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Short communication

Cryopreservation of apple (*Malus* spp.) winter buds

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ABSTRACT

One of the practical approaches for long-term conservation of apple garmplasm is the storage of winter buds in liquid nitrogen. The dormant buds were cryopreserved using two-step freezing method. On dehydration at -20°C, moisture content (MC%) of buds ranged from 37 to 47% after 28 days. On silica gel, the MC% was in the range of 24 and 34% after 4 h desiccation. The cryopreserved buds were rewarmed and patch budded and grafted on the rootstocks in the field. Accessions desiccated on silica gel showed post-thaw recovery. The cryopreserved buds on patch-budding showed 0-10% recovery, while buds on grafting showed higher (up to 25%) regeneration. No natural acclimation at sub-zero temperature can be one reason for low post-thaw recovery across the accessions growing in Indian fields. Winter dormant buds of 35 accessions of *Malus* spp. were cryobanked.

Key words: Apple, climate change, cryopreservation, dormant buds, two-step freezing.

Apple (*Malus* spp.) is an important fruit crop in India, accounting up to 80% of temperate fruit production. Apple genetic resources are important for use in breeding and crop improvement programs. About 750 accessions of apples are maintained in field genebanks in various organizations in the temperate region. Of these, ICAR-NBPGR Regional Station, Shimla is maintaining about 250 accessions. The plants in the field are exposed to natural disasters, climate change, diseases and pests. Therefore, it is safer to duplicate the field germplasm as *in vitro* or cryopreserved backups. Cryopreservation of *Malus* winter vegetative bud by slow cooling using a programmable freezers and direct plunging in LN has been demonstrated (Towill *et al.*, 5). Dormant winter buds survived LN exposure when they were slowly cooled to -30 to -40ºC to allow the freezable water to move from within the cells to the extracellular spaces. Fully acclimated dormant buds at sub-zero temperature for few days have increased levels of dehydration tolerance. Therefore, a high viability of dormant *Malus* buds was recorded after 10 years of storage in liquid nitrogen vapour (Volk *et al*., 7).

The present study was carried out to standardize a protocol to cryopreserve apple dormant buds using two-step freezing method with the aim to cryopreserve apple germplasm maintained in the Shimla field genebank. This method was preferred so as to avoid the use of programmable freezer. Dormant bud woods of 16 apple accessions as mentioned in Table 1 and 2 were used. Fourteen accessions were collected after natural chilling when temperature was below 5°C from the field gene bank of ICAR-NBPGR Regional Station, Shimla. The station is situated at 31°05'924"N latitude, 77°09'580"E longitude and at an elevation of 1925 m amsl. Budwood of two accessions, namely, Blumer Norman and Var. No. 280400 were collected from progeny of orchards of Department of Horticulture, Rohru (2,200 m amsl) and Sangla, Kinnor (3,000 m amsl), respectively. The budwoods were placed in the moss grass to avoid dehydration, while transportation to Tissue Culture and Cryopreservation Unit, ICAR-NBPGR, New Delhi. The twigs of all accessions were stored in freezer (-10°C) until use. Budwood of each accession was cut into 20 segments of 3 cm each and of approximate same diameter, bearing one bud each. In one accession (Blackban Davis EC36439) about 6 cm long segments were also cut. In the preliminary experiments on 10 accessions (Table 1), the cut segments were dehydrated at -15°C for 28 days in a freezer. Segments of other four accessions (Table 2) were desiccated on silica gel for 4 h at room temperature. Moisture content (MC) was determined on the fresh weight basis by oven drying method at 85°C. Segments after desiccation (either in freezer or on silica gel) were placed in cryovials (2-3 in each vial) and transferred to -20°C for 24 h, then transferred to -30°C for next 24 h. For Blumer Norman and Var. No. 2,80,400 (varieties collected from Rohru) were freezing was done at 10°, -15°, -20° and -23°C for 24 h each. After this, the cryovials were arranged in the cryogenic box and quickly placed in the liquid nitrogen vapour phase (-180°C). After overnight storage, the cryovials were removed from cryogenic temperature and thawed at 5°C for 24 h.

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Table 1. Moisture content per cent (MC%) of fresh and desiccated (in freezer) budwood sections of *Malus* spp. prior to cryopreservation.

Genotype	Acc. No.	Fresh MC (%)	MC after desiccation (%)
Apple	EC38372	52.84	45.13
Apple Vered	EC24349	49.21	40.39
Red June	EC28540	45.23	36.69
Blackban Davis	EC36439	44.79	43.73
Vesna	EC44521	46.67	44.47
James Grive	EC492551	46.95	44.81
Vance	EC552612	51.96	46.61
Chaubatia Prince	IC349910	53.12	46.06
Kesari	IC349911	47.83	41.46
Red × Ambri	IC349918	56.54	46.32

Table 2. MC% of fresh and desiccated (on silica gel) budwood sections of *Malus* spp. prior to cryopreservation.

Genotype	Treatment	MC%
Vesna	Fresh	41.35
	Desic. (4 h)	29.72
M. spectabilis	Fresh	37.39
	Desic. (4 h)	30.85
Reineffed Canada	Fresh	45.63
	Desic. (4 h)	34.09
Neema Delicious	Fresh	37.35
	Desic. (4 h)	24.94
Var. No. 280400	Fresh	46.56
	Desic (4 h)	34.95
Blumer Norman	Fresh	46.48
	Desic. (4 h)	36.75

The samples were taken out of the cryovials and placed in the moist peat moss in air tight containers at 6°C. The rewarmed dormant buds were treated with 0.1% Tetrazolium to assess the viability of cryopreserved buds. The samples were transferred to Shimla for patch budding on the rootstock. Ten dormant buds of each cultivar were patch budded/ grafted on to apple rootstocks in the month of March on three rootstocks, *viz*., MM111, MM7, MM106 and *Malus baccata*. Grafting of accessions was also done on seedling rootstocks. The observation on the sprouting of buds and related growth parameters was recorded.

Moisture content percentage of fresh dormant buds of each accession was in the range from 45 to 57%. On dehydration at -20°C, the moisture content of each sample dropped very slowly in all the accessions. After 28 days dehydration the MC of buds ranged from 37 to 47% (Table 1). On silica gel desiccation the MC was in the range of 24 and 34% in 4 h (Table 2). Stushnoff (3) reported that the 50-60% field moisture of dormant buds must be reduced to 20-30% to limit ice formation. Suzuki *et al*. (4) got the maximum of 88.6% regeneration in vegetative buds of pear when the water content of the buds was 41%. The rate of shoot formation decreased significantly after the reduction of water content below 41% as meristems were over dehydrated and injured. The dehydration of vegetative apple buds at -4º to 20-30% water contents significantly enhanced the shoot survival rate (Tyler and Stushnoff, 6). In the present study, the segment bearing buds were green and looked healthy after liquid nitrogen (vapour phase) storage (Fig. 1A). Some samples turned brown after they remained in the moist peat moss for two days at ambient temperature. In most of the cases, phloem and xylem of the budwood appeared to be green and in few cases, phloem and xylem appeared brown but when 0.1% TTC test was done for both brown as well as green budwoods, both showed the red colour, which indicates the survival of tissue after cryopreservation, indicating that tissue was alive after thawing (Fig. 1B).

Various factors influence the survival and regrowth of cryopreserved material. Harvesting season is one of the most important factors which influence the post thaw regrowth after cryopreservation. In general the procedure for cryopreservation using dormant vegetative buds have a problem because the season for collecting these materials is limited (Niino, 2). Partial dehydration up to about 38.5% of the vegetative mulberry buds from shoot tips collected from late autumn to early spring improved the recovery date following the cryopreservation (Niino, 2). These results show that partial dehydration of vegetative buds prior to pre-freezing is a significant factor for normal shoot regeneration following cryopreservation. The cryopreserved patch buds showed 0-10% recovery (Fig. 1C), while up to 25% regeneration was recorded on grafted cryopreserved dormant bud (Fig. 1D). It was also observed that grafts made on to seedling rootstocks remained green for more number of days than clonal rootstocks. For successful cryopreservation, it is important to avoid lethal intracellular freezing. Dehydration of flower buds by wind at 0ºC enhanced the super cooling ability of *Rhododendron* florets in intact buds. Water migration within flower buds was

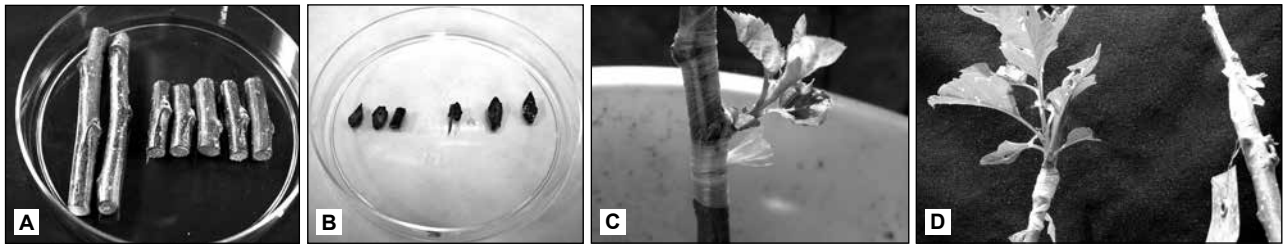

Fig. 1. Apple dormant bud cryopreservation: A. Cryopreserved budwood segments (green and healthy); B. Viability test of cryopreserved apple buds using tetrazolium; C. Regeneration after patch-budding (var. No. 280400); D. Regeneration of new shoot (var. Blumer Norman) after tongue-grafting on rootstock (Left); Dried graft (Right)

observed with some variation, in the ice formation sites and the quantity of migrating water (Ishikawa and Sakai, 1). Recently, untimely flowering of the apple trees growing, in the Himalayan region, was reported. This may have happened due to increase in temperature in the region. For example, in June 2009, at Shimla, mercury crossed the 30°C mark, five above normal and the unprecedented spiral of heat was recorded. Rise in temperature and reduced duration of natural chilling in the field has drastic effects on fruit production. In our cryopreservation experiments, it was observed that the low chilling of budwood material affected the survival of vegetative buds after liquid nitrogen treatment. However, winter dormant buds of 35 accessions of apples were cryobanked using 2-step freezing protocol, *i.e.* silica gel desiccation (4 h), freezing at 10°, -15°, -20°, and -23°C for 24 h each, LN, thawing at 5°C followed by grafting for recovery. The cryogenic method of using vegetative buds appears promising as a routine method for cryopreservation of apple germplasm.

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