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Characterization and cryopreservation of chironji (*Buchanania lanzan)*

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ABSTRACT

Buchanania lanzan Sperg. (Chironji) is an important underutilized fruit species of north, west and central India. Seventy-four diverse accessions were collected from the diversity rich areas of India. Among the collected accessions, 52 have been characterized for fruit and seed characters. Successful cryopreservation of seed kernel and embryonic axes have been achieved using desiccation followed by fast freezing and air desiccation-freezing method respectively, with high viability up to 85% after cryopreservation. Comprehensive base collection of diverse accessions has been successfully established in the Cryogenebank. Morphological characterization with 20 variables representing 9 qualitative and 11 quantitative characters has been undertaken. All the accessions of *B. lanzan* were grouped into eight clusters, showing high level of genetic variability within the species. Two-D plot derived from PCA based on first two components showed four major groups, which was found less similar to the clustering pattern of dendrogram. Morphological characters, viz. fruit shape, length, width, colour, pulp colour, stone shape, stone colour; kernel weight and kernel colour represent maximum variability revealed by first three components identified for developing minimal descriptors. Five accessions, viz. IC-552921, IC-552924, IC-546107, IC-546109 and IC-553215 were identified as superior genotypes, which may be further evaluated and utilized as promising accessions for crop improvement.

Key words: Anacardiaceae, Chironji, Cryopreservation, Morphological characterization

Buchanania lanzan Sperg. (Anacardiaceae) is locally known as *Chironji, Achar, Charoli* and *Chawar* by tribal people of different Indian states Madhya Pradesh, Bihar, Orissa, Andhra Pradesh, Chhattisgarh, Jharkhand, Gujarat, Rajasthan and Maharashtra (Malik *et al.* 2010). It is an important medicinal tropical tree species, which is used for treatment of several diseases (Puri *et al.* 2000, Shukla *et al.* 2001, Kala 2009, Kadavul and Dixit 2009, Malik *et al.* 2010).

Natural populations of this species are vanishing at very fast rate due to deforestation, urbanization and developmental activities being undertaken in the marginal forest lands and tribal inhabited areas, facing genetic erosion and threat of extinction (Singh 2007). No organized cultivation of chironji has been practiced till now and fruits are directly plucked from the naturally wild growing trees. Singh *et al.* (2006) collected 30 accessions of chironji from different regions of Gujarat state and characterized them for physico-chemical characters. Chironji germplasm collected from Uttar Pradesh, Maharashtra, Bihar has been characterized for fruit weight, TSS, acidity, protein content and earliness (Rai 1982). Malik *et al.* (2010) also characterized 52 accessions of chironji based on fruit and kernel characters.

Buchanania lanzan seeds stored at fresh moisture content (16%) showed decline in viability to 35–68%, while stored at 7–10% moisture content showed decline in germinability (58-88%) after 280 days of storage. Based on the desiccation sensitivity and tolerance to freezing, non-orthodox seed storage behaviour has been ascertained for *B. lanzan* seeds (Naithani *et al.* 2004). Cryopreservation, the storage of germplasm at ultra-low temperatures is the only alternative for long term conservation of such species. Several minor fruits have been successfully cryopreserved using various explants (Malik and Chaudhury 2006).

There is an urgent need to collect the available genetic diversity, characterize and appropriately conserve the germplasm for posterity and further utilization. Superior accessions need to be identified for promotion of this highly potential fruit crop. In the present study collection, characterization and conservation of genetic variability have been attempted to identify elite germplasm of the *B. lanzan*.

MATERIALS AND METHODS

Germplasm collection: Specific survey and exploration missions in the diversity-rich areas of Madhya Pradesh, Gujarat, Chhattisgarh and Rajasthan were conducted for the collection of Cchironji germplasm from the naturally wild

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occurring populations during 2010–2017. Collection was made on selective sampling strategy and each collection was allotted a national identity (accession number) and detailed passport information was recorded. A total of 74 accessions were collected from these states, out of which 52 diverse accessions were selected for this study. IC numbers were allotted and related passport information was recorded in the NBPGR data base.

Morphological characterization: Fifty-two accessions have been characterized for fruit, stone and kernel characters to analyze the existing variability in chironji. Mean and standard error values for quantitative data were calculated using five fruits, stones and kernels per accession. A total of 20 quantitative and qualitative characters were considered for morphological characterization.

Moisture content, viability testing and cryobanking of seeds: Whole fruits were brought to the Cryolaboratory at NBPGR, New Delhi from collection sites for cryopreservation experiments and cryobanking. Fruits were depulped and stones were cracked to collect seeds (kernel). Moisture content and viability of kernels was measured before processing for cryostorage. Moisture content was determined gravimetrically using low constant temperature oven method of 17 h drying at 103±2ºC temperature (ISTA 1985).

Extracted kernels were desiccated on the charged silica gel to 5–8% moisture content. For viability testing the kernels were surface decontaminated by treating with 1% NaOCl (Sodium hypo-chlorite) for 10 min followed by three rinses in sterile deionized water before placing for germination. Viability was tested using the top of paper germination method in Petri plates (11 cm diameter). The Petri plates were maintained at 25±2ºC in BOD incubator with 16/8 h-light/dark photoperiod with water replenishment regularly at two-day intervals. The desiccated kernels (10-20) were packed in 5 ml polypropylene cryovials and cryostored using desiccation to different moisture levels followed by fast freezing method. The cryostored samples were thawed in a water bath maintained at 37ºC after 48 h of storage. Germplasm accessions which showed germination values above 60% germinability were further processed for cryostorage using standardized protocols. For cryobanking, a minimum of 50 seeds of each accession were packed in 5 or 50 ml cryovials depending on the number of seeds and placed in the aluminium canisters in the extra-large capacity cryotanks of 960 L capacity (XLC-1830, MVE Cryogenics, USA) in the vapour phase of liquid nitrogen at 180°C.

Air desiccation: Seeds were sterilized using sodium hypochlorite (1.0%) for 10 min followed by rinsing three times with distilled water and excised the embryonic axes. Excised embryonic axes (EA) were kept in batches of 20–25 in the sterile air flow of laminar flow cabinet immediately after excision. Axes were then desiccated for 1–5 h. After each desiccation interval, moisture content and viability of embryonic axes were determined. Desiccated embryonic axes were sealed in 1.0 ml polypropylene cryovials and fast frozen was done by direct plunging in liquid nitrogen (LN). After minimum 24 h of storage, the cryovials were rapidly thawed in water bath at 38°C for 5 min and EA were cultured *in vitro* on two different media within 30 min of retrieval. Medium A contained MS (Murashige and Skoog 1962) macro- and micro-nutrients, vitamins, iron- 2.0/gl, activated charcoal supplemented with 1 mg/l each of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) and in Medium B charcoal was omitted and supplemented with 0.1 mg/l each of BAP and NAA. Cultures were maintained at 25±2°C with 16 h photoperiod under light intensity of 35 µEm-2s-1.

Data analysis: A cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic average (UPGMA) based on Euclidean distance in software NTSYS ver. 2.10e. (Rohlf 2000). Principal Component Analysis (PCA) was also carried out to study correlations among the variables and establish relationship among accessions using same software.

RESULTS AND DISCUSSION

Buchanania lanzan is a medium size tree, up to 40–50 ft height with a straight trunk. Tree shows deciduous nature for short time in summer and new leaves emerge in late May. Leaves are 6–10 inches in length and shape is oblong with obtuse apex; flowers whitish green, sessile. Fruits are drupe, blackish and juicy with moderate sweet and acidic pulp. The trees are highly heterozygous probably due to cross pollinated nature of this species. Elite seedling trees are to be selected from natural populations with desirable characters for improvement of this multipurpose tree species.

Characterization and cluster analysis: All the accessions of *B. lanzan* were characterized for various agro-morphological parameters, viz. fruit, stone and kernel, which showed high variability in qualitative and quantitative characters except fruit juiciness, skin texture, fruit width and stone length. However, maximum variation was attributed to fruit, pulp colour and kernel width. High variation was also observed in quantitative parameters i.e. kernel weight (0.03–0.25 gm; average, 0.08; CV, 50%) and fruit weight (0.21–0.69 gm; average, 0.41 gm; CV, 26.82%). Similar variation within *khirni* germplasm has been recorded using different qualitative and quantitative parameters (Malik *et al.* 2012a).

Fifty two accessions characterized during this study were grouped into eight clusters; cluster I: BL-14; cluster II: BL-6, BL-11, BL-12; cluster-III: BL-13, BL-15; cluster IV: BL-4; cluster V: BL-5; cluster VI: BL-3, BL-7, BL-17; cluster VII: BL-16, BL-36, BL-39; and cluster VIII divided into four sub-clusters, included 38 remaining accessions (Fig 1A). A 2-D plot derived from principal component analysis (PCA) based on first two components showed four major groups (Fig 1B), which was found less similar to the clustering pattern of the dendrogram. In the PCA, first three components accounted 50.49% variability in which maximum variability showed by 1st component (22.45%). In the 2-D plot, all the accessions were grouped in the four groups. Group I and II were the largest groups, which

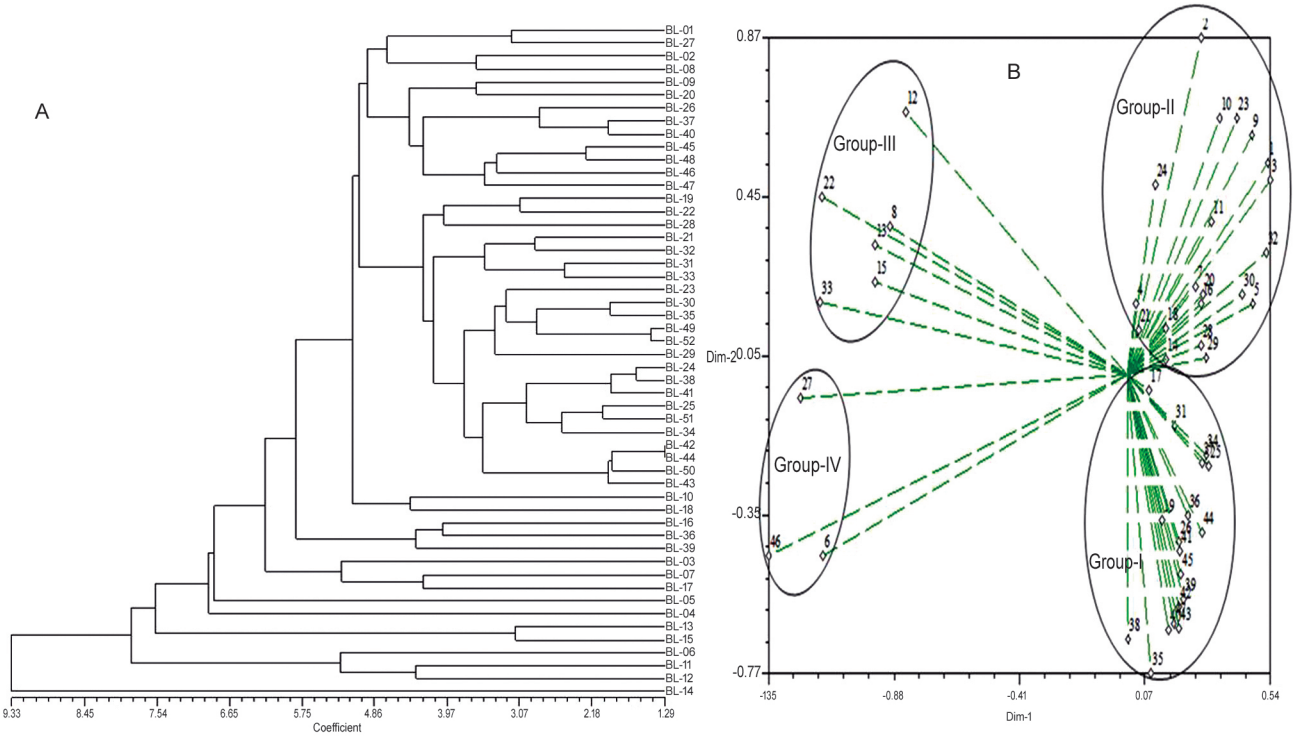

Fig 1 [A] UPGMA dendrogram of 52 accessions of *Buchanania lanzan*, generated based on Euclidean distance [B] 2-D plot of the first and second principal components of 52 accessions of *Buchanania lanzan* using morpho-metric data. The 1st and 2nd principal components are 22.45% and 14.97%, respectively.

were mainly represented by principal component-II (PC-II). These large groups represent the central part of Madhya Pradesh, which exhibit the maximum diversity in this species. However, group III and IV were the smallest groups represented by first principal component. PC-1 represents parameters like fruit shape, pulp colour, stone shape, kernel colour and fruit colour. PC-2 represents character kernel weight, stone colour, fruit colour, fruit shape and fruit length; while fruit colour, fruit width and kernel colour characters were represented by PC-3 (Table 1).

Maximum variability was observed for quantitative parameters like, fruit size and weight. Maximum variation in qualitative attributes was also observed in fruit and pulp colour among all the accessions of chironji. In the dendrogram, BL-14 accession collected from Vadodara (Gujarat) was exceedingly distinct from other accessions as it was clearly separated in cluster I. However, this accession was closer to BL-06 (Vadodara, Gujarat), BL-11 and BL-12 (Chittorgarh, Rajasthan), which formed cluster II. In which two accessions (BL-11 and BL-12) showed maximum similarity that may be due to the adjustant collection area in Chittorgarh (Rajasthan). BL-13 and BL-15 accessions were collected from Vadodara (Gujarat) formed distinct cluster III as both accessions showed similarity in morphological characters like fruit length, width; stone length and width and kernel weight. Accessions BL-04 and BL-05 collected from Vadodara (Gujarat) formed separate cluster IV and V, respectively, as these were, exhibited close association between each other. Three accessions, viz. BL-16, BL-36 and BL-39 grouped to form cluster VII, showing close morphological similarity among each other. Pattern of grouping indicates that most of the accessions belong to same gene pool as collection sites are very close to each other representing single population. Cluster VIII was a major cluster having most of the accessions from Madhya Pradesh. The locations of collection of these accessions were close to each other representing central part of Madhya Pradesh. These accessions were closely related to each other based on morphological characters and exhibited a large natural wild population.

Morphological characters like fruit shape, colour, length and width; pulp colour; stone shape, colour; kernel colour and weight represent maximum variability revealed by first three principal components and therefore, were identified for developing minimal descriptor. All accessions collected from different regions showing high level of genetic variability within species. Accessions, i.e. IC-552921, IC-552924, IC-546107, IC-546109 and IC-553215 were identified as elite, which may be utilized for crop improvement and breeding programmes after detailed evaluation. Morphological characters like fruit, leaf, seed, colour, pulp, pulp firmness, seed surface and TSS content represented maximum variability revealed by first three principal components in *Citrus* (Malik *et al.* 2012c) and papaya fruit (Saran *et al.* 2015).

Cryopreservation of germplasm: Moisture content of freshly harvested seed kernel is above 11% and after desiccation from 5.08–6.86% in the five accessions reported in the present study. Kernels were tested for viability before and after liquid nitrogen exposure. The viability of kernels

Table 1 Eigenvectors of morphological variables explained by first three principal components

Character	PC-1	PC-2	PC-3
FC	1.913	2.1286	6.0468
FS	5.398	1.7085	-3.4328
PC	4.4822	-0.5961	1.4158
SS	3.8708	0.9138	-4.144
SC	-0.0387	4.0802	-1.7042
KS	1.405	-2.3719	1.7504
KC	3.8081	-0.3161	2.1174
FL	-4.1485	1.6864	-0.6216
FW	-4.0857	0.5858	2.2507
FWt	-4.3985	0.7935	0.2028
SL	-2.7396	-0.3087	-2.2073
SW	0.2102	-4.0604	0.3921
ST	-1.8468	-0.3781	-0.4527
SWt	-1.9946	-3.7048	-0.6326
KL	-2.3694	-1.2731	-2.3211
KW	-0.9864	-1.6875	0.1289
KT	2.1196	-2.8221	0.2586
KWt	-0.5986	5.6218	0.9528

(Fruit colour, FC; Fruit shape, FS; Pulp colour, PC; Juiciness, JN; Skin texture, STx; Stone shape, SS; Stone colour, SC; Kernel shape, KS; Kernel colour, KC; Fruit length, FL; Fruit width, FW; Fruit weight, FWt; Stone length, SL; Stone width, SW; Stone thickness, ST; Stone weight, SWt; Kernel length, KL; Kernel width, KW; Kernel thickness, KT; Kernel weight, KWt)

before cryopreservation ranged from 72.22–89.44%. After cryostorage no significant change in the viability was noticed. Kernels showed viability in the range of 65–85% (Table 2). The seedlings obtained from cryostored kernels were visually normal and healthy.

The moisture contents and viability percentages of *B. lanzan* embryonic axes following desiccation under various time factors, with and without cryopreservation is given in Fig 2. The initial moisture content was 15.73%. With increasing duration of desiccation, the moisture content of the axes declined steadily. A major portion of the moisture loss occurred during the first h of desiccation. However, there was not much difference in the moisture loss at 3rd and 4th consecutive desiccation h. Since, the recovery growth of fresh, desiccated and frozen axes was rapid and normal on culture media 'A' in comparison to media 'B'. Reduction in moisture content of axes was, however, accompanied by a decline in the survival rate.

The initial viability values (without desiccation) of embryonic axes were 100% at 15.73% moisture content and declined to 40% at 3.87% moisture content. It was seen that viability fell drastically after 3 h of desiccation at moisture content below 5.46%. Cryopreservation was efficient for

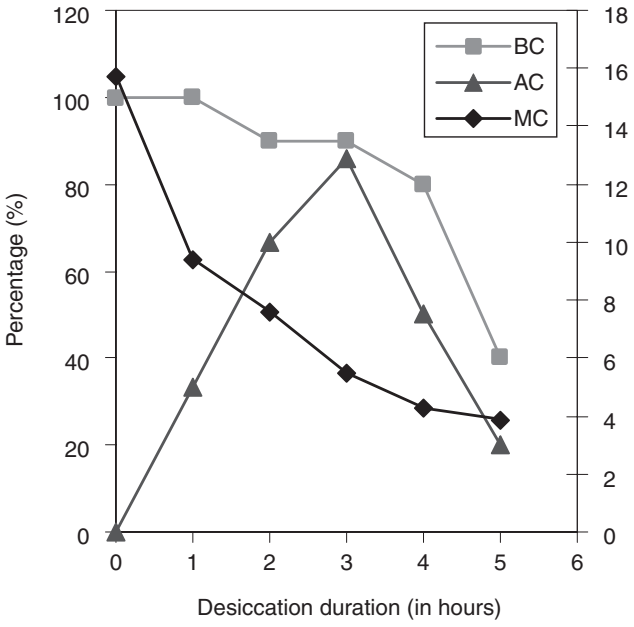

Fig 2 Air desiccation of embryonic axes of *B. lanzan* (AC-After cryo; BC Brfore cryo; MC- Moisture content).

the axes desiccated between 4.26–7.58% moisture content. Freezing of embryonic axes at 5.46% moisture level in LN at -196°C showed a good recovery rate (85.73%), whereas embryonic axes possessing moisture contents above 9.37% and below 3.87% lost viability completely when exposed to LN (Fig 2).

A total of 127 diverse accessions from Rajasthan, Gujarat, Madhya Pradesh, Himachal Pradesh and Jammu and Kashmir have been successfully cryostored as base collection in the cryogenebank. The conserved germplasm represented the invaluable variability existing in natural wild populations of chironji in India (Fig 3A, 3B and 3C).

Collected and characterized accessions of this important tree species were successfully cryopreserved as seed kernels and embryonic axes. Seeds being non-orthodox, successful

Table 2 Moisture content, viability and cryopreservation of chironji seeds

Accession No.	Fresh moisture content %	Viability % control	Moisture content % after desiccation	Viability % after desiccation	
				Before cryopre servation	After cryopre-servation
IC-552919	12.63 (±0.65)	88.89 (±2.78)	5.08 (±0.08)	75.75 (±3.79)	68.78 (±2.02)
IC-552932	11.86 (±0.54)	90.00 (±2.36)	5.18 (±0.10)	72.22 (±3.27)	65.00 (±2.36)
IC-552956	12.29 (±0.52)	85.00 (±2.36)	6.86 (±0.29)	79.22 (±5.27)	68.89 (±4.79)
IC-546109	12.11 (±0.75)	90.00 (±2.36)	6.33 (±0.65)	89.44 (±2.76)	85.00 (±2.36)
IC-553197	12.10 (±0.44)	82.78 (±1.19)	5.49 (±0.03)	76.11 (±3.17)	68.89 (±0.91)

Fig 3 [A] Natural wild population of *B. lanzan*, [B] Seeds of chironji, [C] Embryo showing embryonic axes, [D] Regenerated plantlets of *B. lanzan* after cryopreservation, [E] Rooted plantlets of chironji.

cryopreservation protocol were developed and cryostored as base collection representing sizable diversity in the form of 127 accessions in the National Cryogenebank at NBPGR, New Delhi for posterity and future utilization. The desiccation sensitivity of an explant is the degree of its tolerance to lose free water without associated damage and a decline in viability. In this experiment, we have demonstrated the desiccation sensitivity of embryonic axes of *B. lanzan* at distinct rates of drying. The embryonic axes had high initial moisture content. A relatively high critical moisture content (level below which significant reduction in viability is recorded), a significant decline in viability with the reduction in moisture content, and sensitivity to freezing, are indicative of desiccation and freezing sensitive nature of embryonic axes. Successful cryopreservation using air desiccation-freezing of embryonic axes was achieved first time in this multipurpose tropical tree species (Fig 3D and 3E).

Cryopreservation of germplasm in the form of seeds, embryo and embryonic axes of multipurpose tropical tree species which are predominantly cross pollinated, only ensure the gene pool conservation due to the heterozygous nature of seeds. As most of these species are found natural wild or semi-wild and propagated through seeds in nature, conservation of available genetic variability essentially required for the selection of desired genotypes and which needs to be protected safely and timely. In most of these fruit tree species farmers or local people are propagating progenies using seeds as no commercial cultivars are available and even if few have been identified, planting material is hardly available. Once the promising genotypes are identified in these species, conservation of their vegetative tissues to achieve true-to-type conservation may be attempted using *in vitro* methods. It is, therefore, emphasized that a complementary conservation strategy (Rao 1998), involving the use of more than one relevant approach would be the best option for achieving safe conservation of these multipurpose and underutilized fruit species, some of which facing severe threat of extinction. The *ex situ* conservation of chironji in the cryogenebank is an important alternative conservation strategy which could be complemented with field genebank and dynamic *in situ* conservation approaches in the protected areas.

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