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In vitro storage of some pear genotypes with the minimal growth technique

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Abstract: Shoot cultures of 9 pear genotypes collected from different areas of Azad Jammu and Kashmir (northern Pakistan) were maintained on MS medium supplemented with 30 g L^{-1} of sucrose + 7 g L^{-1} of agar + 1 mg L^{-1} of BAP. For in vitro preservation shoot tips were excised from these cultures and transferred onto different media, i.e. full strength, ¼ strength, ½ strength MS medium, and full strength MS medium containing 2.5% or 3.5% (w/v) mannitol. The cultures were assessed for their survival after storage periods of 3, 6, 9, and 12 months. To test the regenerability of the cultures they were transferred onto fresh culture medium. The genotypes differed significantly in their survival; however, they were statistically similar in terms of regenerability when transferred onto fresh medium. Shoots of the Khurolli and Bagugosha genotypes remained quite healthy, with relatively high survival rates (53.25% and 50.50%, respectively), whereas those of the Desi nakh genotype had the lowest survival rate (41.02%). Full strength MS medium containing 2.5% (w/v) mannitol gave the highest survival and regenerability rates (55.82% and 52.31%, respectively) in cultured shoots. Preservation for the shortest period (3 months) resulted in the highest survival (63.41%) and regeneration (58.81%). Genotypes and storage period interaction showed maximum shoot survival in Kashmiri nakh stored for 3 months, while Raj btung cultures stored for 12 months responded poorly, with the minimum survival rate. Addition of mannitol at the lower concentration of 2.5% (w/v) was an effective technique to lengthen subculture duration, possibly because of a gradual increase in osmotic pressure in the medium, which resulted in reduced availability of water to the growing cultures.

Key words: In vitro preservation, osmoticum, *Pyrus*, reduced medium strength, regenerability, survival

Introduction

Maintenance and preservation of diversity in crop plants is not only important to breeders for crop improvement programs, but also to human beings for fulfilling their nutritional requirements. Valuable resources of pear in wild form and primitive varieties are being displaced and lost as a result of new cultivars, deforestation for fire and timber wood, and long-term exposure to environmental stresses. The importance of preserving these reservoirs of genetic diversity/natural gene pools has not been fully understood, especially in Pakistan. Ex situ preservation of genetic materials, such as field gene banks, seed storage, and botanical gardens, is mostly

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used to safeguard populations in danger of extinction (Rao 2004). Primary collections of *Pyrus* germplasm are conserved primarily in field gene banks (Hummer 1993; Hummer and Sugar 1998). Unfortunately, maintenance of fruit germplasm in the field as living collections is expensive, in terms of labor and land. Moreover, collections are vulnerable to environmental catastrophes and unforeseen problems, such as the risk of destruction by such natural disasters as earthquakes and landslides, to which germplasm are continuously exposed, causing the sudden loss of valuable germplasm.

For safe preservation, the in vitro slow growth storage method was developed and is considered an alternate solution for short- to medium-term storage of fruit germplasm. In vitro culture is an effective method for ex situ conservation of plant genetic diversity (Fay 1994), allowing rapid multiplication from very little plant material and with little impact on wild populations. Slow growth techniques, based on the manipulation of culture conditions and/or culture media, allow cultures to remain viable with a slow growth rate. Furthermore, organized culture systems for regeneration and successful propagation of genetically stable plantlets from cultures are prerequisites for in vitro preservation (Rao 2004). This preservation technique also facilitates the maintenance of pathogen-free plant material and ensures that cultures are not damaged by adverse weather (Withers and Engelmann 1997). Furthermore, virus-tested cultures could provide ideal material for the exchange and distribution of germplasm, as they will be acceptable to plant quarantine authorities (Paroda et al. 1987).

In vitro preservation with the minimal growth technique comprises reducing incubation temperature, modifying/manipulating culture medium (altered nutrient availability), and supplementing with osmotically active compounds, such as mannitol and polyethylene glycol (PEG), to suppress shoot growth (Chen and Dribnenki 2004). These storage techniques are generally applicable to a wide range of fruit tree genotypes to extend the ordinary subculture duration from a few weeks to 12 months. Furthermore, various methods have been used to conserve plant species, such as reducing the concentration of basal media by 25% or 50% (Moriguchi and Yamaki 1989) and by adding mannitol (Wanas et al. 1986). Westcott (1981) effectively achieved slow growth of potato shoots by using 0.2 M mannitol in culture medium. Kartha et al. (1981) preserved coffee shoots for 2 years with the use of ½ strength MS medium without sugar at normal temperature. Further, mannitol has been used successfully to preserve pear rootstocks (Lambardi et al. 2006). These alternate preservation techniques are less costly and safe for conserving germplasms (Epperson et al. 1997). Minimal growth storage is very simple technique and has been studied in several laboratories for *Pyrus* germplasm conservation (Wanas et al. 1986; Wilkins et al. 1988; Moriguchi 1995). Depending on species, these stored plants can be micropropagated rapidly when desired.

Pyrus germplasm preservation using slow growth techniques has not been previously studied in the valuable pear germplasm found in northern Pakistan. The present study is the first effort to extend subculture duration and develop a suitable slow growth storage method by reducing medium strength and adding mannitol to the culture medium for divergent pear genotypes.

Materials and methods

Bud woods of 8 local pear genotypes—Khurolli, Bagugosha, Pathar nakh, Desi nakh, Kotharnul, Frashishi, Kashmiri nakh, and Raj btung—were collected from different areas of Azad Jammu and Kashmir (northern Pakistan) and grafted onto Btangi rootstock in the nursery at the Horticultural Research Institute (HRI), National Agricultural Research Centre (NARC), Islamabad. Explants prepared from shoots of these genotypes and of Btangi were established on MS medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of agar and 1 mg L^{-1} of BAP. Shoot tips were aseptically excised from established cultures of these genotypes, trimmed to a length of 1-1.5 cm, and immediately placed in 200-mL culture jars containing 30 mL of culture medium. Preservation of cultures through modification of medium and addition of mannitol was according to the following treatments:

T_0 = full strength MS medium;

T_1 = ¼ strength MS medium;

T_2 = ½ strength MS medium;

T_3 = full strength MS medium containing 2.5% (w/v) mannitol;

T_4 = full strength MS medium containing 3.5% (w/v) mannitol.

The culture jars were covered and kept at 25 ± 2 °C, with a 16-h photoperiod under white fluorescent light (intensity: 55 μmol m^{-2} s^{-1}), for different periods (3, 6, 9, and 12 months). The experiment was factorial with a completely randomized design (CRD) consisting of 9 genotypes, 5 culture media, and 4 storage periods, with 3 replications. There were 5 shoots in each replication. Data were recorded on survival of the shoot after every storage period. For regenerability of cultures, shoots were removed from the cultures, trimmed to a length of 1-1.5 cm, and transferred to fresh shoot culture medium (full strength MS medium supplemented with 30 g L^{-1} of sucrose, 7 g L^{-1} of agar, and 1 mg L^{-1} of BAP). The cultures were kept at 25 ± 2 °C, with a 16-h photoperiod under white fluorescent light (intensity: 55 μmol m^{-2} s^{-1}). Data regarding regeneration percentages were recorded 1 month after transfer to the culture medium.

Assessment of cultures

Survival of the cultures was assessed on the basis of the criteria described by Reed (1992): dead and brown shoots were considered not to have survived, while those with vigorous growth and healthy leaves were considered to have survived.

$$\text{Survival rate} = \frac{\text{number of shoots survived}}{\text{number of shoots transferred}} \times 100$$

The regenerability of cultures was estimated on the basis of the initiation of shoot growth using the formula:

$$\text{Regeneration rate} = \frac{\text{number of shoots regenerated/ revived growth}}{\text{number of shoots transferred to culture medium}} \times 100$$

Statistical analysis

Data obtained were statistically analyzed using analysis of variance. The treatment means were compared using Duncan's multiple range (DMR) test at 5% probability, using the MSTATC statistical computer package (Michigan State University, East Lancing, MI, USA).

Results

The response of in vitro cultured shoots stored for 3, 6, 9, and 12 months to reduced medium strength and mannitol was assessed on the basis of survival and regeneration rates. The survival rate of each genotype differed significantly; however, non-significant differences were observed between the genotypes in terms of regrowth. The highest survival rate was recorded in Khurolli, followed by Bagugosha and Btangi, and the lowest was observed in Desi nakh (Tables 1 and 2).

The results obtained for all the medium treatments were significantly variable. The maximum survival and regeneration rates were obtained on MS medium supplemented with 2.5% (w/v) mannitol, which significantly differed from other media. The remaining treatments were statistically similar to each other, with significantly lower survival and regeneration rates (Tables 1 and 2).

The storage period also had a significant effect on both parameters (Figure 1a and b). The highest survival and regeneration rates were recorded for 3-month storage, which were significantly different than for the other storage periods. The cultured shoots stored for 12 months had the greatest decrease in both parameters.

The interaction between genotype and medium treatment was statistically non-significant; however, the interaction between genotype and storage period was statistically significant. The maximum survival rate was obtained with the 3-month storage period in Kashmiri nakh. The shoots of Btangi, Frashishi, and Kotharnul were similar and had higher survival rates than other genotypes for the same period of storage (Figure 1a and b). Cultures of Raj Btung, Btangi, Kotharnul, Bagugosha, Pathar nakh, and Kasmiri nakh responded poorly, with minimum survival rates, when stored for 12 months (Figure 1a). As far as regeneration is

Table 1. Survival percentage of pear genotypes as affected by medium strength and mannitol stored for different durations.

Storage periods	Khurolli	Bagugosha	Pathar nakh	Desi nakh	Kotharnul	Btangi	Frashishi	Kashmiri nakh	Raj btung	Mean (T × P)	Mean (treatments)
						T_0 (MS medium full strength)					
3 months	65.66	54.66	73.33	54.00	52.00	54.33	77.00	74.66	38.66	**60.48 abc**	**44.18 b**
6 months	54.66	62.66	38.66	33.33	53.00	40.33	50.66	39.66	65.66	**48.74 d-g**	
9 months	39.00	49.66	37.00	32.00	26.00	43.00	46.66	53.33	49.66	**41.85 fgh**	
12 months	37.66	17.66	30.00	25.33	10.00	11.66	35.00	31.66	31.66	**25.63 ij**	
Mean (G × T)	**49.25**	**46.17**	**44.75**	**36.16**	**35.25**	**37.42**	**52.33**	**49.83**	**46.42**		
						T_1 (MS medium ¼ strength)					
3 months	53.00	52.00	71.00	37.00	80.66	55.00	57.33	63.00	38.00	**56.33 bcd**	**43.23 b**
6 months	56.00	51.33	53.00	52.66	55.66	55.66	37.66	47.66	68.00	**53.07 b-e**	
9 months	63.33	61.00	40.66	31.66	33.00	56.00	20.66	39.33	54.33	**44.44 efg**	
12 months	19.66	11.66	8.66	34.00	30.00	12.00	32.66	7.00	16.00	**19.07 j**	
Mean (G × T)	**48.00**	**44.00**	**43.33**	**38.83**	**49.83**	**44.66**	**37.08**	**39.25**	**44.08**		
						T_2 (MS medium ½ strength)					
3 months	52.66	76.00	57.66	55.66	65.00	76.66	77.00	77.66	75.66	**68.22 a**	**44.73 b**
6 months	55.66	68.66	32.00	37.66	63.00	53.00	52.00	38.00	38.00	**48.66 d-g**	
9 months	67.33	43.66	33.00	41.00	48.33	38.66	15.00	37.00	34.00	**39.78 gh**	
12 months	42.33	28.00	26.66	21.33	15.33	37.33	4.66	14.66	10.00	**22.26 j**	
Mean (G × T)	**54.50**	**54.08**	**37.33**	**38.92**	**47.92**	**51.42**	**37.17**	**41.83**	**39.42**		
						T_3 (MS medium full strength + 2.5% Mannitol)					
3 months	69.00	78.66	52.66	74.00	74.33	76.33	71.33	65.66	52.66	**68.30 a**	**55.82 a**
6 months	58.33	66.00	55.33	62.33	44.00	54.00	50.33	56.00	72.00	**57.59 bcd**	
9 months	65.66	66.00	64.66	50.66	48.33	49.33	54.00	62.33	23.66	**53.85 b-e**	
12 months	53.00	44.66	39.33	50.33	52.66	41.33	32.66	38.66	39.00	**43.52 e-h**	
Mean (G × T)	**61.50**	**63.83**	**53.00**	**59.33**	**54.83**	**55.25**	**52.08**	**55.67**	**46.83**		
						T_4 (MS medium full strength + 3.5% Mannitol)					
3 months	65.33	55.00	78.00	34.33	58.00	76.33	70.33	72.66	63.66	**63.74 ab**	41.94 b
6 months	71.33	53.00	58.00	51.66	48.00	63.00	34.00	35.00	57.33	**52.37 c-f**	
9 months	42.66	57.00	18.33	31.33	15.66	42.00	18.33	39.00	36.00	**33.37 hi**	
12 months	32.66	12.66	18.66	10.00	11.33	15.33	37.00	18.00	8.66	**18.26 j**	
Mean (G × T)	**53.00**	**44.42**	**43.25**	**31.83**	**33.25**	**49.17**	**39.92**	**41.17**	**41.42**		
Mean (genotypes)	**53.25 a**	**50.50 ab**	**44.33 bc**	**41.02 c**	**44.22 bc**	**47.58 abc**	**43.72 bc**	**45.55 bc**	**43.63 bc**		

*Means sharing similar letter(s) in a group are non-significant at α = 5% (DMR test).

**G = Pear genotypes, T = Medium treatments, P = Storage periods

Table 2. Regeneration percentage of pear genotypes as affected by medium strength and mannitol stored for different durations.

Storage periods	Khurolli	Bagugosha	Pathar nakh	Desi nakh	Kotharnul	Btangi	Frashishi	Kashmiri nakh	Raj btung	Mean (T × P)	Mean (treatments)
T_0 (MS medium full strength)											
3 months	57.66	50.00	70.33	50.66	49.00	50.33	73.33	69.33	35.33	**56.22 ab**	**40.07 b**
6 months	47.66	54.00	34.33	30.66	48.66	34.00	48.33	37.00	62.00	**44.07 c-f**	
9 months	35.66	45.00	32.00	28.00	18.66	39.00	42.00	46.00	46.66	**37.00 fg**	
12 months	34.33	12.66	27.66	21.66	8.33	11.33	31.33	27.00	32.66	**23.00 hi**	
Mean (G × T)	**43.83**	**40.42**	**41.08**	**32.75**	**31.17**	**33.66**	**48.75**	**44.83**	**44.17**		
T_1 (MS medium ¼ strength)											
3 months	44.66	46.33	66.66	33.66	78.00	54.00	57.66	61.00	35.33	**53.04 abc**	**39.49 b**
6 months	47.66	46.00	51.00	46.66	55.33	52.33	31.00	44.33	63.00	**48.59 b-e**	
9 months	56.00	56.33	36.33	25.33	25.33	52.66	20.00	37.66	45.33	**39.44 efg**	
12 months	17.33	7.66	6.66	30.66	25.33	11.00	29.66	7.66	16.00	**16.89 i**	
Mean (G × T)	**41.42**	**39.08**	**40.17**	**34.08**	**46.00**	**42.50**	**34.58**	**37.66**	**39.91**		
T_2 (MS medium ½ strength)											
3 months	42.66	69.33	53.33	51.66	62.33	65.66	73.33	73.33	69.33	**62.33 a**	**40.82 b**
6 months	53.00	65.66	25.66	33.66	61.66	48.66	47.66	33.66	32.00	**44.63 c-f**	
9 months	63.66	38.33	29.66	39.33	45.00	34.66	13.66	36.00	34.66	**37.22 fg**	
12 months	38.33	20.66	22.00	20.33	14.00	30.33	4.33	12.33	9.33	**19.07 i**	
Mean (G× T)	**49.42**	**48.50**	**32.67**	**36.25**	**45.75**	**44.83**	**34.75**	**38.83**	**36.33**		
T_3 (MS medium full strength + 2.5% Mannitol)											
3 months	56.66	73.33	44.33	69.33	71.00	72.33	71.33	62.66	49.66	**63.41 a**	**52.31 a**
6 months	52.33	58.66	50.00	58.66	40.66	49.00	50.66	53.33	69.66	**53.66 abc**	
9 months	61.33	60.33	63.00	53.33	44.33	46.33	50.33	58.66	21.33	**51.04 bcd**	
12 months	49.66	43.66	39.66	46.66	49.00	38.33	31.00	37.33	34.66	**41.11 def**	
Mean (G × T)	**55.00**	**59.00**	**49.25**	**57.08**	**51.25**	**51.50**	**50.83**	**53.00**	**43.83**		
T_4 (MS medium full strength + 3.5% Mannitol)											
3 months	53.66	47.66	72.00	30.00	55.33	73.00	64.00	72.33	63.33	**59.04 ab**	**38.32 b**
6 months	66.33	47.66	52.00	49.33	45.33	58.66	33.00	35.33	55.66	**49.04 b-e**	
9 months	37.33	52.00	14.66	29.00	12.66	41.66	14.33	36.00	30.00	**29.74 gh**	
12 months	25.00	9.66	15.66	8.00	10.00	13.33	34.66	14.66	6.00	**15.22 i**	
Mean (G × T)	**45.58**	**39.25**	**38.58**	**29.08**	**30.83**	**45.66**	**36.50**	**39.58**	**38.75**		
Mean (genotypes)	**47.05**	**45.25**	**40.35**	**37.85**	**41.00**	**43.83**	**41.08**	**42.78**	**40.60**		

*Means sharing similar letter(s) in a group are non-significant at α = 5% (DMR test)
**G = Pear genotypes, T = Medium treatments, P = Storage period

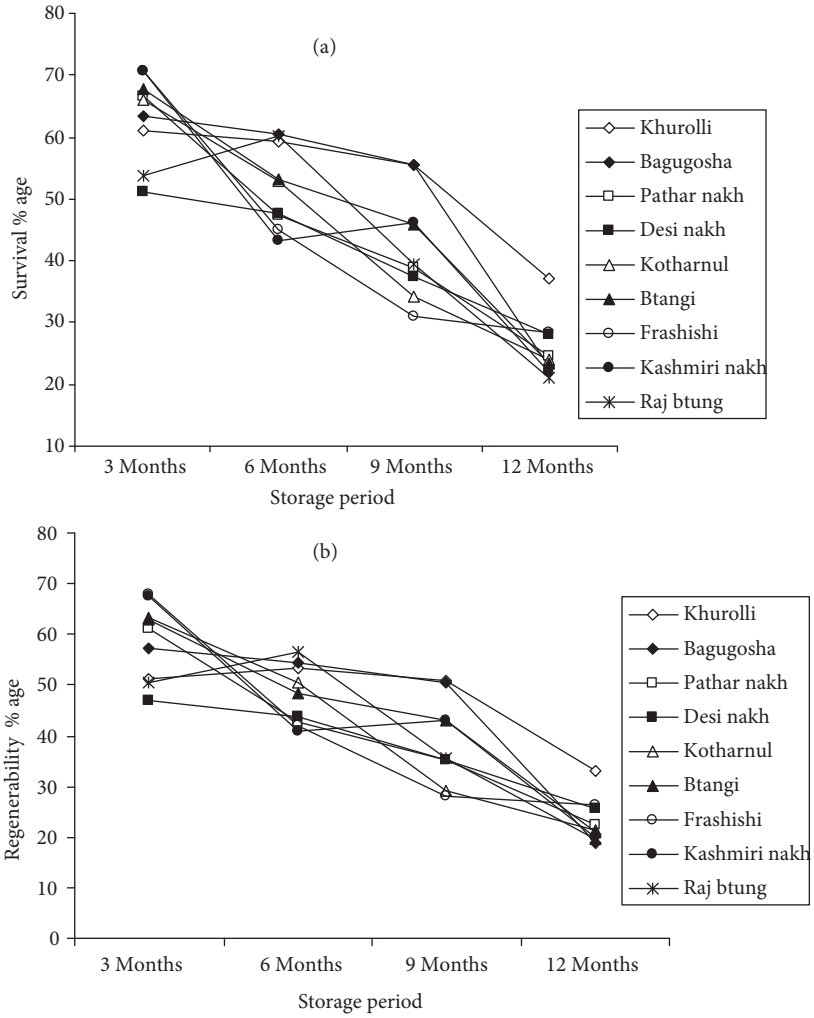

Figure 1. The effect of various storage periods on a) the survival (%) of shoot cultures of 9 pear genotypes and b) Regenerability (%) of shoots after transferring onto fresh shoot culture medium.

concerned, Kashmiri nakh shoots stored for 3 months had the maximum regeneration rate when transferred to the fresh shoot culture medium, whereas Bagugosha, Btangi, Kashmiri nakh, Raj btung, Pathar nakh, and Kotharnul stored for 12 months had a severe reduction in the recovery rate when transferred to fresh shoot culture medium (Figure 1b).

The highest survival and regeneration percentages (63.30% and 63.41%, respectively) were recorded in the cultures stored on medium containing 2.5% (w/v) mannitol for 3 months. The other media treatments were also good for the same time period (3 months). However, the lowest response of the cultures was observed when shoots were stored on medium supplemented with a higher concentration (3.5%, w/v) of mannitol for 12 months (Tables 1 and 2). Moreover, all the other treatments also resulted in a poor response with 12 months of storage.

Discussion

Preservation of plant material in vitro depends upon genotypes, explant types, content of culture medium, and storage period and conditions. Growth medium with reduced availability of nutrients allows explants to develop and grow at a slow rate.

Preservation in vitro with minimal growth can be achieved in a wide range of woody plant species (Wilkins et al. 1988). This is usually accomplished by decreasing the concentration of macro- and micronutrients in the basic medium, or by adding osmoticum (i.e. mannitol).

In the present study the response of different genotypes, in terms of shoot survival, varied according to culture medium modification. The shoots of the genotypes Khurolli and Bagugosha remained quite healthy, with relative high survival rates, depending on the genomic configuration; however, all the genotypes were statistically similar in terms of the regeneration rate and apparently did not show any variability when transferred to fresh medium. These results are in accordance with those of Wilkins et al. (1988), who reported that slow growth storage methods were extremely variable and genotype dependent.

In addition to modifying medium strength, the addition of osmoticum is also effective in suppressing the growth of in vitro cultures under normal incubation conditions (temperature and light) (Lambardi et al. 2006). Mannitol, as an osmoticum in culture medium, facilitates satisfactory conservation of pear rootstocks (Lambardi et al. 2006). In the present study 2 concentrations [2.5% and 3.5% (w/v)] of mannitol were used. The lower concentration of mannitol proved significantly better in maintaining the survival of cultured shoots and also resulted in higher regeneration rates when transferred to culture medium after 6 months of storage. Osmoticum gradually increases the osmotic pressure of the medium and reduces water availability to growing cultures. The higher concentration of mannitol (3.5%) restricted water and nutrient availability to the cultures and quickly reduced culture viability. Poor response in shoots of apple genotypes was observed when medium was supplemented with higher concentrations (3% and 4%) of mannitol (Lambardi et al. 2006; Kovalchuk et al. 2009). It can be concluded that the addition of mannitol to medium is an effective technique to lengthen subculture duration for short-term storage of pear genotypes, when used at the proper concentration; however, higher concentrations of the osmotic compound sharply decreased shoot growth. Wilkins et al. (1988) used osmotic compounds as a mean of suppressing in vitro growth of fruit tree shoot cultures and reported variable results. These results are also partially in agreement with the findings of other researchers (Westcott et al. 1977; Love et al. 1987; Xin 1988), who successfully used mannitol in different concentrations for slow growth conservation of potato and sweet potato germplasms, obtaining survival rates of 70%-100%.

In the present study reduction in MS medium strength and addition of osmoticum resulted in a shoot survival rate greater than 48% when stored for up to 6 months. The regeneration potential of cultured shoots stored for the said period also remained above 44%. The lowest strength medium (¼ strength) was immediately utilized during proliferation of shoots under normal conditions (temperature and light); therefore, depletion of nutrients in the medium caused the shoots to die. Lundergan and Janick (1979) reported that apple shoots were severely affected in vitro by desiccation and nutrient depletion at 26 °C. Similarly, reduced availability of water and nutrients due to the addition of mannitol in medium results in reduced growth of cultures. Some researchers also used low concentrations of nitrate in MS medium for storage of *Vitis* (Moriguchi and Yamaki 1989), *Rubus* (Reed 1993), and apple genotypes (Kovalchuk et al. 2009). However, survival of shoots is also reduced when cultures are stored for longer periods due to depletion of nutrients.

Overall, it appeared that low-concentration MS medium and the lower concentration of mannitol successfully increased the subculture duration of pear genotypes. This short-term in vitro preservation technique is feasible for the maintenance of fruit germplasms in which frequent subcultures enhance the risk of genetic instability (Rani et al. 1995). Furthermore, personnel, energy, and materials costs could be reduced with this type of technique.

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