EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 11
441-446
Year: 2017

CRYOPRESERVATION OF VANILLA POLLEN AND IT'S UTILIZATION IN INTER SPECIFIC HYBRIDIZATION BETWEEN *V. PLANIFOLIA* AND *V. APHYLLA*.

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Article Received on 05/09/2017 **Article Revised on 25/09/2017** **Article Accepted on 15/10/2017**

ABSTRACT
Vanilla from, *Vanilla planifolia* which is native to Central America, is an important spice and plays a major role in global economy. Improvement in *Vanilla planifolia*, is hampered by narrow gene pool in the crop. A few related species occur with useful characters like natural seed set and resistance to fungal diseases. Combining the available gene pool in the genus will help in broadening the genetic base and converging the useful genes into cultivated vanilla from wild species. Interspecific hybridization requires synchronized flowering between the species and or availability of viable pollen. Pollen collected from flowers showed maximum viability (89.4%) on the day of anthesis and that they were 80.1% viable almost 20h prior to anthesis too. Pollen from two asynchronously flowering species of *Vanilla viz*., cultivated *V. planifolia* and its wild relative *V. aphylla*, were cryopreserved after desiccation to % moisture, pretreated with cryoprotectant Dimethyl sulphoxide (5%) and cryopreserved -196ºC in Liquid Nitrogen. This cryopreserved pollen was latter thawed and tested for their viability both *in vitro* and *in vivo*. A germination percentage of 82.1% and 75.4% in *V. planifolia* and *V.aphylla* pollen respectively were observed indicating their viability. This cryopreserved pollen of *V. planifolia* was used successfully to pollinate *V.aphylla* flowers resulting in fruit set. the seeds thus obtaines were sussfully cultured to develop hybrid plantlets. This system is of great importance and can be used for conserving the haploid gene pool of *Vanilla* in cryobanks and their subsequent utility in crop improvement.

KEYWORDS: Cryopreservation, hybridization, germplasm conservation, pollen, *Vanilla aphylla, V.planifolia*.

INTRODUCTION
Vanilla is an important spice and plays a major role in the economy of countries like Madagascar, Mexico, Indonesia, Reunion Islands etc. where it is an important cash crop. The narrow primary gene pool of cultivated vanilla - *V. planifolia*, is threatened due to various factors making the secondary gene pool, i.e., the close relatives of *V. planifolia*, an important source of desirable traits like self-pollination, higher fruit set and disease resistance. Lack of variability is a major bottleneck in crop improvement programmes in vanilla. This can be broadened by combining the available gene pool in the genus *Vanilla* by interspecific hybridization. Different species of vanilla occur at different geographical regions and lack synchrony in flowering seasons, bringing about difficulties in movement of pollen to the receptor species to effect pollination between species. It is in such instances that development of methods for storage of viable pollen for longer periods, attains significance.

Pollen storage has been used in breeding of species with asynchronous flowering. Pollen banks, where pollen is cryopreserved, make pollen easily and readily available for any use.[1,2] Various methods for short term as well as long-term storage of pollen have been reviewed.[3,4,5] Ultra low temperatures can be used for preserving pollen in an unaltered condition with great potential and cryogenic procedures showed promises for long-term preservation of pollen viability. Pollen preservation, cryopreservation and procedures needed for developing pollen genebanks have been examined.[5] Observations on pollen viability of about 80 species stored at low humidity were reported in late 19th –early 20th centuries.[4] There are a large number of crop species, including vegetables, fibre and fruit crops, forages and cereals, for which pollen storage strategies are desirable.[6] Indian Institute of Horticultural Research, Bangalore has established a pollen cryobank which maintains more than 600 tropical pollen accessions collected over different seasons and years, kept under constant cryogenic condition. Cryostorage of pollen in various vegetable and ornamental species such as onion, tomato, *Capsicum* spp, *Solanum* species, rose, gladiolus etc has been reported.[7]

More than 80% of vanilla is produced outside its native area on islands in the Indian Ocean, mainly Indonesia and Madagascar and thus outside the geographical range (Neotropics) of its pollinators (large euglossine bees).[8] Flowering for an average plant in Puerto Rico begins in January, reaches a peak in March, and ends in June. In the Philippines, flowering extends from March to June, with the largest percentage of the flowers appearing in April.[9] In South India, flowering begins by end of December and extends upto March. Moreover, only one or rarely two flowers in a raceme opens in a day, each opening in the morning and closes by the same afternoon and which if not pollinated, is shed the next day. *Vanilla aphylla* flowers retained their freshness till the 2nd day. The entire flowering period of the raceme may last for an average of 24 days.

This study was initiated to develop a cryo-storage protocol for preservation of viable pollen in *Vanilla* species which originated in different geographical regions and habitats, viz., cultivated leafy species *V. planifolia* and wild leafless species, *V. aphylla*, tolerant to *Fusarium* infections[18] and utilizing the cryopreserved pollen in wide crosses.

MATERIALS AND METHODS

Pollen collection and *In vitro germination*

Pollen mass (pollinia) was collected on the day of flower opening, 1 and 2 days before opening of flowers in two species of V*anilla*, *V. planifolia* and *V. aphylla* (Fig 1a & b) and was used to identify the ideal stage for cryopreservation. The pollen germination was assessed by culture on Brewbaker and Kwack (BK) medium[10] and incubation at 25°C in dark. Different sucrose concentrations (5%, 10% and 15%) were tested and germination counts of pollen were made after 18 to 24 h. Pollen germination was considered to occur *in vitro* when a pollen tube had grown at a length twice the diameter of the pollen grain.

Cryostorage of pollen

Pollinia was collected from freshly opened flowers and pollen mass was detached, placed in petridishes and maintained at 25°C. The water content of the pollen was calculated by weighing immediately after collection and after dessication. Pollen mass was then subjected to different pretreatments, like desiccation in laminar air flow cabinet for 5 to 15 mins and desiccation combined with chemical cryoprotection with 5 and 10% of Dimethyl sulfoxide (DMSO). The pretreated pollen samples were transferred to cryovials and plunged into liquid nitrogen (LN2) for durations ranging from 1h to 1week. The cryopreserved pollen samples were thawed by the rapid thawing process by dipping the cryovial in 40°C water bath for 30 minutes after cryogenic storage.

Assessment of pollen viability and fertility

Viability of fresh and cryopreserved pollen samples was assessed by germination *in vitro*, using Brewbaker and Kwack medium and nuclear staining with 2% acetocarmine solution. The pollinial mass excised from flower buds and opened flowers were placed on glass slides. Drops of acetocarmine solution were applied on the pollinial mass, which was teased out. Counts of stained and unstained pollen, observed in ten fields, under a microscope, were made. For the *in vitro* germination test, the pollinial mass was cultured on BK medium. The culture was kept in laboratory conditions. At regular intervals, cultured pollinial mass was teased onto a glass slide, stained with acetocarmine solution and observed under a compound light microscope. Pollen germination counts were made in ten microscope fields.

Pollination with cryopreserved pollen

The fertility of cryopreserved pollen was also tested by controlled field pollinations. Flowers, of the desired female parent, were emasculated. Cryopreserved pollen after thawing was applied on the receptive stigma. Pollinated flowers and fruit set were marked. Fruit were harvested after 2-4 months and the seeds were cultured *in vitro* to assess the viability.

RESULTS AND DISCUSSION

Effect of day of collection and osmotic potential on pollen germination

Collection of pollen is a critical component for ensuring retention of viability. The germination capacity of the fresh pollen grains collected from flowers of three different maturity levels (on the day of flowering, 1 and 2 days before opening of flowers) from two species of Vanilla, *V.planifolia* (Fig. 1) and *V.aphylla* (Fig. 2) were assessed by *in vitro* germination in Brewbaker and Kwack medium. Various media have been reported, but the BK medium[10] formulation still is widely applicable for pollen germinability assays. When a pollen tube had grown more than the diameter of the pollen grain, germination counts were made and addition of sucrose to the medium was essential for pollen germination (Table 1). Pollen germination and growth were assessed by light microscopy. For statistical analysis, the treatment germination percentage was taken as the average of ten observations, each corresponding to a microscope field, with about fifty pollen.

The effects of osmotic potential and *in vitro* pollen germination, was studied (Table 1), by addition of sucrose and varying the range of sucrose concentrations (5%, 10% and 15%). In agreement with many earlier pollen studies[11], 10% sucrose provided the most appropriate osmotic potential for rapid pollen germination for both the species of *Vanilla*, yielding grains with well-rounded morphology and elongated tubes. Osmotic conditions above and below the optimum reduced germination percentages as well as retarded pollen-tube growth. Low osmotic potential, i.e. high water potential, led to shrunken pollen with denser protoplasm and more number of burst pollen. These effects have been reported to be due to entry of water in the pollen, causing endogenous soluble substances and ions to leak out, a phenomenon known as 'imbibition

damage'.[12] Media with highly increased osmotic potential seemed to induce slow water uptake by pollen and allowed better retention of pollen viability.[13]

Table 1. Effect of sucrose and stage of pollen on percentage pollen germination.

Sucrose concentration (%)	Percentage germination					
	V. planifolia			*V. aphylla*		
	Opened flower	1d prior to opening	2d prior to opening	Opened flower	1d prior to opening	2d prior to opening
0	6.0±2.31	5.4	0	7.1 ± 4.01	5.2	0
5	11.9±3.6	11.2	0	10.8 ±3.2	11.0	0
10	89.4±8.0	80.1	0.15	95.1± 2.0	92.2	0
15	73.6±5.91	73.0	0	40.5± 2.0	82.0	0

SE: 4.98; CV: 12.9, CD: 9.088

In both the species of *Vanilla*, 15% sucrose led to a significant reduction in germination, hence 10% seemed ideal in this study. Pollen collected from opened flowers showed maximum percentage of germination (89.4%) and there was no significant difference in germination of pollen collected from flowers one day prior to opening (80.1%). There is a drastic reduction or no in germination of pollen collected from flowers 2 days prior to opening (Table 1) and hence collection of pollen 2 d prior to opening was not continued. Hence only pollen collected from freshly opened flowers was used in all cryopreservation experiments. However observations indicate that flower buds, 1 day prior to opening, could be utilized when two species flowered in different locations and the buds could be transported within 24 hours, to the receptor species to effect fertilization. The flower opens early in the morning and closes in the afternoon, never to re-open again but a viability of 29.59% was also observed in pollen collected from flowers of *V.planifolia* that were left unpollinated and cultured the next day.

Effect of dessication and cryoprotection on germination of cryopreserved pollen

In cryobiology the nature of water's transformation from a liquid solvent to a solid structure during freezing decides the ability to preserve or destroy, thus degree of humidity critically affects viability during storage. Metabolic processes are retarded and respiration is reduced as a consequence of low water content in mature pollen. In this study, simple procedures *viz.*, a dehydration step to avoid intracellular ice-crystal formation and a treatment with cryoprotectant, Dimethylsulfoxide (DMSO), to limit damage during freezing and thawing, were used. Pollen grains were desiccated for different durations in laminar air flow cabinet and desiccation combined with cryoprotection with 5 and 10% of Dimethyl sulfoxide (DMSO). These pretreated pollen samples were transferred to cryovials and were directly plunged into liquid nitrogen for different durations. The cryopreserved pollen samples were thawed by the rapid thawing process by dipping the cryo vial in 40^{o}C water bath for 30 minutes after cryogenic storage. Viability of fresh and cryopreserved pollen samples was assessed using acetocarmine staining and also based on *in vitro* germination. In all treatments the pollen samples survived freezing. The survival rate ranged from 0.65 – 75.42% in *V.aphylla* to 2.38 – 86.5% in *V. planifolia.* The percentage of normal pollen germination in treatments without cryopreservation was 91.4% in *V. planifolia* and 74.01% in *V. aphylla.* Dessication has major role in survival of vanilla pollen and dessication for 5 minutes was most favourable with 73.4% and 75% pollen viability in *V.planifolia* and *V.aphylla* respectively. Significant reduction in pollen germinability was observed on increasing the dessication period, as with germination after freezing. But in both these, 5 minutes dessication, increased the germination (Fig. 4). In the present study cryoprotection with DMSO did not significantly enhance the viability of pollen. However the maximum percentage of germination was observed in pollen desiccated for 5 minutes in the air current of laminar air flow followed by desiccation for 5 minutes and cryoprotection with 5% DMSO. The pollen germination after this treatment was 73.4% and 86.5 % respectively in *V. planifolia* (Fig. 6) and 75% and 75.42% in *V.aphylla* (Fig. 5) which is almost comparable to the control (Table 2). Several abnormalities occurred during pollen growth such as increase in callose deposits resulting in bulging (Fig. 7) and bursting of pollen tube tip, probably due to the shift of pollen tube from autotrophic to heterotrophic phase, whereby nutrient uptake from the culture medium is less and such abnormalities were mostly observed after 20 hours. Branching of pollen tube (Fig. 8), feeding bottle type of pollen tube development etc were also observed. However only 9.5% of the pollen tubes exhibited such abnormalities hence, they may not affect effective hybridizations.

Table 2. Pollen viability in vanilla after cryopreservation as indexed by *in vitro* germination after 20 hours.

Sample	*V. planifolia*		*V. aphylla*	
	Germination (%)	No. of pollen observed	Germination (%)	No. of pollen observed
1. Fresh pollen	91.67	120	84.44	45
Cryopreserved pollen				
2. 5 m dessication + LN2	73.40	94	75.00	132
3. 15 m desiccation + LN2	48.00	75	35.00	140
4. 5% DMSO + LN2	2.38	126	0.65	153
5. 5m desiccation + 5% DMSO + LN2	86.50	89	75.42	118
6. 10m desiccation + 5% DMSO + LN2	38.20	68	53.33	90

Plant material can be stored without alteration for a theoretically unlimited period of time, with no modifications observed at phenotype, biochemical, chromosomal, or molecular levels which could be attributed to cryopreservation.[14,15] The viability of pollen at room temperature lowers and disappears in few days but on the contrary cryopreservation lengthens the pollen viability. In *Citrus*, the pollen of 4 cultivars of *C. limon* has been successfully stored in LN for up to 3 and a half years. Pollen storage of 12 genotypes of the annual soybean and its wild perennial relatives without pre-desiccation at low temperatures (-20ºC and -196ºC) led to retention of pollen viability of annual soybean for 4 months, but pollen of its wild perennial relatives at same storage conditions failed to germinate *in vitro*.[16] Pollen of *Pinus brutia, P. canariensis, P. halepensis, P. pinaster* has been stored at -20^{0}C for one year with the aim of checking the variation of viability. The germination power decreases in *Pinus canariensis* and *P. pinaster*, while in *Pinus brutia* and *P. halepensis* it is increased by low temperature.[17]

Pollination and fruit development with cryopreserved pollen

The i*n vivo* function is the ultimate test of viability hence field fertility trials were conducted. Earlier reports of successful interspecific hybridization between *V.planifolia* and *V.aphylla* and generation of interspecific progenies are available.[18] The interspecific nature of the hybrids have been characterized using RAPD and AFLP profiles, confirming the contribution of male and female parents.[18]

Pollen germination on the stigma and growth in the style was also used as viability test and observed using staining techniques. The pollen germinated on the stigma (Fig. 7) and fruits were developed (Fig. 10). The seeds were collected and were cultured on routine seed germination medium.[18] The seeds germinated (Fig. 11) into plantlets confirming the retention of pollen fertility after cryogenic storage and successful interspecific hybridization in vanilla using cryopreserved pollen. The ability of cryopreserved pollen to effect fertilization did not appear to be adversely affected by cryopreservation as is evident from the fruit set and seed formation among the crosses made using cryostored pollen.

Interspecific hydrization studies have earlier confirmed that pollen storage at ultra-low temperature did not affect haploid production frequency in pearl millet crosses, but greatly reduced the frequency in maize crosses, probably due to the variations in requirements of optimum range of pollen water content for the pollen viability after drying and freezing. Pearl millet pollen is relatively tolerant to drying and freezing, in contrast with maize pollen, which has a narrow water content range for maintaining viability during drying and freezing.[19,20]

Viability and fertility assessment of cryopreserved pollen from *Vanilla* species thus shows that it is possible to use cryogenic methods for conservation and management of the haploid gene pool in this species, facilitating crosses in breeding programmes, distribution and exchange of germplasm and preserving nuclear genes of germplasm. Reversible-inhibition effect of pollen germination has been reported for possible employment in gametophytic selection by exposing pollen grains to a specific compound included in the inhibition medium. After the inhibition period, the resistant grains would germinate faster and/or longer than sensitive ones, favouring the transmission of desirable genes and introgression of characters in subsequent *in vivo* pollination of desired crosses.[21] Further investigations to test these inferences for possible application in vanilla improvement are needed.

This study demonstrates a viable protocol for cryopreservation of pollen for two species of *Vanilla* with high post thaw recovery (75.4 - 82.1%), pollen fertility and successful interspecific hybridisation, by simple manipulation of *in vitro* water uptake by the mature gametophyte effecting to alter the physiology of pollen germination., and the methodology can be implemented as a routine biostorage method to face the problem of asynchronous flowering and several aspects vanilla breeding.

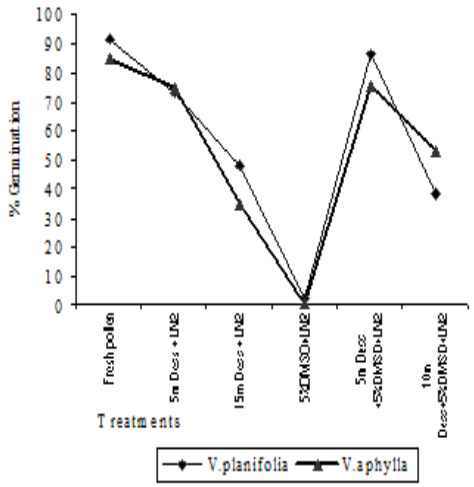

Fig.3. Effect of different treatments on germination of *Vanilla* pollen *in vitro*

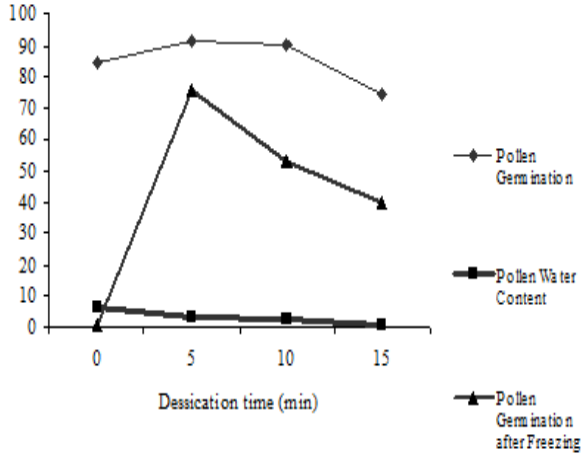

Fig. 4 Effect of pollen water content and freezing on pollen viability

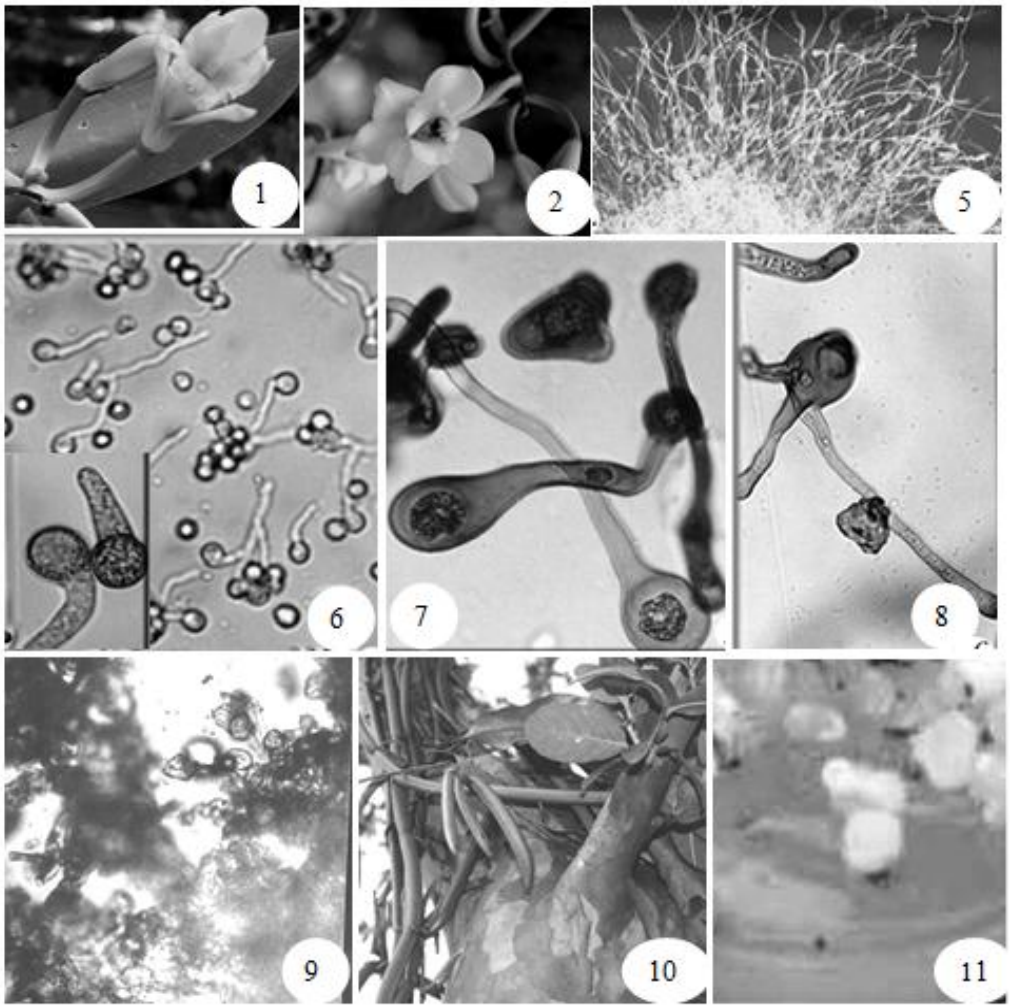

Fig. 1. Flower of *V.planifolia*; 2. Flower of *V.aphylla*; 5. Micrograph of fully elongated pollen tube in *V.aphylla*; 6. Micrograph of pollen germination in *V.planifolia*; 7 Abnormalities in pollen tube growth – tip bulging, 8. Branching of pollen tube; 9 *In vivo* germination of pollen on stigmatic surface; 10. Fruit set after pollination with cryopreserved pollen; 11. Germination of seeds, *in vitro*.

REFERENCES

1. Schoenike RE & Bey CF (1981) Conserving genes through pollen storage. in *Pollen Management Handbook* (ed) Franklin EC, U.S. Dept. of Agriculture Handbook, No 587, Washington D.C. pp. 72-73.
2. Bajaj YPS (1987) Cryopreservation of pollen and pollen embryos and the establishment of pollen banks. *International Review of Cytology*, 107: 397-420.
3. Knowlton EH (1922) Studies in pollen with special reference to longevity. Cornell Univ. *Agric. Exp. Stat. Memoir*. 52: 747–794.
4. Stanley RG & Linskens HF (1974) *Pollen: Biology, Biochemistry and Management*. Berlin, Springer–Verlag.
5. Towill LE & Walters C (2000) Cryopreservation of pollen. In. (eds.) Engelmann F & Takagi H, *Cryopreservation of tropical plant germplasm–Current research progress and application*. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan. pp. 115–129.
6. Barnabas B & Kovacs G (1997) Storage of pollen. In. (eds.) Shivanna KR & Sawhney VK. *Pollen biotechnology for crop production and improvement*. Cambridge University Press. pp. 293–314.
7. Rajasekharan PE & Ganeshan S (2001) Pollen cryopreservation of vegetable and ornamental species: Retrospects and prospects. *Indian J. Plant Genet. Resources*, 14(2): 294–295.
8. FAOSTAT (1998) Statistics database of the FAO. *Acta Hort*. 437: 309–324.
9. David. P. A. 1953. Pollination of vanilla flowers by hand. Philippine Agr. 37: 301-305.
10. Brewbaker JL & Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany*. 50: 859-865.
11. Tupy J, Hrabètová E & Càpková V (1983) Amino acids and bivalent cations in the growth of tobacco pollen in mass culture. *Plant Sci. Lett*. 30: 91-98.
12. Crowe JH, Hoekstra FA & Crowe LM (1989) Membrane phase transitions are responsible for imbibitional damage in dry pollen. Proc. Natl. Acad. Sci. USA, 86: 520-523.
13. Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. *Int. Rev. Cytol*. 107: 01-78.
14. Kartha K (ed.) (1985) Cryopreservation of Plant Cells and Organs. CRC Boca Raton, FL.
15. Engelman F (1997) *In vitro* conservation methods. In (eds.) Ford-Llyod BV, Newburry JH & Callow JA Biotechnology and plant genetic resource conservation and use. Wallingford CABI. 119-162.
16. Tyagi RK & Hymowitz T (2003) Pollen from *Glycine* species survive cryogenic exposure *Cryo Letters*, 24: 119-124.
17. Braggio MG, Cornara L & Dellacha E (1990) Effect of cryopreservation on pollen viability. *Boll Soc Ital Biol Sper*. 66(9): 821-827.
18. Minoo D (2002) Seedling and somaclonal variation and their characterization in Vanilla. Ph.D Thesis. University of Calicut, Kerala, India.
19. Inagaki MN & Mujeeb-Kazi A (1996) Production of polyhaploids of hexaploid wheat using stored pearl millet pollen. In *Proceedings of 5th International Wheat Conference*, Ankara, Turkey. 319-325.
20. Inagaki MN & Mujeeb-Kazi A (1994) Storage of maize pollen for use in haploid production of hexaploid wheat. *Breeding Science*, 44: 387-390.
21. Loguercio LL (2002) Pollen treatment in high osmotic potential: a simple tool for *in vitro* preservation and manipulation of viability in gametophytic populations. *Braz. J. Plant Physiol*, 14(1): 65-70.