Potato J (2019) 46 (1): 39-47

PRELIMINARY INVESTIGATIONS FOR EXPLORING SCOPE OF POLLEN CRYOPRESERVATION FOR CONSERVATION OF POTATO GENETIC RESOURCES

RP Kaur[1], Vanishree Girimalla[2], Vinod Kumar[2], Vinay Bhardwaj[2]

ABSTRACT: Pollen grains are a rich source of diverse alleles in any genepool and represent large genetic diversity in small sample size. Inherent hardiness and stability under harsh conditions make them an effective propagule for germplasm conservation using cryogenic storage. Cryopreservation was used to conserve pollen of six potato varieties namely Kufri Girdhari, Kufri Himalini, Kufri Himsona, Kufri Jyoti, Kufri Kanchan and Kufri Shailja at ICAR-Central Potato Research Institute, Shimla. Pollen collected were packed in gelusil capsules, kept in cryovials and immersed in liquid nitrogen. A single lot was tested after 24 hrs of cryopreservation using pollen germination technique and double staining with Alexanders stain, having malachite green for pollen cell wall staining (indigo-blue colour) and acid fuschine for pollen protoplasm (purple). Under similar conditions, the germination response of pollen of fresh and cryopreserved varieties was statistically at par, with heat-killed pollen as a negative control showing nil germination. The germination response among varieties varied from 4.6% (Kufri Jyoti) to 24.6% (Kufri Himalini). Low germination response was mainly attributable to pollen grains showing no protoplasm and having only indigo-blue staining walls, which may reportedly be due to abnormal meiotic segregation producing non-viable pollen in potato. The pollen tube lengths varied from 10 µm to 500 µm after 3 hours of incubation in medium, indicative of differences in pollen vigour.

KEYWORDS: Cryopreservation, germplasm conservation, pollen, potato genetic resources

INTRODUCTION

Potato has emerged as one of the most important food crops in the 21st century, with its consumption in almost all parts of the world. Potato genetic resources are the key to further improvement in the crop, which face losses due to several biotic and abiotic stresses. With 7 cultivated, 228 wild identified species and more than 4,500 varieties of *Solanum tuberosum* ssp. *tuberosum*, it has been reported to have the richest genetic diversity (FAO, 2007; Hils and Pieterse, 2009). Potato genetic resources are conserved as tubers *(ex situ* and *in situ*), as *in vitro* plants and as shoot tips under long-term cryopreservation. However, *in situ* conservation has a limited scope for countries where potato does not grow naturally whereas *ex situ* conservation strategies are prone to issues related to disease and other stresses. *In vitro* conservation of genetic resources under slow growth and cryopreservation of shoot tips is a tedious process and marred by low regeneration rates, subject to genetic instability, and depicting varietal differences which limit their utilization in potato improvement programmes (Kaczmarczyk *et al*., 2011).

Another option readily available for conservation of genetic resources is the cryopreservation of pollen grains at -196°C in liquid nitrogen (LN). Pollen grains are known to be a rich source of diverse alleles in any gene-pool, holding enormous genetic diversity in a small sample size and simultaneously offering an effective propagule for germplasm conservation. Viability is preserved at cryogenic temperatures as metabolism is completely blocked. More so, pollen can be directly utilized in any breeding program,

[1]ICAR-Central Potato Research Station, Jalandhar - 144 003, Punjab, India
[2]ICAR-Central Potato Research Institute, Shimla - 171 001, HP, India
Email: kaur.rp@gmail.com

along with ease of transport, germplasm exchange (owing to their small size and non-transmission of viruses except PSTVd), with no requirement of male parents to be grown at the site of hybridization. Although pollen do not offer true-to-type plants but only the alleles, these offer the benefit of their direct channelization into breeding programmes, without any regeneration to unveil the genetic potential. Pollen cryopreservation is one of the simplest methods to conserve genetic resources. The procedure is simple as compared to those of cells and tissues due to the inherent hardiness of pollen grains. Further, scope of development of diploid plants by regeneration of pollen from cultivated tetraploid *Solanum tuberosum* may be explored. Successful pollen cryopreservation has been reported in several crop species like brinjal, tomato, potato and several other horticultural crops (Choudhary *et al.*, 2010; Karipidis and Douma, 2011). Retention of pollen viability after cryopreservation is of utmost importance and is dependent on the pollen handling procedure. It can be tested by several procedures of which pollen germination and hybridization are most important. Double staining of *in vitro* germinated pollen grains using Alexander's stain (Alexander, 1969) is the most widely used and reliable method for ascertaining pollen viability. The present study was done to cryopreserve 6 varieties of potato, in order to undertake cryobanking of pollen of Indian potato genetic resources.

MATERIALS AND METHODS

Pollen Collection and Handling

Flowers of six potato varieties namely Kufri Girdhari, Kufri Himalini, Kufri Himsona, Kufri Kanchan, Kufri Jyoti and Kufri Shailja were collected from the seed production plots of ICAR-Central Potato Research Station, Kufri, Himachal Pradesh, India in July, 2015. The pollen were collected from about-to-open flower buds and freshly opened flowers. The anthers of the potato flowers were plucked off with forceps and spread on a butter paper, and kept for drying under 40 W bulbs for 1-2 h and subsequently overnight (16 h) in a well-ventilated room at 22-25°C to allow shedding of pollen from anthers. Subsequently, they were transferred to nylon sieves and shed pollen were separated out by vigorous shaking onto a butter paper. These were packed into gelusil capsules, inserted into cryovials and plunged into LN in different lots. Each lot or replicate comprised of one pollen capsule of each variety to be withdrawn at separate point of time for testing/ utilization etc. A single lot was withdrawn after 24 h to ascertain pollen viability by pollen germination technique and hybridization. The lot was kept at room temperature for 1 h before testing for viability.

Pollen Germination Test for Pollen Viability

The viability of the pollen grains was tested by pollen germination in Brewbaker and Kwack medium (Brewbacker and Kwack, 1963) in 2.0 ml Eppendorf tubes. The sugar concentration, pH of the medium and duration of incubation, for Indian potato varieties, were followed as standardized. Approximately 10 mg pollen grain at the tip of a narrow spatula were incubated in the 200 µl germination medium. After 3 h of incubation at ambient temperature, 20 µl of the germination medium with germinated pollen grains was transferred to a glass slide with a cut microtip, to prevent damage to the growing pollen tubes and stained with Alexander's staining solution (5 µl). Pollen germination in cryopreserved pollen along with the controls was observed under a light microscope. Three slides of each composite sample were considered with more than

100 pollen grains per view. Viable pollen grains were visible with emerging bright purple protuberance from the pollen grains. Pollen grains having pollen tube length equal to or greater than the pollen diameter were considered as viable/ germinated. The length of pollen tubes were also measured from 10 random pollen tubes per slide in three replicates. The germination percentage of pollen grains for different treatments were statistically analyzed using analysis of variance technique using Sigma Plot 12.0 and treatment least square means obtained were compared using least significant difference (LSD) at 5% level of probability. Suitable positive and negative controls as freshly collected pollen and heat-killed pollen, respectively were used to draw suitable conclusions.

Hybridization

The cryopreserved pollen, as well as the pollen of the controls (fresh and heat-killed pollen) were used in carrying out hybridization with compatible parents to ascertain viability. The hybridization work was carried out at the ICAR-Central Potato Research Station, Kufri, Shimla, HP, India. Flowers of potato varieties namely Kufri Swarna, Kufri Lalima, Kufri Pukhraj, Kufri Bahar and Kufri Lauvkar were emasculated carefully on the first day. The pollen of each of the varieties, fresh and cryopreserved, were carefully applied to the emasculated female parents of the varieties in the next morning. A total of 16-30 flowers on 3-6 bunches per female parent in each of the treatments were pollinated. Successful berry formation and number of seeds formed on the hybridized bunches were recorded.

RESULTS AND DISCUSSION

The different varieties exhibited varying degree of pollen shed, with Kufri Himalini, Kufri Himsona and Kufri Girdhari showing ample pollen production, and Kufri Kanchan, Kufri Shailja and Kufri Jyoti producing very limited amount of pollen and therefore requiring larger number of flowers for ample pollen collection. The methodology of sieve filtration followed in the experiment was used to successfully get larger amount of pollen from the flowers, for use in cryopreservation. The pollen were collected in bulk from flowers in different stages of development ranging from about-to-open buds to freshly opened flowers (**Fig. 1a**). The pollen morphology observed under the microscope revealed an oval tetra-colpate structure of approximately 23 µm diameter (**Fig. 1b**) and mono-siphonic to rarely tetra-siphonic germination types (**Fig. 1c, 1d**). Abnormal pollen germination with two pollen having a common pollen tube were also observed (**Fig. 1e**). Meiotic abnormalities are a common feature in *Solanum tuberosum* due to its allotetraploid nature where both mono-somic and di-somic segregations have been reported (Jansky, 2006). Floral abnormalities were also observed with partly fused anthers and petals especially in variety Kufri Himalini and Kufri Himsona. Deformation in potato flowers attributable to cytoplasmic factors has also been reported.

Pollen handling is one of the major causes affecting the viability of pollen along with stage of pollen development, environmental conditions (day length and temperature) and the genotype (with respect to male sterility and compatible pollen-stigma interaction. Plump anthers collected from freshly-opened flowers yielded pollen with better viability and vigour in terms of longer pollen tube growth as compared to pollen collected from 1-2 days open flowers. Potato pollen are bi-nucleate at the time of anthesis and have been reported to be rather susceptible to temperature stress, exposure of pollen of

Fig. 1. a: Dehiscing anthers release pollen for collection; b: Tetra-copate pollen grain; c: di-siphonic pollen grain; d: Tetra-siphonic pollen grain; e: abnormally fused pollen tubes arising out of two different pollen grains; f: in vivo pollen germination on compatible stigma.

different potato varieties to 30°C for up to 30 min has been reported to strongly decrease pollen germination by 50% resulting in lesser seed set. Further its viability is lost in 4-6 days at room temperatures, 5-8 weeks at 2-4°C and in 7-8 months in a freezer (-10°C) (Luthra *et al*., 2006). Reduction in potato pollen viability over time lasting upto 10-14 days at 15-20% RH has been reported. Pollen desiccation has also been reported to affect pollen cryopreservation which showed non-significant effects on viability in our previous study. It is, therefore, inferred that probably the overnight drying at 22-25°C involving pre-drying under 40 W bulbs in a well-ventilated room for shedding of pollen provided ample desiccation for effective cryopreservation. Longer exposure of pollen to 40 W light bulbs or keeping at 22-25°C for more than 16 h drastically reduced the pollen viability and pollen tubes were observed to burst when incubated in pollen germination medium. Efforts were directed to keep the anthers at 22-25°C for a minimum period of time, so as to retain maximum viability.

In vivo study of pollen germination on compatible stigma revealed that pollen grains germinate within the first half-an-hour of hybridization (**Fig. 1f**), however fertilization has been reported to take up to 36 h (Heslop-Harrison, 1992) and berry formation up to 6 wks. *In vitro* germination of viable pollen occur within 30 mins of incubation, however optimum results were obtained at 3 h of incubation, when all viable pollen germinated. Microscopic study of *in vitro* germinated pollen revealed that the pollen grains could be differentiated on the basis of their shape, staining variation

and their germination ability into a) viable pollen with germinating pollen tubes of variable sizes, b) putative viable pollen showing no germination and c) non-viable pollen showing only stained pollen walls and somewhat shriveled shape (**Fig. 2**). These three observable pollen classes were calculated on the basis of visual evaluation using Image J and expressed in the percentage composition of each class out of total over replicate observations taken (**Fig. 3**).

The ability to differentiate viable germinating pollen from putative viable non-germinating pollen is a major merit of

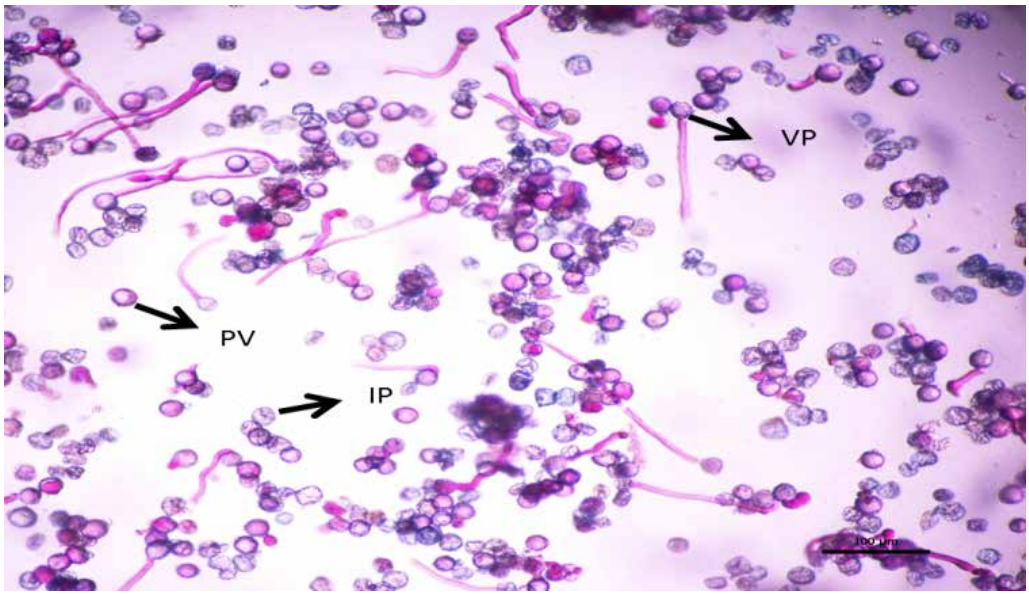

Fig. 2. Pollen types in potato pollen showing the different classes of pollen observed where VP is viable pollen, PV is putative viable pollen and IP is inviable pollen.

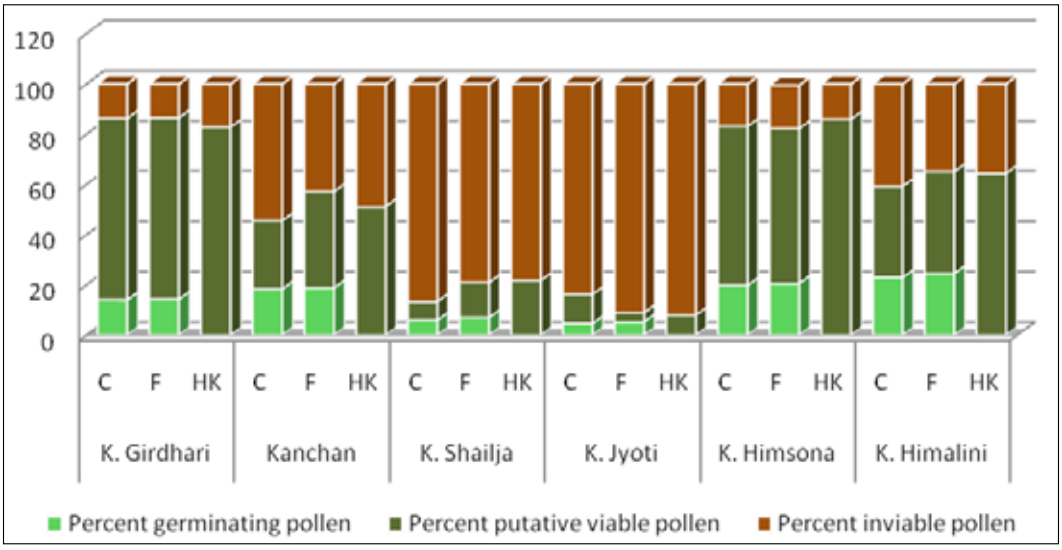

Fig. 3. Percentage pollen types observed in different varieties and pollen status (germinating pollen, putative viable pollen and in-viable pollen), where C is cryopreserved, F is fresh anf HK is heat killed pollen considered in the study.

using *in vitro* pollen germination method over conventionally used pollen staining method for viability testing of pollen grains, which has been reported to inflate the viability results of potato genotypes (Tomé *et al*., 2007). The occurrence of putative viable pollen in our experiment is indicative of the same observation where germinating pollen grains in the medium were observed to be less than the total number of viable pollen observed on the basis of staining in all the varieties. This observation is in agreement to the results reported by Choudhary *et al.* (2010) and Tomé *et al*. (2007) where more reliable pollen viability results were obtained from pollen germination. The occurrence of non-viable viable pollen can be explained on the basis of male sterility and abnormal meiotic segregation in potato.

The genotypic variation in different pollen types among the six varieties was evident. The proportion of the viable germinating pollen varied in varieties from 4.6% in Kufri Jyoti to 24.6% in Kufri Himalini. The highest number of non-viable pollen were observed in Kufri Jyoti (91%) followed by Kufri Shailja (79%). Similarly, maximum putative viable pollen were observed in Kufri Girdhari followed by Kufri Himsona, this class of pollen contributes to non-viability in pollen as a part of male sterility as no pollen tube is observed to originate even after 6 h of culture in germination medium. Occurrence of such varietal differences for pollen viability within species have also been reported by in other crops like *Glycine* coconut *Rosa* Mango, Litchi, Vitis, Dendrobium. Similarly, variations in pollen production and viability rates between the clones of wild and cultivated species of potato have also been reported by Tomé *et al*., 2007. These variations impair the choice of male parents in breeding studies, where parents are generally characterized on their fertility as male and female. Male sterility in tuber-bearing cultivated potato varieties is as an important reproductive barrier guaranteeing species integrity and associated with nuclear-cytoplasm interactions (Camadro *et al*., 2004).

The length of the pollen tube after 3 h of incubation in germination medium ranged from just emerged to 500 µm which was quite extraneous to be included in the study. Since, flowers were collected randomly from similar stage of development (about-to-open and freshly opened) they are assumed to be representative of the genotype holding viable pollen. Therefore, differences in pollen tube growth and observation of putative viable pollen signify differences in the vigour of the pollen grains, attributable to physiological and genetic causes.

Comparison between cryopreserved vs. heat killed and fresh vs. heat killed pollen grains for pollen germination showed significant differences, whereas fresh vs. cryopreserved pollen showed non-significant differences (**Table 1)**. This implies successful cryopreservation of pollen where viability is retained after 24- hrs of cryopreservation and suggestive of feasibility of long-term cryopreservation of pollen as pollen once cryopreserved at temperatures below -160°C would theoretically have infinite periods of longevity (Mazur, 1984 and Towill, 1985). Stable cryopreservation of pollen without loss of viability have been reported in tomato, *Vitis*, *Glycine*, Dendrobium and potato. Improved fertility after cryogenic treatment to pollen have been reported by Karun *et al.* (2014).

The results of the hybridization carried out using cryopreserved pollen as well as the control have been depicted in **Table 2**. The cryopreserved pollen was observed to effect successful berry formation in compatible crosses when crossed with different female

Table 1. Least square means for pollen germination, percent putative viable pollen and percent inviable pollen showing no germination over pollen status (cryopreserved, fresh and heat-killed); varieties and their interaction effects. Standard errors have been given.

Pollen status × Variety	Percent Pollen germinated	Percent putative viable pollen grains showing no germination	Percent inviable pollen grains
Cryopreserved × K. Girdhari	13.941	72.371	13.688
Cryopreserved × K. Kanchan	18.348	27.278	54.374
Cryopreserved × K. Shailja	5.912	7.383	86.705
Cryopreserved × K. Jyoti	4.601	11.621	83.778
Cryopreserved × K. Himsona	19.774	63.418	16.808
Cryopreserved × K. Himalini	22.963	36.281	40.759
Fresh × K. Girdhari	14.213	72.294	13.493
Fresh × K. Kanchan	18.8	38.315	42.885
Fresh × K. Shailja	6.97	13.936	79.094
Fresh × K. Jyoti	5.279	3.719	91.002
Fresh × K. Himsona	20.58	61.7	17.22
Fresh × K. Himalini	24.605	40.513	34.882
Heat Killed × K. Girdhari	0	82.84	17.16
Heat Killed × K. Kanchan	0	51.115	48.885
Heat Killed × K. Shailja	0	21.756	78.244
Heat Killed × K. Jyoti	0	7.995	92.005
Heat Killed × K. Himsona	0	85.987	14.013
Heat Killed × K. Himalini	0	64.367	35.633
Standard Error of LS Mean	0.626	3.327	3.32
LSD (0.05)	2.539	9.434	9.415
Cryopreserved**	14.256	36.392	49.352
Fresh**	15.074	38.413	46.429
Heat Killed**	0	52.344	47.657
Standard Error of LS Mean	0.361	1.358	1.356
LSD (0.05)	1.037	3.852	3.843

*Excluded in analysis
**depicts mean over all varieties in treatment

parents including Kufri Lauvkar, Kufri Swarna, Kufri Pukhraj, Kufri Lalima and Kufri Bahar. Heat killed pollen was not used as it was physiologically non-viable as observed in the pollen germination tests. The cryopreserved pollen was observed to yield berries successfully which conclusively proves its viability. Kufri Jyoti and Kufri Shailja yielded lesser number of berries evident from *in vitro* pollen germination tests. However, not all pollination conducted in hybridization were successful and berries were formed in approximately 8% of the hybridization carried out in both the treatments, which may be attributed to environment and human error. Successful formation of berries and seed-set for each variety in fresh and cryopreserved pollen confirms the viability of the pollen grains. The number of seeds set is a direct indicator of number of successful fertilization events that occurred during hybridization. Viability of cryopreserved pollen through

Table 2. Pollen hybridization using fresh and cryopreserved pollen; bold numerals depict number of berries formed on number of pollinated flowers (unbold numerals) and number of seeds formed in parenthesis.

	Pollen parents											
Female parents	KHL (F)	KHS (F)	KG (F)	KSH (F)	KJ (F)	KK (F)	KHL (C)	KHS (C)	KG (C)	KSH (C)	KJ (C)	KK (C)
Kufri Lauvkar	17/2 (300)	21/1 (210)	23/3 (600)	22/3 (560)	31/4 (970)	21/2 (340)	20/1 (170)	20/2 (430)	22/2 (450)	19/0 (0)	23/1 (200)	25/3 (720)
Kufri Swarna	32/3 (450)	22/5 (1020)	26/4 (860)	21/2 (580)	26/2 (450)	18/2 (440)	18/3 (730)	23/3 (650)	26/2 (360)	17/2 (510)	22/2 (500)	23/2 (520)
Kufri Pukhraj	27/4 (750)	31/4 (670)	22/2 (550)	18/1 (220)	16/0 (0)	17/3 (630)	21/2 (410)	19/0 (0)	17/1 (180)	15/1 (240)	21/3 (620)	28/2 (430)
Kufri Lalima	16/2 (400)	22/1 (160)	20/2 (600)	21/2 (710)	18/3 (650)	22/5 (850)	31/1 (230)	25/2 (350)	20/3 (730)	14/1 (240)	31/1 (250)	21/2 (380)
Kufri Bahar	24/2 (420)	25/2 (580)	16/1 (300)	17/1 (180)	12/0 (0)	21/2 (310)	22/3 (580)	21/0 (0)	28/1 (150)	28/2 (300)	32/2 (510)	27/4 (1110)
Total	116/13 (2,320)	121/13 (2640)	107/12 (2910)	99/9 (2,250)	103/9 (2070)	99/14 (2570)	112/10 (2120)	108/7 (1430)	113/9 (1,870)	93/6 (1,290)	129/9 (2,080)	124/13 (3,160)
			546/45 (12,190)						679/54 (11,950)			

Where KHL, KHS, KG, KSH, KJ and KK are varieties Kufri Himalini, Kufri Himsona, Kufri Girdhari, Kufri Shailja, Kufri Jyoti and Kufri Kanchan
F and C depict pollen states fresh and cryopreserved

successful fertilization was ascertained in Rose, Glycine, Mango, Litchi and Vitis.

CONCLUSIONS

The study indicates the successful cryopreservation of pollen grains of Indian potato varieties which may be suitably extended to other potato genetic resources. With limited reports of pollen cryopreservation of potato genetic resources, there is much scope of application of this technique in varied areas of potato improvement from genetic resources conservation, germplasm exchange to supplementing of breeding programs. The viability results in our study were suitably confirmed using hybridization confirming its fertility and assuring its prognostic long-term storage. Genotypic effects related to male sterility, amounts of pollen production in a variety/ accession and their viability were also observed in the present study during pollen extraction and as varietal differences for viability. These parameters strongly hinder the optimum utilization of this important resource for the conservation of genetic resources. The pollen extraction and handling are the most crucial factors determining the quality of pollen in cryopreservation. Storage of good quality pollen with high viability yields ensures retrieval of the same higher quality from cryogenic storage and effects effective subsequent use. In view, of this future research in the area may be targeted to achieving more efficient pollen handling protocols whereby pollen may be extract at the earliest fertile development stage before its cryopreservation for retaining maximum viability and fertility.

LITERATURE CITED

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. *Stain Tech* **44**: 117-22.

Bajaj YPS (1987) Cryopreservation of Pollen and Pollen Embryos, and the Establishment of Pollen Banks. *Intl Review Cyt* **107**: 397-20.

Brewbacker JL and BH Kwack (1963) The essential role of calcium ion in pollen germination and pollen tube growth. *Amer J Bot* **50**: 859.

Camadro EL, Carputo D, Peloquin SJ (2004) Substitutes for genome differentiation in tuber-bearing Solanum: interspecific pollen-pistil incompatibility,

nuclear-cytoplasmic male sterility, and endosperm. *Theor Appl Genet* **109**: 1369–76.

FAO (2007) The Potato. http://www.potato2008.org/en/potato/.

Ganeshan S, PE Rajasekharan, S Shashikumar and W Decruze (2008) Cryopreservation of Pollen. In: Reed BM (eds.) *Plant Cryopreservation: A Practical Guide*. Springer, New York: 443-464p.

Heslop-Harrison J (1992) Cytological Techniques to assess Pollen Quality. In: Cresti M, Tiezzi A (eds.) *Sexual Plant Reproduction*. Springer, Berlin Heidelberg: 41-48p.

Hills U and L Pieterse (2009) World catalogue of potato varieties 2009/10. *Agrimedia*, Clenze: 323p.

Howard HW (1958) The storage of potato pollen. *Amer Potato J* **35**: 676-78.

Jansky S (2006) Overcoming hybridization barriers in potato. *Plant Breed* **125**: 1-12.

Kaczmarczyk A, VM Rokka and ERJ Keller (2011) Potato Shoot Tip Cryopreservation. A Review. *Potato Res* **54**: 45-79.

Karipidis CH and D Douma (2011) Tomato Pollen Storage at Freeze and Cryogenic Temperature – Effects on Viability and Fecundity. *Acta Hort* **908**: 257-63.

Luthra SK, SK Pandey, BP Singh, GS Kang, SV Singh and PC Pande (2006) Potato Breeding in India, *CPRI Technical Bulletin No. 74*. Central Potato Research Institute, Shimla, Himachal Pradesh, India, 90p.

Mazur E (1984) Freezing of living cells: mechanisms and implications. *Amer J Physiol* **247**: 125-42.

Tomé LGO, LC Davide, CABP Pinto, AA Alves and CC Salgado (2007) Pollen viability and meiotic analysis of *Solanum commersonii commersonii* Dun., *Solanum commersonii malmeanum* Bitt. and *Solanum tuberosum* L. *Crop Breed App Biotechnol* **7**: 387-93.

Towill LE (1985) Low temperature and freeze/vacuum-drying preservation of pollen. In: Kartha KK (eds.) *Cryopreservation of Plant Cells and Organs*. CRC Press Inc., Boca Raton, FL, USA, pp. 171-198.

MS received : 31 December 2018; Accepted: 03 July 2019