Pak. J. Bot., 42(3): 1639-1650, 2010.

IN VITRO PRESERVATION OF *PYRUS* GERMPLASM WITH MINIMAL GROWTH USING DIFFERENT TEMPERATURE REGIMES

MAQSOOD AHMED[1*], MUHAMMAD AKBAR ANJUM[2], ASAD HUSSAIN SHAH[3] AND ABDUL HAMID[1]

[1]Department of Horticulture, Faculty of Agriculture, Rawalakot, University of Azad Jammu and Kashmir, Pakistan
[2]University College of Agriculture, Bahauddin Zakariya University, Multan, Pakistan
[3]Department of Plant Breeding and Molecular Genetics, Faculty of Agriculture Rawalakot, University of Azad Jammu and Kashmir, Pakistan

Abstract

In vitro cultures of 9 pear genotypes collected from different areas of Azad Jammu & Kashmir were established on MS medium supplemented with 30 g l^{-1} sucrose, 7 g l^{-1} agar and 1 mg l^{-} BAP. Proliferated shoots of these genotypes were excised aseptically, cultured on fresh medium and tested for their survival and regenerative ability at various incubating temperatures regimes i.e., 25, 15, 10 and 5ºC, stored for 3, 6, 9 and 12 months. Significant differences were found in all genotypes for the both growth parameters and the highest survival (65.25 %) and regenerability percentage (61.21) were recorded in shoots of Frashishi. The least survival (58.92 %) and regenerability percentage (55.21) were observed in shoots of Btangi. As far as temperatures treatment and storage periods are concerned, the highest survival (84.15 %) and regenerability percentage (77.63) were found in the cultures stored at the lowest temperature (5ºC) for the period of 3 months, whereas, the 32.37 % and 28.37 % survival and regeneration were recorded in the cultures stored at 25ºC for 12 months. The interaction among three factors (genotypes, temperature and storage periods) revealed that the highest survival percentage (99.33) and regeneration percentage (91.66) were in the cultures of Bagugosha and Raj btung, stored at 5ºC for 3 months. Incubating temperature treatments i.e., 5ºC and 10ºC, proved successful for preservation of apical shoots for 3 months, however, the shoots kept at higher temperature for longer period survived with poor re-growth. Hence the shoot tips of pear genotypes can be successfully stored *In vitro* for short to medium terms at reduced incubation temperatures.

Introduction

Conservation and sustainable use of plant genetic resources is important to meet the demand for future food security. The biodiversity in plant resources is threatened to extinct as a result of deforestation, developmental activities, introduction of new varieties and modernization in agriculture on worldwide scale. The security of genetic resource collections requires their conservation by several techniques. Duplicate collections of field-grown pear genetic resources are now conserved in medium- and long-term storages (Reed *et al*., 1998). Conservation approaches namely *in situ* and *ex situ* are mostly used to safeguard populations in danger of extinction (Rao, 2004). The fruit species are usually conserved in field gene banks, unfortunately, field tree collections are vulnerable to environmental catastrophes such as high wind, rain, drought, freezing, pests and diseases outbreaks. Moreover, risk of destruction by natural disasters like earth quake and land sliding to which germplasm are continuously exposed, causes sudden loss of valuable germplasm.

Large collection of pear (*Pyrus communis*) and apple (*Malus domestica* [*M. pumila*]) genetic resources have been preserved through *in vitro* cultures as alternative techniques to the field gene banks (Sedlak *et al*., 2001). *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, with low impact on wild populations. Furthermore, the organized culture systems have a high degree of genetic stability and cultures can be readily brought back to normal culture conditions to produce plants on desire. Organized culture systems for regeneration and successful propagation of genetically stable plantlets are prerequisites for *In vitro* preservation (Rao, 2004). This preservation technique also favours to maintain pathogen free plant materials, safely distribution and cultures are not damaged by adverse weather conditions (Withers & Engelmann, 1997). There are two types of *In vitro* preservation methods used in tissue culture. These include; a) growth retardation (slows growth or minimal growth) method and b) cryopreservation or ultra low temperature preservation (Scowcroft, 1984; Sedlak *et al*., 2001). In the first method preservation of genetic resources for short to medium terms (from several months to a few years), the latter method is for the preserving for long time (for several decades or longer). These alternate preservation techniques are less costly and safe to conserve germplasm (Epperson *et al*., 1997).

The minimal growth storage techniques are generally applicable to wide range of fruit tree genotypes to extend the ordinary subculture duration for a few weeks to 12 months for future needs and to preserve germplasm as *in vitro* gene bank in limited spaces. Slow growth storage technique is commercially used in laboratories to maintain stock cultures through low temperatures.To achieve this purpose, various methods have been used such as by reducing concentration of basal medium (Moriguchi & Yamaki, 1989), adding mannitol as osmoticum (Wanas *et al*., 1986), and removal of cytokines and addition of growth retardants (Gunning & Lagerstedt, 1985), at normal temperature. However, the most reliable and simple method for growth retardation is lowering the culture temperature. Attempts have been made in several laboratories to conserve *Pyrus* germplasm by using this technique (Wanas *et al*., 1986; Wilkins *et al*., 1988; Moriguchi *et al*., 1990; Moriguchi, 1995). Depending on species, these stored plants can be micropropagated rapidly when desired. Medium term (3 months to 3 years) storage conditions for *in vitro* cultures of temperate genera are typically 4 or 5 °C in darkness (Druart, 1985, Marino *et al*., 1985; Wilkins *et al*., 1988; Reed, 1992). Ludergan & Janick (1979) described the reduced temperature (1°C and 4°C) storage of shoots of apple scion variety (Golden Delicious) for one year. Barlass & Skene (1983) have reported successful storage of 7 species of *Vitis* (grapes) for periods up to 12 months at 9°C. Marino *et al*., (1985) have reported successful storage of shoots of three Prunus rootstock genotypes for several months at reduced temperature. The shoots of peach and cherry could be preserved for 120 days at 4°C. Twenty pear (*Pyrus*) cultivars of seven species cultured on Murashige & Skoog (MS) basal nutrient medium and stored *in vitro* culture methods like meristem cryopreservation as long term and medium-term storage techniques involving low temperatures (1-4°C) for germplasm preservation programmes (Bell & Reed, 2002).

Keeping in view the existing genetic diversity in *Pyrus* germplasm in Azad Jammu and Kashmir, slow growth storage techniques by using low temperatures regimes was first attempted to conserve the germplasm for short to medium term storage with aiming to develop slow growth storage technique and extending sub-culturing duration.

Materials and Methods

Sterilization of plant materials: For establishment of cultures, apical shoots of 3 to 4 cm in length were excised with the help of scalpels from the plants pear genotypes growing in the nursery in early spring and collected in 500 ml Erlenmeyer flasks containing water to avoid desiccation. The excised shoots were washed in running tap water for 10 minutes. These were then submerged in 1 % Sodium hypochlorite solution containing 2-3 drops of Tween 20 for 12 minutes.

These were then rinsed three times with sterile distilled water to remove the traces of disinfectant, every rinse for 5 minutes. The cut ends of the explants that had become dead due to exposure to disinfectant were cut with a scalpel before inoculation on culture medium. Surface sterilization was carried out under aseptic conditions in a laminar air-flow cabinet.

Proliferated shoots of 9 pear genotypes viz., Khurolli, Bagugosha, Pathar nakh, Desi nakh, Kotharnul, Btangi, Frashishi, Kashmiri nakh and Raj btung were excised aseptically from already established *in vitro* cultures. For *In vitro* preservation, fresh culture medium i.e., MS (Murashige & Skoog, 1962) supplemented with 30 g l-1 sucrose, 7 g l-1 agar and 1 mg l-1 BAP were used (pH 5.7 + 0.1). Excised shoot tips were trimmed to 1.0-1.5 cm and cultured in 200 ml culture jars containing 30 ml of prepared medium. The culture jars were closed with their autoclavable plastic lids. The cultures were stored in different growth chambers at various temperatures (i.e., 25, 15, 10 and 5ºC) for different periods (i.e., 3, 6, 9, and 12 months) in 16 h photoperiod under white fluorescent light intensity of 55 µmol m-2 s-1. The experiment was laid out in completely randomized design (CRD) with factorial arrangement having nine genotypes, four storage temperatures and three storage periods with three replications. These were at least 10 shoots in each replication. Data were recorded on survival and regeneration percentage of shoots after every storage period.

Survival of the cultures was assessed on the basis of criteria as suggested by Reed (1992) as dead and brown shoots were considered as unsurvived while those with vigorous growth and having healthy leaves were considered as survived.

$$\text{Survival percentage} = \frac{\text{Number of shoots survived}}{\text{Number of shoots transferred}} \times 100$$

For regenerability of the cultures, shoots were removed from the cultures, trimmed off to a length of 1 to 1.5 cm without damage and transferred onto fresh shoot proliferation medium. The cultures were kept at 25 + 2°C in 16 h photoperiod under white fluorescent light intensity of 55 µmol m-2 s-1. The data regarding regeneration percentage were recorded after one month on the basis of growth initiation by using the formula:

$$\text{Regeneration percentage} = \frac{\text{Number of shoots regenerated / revived growth}}{\text{Number of shoots transferred on culture medium}} \times 100$$

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

Results

All the genotypes differed significantly for the survival and regenerability. The maximum survival percentage was recorded in shoots of Frashishi, followed by Desi nakh, Khurolli and Kotharnul. However, these genotypes were statistically at par with each other. The least survival was observed in shoots of Btangi followed by Pathar nakh, Bagugosha, Kashmiri nakh and Raj btung. All these genotypes behaved statistically alike. Almost similar pattern was observed for the parameter of regeneration percentage. However, shoots of a few genotypes differed for higher and lower rate in both parameters (Tables 1, 2).

As far as temperature treatments are concerned, it is obvious that as the storage temperature decreased, the survival and regeneration percentage of the cultures were increased. All the storage temperatures differed significantly from each other (Table 2). The highest survival and regenerability percentages were found in the cultures stored at the lowest temperature (5ºC). Declined trend was observed in cultures for both parameters when incubating temperature increased to normal one (25ºC) (Tables 1, 2).

The mean values for storage periods indicated that as the storage period was increased, survival as well as regeneration percentage of the cultures was progressively decreased. The maximum survival of cultures was recorded when these were stored for 3 months, followed by those stored for 6 months. Both the storage periods were statistically at par. The minimum survival and regenerability was recorded when the cultures were stored for 12 months (Fig. 1a, b).

The interaction between the genotypes and the temperature treatments indicated the highest survival and regenerability percentage in the cultures of Bagugosha and Raj btung when kept at 5ºC. On the other hand, Bagugosha when stored at the highest temperature (25ºC) showed the lowest survival as well as regeneration percentage (Tables 1, 2).

Interaction between genotypes and storage period showed that the highest survival and regeneration percentage was in Khurolli cultures when stored for 3 months. The minimum survival was recorded in the cultures of Khurolli, when stored for the maximum duration i.e., 12 months. On other hand the minimum regeneration percentage was observed in the shoots of Kashmiri nakh stored for 9 months (Fig. 1a, b).

The maximum survival and regeneration percentage was found when cultures were stored for 3 months at the lowest temperature treatment (5ºC). This treatment combination significantly differed from all other treatment combinations. On other hand, the lowest survival was recorded when cultures were kept at the highest temperature treatment (25ºC) for 12 months of storage period, whereas, the lowest regeneration rate was achieved when the shoots were kept at same temperature treatment for 12 or 9 months, which differed significantly from all other treatment combinations (Table 1, 2).

As far as data regarding interaction among genotypes, temperature treatments and storage periods is considered, the highest survival percentage was recorded in the cultures of Bagugosha stored at 5ºC for 3 months. This was followed by Raj btung, Kashmiri nakh, Khurolli and Frashishi stored at the same temperature and for the same period. The lowest survival rate was found in the cultures of Khurolli stored at 25ºC for 12 months, followed by those of Kashmiri nakh stored at the same temperature but for 9 months, of Pathar nakh stored at the same temperature for 12 months, and those of Bagugosha stored at the same temperature for 12 months and 9 months. All these treatment combinations were statistically similar to each other (Table 1).

The overall interaction among genotypes, temperature treatments and storage periods for regenerability of shoots revealed that the maximum regeneration percentage was recorded in the shoots of Raj btung kept at 5ºC for 3 months. This treatment combination was statistically alike to several treatment combinations. The minimum regeneration percentage was found in the shoots of Kashmiri nakh stored at normal temperature (25ºC) for 6 months, which was also statistically similar with several other treatment combinations (Table 1).

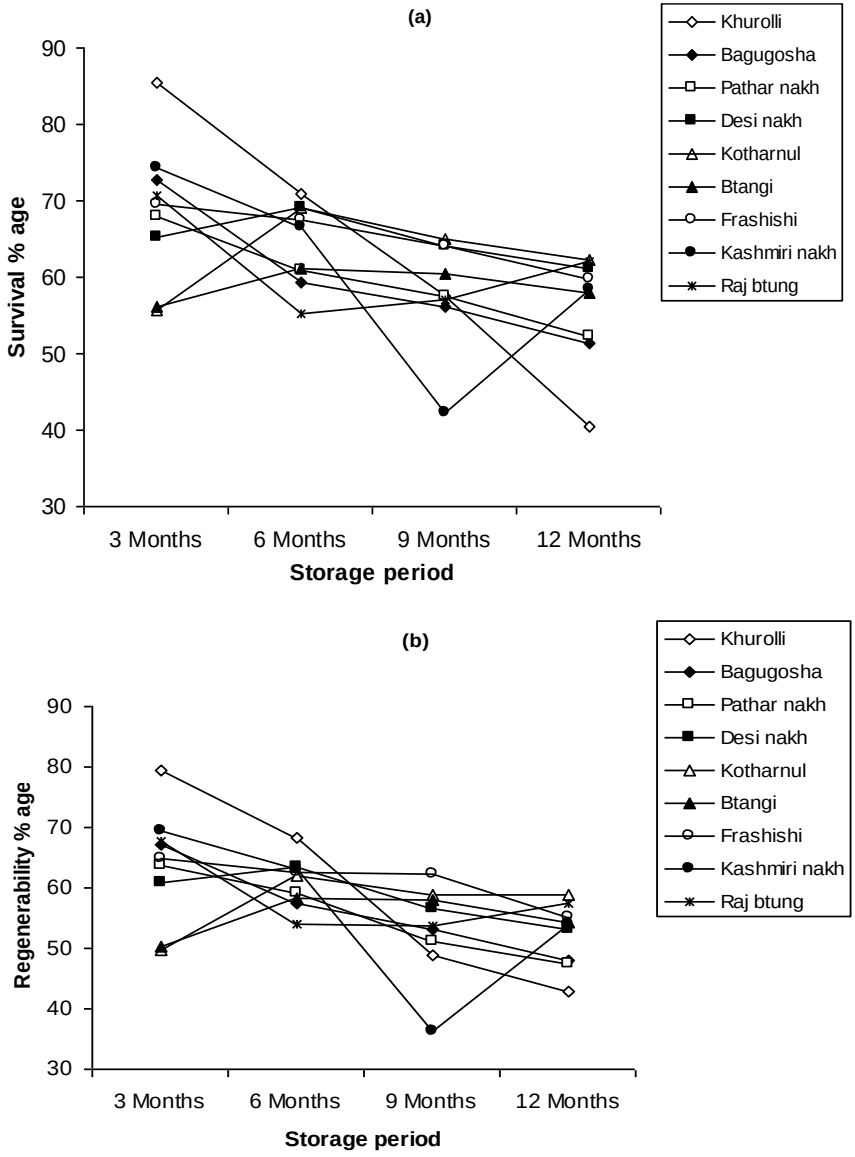

Fig. 1a & b. Effect of various storage periods on survival percentage of shoot cultures of nine genotypes during storage and regenerability percentage of shoots after transferring onto fresh medium.

Discussion

Low temperature has been successfully applied to *in vitro* cultures of various plant species for short and medium term storage. Tahtamouni & Shibli, (1999) preserved micro shoots of wild pear (*Pyrus syriaca*) through slow growth (low temperature) technique. *In vitro* culture viability indicated that preservation at 8°C was able to decrease growth and maintain explant health with better survival and regrowth percentages. Moreover, in

previous study, temperature in the range of 5 to 10ºC has been found suitable for short term *In vitro* storage of meristem cultures of several temperate species (Lundergan & Janick, 1979). In the present study, the effect of different temperature regimes on the shoot tips of nine pear genotypes cultured on shoot proliferation medium for different periods (3, 6, 9 and 12 months) was assessed on their survival and regenerability. Lower temperatures, especially 5ºC, and 10ºC, proved successful for preservation of apical shoots for duration of 3 and 6 months. The shoots stored at higher temperature for longer periods resulted in lower survival percentage with poor regrowth. These results confirmed the findings of other workers who reported that meristem cultures of pear (Wanas *et al.,* 1986), apple (Lundergan & Janick, 1979) and apple rootstocks (Orlilkowska, 1992) can be stored *in vitro* at low temperatures. However, single node shoots of apple genotypes showed high survival percentages for up to an year when stored at 4ºC and regrowing easily when shifted onto proliferated conditions but resuming capability of shoots dropped afterwards (Negri *et al.*, 2000). Data relative to survival percentage of 4 genotypes i.e., Frashishi, Desi nakh, Khurolli and Kotharnul showed more stability and maintained better viability as compared to other genotypes studied. As already reported by Kovalchuk *et al.,* (2009) *In vitro* storage 10 out of 16 genotypes of apple survived longer period of time at low temperature. Variability in survival rates of the genotypes might be due to their genetic makeup. These findings are also in accordance with the results of (Wilkins *et al.,* 1988) who observed that the variability in survival rate during *In vitro* preservation of apple varieties was variety dependent. In present study, these 4 of the 9 genotypes might be cold hardiness and acclimatized with passage of storage time while remaining had less resistance against low temperature. This proved the cold hardiness behaviour of these genotypes in accord with (Kushnarenko *et al.,* 2008) of *in vitro* apple shoots storage. Genetic variation among genotypes for survival as well as ability to start re-growth after *In vitro* preservation has also been reported in European and Japanese pears. According to (Moriguchi *et al.,* 1990), the shoots of European pear (*P. communis*) survived at 5°C for 12 months while none of the three varieties of Japanese pear (*P. pyrifolia*) survived at any temperature of 5, 10 or 15°C for 12 months.

In the present study, the temperature treatments of 5 and 10ºC were found suitable for preservation of shoots of various genotypes tested, but genotypes stored at 5ºC had significantly better survival rate and showed re-growth after transferring to regeneration medium at normal temperature of 25ºC. The shoots stored at higher temperatures (15 and 25ºC) had poor survival and needed frequent subculturing due to depletion of nutrients. Both, survival and shoot regeneration rates were the highest when shoots were stored at 5ºC and these decreased when temperature increased. Furthermore, it was also noted visually that growth rate of shoots was very slow at 5ºC storage temperature, as compared to those cultures kept at higher temperatures.

As far as duration of preservation is concerned, 3 and 6 months of storage periods were proved better than longer durations. With increase in storage period, survival rate as well as regenerability was reduced significantly. In the cultures, which were stored for the period of 9 or 12 months, their survival and regenerating percentage remained below 60 percent. Results indicate that the durations up to 6 months are more effective for short term preservation of pear genotypes as compared to longer period of storage. The present results are supported by the findings of Oka and Niino, (1997) who found that when pear (*P. serotina* [*P. pyrifolia*] cv. Senryo) shoots were grown on MS medium at 10 and 5ºC, the survival rates of the shoots gradually declined after 16 weeks and 32 weeks, respectively. This decrease in survival rate might be due to depletion of nutrients in medium which were utilized by the shoots during earlier period of culture. The results of

the present study regarding length of storage duration are in line with Wanas *et al.*, (1986) who reported that the shoots of *Pyrus communis* cv. 'Conference' showed reduced proliferation potential after 12 months storage in comparison with after 6 months storage. Regrowth of cultured shoots depends upon subsequent health of the shoots in previous medium. The present investigation revealed that the higher survival rates had positive effect on regeneration percentage of the shoots. Reduced temperature treatments (5ºC to 10ºC) are suitable possibly because cultures could continue to proliferate slowly. However, the temperatures above this range do not show better results in term of survival as well as re-growth of shoots. Temperatures below and above this range might be critical for minimal growth of pear shoots due to browning / low temperature killing and declined shoot survival rate (Oka & Niino, 1997). At higher temperature proliferation of shoots increased which ultimately resulted in depletion of nutrients in medium and hence low survival rate. That is why high temperature became limiting factor for pear genotypes in this study. This phenomenon is supported by earlier study of (Lundergan & Janick, 1979) who observed high losses due to contamination, desiccation and nutrient depletion at 26ºC. Shoots of pear genotypes can survive successfully at low temperature due to hardy and temperate nature. In the present study, *in vitro* shoots cultures of nine pear genotypes were successfully stored at 5ºC and survival rate and re-growth after transferring to regeneration medium remained high. Therefore, it is concluded that the shoot tips of pear genotypes could successfully be preserved *in vitro* by lowering culture temperatures for short to medium term storage.

Conclusion

This study showed that the shoots of nine pear genotypes can be maintained successfully for several months by *in vitro* slow growth preservation technique under described conditions (low temperature). Low temperature treatments (5 & 10ºC) were more effective due to hardy and temperate nature of genotypes. However, satisfactory results were achieved at constant and slightly altering temperature treatments for lengthening subculture duration regarding survival and recovery rate. Genotypes showed variable response with interaction of temperature treatments and storage periods. Therefore, this technique could be useful for *in vitro* pear germplasm collections as duplicate storage technique with minimum cost and least risk of genetic instability.

Acknowledgments

This research was supported by Plant Genetic Resources Institute, National Agricultural Research Center, Islamabad which enabled the author to carry out this investigation. The authors gratefully acknowledged to Dr. G.M. Sajid (*In vitro* germplasm preservation laboratory, PGRI) for his valuable suggestions.

References

Barlass, M. and K.G.M. Skene. 1983. Long term storage of grape *in vitro*. Plant Genetic Resour Newl, 53: 19-21.

Bell, R.L. and B.M. Reed. 2002. *In vitro* tissue culture of pear: advances in techniques for micropropagation and germplasm preservation. *Acta Horti*., 596: 412-418.

Druart, P. 1985. *In vitro* germplasm preservation technique for fruit trees. In: *In vitro* Techniques: Propagation and Long Term Storage. (Ed.): M.A. Schafer. Martinus Nijhoff Publishers, Dordrecht, Netherland, pp. 167-171.

Epperson, J.E., D.H. Pachioo and C.L. Guevera. 1997. A cost analysis of maintaining cassava plant genetic resources. *Crop Sci*., 37: 1641-1649

Fay, M.F. 1994. In what situation is *in vitro* culture appropriate to plant conservation? Biodiversity and Conservation, 3: 176-183.

Gunning, J. and H.B.Lagerstedt. 1985. Long term storage techniques for *in vitro* plant germplasm. Proceeding of the International Plant Propagation Society, pp. 199-205.

Kovalchuk, I., Lyudvikova, Y., Volgina, M. and B.M. Reed. 2009. Medium, container and genotype all influence *in vitro* apple germplasm. *Plant Cell Tiss Organ Cult*., 96: 127-136.

Kushnarenko, S.V., Romadanova, N.V. and B.M. Reed. 2008. Cold acclimatization improves regrowth of cryopreserved apple shoot tips. Cryo Letters (In press).

Lundergan, C. and J. Janick. 1979. Low temperature storage of *In vitro* apple shoots. *Hort Science*, 14: 514

Marino, G., P. Rosati and F. Sagrati. 1985. Storage of *in vitro* cultures of *Prunus* rootstocks. *Plant Cell Tissue Org. Cult*., 5: 73-78.

Moriguchi, T.1995. Cryopreservations and minimum growth storage of pear (*Pyrus* species). In: Bajaj, Y.P.S. (ed.). Biotechology in Agriculture and Forestry, Vol. 32. Springer Verlag, Berlin, Heidelberg, pp. 114-128.

Moriguchi, T., S. Kozaki, S. Yamaki and T. Sanada. 1990. Low temperature storage of pear shoots *in vitro*. *Bull. Fruit Tree Res. Stat*, 17: 11-18.

Moriguchi, T. and S. Yamaki. 1989. Prolonged storage of grapes nodal culture using a low concentration of ammonium nitrate. *Hort Science*, 24: 372-373.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Planta*, 15: 473-497.

Negri, V., N. Tosti and A. Standardi. 2000. Slow growth storage of single node shoots of apple genotypes. *Plant Cell Tissue Org. Cult*., 62: 159-162.

Oka, S. and T. Niino. 1997. Long term storage of pear (*Pyrus* sp.) shoot cultures *in vitro* by minimal growth method. *Japan Agr. Res. Quart*, 31: 1-7.

Orlilkowska. T. 1992. Effect of *in vitro* storage at 4ºC on survival and proliferation of two apple rootstocks. *Plant Cell Tissue Org. Cult*., 31: 1-7.

Rao N.K. 2004. Plant genetic resources: Advancing conservation and use through biotechnology. African J. Biotech, 3: 136-145.

Reed, B.M. 1992. Cold storage of strawberries *in vitro*: A comparison of three storage systems. *Fruit Var. J*., 46: 93-102.

Reed, B.M., C.L. Paynter, J. DeNoma and Y.J. Chang. 1998. Techniques for medium- and long-term storage of pear (*Pyrus* L.) genetic resources. *Plant Genetic Resour. Newl*., 115: 1-5.

Scowcroft, W.R. 1984. Genetic variability in tissue culture: Impact on germplasm conservation and utilization. IBPGR Technical Report, International Board for Plant Genetic Resources, Rome, Italy, pp. 41.

Sedlak, J., F. Paprstein, A. Bilavcik and J. Zamecnik. 2001. Adaptation of apple and pear plants to *in vitro* conditions and to low temperature. *Acta Horti*., 560: 457-460.

Tahtamouni, R.W. and R.A. Shibli. 1999. Preservation at low temperature and cryopreservation in wild pear (*Pyrus* syriaca). *Adv. Hortic. Sci*., 13: 156-160.

Wanas, W.H., J.A. Callow and L.A. Withers. 1986. Growth limitation for the conservation of pear genotypes. In: Plant Tissue Culture and Its Agricultural Applications. Butterworth, London, pp. 285-290.

Wilkins, C.P., Dodds, J.H. and H.J. Newbury. 1988. Tissue culture conservation of fruit trees. FAO/International Board of Plant Genetic Resources Newsletter, 73/74: 9-20.

Withers, L.A. and F. Engelmann. 1997. *In vitro* conservation of plant genetic resources. In: Biotechnology in Agriculture. (Ed.): A. Altman. Marcel Dekker Inc., New York, pp. 57-88.

(Received for publication 13 July 2009)