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Cryobase establishment of Indian almonds using embryonic axes

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ABSTRACT Almond, *Prunus dulcis* is one of the most commercially important nut crops of temperate hilly regions of India. Indian states of Jammu and Kashmir, Himachal Pradesh and Uttaranchal have been cultivating several landraces, seedling selections, and wild types and cultivars, both indigenous and exotic since long which need protection as a safety back to field gene banks. Morphological characterization using seven carpological characters among selected 25 almond cultivars of the three states were done to estimate genetic variability and establish genetic relationships of indigenous and exotic cultivars. High morphological variability (86%) among the cultivars/selections of almonds were observed. Almond seeds are characterized by poor germination due to low viability, dormancy and sensitivity to subzero temperatures were cryobanked using embryonic axes (EA) for cost effective conservation. After cryo exposure of 48h, the EA viability ranged from 79 to 100% with 36% of the accessions retaining viability between 95 to 100%. A 240 accessions with 3.5 to 6 percent moisture levels have been successfully cryostored. Evaluation of EA viability for durations of up to 15 years of cryostorage ascertained the applicability of the developed protocol for diverse almond germplasm with retention of original high viability and successful transfer of plantlets to field.

Keywords: *Prunus dulcis*, genetic diversity, cryobanking, embryonic axes, UPGMA

Almond *Prunus dulcis* (Miller) D.A. Webb., of family Rosaceae, is a large genus of deciduous or evergreen trees and shrubs, distributed chiefly in the temperate regions of the northern hemisphere and is valued for its edible fruits or seeds or for ornamental flowers. The cultivated sweet almond is diploid and now classified as *P. dulcis* (Miller) D.A. Webb. with *P. amygdalus* as a synonym. With its origin traced to central to south western asia [1] it is probably the longest of the cultivated nut crops. Most of the plantations grown world over had been of seedling origin resulting in very low productivity and hence during last few decades defined cultivars are in use to boost productivity. Commercially important characters for cultivars or selections are kernel size, quality and also shell hardness and shelling percentage. 'Nonpariel' and 'Mission' are the most prominent cultivars in use globally.

Almond is one of the most important nut crops of temperate hilly regions of India, mainly grown commercially in Jammu and Kashmir, Himachal Pradesh and Uttaranchal [2]. The demand for temperate fruits and nuts is expected to rise to the tune of 60 lakh tonnes by 2020 [3].

In the Himalayas especially in the Khasi and Jaintia hills, almonds are being used locally as rootstocks for commercial stone fruits. Rich diversity of seedling populations which are highly variable is found in Kashmir (J&K) and Himachal Pradesh. Several cultivars developed in India and also those introduced as exotic collections *e.g.* Prianyi and Primorskij from Russia, White Brandis from Australia and others like Merced, Nonpareil, California, Paper shell, Ne Plus Ultra now present good choice to farmers for improved cultivation. Almond is cultivated on an area of 21,300 ha with an annual production of 15,620 mt and the productivity is 0.73 t/ha [4].

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The main two types- bitter and sweet almonds are grown as orchard crops. The sweet almonds are classified into 3 groups namely hard shell types, soft shell types and paper shell types, depending upon the thickness of the shell. Introduced cvs of Nonpareil and thin shell (Paper-shell types); IXL and Ne Plus Ultra (Soft-shell types) and Drake (Hard-shell types) are being grown in orchards along with the seedling trees of local origin. Important cultivars recommended for India are Waris, Makhdoom, Shalimar, Parbhat, Nauni Selection, Merced, Prianyi, Primorskij, Nonpareil, California Paper shell, Ne Plus Ultra and Drake [5-7].

It is important to characterize and conserve this vast genetically unique and important diversity for long-term using cost effective methods. In India Central Institute of Temperate Horticulture (CITH), Srinagar, National Bureau of Plant Genetic Resources (NBPGR), New Delhi, Dr. YS Parmar University of Horticulture and Forestry (YSPUH&F), Solan and Sher-E-Kashmir University of Agricultural Sciences & Technology (SKUAST), Srinagar are the leading Institutes carrying out genetic resources management of *Prunus*. Cooperative programmes for networks for PGR maintenance of *Prunus* are operational in European Union are good examples for consolidation, characterization and conservation [8]. INRA (Institute National de la Recherche Agronomique) Bordeaux reported cryopreservation of different stone fruits using embryonic axes, shoot tips and somatic embryos [9].

Prunus seeds have been reported to show poor germination due to low seed viability, seed dormancy and pathogens. They are desiccation tolerant but do not store well at subzero temperature and have short life. Based on this seed storage behavior, seed conservation is difficult due to several factors [10]. To overcome the problem of large seed size and associated germination problems, attempts on embryonic axes conservation has been made [11]. In view of inherent problems associated with seed handling for germination and storage and in view of large seed size, attempts have been directed in several *Prunus* species to use excised embryonic axes, containing high proportion of meristematic tissues for cryobanking. Embryonic axes have been successfully cryopreserved in *Prunus dulcis* [12], *P. armeniaca* [13], *P. persica* [14] and *P. avium* [15].

MATERIALS AND METHODS

Plant materials

A total of 25 (10 exotic and 15 indigenous) cultivars of *P. dulcis* collected from field gene bank of NBPGR regional station, Shimla, Himachal Pradesh were used for morphological and cryoprotocol development studies (Table 1). Further for establishing cryobase collection nuts were received from different collaborating institutes of the country over a period of 15 years, from 1997 to 2006, which belonged to indigenous sources mainly landraces and bore an indigenous collection number (IC number) or exotic sources and bore exotic collection numbers (EC number). Institutes that contributed the germplasm were Dr. YS Parmar University of Horticulture and Forestry (YSPUH&F), Sher-E-Kashmir University of Agricultural Sciences & Technology and from NBPGR regional rtation, Srinagar. Few collections were made by authors from districts of Kinnaur following selective sampling strategy where samples collected from single plant of a cultivar/ selection were treated as individual accession. Detailed passport information of each accession was recorded in NBPGR database.

Morphological characterization

Morphological characterization of selected set of 25 cultivars of *P. dulcis* belonging to bitter types, sweet types, released varieties and exotic introductions, was done using minimal descriptors of agri-horticultural crops developed for fruit crops by National Bureau of Plant Genetic Resources (NBPGR), New Delhi [16]. Characterization data of seven nut and kernel (carpological) characters were recorded (Table 1). A pair-wise similarity matrix was generated based on simple matching coefficient method using software NTSYS ver. 2.10e. [17]. A cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic average (UPGMA) based on simple matching coefficient in NTSYS software. Principal Component Analysis (PCA) was also carried out to study correlations among the variables and establish relationships among cultivars using the same software. Two-way Mantel test [18] for goodness of fit for UPGMA cluster was also performed using the same software.

Cryopreservation

In the present study, the cryopreservation protocol of embryonic axes of *Prunus dulcis* developed earlier [12] and also detailed later [19] has been adopted and extended to range of germplasms. Almond nuts collected during various exploration missions in the high altitude areas of Indian states of Himachal Pradesh, Jammu and Kashmir and Uttaranchal and also received from field gene banks of institutes on receipt in the cryolab at NBPGR, New Delhi were kept at 10^{0}C temperature till the initiation of experiments. The nuts were cracked to collect the seeds (kernels). For viability testing the seeds/kernels were cut in the middle and half seeds enclosing the embryonic axes were surface sterilized by treating with 1% NaOCl (Sodium hypochlorite) for 10 min followed by three rinses with sterile deionised water before placing for germination.

Embryonic axes (EA) were excised aseptically from surface sterilized half seeds in the laminar air flow cabinet. The embryonic axes, fresh, as well as after desiccation and cryopreservation, were cultured on Murashige and Skoog's [20] medium 'A' supplemented with 1 mgl^{-1} each of 6-Benzyloaminopurine (BAP) and á-Naphthalene acetic acid (NAA) and medium 'B' similar to 'A' except additionally supplemented with 2 gl^{-1} activated charcoal. Cultures were maintained at 25±2^{0}C with a 16 h photoperiod under light intensity of 35μEm^{-2}s^{-1}. Axes that formed a well-defined root and shoot were considered viable.

Moisture content of embryonic axes (EA) was determined gravimetrically using low constant temperature oven method of 17 hours drying at 103 ± 2^{0}C [21]. Moisture content was obtained from 3 replications using 5-10 embryonic axes each and expressed on fresh weight basis. EA with moisture contents higher than 7% were needed to be desiccated for 1-2 h in laminar flow while spread over sterile filter paper discs in batches of forty to achieve moisture levels between about 4 to 7% before liquid nitrogen (LN) exposure. Germplasm accessions which showed satisfactory germination (above 80% germinability) were further processed for cryostorage. EA were packed in 2 ml polypropylene cryovials and lowered in vapour phase of liquid nitrogen (LN), at temperatures between -170°C to -180°C, which led to slow freezing.

Thawing and assessment of recovery

Survival of embryonic axes was recorded on the first testing after 48h of transfer to vapour phase. Later, testing intervals were increased to years ranging from 6 to 15 years as per experimental schedules. Cryovials were rapidly thawed in a water bath at 37°C for 5 min and subsequently explants were used for regeneration by *in vitro* culturing using both medium 'A' and 'B'. Recording of initiation of regrowth was done within 6 days of culturing by assessing the percentage of shoot and root emergence and termed survivability. After 3 months in culture, the development of the surviving axes was examined and regrowth percentage was recorded. Healthy plantlets were transferred to pots in soilrite.

Establishment of cryobase collection

For cryobanking of germplasm, a minimum 60 embryonic axes of each of the accessions were packed as 15 axes per cryovial creating minimum four replicates with final number depending on the total number of explants. These cryovials were cryostored after placing them the aluminum canisters in the extra large capacity cryotanks of 960 liters capacity (XLC-1830, MVE Cryogenics, USA) while mounted on aluminum canes in the vapour phase of liquid nitrogen. Each cryovial and cryocontainer was labeled properly. Passport data of individual accession along with storage conditions and retrieval requirements was entered in the cryogenebank database. Diversity augmented has been successfully cryopreserved as a base collection in the National Cryogenebank at NBPGR, New Delhi.

RESULTS AND DISCUSSION

Morphological characterization

Almond germplasm have been characterized based on carpological traits of nut and kernel characters and data presented (Table 1). Shell was generally paper shelled, hard and very hard and rarely soft. Nut length varied from 1.67 cm to 4.16 cm. Minimum nut length was 1.67 cm in Bitter Almond-4 followed by 2.20 cm in Dhaber and Drake cultivars and maximum was 4.16 cm in Primorskij, followed by 4.12 cm and 4.00 cm in Sharbo Selection and Ne Plus Ultra cultivars, respectively. Nut breadth ranged from 0.90 cm to 2.58 cm. Maximum nut breadth of 2.58 cm in Sharbo Selection was followed by 2.40 cm and 2.17 cm in Ne Plus Ultra and White Brandis cultivars, respectively and minimum of 0.90 cm in Bitter Almond-1 was followed by 1.03 cm and 1.15 cm in Bitter Almond-4 and Bitter Almond-2 cultivars, respectively.

Kernel was rarely smooth and mostly rough and wrinkled in shape. Kernel length varied from 1.00 cm to 2.90 cm. Minimum kernel length was 1.00 cm in Bitter Almond-4 followed by 1.40 cm and 1.45 cm in JKS-184 and Drake cultivars/selections, respectively and maximum was 2.90 cm in Primorskij, Sharbo Selection and Telangi Selection. Kernel breadth ranged from 0.60 cm to 1.60 cm. Maximum kernel breadth of 1.60 cm in Sharbo Selection was followed by 1.40 cm in Tree no-109, Katha and White Brandis cultivars. The minimum breadth was 0.60 cm in Bitter Almond-1 and Bitter Almond-4 followed by 0.70 cm in Drake cultivar. Kernel weight varied from 0.50 g to 1.26 g. Maximum kernel weight was 1.26 g in Pethick's Wonder followed by 1.11 gm and 1.07 g in Sharbo Selection and IXL, respectively and minimum was 0.50 g in JKS-184 followed by 0.54 g and 0.56 g in, Dhaber and Bitter Almond-4 cultivars respectively. Bitter Almond-4 showed smallest nuts whereas Sharbo Selection nuts were the largest. A pair wise similarity values among the cultivars of *P. dulcis* ranged

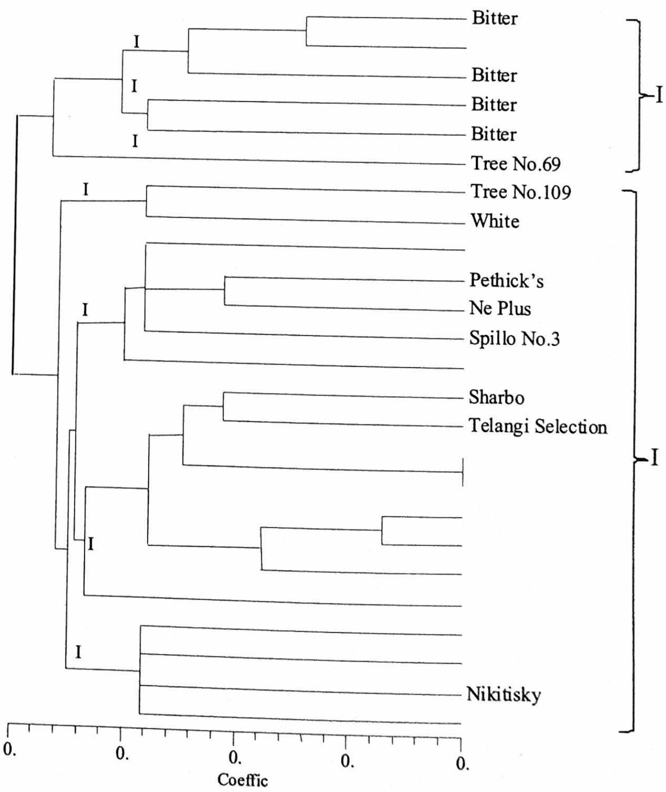

Fig. 1. Dendrogram generated based on morphological traits of 25 cultivars of *Prunus dulcis* using UPGMA method

Table 1. Morphological characterization of diverse indigenous and exotic almond germplasms

Cultivar/ selection	Accession No.	Shell softness	Nut size		Kernel shape	Kernel size		Kernel weight (g)
			Length (cm)	Breadth (cm)		Length (cm)	Breadth (cm)	
IXL	EC024664	Soft	3.50 (±0.19)	1.70 (±0.05)	Rough	2.35 (±0.09)	0.90 (±0.05)	1.07 (±0.02)
Ne Plus Ultra	EC024665	Hard	4.00 (±0.47)	2.40 (±0.19)	Wrinkled	2.20 (±0.05)	1.30 (±0.14)	0.95 (±0.03)
Drake	EC032482	Very hard	2.20 (±0.19)	1.20 (±0.09)	Smooth	1.45 (±0.14)	0.70 (±0.05)	0.71 (±0.02)
White Brandis	EC033950	Paper shelled	3.40 (±0.19)	2.17 (±0.08)	Rough	2.30 (±0.05)	1.40 (±0.19)	0.81 (±0.04)
Non Pariel	EC033951	Paper shelled	3.30 (±0.09)	1.73 (±0.13)	Smooth	2.20 (±0.05)	1.10 (±0.05)	0.82 (±0.08)
Texas	EC038651	Paper shelled	3.23 (±0.06)	1.78 (±0.10)	Wrinkled	2.50 (±0.09)	1.00 (±0.14)	0.72 (±0.01)
Nikitisky	EC042200	Very hard	2.50 (±0.09)	1.30 (±0.14)	Rough	1.60 (±0.09)	1.00 (±0.14)	0.61 (±0.04)
Primorskij	EC042202	Paper shelled	4.16 (±0.03)	1.93 (±0.03)	Rough	2.90 (±0.05)	1.20 (±0.09)	0.85 (±0.05)
Merced	EC087863	Paper shelled	3.56 (±0.07)	1.93 (±0.03)	Rough	2.60 (±0.09)	1.20 (±0.09)	0.78 (±0.05)
Pethick's Wonder	EC094309	Hard	3.45 (±0.07)	1.86 (±0.07)	Rough	2.50 (±0.09)	1.30 (±0.14)	1.26 (±0.34)
Bitter Almond-1	IC202680	Very hard	2.85 (±0.02)	0.90 (±0.05)	Rough	2.00 (±0.09)	0.60 (±0.02)	0.71 (±0.01)
Bitter Almond-2	IC202681	Very hard	2.98 (±0.04)	1.15 (±0.07)	Wrinkled	2.10 (±0.14)	0.80 (±0.09)	0.77 (±0.04)
JKS 184	IC202683	Very hard	2.40 (±0.09)	1.40 (±0.19)	Rough	1.40 (±0.19)	0.90 (±0.05)	0.50 (±0.05)
Bitter Almond-3	IC202684	Very hard	3.24 (±0.03)	1.67 (±0.03)	Wrinkled	2.50 (±0.09)	1.00 (±0.14)	0.83 (±0.07)
Bitter Almond-4	IC204472	Very hard	1.67 (±0.03)	1.03 (±0.03)	Wrinkled	1.00 (±0.14)	0.60 (±0.02)	0.56 (±0.06)
Katha	IC255390	Very hard	2.90 (±0.05)	2.00 (±0.09)	Rough	2.00 (±0.09)	1.40 (±0.19)	0.64 (±0.03)
Telangi Selection	IC255392	Paper shelled	3.45 (±0.07)	1.85 (±0.07)	Rough	2.90 (±0.05)	1.20 (±0.09)	0.96 (±0.02)
Dhaber	IC255394	Hard	2.20 (±0.05)	1.60 (±0.09)	Rough	1.50 (±0.05)	1.00 (±0.14)	0.54 (±0.02)
Tree No. 109	IC255395	Paper shelled	3.18 (±0.01)	1.92 (±0.04)	Smooth	2.70 (±0.14)	1.40 (±0.19)	0.71 (±0.01)
Tree No. 69	IC255396	Very hard	2.70 (±0.09)	1.90 (±0.04)	Smooth	1.90 (±0.04)	1.20 (±0.09)	0.74 (±0.03)
Spillo No. 3	IC255397	Hard	2.70 (±0.14)	1.50 (±0.05)	Rough	1.80 (±0.19)	1.10 (±0.05)	0.63 (±0.03)
Sharbo Selection	IC255399	Paper shelled	4.12 (±0.01)	2.58 (±0.10)	Rough	2.90 (±0.05)	1.60 (±0.09)	1.11 (±0.04)
Makhdoom	IC263077	Soft	3.33 (±0.03)	1.96 (±0.02)	Rough	2.30 (±0.14)	1.10 (±0.05)	0.59 (±0.02)
Shalimar	IC263078	Paper shelled	3.60 (±0.19)	1.76 (±0.11)	Rough	2.60 (±0.28)	1.00 (±0.14)	0.71 (±0.01)
Waris	IC263080	Soft	3.33 (±0.03)	2.10 (±0.05)	Rough	2.40 (±0.19)	1.20 (±0.09)	0.63 (±0.01)

Table 2. Eigen values, percentage of proportions and cumulative for 6 principal co-ordinate axes, derived from morphological data of 25 cultivars of *P. dulcis*

S. No.	Eigenvalue	Percent	Cumulative
1	14.47	60.28	60.28
2	04.51	18.80	79.09
3	02.48	10.35	89.44
4	01.35	05.61	95.04
5	00.62	02.57	97.62
6	00.44	01.84	99.46

from 0.05 to 0.86 with an average of 0.14 based on morpho-metric data.

A dendrogram generated based on morpho-metric data grouped all the 25 cultivars into two major clusters (Fig. 1). First cluster comprised of 6 cultivars or selections which got subdivided into three sub-clusters. Sub-cluster Ia comprised of three cultivars or selections namely Bitter Almond-1, Texas and Bitter Almond-4. Within the first cluster Bitter almond-1 and Texas were most similar morphologically showing a similarity value of 0.57. Sub-cluster Ib comprised two cultivars *viz.*, Bitter Almond-2 and Bitter almond-3 with 0.29 similarity value.

Sub-cluster IC comprised only one cultivar namely Tree No 69 which was distinct from all cultivars of this first cluster with 86% diversity. Second cluster was bigger comprising of 19 cultivars/selections. It is also subdivided into four sub-clusters. Sub-cluster IIa, comprised of two cultivars *viz.*, Tree No 109 and White Brandis with similarity value of 0.29. Subcluster IIb comprised of five selections or cultivars naming Dhaber, Spillo No-3, Nonpariel, Ne Plus Ultra and Pethick's Wonder. Within this subcluster Ne Plus Ultra and Pethick's Wonder were most similar to each other with 0.43 similarity values. Dhaber and Spillo No 3 were also similar to each other with similarity value of 0.29. Nonpariel was the most distinct cultivar within this sub cluster. Sub-cluster IIc was the biggest one comprising 8 cultivars/selections *viz.*, Sharbo Selection, Telangi Selection, Merced, Primorskij, Makhdoom, Waris, Shalimar and IXL. In this cluster Sharbo Selection and Telangi Selection were similar to each other with similarity value of 0.43. Within this sub-cluster Merced and Primorskij were most similar to each other with similarity value of 0.86. Makhdoom, Waris and Shalimar were in a separate group in this sub-cluster and IXL was the most distinct from rest of the other cultivars. Sub-cluster IId comprised four cultivars or selections *viz.* Katha, Drake, Nikitisky and JKS 184. Within this subcluster all four cultivars were similar to each other with similarity value of 0.29.

Based on Mantel Z-statistics the correlation coefficient (r) was estimated as 0.82. A value of 0.82 is considered a good fit of the UPGMA cluster pattern to the data. Two- dimensional plot generated from PCA showed four groups which was found most similar to the clustering pattern of UPGMA dendrogram (Fig. 2). In 2-D plot, Bitter Almond-4 was grouped together with four other cultivars *viz.* Bitter Almond-1, Bitter Almond-2, Bitter Almond-3 and Texas in one cluster. Drake, Spillo No-3 and Tree No-69 were grouped with Nikitisky, JKS-184, Katha and Dhaber but in dendrogram both of them were in separate cluster.

The analysis gave 25 principal components out of which first 6 principal components contributed 99.46% of the total variability (Table 2). The first 5 principal components accounted for 97.62% of the total variation and the first three accounted for 89.44% of the variation, in which maximum variation was contributed by first component (60.28%) followed by second component (18.80%), and third component (10.35%). The first PC was highly influenced by morphological characters *viz.* shell softness and kernel shape. In second PC, the traits contributing to the total variability were shell softness, nut length, nut breadth and kernel breadth. The third PC was influenced by shell softness, nut length, kernel length and kernel weight (Table 3).

Morphological analysis using seven carpological characters among the 25 almond cultivars was successfully employed to estimate genetic variability and to establish genetic relationships of indigenous and exotic cultivars. Genetic similarity calculated revealed very low level of similarity (0.14). A high level of morphological variability (86%) among the cultivars/selections of almonds was observed. Mousavi *et al.* [23] also reported

Table 3. Eigen vectors of morphological variables explained by first three principal components

Characters of cultivars/selections	Components PC-1	PC-2	PC-3
SS	0.3418	0.0462	0.0092
NL	-0.1795	0.0184	0.028
NB	-0.2521	0.0921	-0.0929
KS	0.0693	-0.2106	-0.0608
KL	-0.2441	-0.0331	0.0793
KB	-0.2472	0.0944	-0.0704
KW	-0.1552	-0.0617	0.0729

SS-Shell softness, NL-Nut length, NB-Nut breadth, KS-Kernel shape, KL-Kernel length, KB-Kernel breadth, KW-Kernel weight

Grouping of all the 25 cultivars in the dendrogram was congruent with the morphology of the cultivars. Merced and Primorskij cultivars were grouped together in the same cluster, showing close genetic relationship as both cultivars are exotic having similar morphological traits, whereas Tree No-69 was most distinct in the cluster-I. The first principal component was highly influenced by characteristics of the morphology *viz.* shell softness and kernel shape, while nut length, nut breadth, kernel length, kernel breadth and kernel weight were influenced by the second and third principal components.

Studies on morphological traits analysis in almond germplasm by Gouta *et al.* [24] revealed that the major fruit weight and size variables such as nut weight, nut length, nut thickness, nut size, kernel length, kernel weight and kernel thickness were strongly associated with the first principal component.

In the present study morphological characters like shell softness, nut length, nut breadth, kernel shape, kernel length, kernel breadth and kernel weight represented maximum variability as revealed by first three principal components. High variability ranging from low (0.09) to high (0.96) among the cultivars of almond germplasm based on morphological characterization.

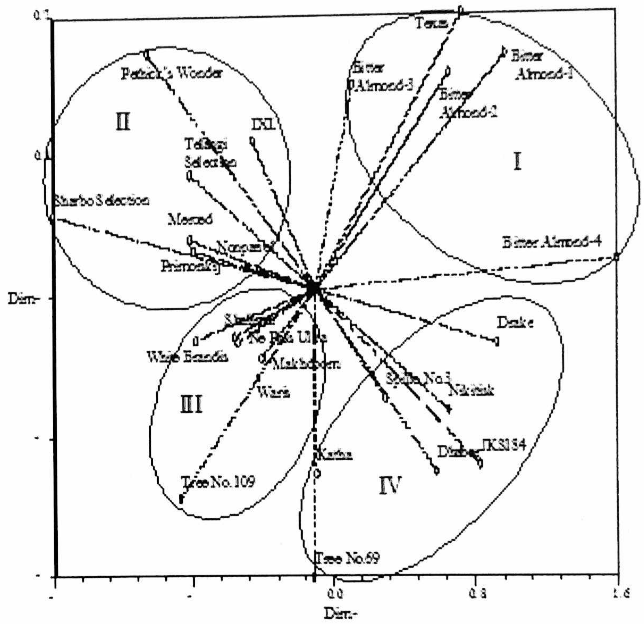

Fig. 2. 2-D plot of 25 cultivars of *P. dulcis* generated based on PCA of morphological data

Cryopreservation of embryonic axes: standardization using representative set

Moisture content (MC) of freshly excised embryonic axes of different accessions varied from 3.5 to 8%, necessitating short desiccation duration of 1-2 h in laminar flow in few to adjust the MC in all of them to between 3.5 to 7% before cryopreservation (Table 4). Embryonic axes of different cultivars and selections of almond were tested for viability *in vitro* before and after minimum 48 h of cryostorage. The germinability of unfrozen controls varied from 80 to 100% with 52% of

Table 4. Viability of embryonic axes of *Prunus dulcis* germplasm of diverse indigenous and exotic accessions tested after 48 hours of liquid nitrogen exposure

Cultivars/ Selections	Moisture content (%) Mean	Viability (%)	
		Before cryopreservation Mean	After cryopreservation Mean
IXL	3.50 (± 0.09)	90.00 (± 4.71)	86.00 (± 1.86)
Ne Plus Ultra	3.66 (± 0.14)	100.00 (± 0.00)	100.00 (± 0.00)
Drake	3.56 (± 0.04)	90.00 (± 4.71)	85.00 (± 2.36)
White Brandis	3.50 (± 0.06)	90.00 (± 4.71)	85.00 (± 2.36)
Nonpareil	3.50 (± 0.06)	100.00 (± 0.00)	98.00 (± 2.36)
Texas	3.63 (± 0.14)	95.00 (± 2.36)	95.00 (± 2.36)
Nikitisky	6.92 (± 0.05)	100.00 (± 0.00)	100.00 (± 0.00)
Primorskij	4.10 (± 0.09)	90.00 (± 4.71)	86.00 (± 1.86)
Merced	3.51 (± 0.05)	85.00 (± 2.36)	79.00 (± 1.41)
Pethick's Wonder	3.92 (± 0.14)	90.00 (± 4.71)	88.00 (± 3.39)
Bitter Almond-1	3.89 (± 0.09)	100.00 (± 0.00)	90.00 (± 4.71)
Bitter Almond-2	4.33 (± 0.04)	90.00 (± 4.71)	88.00 (± 3.39)
JKS 184	5.73 (± 0.06)	95.00 (± 2.36)	92.00 (± 2.36)
Bitter Almond-3	4.54 (± 0.14)	100.00 (± 0.00)	95.00 (± 2.36)
Bitter Almond-4	3.46 (± 0.06)	100.00 (± 0.00)	100.00 (± 0.00)
Katha	4.05 (± 0.04)	100.00 (± 0.00)	100.00 (± 0.00)
Telangi Selection	3.95 (± 0.08)	90.00 (± 4.71)	85.00 (± 2.36)
Dhaber	3.59 (± 0.08)	90.00 (± 4.71)	85.00 (± 2.36)
Tree No. 109	3.58 (± 0.14)	100.00 (± 0.00)	95.00 (± 2.36)
Tree No. 69	4.19 (± 0.04)	90.00 (± 4.71)	88.00 (± 3.39)
Spillo No. 3	5.60 (± 0.08)	80.00 (± 4.71)	79.00 (± 2.36)
Sharbo Selection	4.23 (± 0.06)	100.00 (± 0.00)	100.00 (± 0.00)
Makhdoom	5.15 (± 0.06)	90.00 (± 4.71)	85.00 (± 2.36)
Shalimar	3.63 (± 0.14)	95.00 (± 2.36)	90.00 (± 4.71)
Waris	3.96 (± 0.04)	90.00 (± 4.71)	85.00 (± 2.36)

Figures in parentheses are SEM values

the accessions showing high viability between 95 to 100%. After LN storage for 48h the viability ranged from 79 to 100% with 36% of the accessions retaining viability between 95 to 100%. Those accessions which showed a decline in viability on LN exposure, it amounted to only about 2-6% when compared to their respective unfrozen controls. The plantlets obtained *in vitro* from cryostored embryonic axes showed direct regrowth without any intervening callus (Fig. 3A-D). No differences in growth of plants were observed among the two media 'A' and 'B'. Initiation of growth was apparent within 6 days of *in vitro* inoculation in almost all cultivars except in Bitter Almond-4 where it took 10-12 days. Within 10 days the roots initiated the growth attaining a length of 5-6 cm by 45 days of culture. After about 64 days of regrowth, all healthy plantlets with fully

Table 5. Survival percentage of cryostored embryonic axes of almond accessions after different storage durations

IC/EC No (Cultivars/Landraces)	Viability (%) after initial 48 hour of cryostorage	Extended cryostorage duration (in years)	Viability (%) after extended cryostorage periods
IC547768 (Landrace)	90.00 (±4.71)	6	91.33 (±2.72)
IC547774 (Landrace)	90.00 (±4.71)	6	90.00 (±4.71)
IC547777 (Landrace)	100.00 (±0.00)	6	100.00 (±0.00)
IC561541 (Landrace)	100.00 (±0.00)	6	100.00 (±0.00)
EC033950 (White Brandis)	85.00 (±2.36)	10	80.00 (±2.36)
IC263080 (Waris)	85.00 (±4.71)	10	85.00 (±4.71)
IC380192 (Landrace)	80.00 (±2.36)	10	80.00 (±2.36)
IC380197 (Landrace)	100.00 (±0.00)	10	100.00 (±0.00)
IC547764 (Landrace)	100.00 (±0.00)	10	100.00 (±0.00)
IC255399 (Sharbo Selection)	100.00 (±0.00)	15	100.00 (±0.00)
EC032482 (Dhaber)	85.00 (±4.71)	15	85.00 (±4.71)
IC255390 (Katha)	100.00 (±0.00)	15	100.00 (±0.00)

Figures in parentheses are SEM values

grown roots and fully opened leaves were transferred to soilrite (Fig. 3E). In all the cases recovery percentage was equivalent to survival and hence viability values depicted in tables are the final regrowth. Conservation of cultivated plants with high economic potential is a priority and when cultivars are conserved together with all its landraces, seedling selections, and wild types it represents diversity readily available for exploitation. Cryobanked materials are the safety back up of field gene banks and *in vitro* repositories which are prone to biotic and abiotic stresses. Various conservation strategies for *Prunus* species available and presently in use at national and international level has been compiled [25]. It is advocated to develop a long-term storage methodology for difficult-to-handle seeds of *Prunus* species, where seeds are relatively large, do not store well at subzero temperatures, viability declines within 18-24 months following harvest, and need chilling up to six months for dormancy breaking. A decline in viability on LN exposure, it amounted to only about 2-6% when compared to their respective unfrozen controls. The plantlets obtained *in vitro* from cryostored embryonic axes showed direct regrowth without any intervening callus (Fig. 3A-D). No differences in growth of plants were observed among the two media 'A' and 'B'. Initiation of growth was apparent within 6 days of *in vitro* inoculation in almost all cultivars except in Bitter Almond-4 where it took 10-12 days. Within 10 days the roots initiated the growth attaining a length of 5-6 cm by 45 days of culture. After about 64 days of regrowth, all healthy plantlets with fully grown roots and fully opened leaves were transferred to soilrite (Fig. 3E). In all the cases recovery percentage was equivalent to survival and hence viability values depicted in tables are the final regrowth. Conservation of cultivated plants with high economic potential is a priority and when cultivars are conserved together with all its landraces,

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In such species, the embryonic axes being small in size and with potential to regenerate whole plant from its root and shoot meristem have been manipulated to survive freezing temperature of liquid nitrogen. Long term storage of *Prunus* spp. by cryostorage of embryonic axes has been recommended [8, 10] and successful cryopreservation of embryonic axes of *Prunus persica* [14] and of *Prunus amygdalus* [12] has been reported.

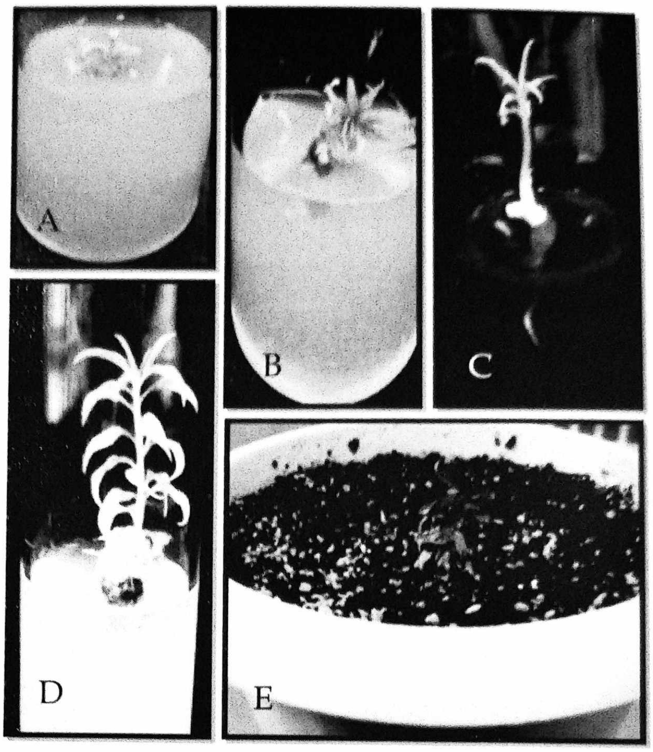

Fig. 3. In vitro regeneration of *P. dulcis* plantlets in medium 'A' raised from cryopreserved embryonic axes. [A] Shoot growth initiation after 15 days of culturing, [B] Plantlet after 25 days of culturing, [C] Well grown rooted plantlet after 35 days of culture in medium 'B', [D] 60 days old healthy plantlet and [E] Plantlet after one week of transfer to soilrite

Long-term cryobanking

Using the air desiccation-freezing protocol on embryonic axes, 240 diverse accessions of almond germplasm have been cryobanked for up to six to 15 years (Table 5). Apart from the germplasm listed (Table 1), prominent Indian cultivars *viz.* Aari, Ashiyana, Bajhal, Elaichee, Gulbadam, Gulbadam No-2, Gulfam, Kashamaha, Kathia, Mirg, Nauni Selection, Neelofer, Thin- shell, Tree No-2 and Zira have also been conserved in National Cryogenebank as a cryobase collection of available diversity of almond of the country. Cryobase collection is presently comprised of landraces (141 accessions), cultivars (40 accessions) and seedling selections (56 accessions) originating from 3 districts of Himachal Pradesh, one district of Uttaranchal and 3 districts of Jammu and Kashmir states. Several of them are in frequent use as rootstock. Variability in range of oil contents in representative cryostored accessions was studied in 39 accessions (25 accessions from district Budgaon and 14 accessions from district Pulwama) of state of Jammu and Kashmir based on methodology developed by Mandal *et al.* [22]. The percent oil ranged between 48.9 to 67.9 % where Budgaon accessions showed values from 48.9 to 67.9% and Pulwama accessions from 55 to 64%. The conserved germplasm thus represents the invaluable variability existing in cultivated and wild populations of almond in India and with diverse range of oil contents. Apart from 25 accessions retested for viability after 48 h cryostorage (Table 4), 12 cultivars or selections were assessed for viability intermittently after longer cryostorage periods of 6, 10 and 15 years (Table 5). After these long-term storage periods, all samples retained their respective viabilities that had ranged from 80-100%, except a 5% decline apparent in White Brandis after 10 years of cryostorage. Hence, expecting ensurance of high original viabilities of diverse germplasm after longer periods. In present studies, embryonic axes of almond have been desiccated with relative simple procedure without use of any chemicals before storage in LN. *In vitro* viability testing, a standard recommended test for explants like embryonic axes which are in sterilized state have been used to obtain uniform results. The level of survival and recovery of embryonic axes tissues tested after short and long duration has been fairly uniform and predictive over long duration. Very rarely root growth has been stunted or abnormal. The slow growth of cryopreserved axes during initial two weeks when compared to controls have been consistently observed which is in line with observations made with other species. In earlier studies large seeded temperate tree species germplasm cryostored as EA have been retested after an initial exposure of few hours to days [10, 12, 14]. Retesting after a longer duration of about a decade (8.5 to 9.5 years) have been reported with 80-100% survival only in *Juglans nigra, Aesculus glabra* and *A. hippocastanum* [26]. Present studies report evaluation of viability for longer durations of up to 15 years and ascertains the applicability of the developed protocol for diverse almond germplasm with retention of original high viability and successful transfer of plantlets to field.

Oil content of 48.9 to 67.9 % in selected accessions of almond germplasm collected from Budgaon and Pulwama district of Jammu and Kashmir [27] are similar to reported values of 51.5 to 66.8% of earlier studies [28]. Main fatty acids in kernel oil reported for germplasm from these districts [27] were oleic acid (56.95-78.76%), linoleic acid (13.69-32.95%), palmitic acid (5.05-9.08%) and stearic acid (0.66-3.61%). This was similar to earlier reports of 62.9%-77.3% for oleic acid, 14 to 26.8% for linoleic acid 4.9 to 7% for palmitic acid 1.5 to 3.4% for stearic acid by Kodad *et al.* [28]. Oilseeds are generally considered as poor storers. Several studies have shown that seeds containing high proportion of saturated medium and long chain fatty acids are problematic for storage at low temperatures especially when in dry state due to lipid crystallization [29-30]. Water content has a large role in this crystallization phenomenon which proves lethal especially in intermediate seed species. In almond, rich in unsaturated fatty acids, only consideration is thus only for water content per se and not for fatty acids for good storability.

According to a report from Tunisia [24], more than 3 million almond trees were lost due to severe drought which has led to loss of valuable genepool in their country. In present studies efforts have been made to establish cryobase collection of available diversity of almond of the country. Protocol for material handling and processing has been devised. Storage considerations such as survival criteria, number of propagules stored, and actual temperature of storage, response of diverse germplasm and management of data generated.

The present studies indicated that fairly uniform simple procedure can be followed for routine cryobanking for almond comprising 240 accessions where EA with 3.5 to 6% moisture levels can be cryoconserved and they are expected to have extended storage lives of several thousands of years [31]. Several studies on re-calcitrant seeds have reported that excised embryos and embryonic axes are more tolerant to desiccation and subsequent cryo exposure than whole seeds [13, 19, 32-34]. Smaller size of explants survive cryo exposure well, since in large explants, the hydration and thermal gradients created within the samples during pre-treatment with physical desiccation or cryoprotectants and during freezing, respectively, are reported to result in structural damage [33].

Variability available in India includes the sweet almonds in 3 groups- hard shell, soft shell and paper shell types, some accessions of seedling origin and some well known introductions like Nonpareil, IXL, Ne Plus Ultra and Drake and also cultivars developed from India. In the base collection, the representative accessions from each of these types have been cryopreserved. In cryobank, the space requirement and cost involvement for storage of such a large number of accessions when compared to that required in field gene bank is negligible [33]. As per our estimates 240 accessions have occupied a small space of about 0.9 sq ft in the cryogenebank at NBPGR which is just 1% space of that required for storing whole seeds. High direct recovery growth of embryonic axes without intervening callus after cryopreservation indicates the effectiveness of these techniques for long-term conservation. During cryostorage regeneration requirement inherent with decline in viability, if stored at seed gene bank temperatures of -20°C, is rare. Hence it proves to be the most advantageous and essential for tree species as any decline in viability would entail a regeneration schemes, where fresh seed harvest would take more than a decade. For re-calcitrant seed species that can only be conserved as live plants, cryobanking has proven to be the method of choice. The facility offers safe storage for a substantial range of almond genetic diversity in India, avoiding some of the challenges associated with field gene banks. While establishing base collection for crop species, genetic variability existing in the country should be assessed and efforts must be made to conserve maximum genetic diversity.

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