagation, conservation and its economic importance in the country, the present study was designed to meet the relevant objectives. These studies will have an impact on germplasm storage and movement for future grape genetic improvement strategies.

Materials and methods

Plant material

The explants were taken from the field gene bank at the premises of the Institute of Agricultural Biotechnology and Genetic Resources (IABGR), National Agriculture Research Centre (NARC), Islamabad. Seven accessions of grape namely 4492(1) Kowar Naray, 4489(1) Kowar Jangli, 4472(12) Harker Agoon, 4472(9) Lok Hamochan Agoon, 4494(1) Kowar Sufaid, 4472(18) Singh Lok Agoon and 4698 were used for culture establishment and three accessions namely 4472(1) Gang Agoon Sufaid, 4497(7) Haita and Wild Grape (WG) were used for *in vitro* conservation.

Media composition, sterilization and dispensing

In previous studies it was found that grapes, although being woody in nature, do not respond well to Woody Plant Medium (WPM). They were also found to show better response on 75% levels of MS salts than on the 100% levels of MS salts. Therefore, in the current studies, all the grape germplasm were grown on 75% MS salts supplemented with standard concentrations of sucrose and vitamins of MS medium and a multitude of hormonal regimes for *in vitro* culture establishment and other studies. All the inorganic salts, vitamins and hormones used in the experiment were from Sigma and Wako Chemical Company. The Test tubes containing the media were sterilized by autoclaving at 121^{o}C either for 9 minutes. Wet heating in autoclave killed the bacteria and fungal spores. For establishment of germplasm, the test tubes with autoclavable plastic caps were used because buds require a small area for establishment and to avoid contamination.

Establishment of *in vitro* cultures

A series of experiments were carried out to study the *in vitro* effect of duration of surface disinfectant ($HgCl_2$) on various culture establishment parameters (mortality, viability and contamination) and culture growth parameters (shoot number, shoot length and number of nodes per plant) of different grape germplasm accessions. The nodal segments or lateral buds from a fresh shoot obtained from field are exposed to various pathogenic elements such as viruses, bacteria, and fungi. These pathogens restrict and hinder the growth of plants by releasing toxic substances or accelerating the process of death and decay of plants. It is therefore imperative that the nodal segments or buds were surface sterilized to free them from surface contaminants as reported by A.J.ABBOT (1977) [11].

Surface sterilization was carried out under aseptic conditions of clean bench (Hitachi). The nodal segments were excised from the field grown grape plants with the help of scalpel and collected in the flasks containing sterile water in order to avoid desiccation. Nodal segments were trimmed to a bud size and were rinsed in running tap water for 60 minutes. Buds were soaked in 0.1 % mercuric chloride ($HgCl_2$) solution for one, two, three or four minutes in clean bench for disinfection and shaken frequently and were rinsed with autoclaved distilled water to remove the traces of the disinfectant; the rinsing was repeated 3-4 times each for 2-3 minutes. The cut end or edges of the explants that had become dead due to exposure to the disinfectant were removed before it was inoculated on to the appropriate media. Once the explants have been disinfected, they were transferred on to a medium of defined composition under aseptic conditions. The cultures were incubated in the growth room maintained at a temperature of 20 ± 2 ^{o}C, illuminated with 1000-2000 lux of light, maintained under a photoperiod of 16 hours and data were recorded after 4-6 weeks.

Osmotics mediated growth retardation

Three accessions of grape namely 4472(1) Gang Agoon Sufaid, 4497(7) Haita and Wild Grape (WG) taken from *in vitro* gene bank were conserved using minimal growth approach. Each of the two osmotica (sorbitol and mannitol) was separately supplemented in the 75% MS media at different concentrations i.e. 0, 0.01, 0.05, 0.2, or 1.0 gm/l. These levels of growth retardants were incorporated into the media containing seventy-five percent MS salts, myo-inositol (0.1 g/L), vitamin stock (1 ml/L), 3% sucrose and 0.8% agar. The cultures were incubated in the growth room maintained at a temperature of 20 ± 2 ^{o}C, illuminated with 1000-2000 lux of light, maintained under a photoperiod of 16 hours and data recorded. The growth retardation effect of the osmotics was compared between the accessions and the level and type of the osmotic used.

Measurement of Data

Viability, mortality, contamination, shoot number, shoot length, number of nodes, shoot mass, root mass and chlorophyll content are the parameters on which data were taken after the 4-6 weeks. Number of replicates was different for each experiment ranging from 3-10 in completely randomized design. The data thus collected were subjected to statistical analysis after R.G.D.STEEL and J.H TORRIE (1980) [12].

Results and Discussion

A number of conventional and modern techniques were being carried out to improve grape germplasm for higher yields, disease resistance, quality enhancement and improved nutritional value. Among these techniques, tissue culture technique occupies a central position. The ability to grow plant cells and tissues in culture and to control their development represents the basis of many practical applications in agriculture, horticulture and industrial chemistry and is a prerequisite for plant genetic engineering. Crop improvement by

conventional methods is slow and depends on the sexual recombination of selected plants to regenerate new genotypes that can be propagated through seeds. *In vitro* cultivation of plant cells and tissues and the new molecular biological procedures, had a pronounced impact on both plant breeding and vegetative propagation (micro propagation) and has also simplified the storage and conservation of germplasm. Germplasm storage and conservation of vegetatively propagated species require special and unique conditions as they are either seedless or their seed is not used in raising crops due to genetic inherent heterogeneity. Germplasm storage is a vital component of the systematic germplasm improvement systems such as trans-boundary movement and germplasm exchange. Hence, *in vitro* conservation offers an alternative to field gene banks. Therefore, we attempted to carry out the present study to relate the best possible conditions to successful *in vitro* culture establishment and conservation.

***In vitro* culture establishment**

MS salt concentration in the subsequent experiments was reduced to 75% levels because from the previous studies it is clear that full strength MS media is not suitable for the culture growth of grapes, as the high salt concentration resulted in leaf senescence [13]. Similar observations were reported by Dimitrova *et al.,* (2000) on reducing the macronutrients to only 40% of the standard level in MS media and to increase the micronutrients to 120% levels [14]. Due to scarcity of the planting material available for culture establishment, every effort was made to ensure the survival of cultures through the process of surface sterilization. Duration of treatment with the disinfectant is very critical, to strike a balance between the mortality due to the excessive disinfectant treatment and contamination due to incomplete disinfections. For this reason, mercuric chloride treatments of different durations were used. In some of the accessions, there was an increase in mortality of cultures as the duration of treatment was increased but in other accessions, there was no such response. This may be attributed to that explants were taken from field grown plants, which may be infected with the pathogens to varying extents and also number of explants available were very few. Dalal *et al.* (1999) have also reported that the explants source and the cultivars both had significant effects on the culture survival [15]. Therefore, careful attempts were made to ensure that at least some buds survive the harsh treatments to save the valuable material. In our study, grape accession, 4472(9), showed maximum viability 58.3% with 4 min treatment of $HgCl_2$ with 0% mortality. Maximum shoot number (2.0), shoot length (3.8 cm) and no of nodes per plant (4.0) was achieved with 1 min treatment. In case of other accession, 4472(12), maximum viability (80%) was achieved with 3 min treatment of $HgCl_2$ with 10% mortality and contamination. Maximum shoot number (1.5) was obtained with 2 min treatment and shoot length (4.26) with 4 minute treatment. Grape accession 4489(1) showed maximum viability (75%) and minimum contamination (16.6%) with highest shoot length (2.98 cm) and no. of nodes (2.1) for 3 min treatment of $HgCl_2$. In another grape accession, 4492(1), maximum viability of 88.8% with 0% mortality, highest shoot number (1.25) and shoot length (3.25 cm) was achieved when treated for 2 min treatment of $HgCl_2$. Highest no. of nodes (2.66) were obtained in 4 min treatment of $HgCl_2$ with minimum contamination of 10%. Grape accession namely, 4494(1) was registered with maximum viability of 83.3% with 8.3% mortality and contamination and highest shoot number (1.3) for 4 min treatment. Highest shoot length of 3.27 cm and no. of nodes (2.5) was achieved with 2 min treatment of $HgCl_2$. In case of the accession (4698), surface sterilization was carried out with $HgCl_2$ either for 1, 2, 3 or 4 minutes. The maximum viability (50%) was achieved with 4 minutes and 0% mortality with 2 minutes. The highest shoot number (1), shoot length and no. of nodes were obtained with 1 minute treatment of $HgCl_2$. (Table 1, Figure 1(A and B). Among all the accessions, grape accession, 4472(18) showed maximum viability of 90% with 0% contamination and 10% mortality as a result of treatment for 2 min treatment of $HgCl_2$. Highest shoot length and no. of nodes (4.18 cm and 3.6) was achieved with 1 min treatment.

Table 1. Effect of duration of treatment of the grape germplasm accessions with $HgCl_2$ on viability, contamination, mortality, shoot number, shoot length and no. of nodes.

Accession	Treatments with $HgCl_2$ (min)	Viability (%)	Contamination (%)	Mortality (%)	Shoot number explant^{-1}	Shoot length (cm)	No. of nodes plant^{-1}
4472(9)	**1**	30	50	20	2	3.86	4
	2	30	60	10	1	2.93	4
	3	30	60	10	1	2.2	1
	4	58.3	41.6	0	1.2	3.16	2.66
4472(12)	**1**	60	30	10	1	3.15	1.5
	2	50	20	30	1.5	4	1.33
	3	80	10	10	1.4	3.85	1.57
	4	57	14.3	28.5	1.25	4.26	2.6
4489(1)	**1**	25	75	0	2	2.25	1.75
	2	7.6	46	46	1	3.5	2
	3	75	16.6	8.3	1.62	2.98	2.1
	4	50	28.5	21.4	1.5	2.65	1.11
4492(1)	**1**	77	11.1	11.1	1	2.7	2
	2	88.8	11.1	0	1.25	3.25	2.5
	3	55.5	11.1	33.3	1	3.15	2
	4	70	10	20	1	2.43	2.66
4472(18)	**1**	60	20	20	1	4.18	3.6
	2	90	0	10	1	3.95	2.5
	3	40	20	40	1.25	3.22	2.2
	4	58	8.3	33.3	1.25	3.12	1.25
4494(1)	**1**	40	50	10	0	0	0
	2	40	30	30	1	3.27	2.5
	3	40	30	30	1	3.25	2
	4	83.3	8.3	8.3	1.33	3.25	1.88
4698	**1**	10	60	30	1	2	1
	2	40	60	0	0	0	0
	3	30	40	30	0	0	0
	4	50	41.6	8.3	0	0	0

On the whole, response of cultures to different treatments was found to be dependent on the accession and the duration of the treatment. Pierik (1987) observed that various factors related with plant materials from which explant is taken affect the growth *in vitro* viz: genotype, plant stage, physiological state and age of tissue or explants, general health of the plant, position of explants within the plant, size of the explants and methods of inoculation [16]. Murashige and Skoog (1962) also reported that genetic makeup is a decisive factor at every stage in the life cycle of a plant [17]. The expression of genetic makeup also depends on physical and chemical conditions available during growth *in vitro*. Moreover, tissues from different parts of the same plants may indicate different requirement for normal growth in the medium. Abracheva *et al.,* (1992) have observed that larger explants gave the regenerants with the highest vigor [18]. In one of the accessions namely, 4489(1), the mortality did not depend upon the duration of disinfection treatment as no mortality was obtained even in the shortest (1 min) duration of treatment tested in this study. The viability was maximum with 2 or 3 min treatment with $HgCl_2$ in most accessions. Highest contamination of culture was observed with the shortest duration of treatment and vice versa. In some cases, the grape accessions which produced the highest shoot length could not produce the highest number of nodes. Silva *et at* (2000) have reported significant differences mainly concerning shoot and root development and number of nodes among the five varieties of the grapevine root stock [19].

***In vitro* conservation via growth retardation**

The field gene bank collections and plantations are prone to natural disasters such as disease epidemics and therefore, *in vitro* gene bank establishments are vital to safeguard against the germplasm extinctions. An efficient system for culture establishment and culture revival is critical for germplasm management of an extensive collection of germplasm. Growth retardation induced by osmotics such as Sorbitol and Mannitol may prove to be very useful tool at hand to manage the germplasm conservation on a large scale as this will save space, labor and manpower. The advantage of this approach is that cultures can be readily brought back to normal culture conditions to produce plants on demand. However, the need for frequent subculturing may pose a great disadvantage including contamination of cultures as well as imposition of selection pressure with subsequent change in genetic make-up due to somaclonal variation. In our study in case of the grape accession Gang Agoon Sufaid [4472(1)], there was no mortality and very low contamination of cultures for either of treatments of sorbitol tested (Table.2 and Fig.1(C).

There was also decline in shoot length and no. of nodes along with the increase in concentration of sorbitol. In grape accession WG (Wild Grape) mortality and contamination was very low, other growth parameters such as shoot number, shoot length, no. of nodes, chlorophyll content, shoot mass and root mass showed growth retardation as the concentration of sorbitol was increased from 0g/l to 1.0g/l. Grape accession Haita [4497(7)] showed no mortality and very low contamination. There was also decline in shoot number, chlorophyll content, shoot mass and root mass as the concentration of osmotics was increased from 0g/l to 1.0g/l.

There was decline in culture growth along with the increase in the concentration of mannitol in case of accession Gang Agoon Sufaid [4472(1)]. Very low contamination and zero mortality were documented. The retardation effect in shoot length, no. of nodes and shoot mass was found to be highest at the uppermost concentration of mannitol (1.0g/l). All the growth parameters such as shoot number, shoot length, no. of nodes, chlorophyll content, shoot mass and root mass for grape accession, WG, showed decline in culture growth at the highest concentration of mannitol (1.0g/l). In grape accession, Haita [4497(7)], 100% viability was observed in all the cultures for either of the treatments of mannitol tested. Also growth reduction was observed as the concentration of mannitol was increased from 0g/l to 1.0g/l. (Table.3 and Fig.1 (D).

In our findings, degree of retardation was directly proportional to the concentration of the osmotic regulator. The retardation effect was found to be highest at the highest concentration of either sorbitol or mannitol. The latter proved slightly better than the former in imparting growth retardation to the accession under study. All the growth parameters including shoot number, shoot length, no. of nodes, chlorophyll content and shoot mass exhibited reduction in both the osmotica i.e., sorbitol and mannitol. We noticed no deterioration in cultures of any accession after 8 weeks in culture as they had to be harvested to record data on growth parameters and therefore it seems appropriate that they can be stored a long more than 30 weeks. Similar results have been reported by Ishtiaq *et al.* (2001) in case of potato cultures of a number of accessions obtained from the International Potato Centre, Lima, Peru [20]. These treatments may improve even further the effectiveness of conservation protocols if they are used in conjunction with other treatments such as growing the cultures at low temperatures. Conservation mediated by osmotics may be more economical than cryopreservation. Our findings are also in conformity with the results of Lemos *et al* (2002) who used mannitol and Sorbitol as osmotic regulators along with other reagents such as sucrose and abscissic acid and found that they have a positive effect on conservation of germplasm without any sub culturing for 52 weeks [21].

Table 2. Growth retardation induced by varying levels of sorbitol for short term *in vitro* conservation of grape germplasm accessions.

Accessions	Treatments	Mortality (%)	Viability (%)	Contamination (%)	Shoot number per explant	Shoot length(cm)	No.of nodes per plant	Chlorophyll Contents	Shoot mass(g)	Root mass(g)
Gang Agoon Sufaid	S 0	0	100	0	1	6.48	6.0	33.32	0.35	0.30
	S 0.01	0	80	20	1.25	6.80	5.8	33.89	0.55	0.83
	S 0.05	0	100	0	1	5.08	3.4	32.0	0.46	0.16
	S 0.2	0	100	0	1	6.70	5.60	26.62	0.38	0.19
	S 1.0	0	100	0	1.4	5.41	5.57	35.7	0.40	0.59
	Mean	**0**	**96**	**4**	**1.13**	**6.09**	**5.27**	**32.30**	**0.42**	**0.37**
	Range	**0**	**80-100**	**0-20**	**1-1.4**	**5.08-6.80**	**3.4-6**	**26.62-35.7**	**0.35-0.55**	**0.16-0.83**
Wild Grape	S 0	0	80	20	1.75	6.0	6.0	29.8	0.43	0.19
	S 0.01	0	100	0	1.2	5.65	5.5	23.4	0.19	0
	S 0.05	0	100	0	2.0	4.05	4.6	33.65	0.35	0.30
	S 0.2	20	60	20	2.0	3.83	2.66	27.2	0.65	0.11
	S 1.0	0	100	0	1.2	3.63	4.83	27.9	0.18	0.11
	Mean	**4**	**88**	**8**	**1.63**	**4.63**	**4.71**	**21.39**	**0.36**	**0.14**
	Range	**0-20**	**60-100**	**0-20**	**1.2-2**	**3.63-6**	**2.66-6**	**23.4-33.5**	**0.18-0.65**	**0-0.30**
Haita	S 0	0	100	0	1.8	3.91	5.0	26.88	0.23	0.23
	S 0.01	0	100	0	1.8	4.01	6.62	27.97	0.40	0.31
	S 0.05	0	100	0	2	3.40	4.70	27.84	0.29	0.12
	S 0.2	0	100	0	2	4.71	5.40	28.98	0.46	0.41
	S 1.0	0	60	40	1	4.0	5.0	14.8	0.21	0.02
	Mean	**0**	**92**	**8**	**1.72**	**4.0**	**5.34**	**25.29**	**0.31**	**0.21**
	Range	**0**	**60-100**	**0-40**	**1-2**	**3.4-4.71**	**4.7-6.62**	**14.8-28.98**	**0.21-0.46**	**0.02-0.41**

Table 3. Growth retardation induced by varying levels of mannitol for short term *in vitro* conservation of grape germplasm accessions.

Accessions	Treatments	Mortality (%)	Viability (%)	Contamination(%)	Shoot number per explant	Shoot length(cm)	No.of nodes per plant	Chlorophyll Contents	Shoot mass(g)	Root mass(g)
Gang Agoon Sufaid	M 0	0	100	0	1	6.82	6.4	31.0	0.33	0.30
	M 0.01	0	100	0	1.2	6.4	5	33.42	0.40	0.28
	M 0.05	0	80	20	1.25	6.34	4.0	30.59	0.31	0.17
	M 0.2	0	100	0	1.4	4.17	3.85	20.73	0.28	0.08
	M 1.0	0	100	0	1	4.06	3.20	21.45	0.15	0.28
	Mean	**0**	**96**	**4.0**	**1.17**	**5.55**	**4.49**	**22.03**	**0.29**	**0.22**
	Range	**0**	**80-100**	**0-20**	**1-1.40**	**4.06-6.82**	**3.20-6.40**	**20.13-33.42**	**0.15-0.40**	**0.08-0.30**
Wild Grape	M 0	0	100	0	1.6	3.13	3.87	25.26	0.18	0.07
	M 0.01	0	100	0	2.0	4.45	4.80	30.7	0.44	0.34
	M 0.05	0	100	0	1.4	3.74	4.42	26.72	0.25	0
	M 0.2	0	100	0	1.6	2.68	3.25	27.27	0.22	0.06
	M 1.0	0	100	0	1.2	2.53	2.33	19.7	0.16	0
	Mean	**0**	**100**	**0**	**1.56**	**3.30**	**2.96**	**25.93**	**0.25**	**0.09**
	Range	**0**	**100**	**0**	**1.2-2.0**	**2.68-4.45**	**2.33-4.80**	**19.7-30.7**	**0.16-0.44**	**0.-0.34**
Haita	M 0	0	100	0	1.4	3.5	4.85	20.02	0.27	0.1
	M 0.01	0	100	0	2.2	4.5	7.81	27.98	0.72	0.27
	M 0.05	0	100	0	2.2	3.71	4.18	19.8	0.57	0.16
	M 0.2	0	100	0	2.4	2.90	3.33	11.28	0.23	0
	M 1.0	0	100	0	1.4	2.72	3.0	14.4	0.26	0
	Mean	**0**	**100**	**0**	**1.92**	**3.46**	**4.63**	**18.69**	**8.42**	**0.11**
	Range	**0**	**100**	**0**	**1.4-2.4**	**2.72-4.50**	**3.0-7.81**	**11.28-27.98**	**0.23-0.72**	**0-0.27**

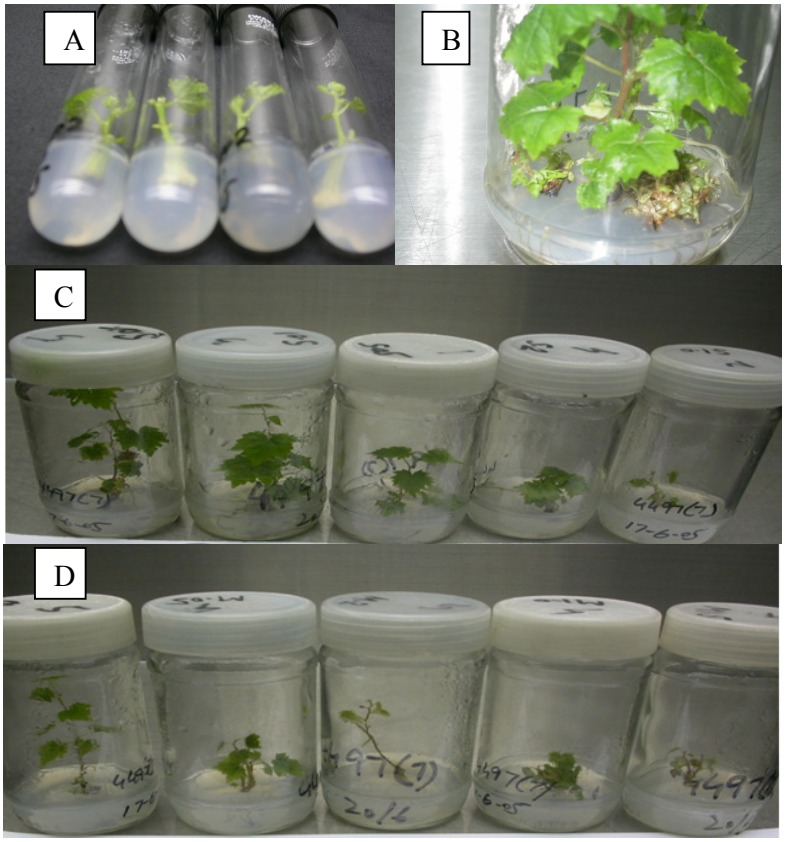

Figure 1: Grape germplasm accessions at their early stages of establishment (A and B), Grape germplasm conservation through retardation induced by various levels of sorbitol and mannitol (C and D) respectively increasing from left to right.

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