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AN IMPROVED POLLEN COLLECTION AND CRYOPRESERVATION METHOD FOR HIGHLY RECALCITRANT TROPICAL FRUIT SPECIES OF MANGO (*Mangifera indica* L.) AND LITCHI (*Litchi chinensis* Sonn.)

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Abstract

An improved method for pollen collection from freshly dehiscing anthers of mango (*Mangifera indica* L.) and litchi (*Litchi chinensis* Sonn.) using the organic solvent cyclohexane has been devised. Using this method pollen quantity sufficient for large scale pollinations could be collected and stored for future use. Transport of pollen in viable conditions over long distances, from site of collection (field genebank) to cryolab was successfully devised for both these fruit species. Cryopreservation was successfully applied to achieve long-term pollen storage over periods of up to four years. Pollen viability was tested using *in vitro* germination, the fluorochromatic reaction (FCR) method and by fruit set following field pollination. On retesting, four year cryostored pollen of different mango and litchi varieties showed high percentage viability as good as fresh control pollens. Pollens of more than 180 cultivars of mango and 19 cultivars of litchi have been stored in the cryogenebank using the technology developed, thus facilitating breeding programmes over the long-term.

Keywords: mango, litchi, cryopreservation, pollen, viability, cyclohexane

INTRODUCTION

Mango (*Mangifera indica* L.; family Anacardiaceae) is one of the most important fruit crops of India with its origin traced to the Indian subcontinent, especially Assam and North-Eastern regions (14). It is an open pollinated crop and more than a thousand types are available in India with extensive genetic diversity (17). For breeding new varieties with specific qualities using improved genetic material, the existing natural variability offers a vast genetic resource of desirable genes. Mango hybridization programmes have been undertaken by some Indian research organizations over the last two decades to exploit this available

variability. However, due to asynchrony in flowering and due to the location of putative parents in different geographical areas within the country especially among North Indian and South Indian varieties, several cross combinations could not be attempted. Flowering periods ranged from December to April depending on agroclimatic zones. The hybridization programmes in mango are on several occasions hampered not only because of spatial and temporal isolation of the parents but also due to the lack of an effective technology to maintain the viability of pollen while it is collected and transported between locations.

The litchi or lychee (*Litchi chinensis* Sonn.; family Sapindaceae) is one of the important subtropical commercial fruits with its origin in South China and adjoining areas (16). About 51 cultivars are reported from India, the second largest producer of litchi in the world. Improvement programmes are needed for development of precocious and prolific bearing dwarf cultivars with good fruit quality and small and flat seeds, cracking resistant, pest and disease resistant and early maturity cultivars (cvs). Such programmes depend on the application of hybridization. In general three different types of flowers, i.e. male, pseudo-hermaphrodite and female, are encountered in litchi; only the female flowers set fruit. The pollen germinability reportedly decreases rapidly after anther dehiscence due to poor storage capability (15, 25). There are no long-term storage methods available so far for this genus. Storage of viable pollen of litchi cvs for the long-term would be highly desirable for breeding programmes.

As mango pollen is somewhat sticky and difficult to separate from the anther, development of an improved method for pollen collection is a prerequisite for effective storage. In litchi, pollen grains from male flowers have reportedly less viability than those from pseudo-hermaphrodite. It is thus essential to collect sufficient pollen to ensure a high proportion of viable pollen. Suitable methods for transport of pollen over long distances, e.g. that take 2-3 days, also needs to be standardized. Even after storage, pollen may have to be transported in a viable condition to the site of the female parent for pollinations. In the light of these requirements, the present studies were undertaken on selected genotypes of mango and litchi with the objectives of enhancing the quality of collected pollen, maximising survival on transport, cryostorage and thawing, optimising retesting of pollen viability in the lab, and safe transport to enable field pollinations.

MATERIALS AND METHODS

Pollen collection and transportation

Mango pollen collections were made from the germplasm grown at the Field Genebank at the Central Institute of Subtropical Horticulture (CISH), Lucknow during the mango flowering period in March, during the years 2003-2006. Litchi pollen were collected from the Field Genebank of Central Horticultural Experiment Station (CHES) at Ranchi during mid-flowering (1st week of March) in 2005 and 2006. Anthers were collected at dehiscence time between 8 and 10 a.m. Using forceps, the anthers were transferred to a 10 ml glass vial. After sufficient numbers of anthers were collected, 3 ml of cyclohexane (GR, Merck, Germany) was poured over them into the vial and shaken vigorously for 30-50 s. The solvent along with the suspended pollen grains and the anthers was then syringed out using a glass syringe, with the needle removed, and passed through a filter disc (pore size 0.22 μm) placed in a millipore filter assembly**.** The pollen grains along with the empty anthers

were retained on the filter disc that were then allowed to dry for a few minutes. The anthers were removed from the filter disc; the disc containing pollen grains was cut into 2 equal pieces and placed in separate polypropylene cryovials. These vials were transported in an ice box (holding ice at about 5ºC) to the cryolab at NBPGR, New Delhi (about 400 kms from Lucknow and about 1000 kms from Ranchi) within 2 days of collection.

Pollen cryostorage

In the cryolab the lids of cryovials containing pollen-loaded discs were removed and the vials kept over charged silica gel in a desiccator to partially desiccate the pollen. After 1 h the lids were closed and the cryovials plunged in liquid nitrogen (LN) to achieve fast freezing. After 24 h, the cryovials were shifted to the vapor phase of LN at temperatures between –170ºC to –180ºC in large capacity cryotanks XLC 1830 (MVE Cryogenics, USA). Eight to ten replicates of each pollen sample were cryostored. After storage for various periods, the cryovials and stored pollen of different mango and litchi cultivars were thawed in a water bath at +38ºC for 30 s and viability tested by a minimum of two of the following three methods.

Field pollinations

The cryovials containing stored mango pollen were transported at about 5ºC in a Mini cooler (Nalgene Co., USA), arriving at CISH, Lucknow within 2-3 days for field pollination. For effective comparison, one set of mango pollen was tested for fruit set at the Indian Agricultural Research Institute (IARI), New Delhi fields. In the fields the filter disc coated with pollen was taken out from the vial with a forceps and gently rubbed on the receptive stigmas of different cultivars to bring about pollination. The pollinated flowers were immediately bagged with wax paper. The details of fruit set were noted after 2-4 weeks. For controls, fresh pollen extracted as pollen-anther mixture (without using any solvent, i.e. cyclohexane), was used for pollinations. About four to five flowers each from at least 25 panicles were hand-pollinated. In all a total of 100-125 flowers were used for pollination in a cross each year. This was done considering the fact that a mango panicle rarely carries more than one fruit to maturity (24).

In vitro *pollen germination*

Litchi pollen viability was tested using an *in vitro* germinability test following the method reported by Shukla *et al.* (22) where two liquid culture media, A and B, were used. The media were composed of 15% sucrose, 20 ppm boric acid and IAA at two concentrations, namely 10 ppm (in medium A) and 20 ppm (in medium B). Before culturing for germination, pollen were subjected to controlled hydration in humid chambers (90-100% humidity) for 30 min. In the same chambers pollens were cultured in two replicate liquid culture drops. After 4 h, pollen showing tube lengths longer than the pollen diameter were scored as viable with 400-500 pollen in two replicate drops scored in total. Pollens germinability after cryostorage were assessed using the same protocols used for fresh pollen. Images were recorded using Carl Zeiss AX10 Microscope.

Fluorochromatic reaction (FCR) test

The FCR method developed by Heslop-Harrison and Heslop-Harrison (6) was used both for mango and litchi pollen, basically to assess membrane integrity. Pollen samples were exposed to controlled hydration before testing with fluorescein diacetate (FDA) by

maintaining in a humid chamber for 10 min (10). To a solution containing sucrose (10%) and calcium nitrate (2.36 mg ml^{-1}) drops of stock solution of fluorescein diacetate in acetone (2 mg ml^{-1}) was added until the resulting mixture showed persistent tubidity. This mixture was used for dispensing pollen on a microslide. The brightly fluorescing pollen grains as observed in fluorescence microscope within 3-5 min of staining were scored as viable. A minimum 10 optical fields in each of two drops of staining solution were scored, with about 300-500 pollen scored in total.

Statistical analyses of data

The results for all the viability tests presented are the mean ± SE of at least 10 observations for FCR and germinability tests and for field pollinations data collected at two sites, i.e CISH, Lucknow and IARI, New Delhi were pooled. Data of Table 1 was subjected to Univariate Analyses of Variance using SPSS 10.0 software. Post-hoc analyses of data was performed by Scheffe's test.

RESULTS

In the present studies the organic solvent cyclohexane has been effectively used to extract and separate large quantities of pollen from the anthers of two species. Cyclohexane removed the sticky lipids from the pollen surface and thus separated individual pollen grains from the anthers, as evident by largely empty anthers of mango and litchi when observed under the microscope. It allowed for the collection of a large amount of pollen on the filter discs. Additionally, handling of the filter discs with adhered pollen, instead of applying the pollen with a brush, during field pollination was much easier in mango and facilitated pollination of a large number of stigmas from the same disc in a shorter time. Pollen loss was minimum using this technique.

Pollen of mango cultivars Amrapali, Bombay Green, Neelum and Bangalora cryostored for various periods were used for pollinating cvs Neelum and Amrapali, as the latter two are self-incompatible. Fresh controls showed fruit set ranging from 12 to 20% (Table 1). Fruit set was achieved in all the cross combinations tried with cryostored pollen and tested for upto 4 years storage. However, the results were found to vary with year and cross combination. Fresh Amrapali pollen on crossing with Neelum led to 20% fruit set. After cryostorage for 2 days (data not shown) and subsequently 1 year, fruit set of about 15% was uniformly achieved. A higher fruit set of 18% was achieved using 4 year cryostored pollen. Fresh Bombay Green pollen led to fruit set of c.16% when crossed with both Amrapali and Neelum. One year cryostored pollen in crosses with Amrapali and Neelum, however, showed a decline in fruit set to 3.4% and 8.3%, respectively. Surprisingly 3 years later, the same cryostored pollen (after 4 years storage) led to higher fruit sets of 14% and 19%, respectively. A different pattern was apparent in case of 1 and 4 year cryostored Neelum pollen when pollinated with Amrapali. A high fruit set of 21% was achieved by 1 year storage, which is higher when compared to 14% achieved with fresh pollen. Whereas a low fruit set of 2% was achieved with pollen after further 3 years cryostorage. Fresh Bangalora pollen led to 12% fruit set, which declined to 4% and 5% using 1 and 4 year cryostored pollen, respectively.

In another set of experiments (Table 2), fruit setting using a fresh pollen-anther mixture and a fresh cyclohexane extracted pollen was compared in cv Amrapali, Neelum and Bombay Green in cross combination with females Neelum and Amrapali. In comparison

to results obtained with control pollen-anther mixture, using cyclohexane extracted pollen the fruit set was 20% for Amrapali and 17% for Bombay Green whereas for Neelum pollen it was slightly lower (14%). Although no definite pattern was observed, these results confirmed that treatment of pollen with cyclohexane did not affect its fertilizing ability.

In mango, the FCR method was simultaneously used for viability testing of samples. Viability of 4-year-cryostored pollen of different cultivars (namely Amrapali, Bombay Green, Neelum, Bangalora and Kishan Bhog) was tested and compared with that of fresh controls (Table 3). The viability of fresh controls ranged from 55% in Bombay Green to 86% in Amrapali. After cryostorage, the pollen viability values ranged from 57% to 87% amongst the cultivars, with individual samples showing no significant change in viability values compared to the controls, indicating successful storage.

Table 1. Effect of cryostorage on mango cultivars (cvs) pollen viability as revealed by fruit set percentage resulting from field pollinations. Means with same superscript are not significantly different from each other at a 5% level according to Scheffe's test.

Mango CVS Pollen parent x female parent	Length of cryostorage (Years)	Fruit set (%)
Amrapali x Neelum	0	20.00^{ab}
	1	15.20^{abcd}
	4	18.20^{ab}
Bombay Green x Amrapali	0	16.10^{abc}
	1	3.40^{e}
	4	13.60^{abcde}
Bombay Green x Neelum	0	16.50^{abc}
	1	8.30^{bcde}
	4	18.90^{ab}
Neelum x Amrapali	0	13.60^{abcde}
	1	20.80^{a}
	4	2.04^{e}
Bangalora x Neelum	0	11.80^{abcde}
	1	4.10^{de}
	4	4.83^{cde}

Table 2. Effect of pollen extraction method on mango fruit setting percentage resulting from pollination using fresh pollen.

Mango cvs of male parent		Cvs of female parent	Fruit setting percentage (±SE)	
			Using pollen-anther mixture	Using pollen extracted with cyclohexane
Amrapali	X	Neelum	14.2 (±1.0)	20.0 (±1.0)
Neelum	X	Amrapali	15.3 (±0.8)	13.6 (±0.3)
Bombay Green	X	Neelum	16.1 (±1.1)	16.5 (±0.3)

Similarly in litchi, viability of 4-years cryostored pollen of different cultivars, namely CHES-6, Chaina and Kasba, was tested by the FCR method and compared with that of fresh controls (Table 4). Fresh controls differed in viability from 49% to 70%. The cryostored pollen invariably showed similar viability values as the controls which ranged from 47 to 72%, demonstrating the retention of enzyme activity and membrane intactness in cryostored pollen and thus no loss of viability. Simultaneously, an *in vitro* germination test was conducted on fresh and cryostored pollen of these accessions on two different germination media (A & B) for effective comparison with FCR test results. Viability values for fresh pollen ranged 19% to 21% using medium A which improved by 7-10% using medium B, achieving a maximum of 31% germination in cultivar Kasba. *In vitro* germination of 4-year cryostored pollen using media B showed germinability values as good as fresh controls: 26 % in Chaina (Table 4; Fig 1 B), 28% in Kasba (Table 4; Fig 1 C) and 32 % in CHES-6 (Table 4; Fig 1 A). Irrespective of the viability test conducted there was no decline in pollen viability when tested for three cultivars of litchi.

Table 3. Viability of 4 year cryostored mango pollen as judged by FCR test.

Mango cultivars	% Fluorescing pollen (±SE)	
	Fresh	4-year cryostored
Amrapali	85.5 (±1.6)	87.0 (±0.6)
Bombay Green	55.0 (±2.1)	56.8 (±0.7)
Neelum	75.0 (±6.1)	70.6 (±0.5)
Bangalora	71.7 (±3.7)	73.2 (±1.7)
Kishan Bhog	82.5 (±2.3)	80.0 (±2.1)

Table 4. Pollen viability testing by *in vitro* germination and FCR test in different Litchi (*Litchi chinensis*) cvs before and after storage.

Cvs	Viability (%) of fresh pollen (±SE)			Viability (%) of pollen after 4 year cryostorage (±SE)		
	In vitro germination using		FCR test	*In vitro* germination using		FCR test
	Media A	Media B		Media A	Media B	
CHES-6	20.8 (±0.7)	28.0 (±1.0)	69.9 (±2.9)	22.9 (±1.2)	31.5 (±0.8)	72.2 (±2.3)
Chaina	18.5 (±0.6)	25.0 (±0.7)	48.7 (±1.3)	16.7 (±0.6)	25.5 (±1.4)	46.5 (±0.9)
Kasba	20.0 (±2.9)	30.5 (±0.9)	58.7 (±0.9)	18.8 (±0.3)	27.7 (±1.3)	60.0 (±5.4)

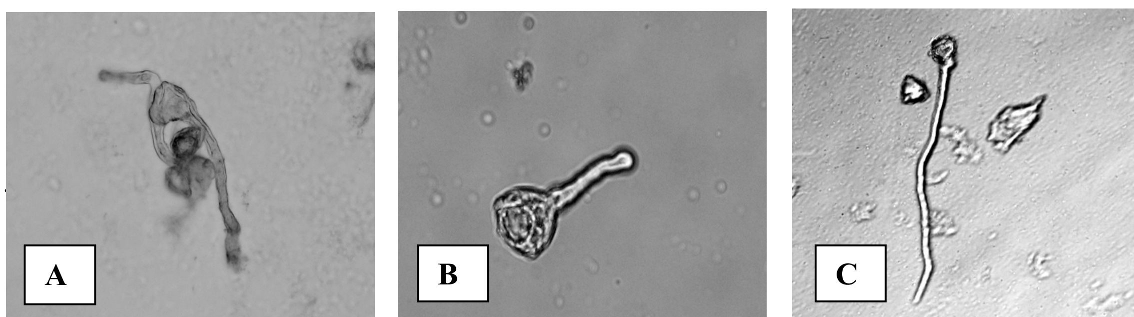

Figure 1. *In vitro* germinated litchi pollen after 4 years cryostorage of different cultivars: A- CHES-6; B- Chaina, C- Kasba

DISCUSSION

Collection of pollen in viable condition and in sufficient quantities is the primary requirement for any breeding and storage work. Large amounts of pollen are needed for pollination, viability testing, storage and future distributions. Hence replicate samples needed to be stored. Use of organic solvents such as cyclohexane, diethyl ether, acetone and benzene have been used in the past for pollen storage at 5ºC for durations of 2 to 3 months especially for 2-celled pollen systems (8, 11, 13). Pollen stored successfully in non-polar solvents such as cyclohexane, hexane and diethyl ether are known to result in very little leaching of membrane phospholipids, sugars and amino acids into the solvent (9). In polar solvents, however, extensive leaching of these compounds leads to loss of pollen viability. In studies by Mishra and Shivanna (13) cyclohexane has been found to be the best solvent for legume pollen storage and was recommended for short-term storage. In the present study, the use of solvent for pollen collection proved to be an improved method in

comparison to that reported earlier (3) for anthers and pollen-anther mixtures in mango. In studies by Jain and Shivanna (10) seed set obtained with pollen samples of *Crotalaria retusa* stored in cyclohexane for 6 months was as good as with fresh pollen, which was due to maintenance of osmotically active membranes that prevented excessive leaching of metabolites. In the present studies cyclohexane enabled the maximum extraction of pollen with high viability, as tested by germinability, vital dye staining and fertilizing ability.

Pollen longevity has been reported to be extended by using lower temperatures such as 5ºC, –20ºC, –80ºC and –196ºC and low moisture contents. The actual longevities at 5ºC, –20ºC, –80ºC temperatures are known to differ considerably among species and genotypes (12, 29). The pollen once cryostored at temperatures below –160ºC would theoretically have 'infinite' periods of longevity (26) thus assuring availability of viable pollen for long periods. In our studies mango pollen fertility was retained during cryostorage as pollen of all four cultivars tested could successfully effect fertilization. High viability retention in cryostored pollen was also confirmed by the FCR test. It is thus expected that pollen of other cultivars of *Mangifera indica* could be cryostored with good viability following similar procedures The retention of mango pollen viability for up to 5 years under cryostorage has already been demonstrated in studies undertaken by the Indian Institute of Horticultural Research (IIHR), Bangalore (4). However, for litchi pollen no reports on cryostorage have previously been reported.

In the present studies, the hand pollination method used is known to result in as high as 3.85% fruit set (24). It has been observed that in nature 50% of the mango flowers do not receive any pollen (9). In the present study we ensured that all flowers received pollen. This would have resulted in higher efficiency of fruit setting. However, in spite of high fruit formation, there was a heavy fruit drop leading to low retention of crossed fruits. This phenomenon has been observed routinely in mango (9). Variability in fruit set observed using the cryostored pollen over the years could be due to seasonal fluctuations in field conditions and natural alternate bearing behaviour well known in mango. Use of bulk pollen for supplementary pollinations in orchards for sustaining or enhancing the yield is well known (28). Higher fruit sets using cryostored pollen could be due to dusting of a large quantity of viable pollen on the individual stigma.

Major work on mango hybridization to develop new cultivars by inter varietal crosses has been carried out in India (18). Potential use of wild mango species has been equally emphasized and elaborated (9). In view of the large genepool of *Mangifera indica* and availability of related wild spp. *M. andamanica*, *M. sylvatica*, *M. khasiana* and *M. camptosperma*, there is a wide scope of genetic improvement work and the methods devised in the present work would substantially aid this.

Pollen germinability in litchi has been reported to be reduced at ambient temperatures 1-2 days after anther dehiscence (23). It was thus imperative to devise a rapid collection and suitable storage method. Using the acetocarmine stainability test in litchi, Singh and Singh (25) observed 98% pollen viability after 11 months of storage at 7ºC and 25% RH, and 65.7% viability after 24 months of storage at 0ºC and 25% RH. Pollen viability testing methods using nonvital stains, such as acetocarmine, merely imparts color to the contents of the pollen irrespective of whether it is live or dead. In contrast, *in vitro* germination generally shows a correlation with fruit set in several species (21). The FDA test is a comparatively reliable test as it assesses the esterase enzyme activity and intactness of the cell membranes. However FCR test values are invariably an overestimate of the actual viability compared with *in vitro* germination (20). In species where the *in vitro* germination response is optimal, a close

correlation is observed between viability values using the FDA test and *in vitro* germination. If the germination medium is not optimal for a species, then FDA results are a significantly better index of viability than the *in vitro* germination (19).

It has been observed in several cases that if pollen germination *in vitro* is more than 10-30%, normal fertilization and seed set comparable to fresh pollen often occurs (7). Hence litchi pollen stored with 30% or lower germinability are expected to effect normal fertilization whenever used in field pollinations.

The present studies elaborate an improved method for pollen collection, transportation over a long distance before cryostorage and transportation back to the site of the female parent in a viable condition. This methodology opens up immense possibility for breeding programmes covering diverse parts of the country. A further refinement of the method reported here would be to carry a small cryocan (dry shipper) containing sufficient liquid nitrogen (LN) to last 2-3 weeks along with small glassware, filter assembly and solvent to the place of pollen collection and use it for cryostorage. This cryocan can then be transported to the site of female parent and pollination can be effected after thawing.

Our earlier studies have clearly shown that it is feasible to cryogenically store the pollen grains of oil palm for periods beyond 8 years without any significant loss in their viability and germinability (27) and may be used effectively for pollinations. Based on the technique developed presently more than 180 cvs of mango and 19 accessions of litchi have been successfully crystored in the National Cryogenebank at NBPGR, ensuring availability of viable pollen for infinite periods. An extensive programme for cryostorage of mango and litchi germplasm from the field genebanks, in addition to germplasm of rare and wild species available throughout the country, is envisaged in the near future. There are about 51 commercial cultivars of litchi being grown in Indian states that have been targeted for cryostorage. The duplicate sets of pollen collected can be stored for posterity in the cryogenebank to ensure availability of pollen with high viability throughout the year. Pollen preservation has particular relevance in crops which are not amenable to *in vitro* storage and/ or also produce difficult-to-store intermediate and recalcitrant seeds. In mango and litchi there are as yet no medium- or long-term conservation efforts to compliment the germplasm conservation other than that in field genebanks available in the country.

At present the recovery of plants from stored pollen is not directly possible and desirable genes contained in them can be recovered only through pollinations. However, storage of pollen assumes extra importance in view of reports of *in vitro* plant regeneration from cryostored pollen as reported in *Brassica napus* (1), androgenesis and plant regeneration from isolated microspores, as reported in *Coffea arabica* (5), and development of cryotechnology for anthers of *Ceratonia siliqua* (2). These studies used the pollen mostly at late uninucleate to early binucleate stages. In the light of these studies, hopefully, in the near future, it would be feasible to raise whole plants from cryostored pollen, stored at defined maturity stages, that would have direct relevance for the germplasm conservation of mango and litchi.

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REFERENCES

1. Chen JL & Beversdorf WD (1992) *Plant Cell Tissue and Organ Culture* **31**, 141-149.
2. Custodio L & Romano A (2006) in *Proc. Vth IS on* In Vitro *Culture and Hort. Breeding* (eds) MG Fari, I Hold & GyD Bisztray, Acta Hort **725**, ISHS 2006, pp 863-867.
3. Ganeshan S (1998) in *Tropical Fruits in Asia: Diversity, Maintenance, Conservation and Use* (eds) RK Arora & VR Rao, IPGRI-ICAR-UTFANET Regional Training Course on the Conservation and Use of Germplasm of Tropical Fruits in Asia, held at IIHR Bangalore, India, 18-31 May, 1997, International Plant Genetic Resources Institute, Office for South Asia, New Delhi, India, pp 120-126.
4. Ganeshan S (2003) in In Vitro *Conservation and Cryopreservation of Tropical Fruit Species* (eds) R Chaudhury, R Pandey, SK Malik & Bhag Mal, IPGRI, Office for South Asia, New Delhi, India/NBPGR, New Delhi, India, pp 215-227.
5. Herrera JC, Moreno LG, Acuna JR, De Pena M & Osorio D (2002) *Plant Cell Tissue and Organ Culture* **71**, 89-92.
6. Heslop-Harrison J & Heslop-Harrison Y (1970) *Stain Technol* **45**, 115-120.
7. Honda K, Watnabe H & Tsutsui K (2002) *Euphytica* **126**, 315-320.
8. Iwanami Y (1984) *Experientia* **40**, 568-569.
9. Iyer CPA & Degani C (1997) in *The Mango: Botany, Production and Uses* (ed) RE Litz, Commonwealth Agricultural Bureaux International, United Kingdom, pp 49-68.
10. Jain A & Shivanna KR (1988) *Ann Bot* **61**, 325-330.
11. Jain A & Shivanna KR (1990) in *Proc Intl Cong Plant Physiology* (eds) SK Sinha, PV Sane, SC Bhargava & PK Agarwal, Soc Plant Physiol Biochem, New Delhi, pp 1341-1349.
12. Mishra R (1984) in *Ph.D. thesis*, Delhi University, India.
13. Mishra R & Shivanna KR (1982) *Euphytica* **31**, 991-995.
14. Mukherjee SK (1997) in *The Mango: Botany, Production and Uses,* (ed) R Litz, Commonwealth Agricultural Bureaux International, United Kingdom, pp 1-19.
15. Pivovaro SZ (1974) in *M.Sc thesis*, Hebrew Univ. of Rehovat, pp 139.
16. Rai M, Nath V & Dey P (eds) (2001) *Litchi,* CHES, Plandu, Rajaulatu, Ranchi, Jharkhand, 100 pp.
17. Ram S & Rajan S (eds) (2003) *Status Report on Genetic Resource of Mango in Asia-Pacific Region*, International Plant Genetic Resources Institute, Office for South Asia, Pusa campus, New Delhi, India, 196 pp.
18. Ramanatha Rao V & Bhag Mal (eds) (2000) in *Tropical Fruit Species in India: Diversity and Conservation Strategies*, Presented at the International Symposium on Tropical and Subtropical Fruits, 26 Nov-1 Dec 2000, Cairns, Australia.
19. Shivanna KR (ed) (2003) *Pollen Biology and Biotechnology*, Oxford and IBH, New Delhi, 301 pp.
20. Shivanna KR & Heslop-Harrison J (1981) *Ann Bot* **47,** 759-770.
21. Shivanna KR & Rangaswamy NS (1992) *Pollen Biology: A Laboratory Manual*, Springer-Verlag, Berlin, 119 pp.
22. Shukla KS, Misra RL, Kaul MK & Prasad A (1978) *Haryana J Hort Sci* **7**, 162-164.
23. Singh HP (1998) in *Tropical Fruits in Asia, Diversity, Maintenance, Conservation and Use* (eds) RK Arora & V Ramanatha Rao, IPGRI, pp 185-195.
24. Singh RN, Sharma DK & Majumdar PK (1980) *Scientia Horticulturae* **12**, 299-301.
25. Singh SN & Singh SP (1952) *Journal of Agriculture and Animal Husbandry* **2**, 3-11.

26. Stanwood PC (1985) in *Cryopreservation of Plant Cells and Organs* (ed) KK Kartha, CRC Press, Boca Raton, Florida, pp 199-226.
27. Tandon R, Chaudhury R & Shivanna KR (2007) *Current Science* 92, 182-183.
28. Towill LE (2002) in *Biotechnology in Agriculture and Forestry Vol 50* (eds) LE Towill & YPS Bajaj, Cryopreservation of Plant Germplasm II. Springer- Verlag Berlin Heidelberg, pp 3-19.
29. Towill LE & Walters C (eds) (2000) in *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application,* Japan International Research Center for Agriculture Sciences, Tsukuba, Japan/IPGRI, Rome, Italy, pp 115-129.

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