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Pollen storage studies in *Oroxylum indicum* (L.) Vent: A threatened medicinal species of Western Ghats

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Abstract
Pollen storage studies in *Oroxylum indicum* (L.) Vent. was conducted at "*In vitro* and Cryopreservation laboratory", Division of Floriculture and medicinal crops, ICAR-Indian Institute of Horticultural Research, Bengaluru during 2020-21. Fresh pollens from 12 different accessions of *O. indicum* were collected from the Field Gene Bank of RET medicinal plants, IIHR, Bengaluru and pollens were preserved in different storage temperatures *viz*., 5 °C, -20 °C and -196 °C for 6 months. Initial viability assessment and post storage viability after retrieval was evaluated. The maximum pollen germination percentage was recorded in cryopreserved pollen after six months of storage. The fresh pollen viability was recorded and highest (98.25±0.25%) was noticed in Accession ASRET-2 where cryopreserved pollen recorded germination percentage of 86.65±0.45.

Keywords: *Oroxylum indicum*, pollen storage, cryopreservation, pollen germination

1. Introduction
Oroxylum indicum (Bignoniaceae) is commonly called as Indian trumpet flower or broken bones. It belongs to monotypic genus, evergreen with 20 m growth height and native to Indo-Malayan region. Roots are used in traditional Ayurveda whereas root bark is one of the ingredients in dashmoola. The tree species is pollinated by bats (*Eonycteris spelaea*) and flowers are nocturnal blooming from July to September (Sharma and Jain, 2016) [11]. It is used for anti-inflammatory and anti-cancerous properties both in folk and modern medicines. It is in the status of "near threatened" by IUCN red listed species due to destructive methods of harvesting. The existence of the species in nature is highly threatened and has been categorized as "vulnerable" by FRLHT (Foundation for revitalization of local health tradition) (Ravikumar and Ved, 2002).
With the increase in demand for the crude drugs, plants are exploited in nature due to uncontrolled harvesting and unmonitored trade, threatening its survival. Depletion of genetic resources has hampered its utilization for breeding purpose. Pollen storage is an excellent way of preservation without loss of its essential capabilities. Pollen storage is the major means of complementary conservation of Plant Genetic Resources in *O. indicum*. Cryopreservation is the method of long-term conservation of plant meristems, tissues, seeds or pollen to retain its normal function. Pollen, the male gametophyte can be stored in cryo-biological system for its extended uses in conservation, species restoration and in breeding programs.

2. Materials and Methods
The experiments were carried out at "*In vitro* and Cryopreservation laboratory", ICAR-Indian Institute of Horticultural Research, Hesaraghatta, Bengaluru during the year 2020-21.

2.1 Anthesis and pollen collection
The 12 accessions of *O. indicum* were taken under study and flowers were freshly harvested from the ten year old trees maintained in the Field Gene Bank (FGB) of RET medicinal plants at the Division of Floriculture and Medicinal crops, ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru. Pollen grains were extracted at 10.00 am from the matured and unopened flower bud which would anthesis in the evening (Srithongchuay *et al*., 2008) [12]. Petals are separated and copious amount of pollen (white colored) was extracted on the butter paper from the slit end of anther using needle. Pollen collected was bulked to get uniform *in vitro* germination.

2.2 In vitro pollen viability tests

In vitro pollen germination of *Oroxylum* is carried out to assess the pollen viability and vigor *i.e.*, rate of growth of pollen tube in the artificial nutrient media over a period of time. *In vitro* viability assessment was carried out through hanging drop method using pollen media *i.e.*, Brewbaker's and Kwach (PGM) media which is enriched with 15% sucrose concentration (Brewbaker and Kwach, 1963) [2]. Pollen grains which is dried, is dusted over the micro cover slip using a paint brush. A drop of pollen media is placed and pollen is spread with a slim brush. The periphery of the micro cover slip is lined with petroleum jelly and placed inversely on the glass slide.

The germinability was observed after 3 h incubation under 10× magnification of a compound microscope. Alexander stain is added over the germinated pollen where stain differentiates the aborted and non-aborted pollen grains (Alexander, 1980) [1]. Pollen grains whose tube length longer than the grain diameter was considered as germinated and viable. Pollen germination percentage was determined by dividing the number of germinated pollen per field of view by the total number of pollen per field of view and expressed as a percentage.

2.3 Pollen storage and pollen retrieval

Pollen moisture content was determined before cryopreservation and it was found to be 34%. It is dried using zeolite granules and relative moisture content was reduced to less than 10%. The pollens were transferred to butter paper which were packed in air tight aluminum pouches and preserved at different storage temperatures *viz.*, 5 °C, -20 °C and -196 °C. Pollen samples were stored for 6 months and analyzed for viability at monthly intervals. For pollen retrieval, pollen samples were warmed to ambient temperature for 1 h and post viability assessment were carried out.

2.4 Scanning Electron Microscopy of fresh and stored pollens

Fresh pollen and stored pollen under different temperature regimes were dehydrated in zeolite granules for 1 hour to reduce the moisture content. Further, these pollens were placed directly onto copper double sided tape on the disc surface of polished aluminum stabs and passed through a vacuum evaporator under the scanning electron microscope (Model No. TM 3030 Plus Scanning Electron Microscope). Pollen images were taken at 800x resolution. Morphological measurements such as pollen length (µm), pollen width (µm) and pollen perimeter (µm) were recorded in Fiji J software (Image J win 64).

2.5 Statistical analysis

The experiments were laid out in Completely Randomized Design (CRD) by following the procedure outlined by Panse and Sukhatme (1967) [6]. The data was subjected to ANOVA with critical difference values tabulated at one per cent level of significance of the corresponding degree of freedom and the means were compared using Duncan's multiple range test (DMRT).

3. Results

3.1 Initial viability assessment

Results revealed that significantly higher pollen germination was observed in 15% sucrose solution after 2 h of incubation at ambient temperature. Among 12 different accessions studied, maximum pollen germination of 98.25±0.25 per cent was elicited in Accession ASRET-2 which was on par with Accession ASRET-1 with percentage of 96.90±0.40. Lowest per cent of pollen germination was noticed in Accession ASRET-3 with per cent germination of 80.10±0.19.

Table 1: Fresh pollen germination of different accessions of *O. indicum*

Accessions	Fresh pollen germination (%)
ASRET-1	96.90 ± 0.40^{a}
ASRET-2	98.25 ± 0.25^{a}
ASRET-3	80.10 ± 0.19^{g}
ASRET-4	87.05 ± 0.44^{e}
ASRET-7	96.75 ± 0.85^{a}
ASRET-9	90.10 ± 0.40^{d}
ASRET-17	82.45 ± 0.25^{f}
ASRET-18	95.10 ± 0.19^{b}
ASRET-19	94.25 ± 0.55^{bc}
ASRET-20	94.65 ± 1.05^{bc}
ASRET-23	89.05 ± 0.04^{d}
ASRET-24	93.15 ± 0.25^{c}
S. Em. ±	0.50
C. D.	2.15

3.2 Effect of 5 °C storage on viability *in vitro*

In vitro germination profiles of pollen stored at 5°C were compared with germination profiles of fresh pollen. The data revealed that there is non-significance among the germination percentage of accessions stored at 5°C and is not statistically comparable with the fresh pollen. A significant reduction in pollen germination was noticed after one month of storage which subsequently reduced to null after three months of storage in accessions ASRET *viz.*, 3, 4, 9, 17, 18, 19 and 20.

3.3 Effect of -20 °C storage on viability *in vitro*

Pollen samples stored at -20 °C continuously decreased their capacity to germinate *in vitro* after storage where pollen germination percentage reduced to less than 5% at 4 months, after which the viability was completely lost at the end of 4 months of storage. The germination profiles of pollens stored at -20°C is not statistically comparable with fresh pollens.

Table 2: Effect of storage temperature 5 °C and -20 °C on pollen germination of different accessions of *O. indicum*

Accessions	5 °C			-20 °C			
	Months after storage (%)			Months after storage (%)			
	1	2	3	1	2	3	4
ASRET-1	62.25 ± 0.55^{c}	54.35 ± 0.45^{b}	7.30 ± 0.30^{b}	77.20 ± 0.30^{b}	58.95 ± 0.25^{b}	33.05 ± 0.35^{b}	2.95 ± 0.05^{c}
ASRET-2	76.80 ± 0.29^{a}	57.00 ± 0.60^{a}	8.85 ± 0.15^{a}	83.05 ± 0.44^{a}	64.20 ± 0.70^{a}	37.70 ± 0.10^{a}	4.95 ± 0.05^{a}
ASRET-3	34.30 ± 0.90^{k}	10.82 ± 0.42^{l}	0.00 ± 0.00^{f}	42.15 ± 0.35^{j}	23.35 ± 0.55^{j}	9.85 ± 0.35^{h}	0.0 ± 0.0^{e}
ASRET-4	51.05 ± 0.14^{f}	25.5 ± 0.50^{h}	0.00 ± 0.00^{f}	53.10 ± 0.40^{g}	39.60 ± 0.40^{f}	19.40 ± 0.20^{e}	0.0 ± 0.0^{e}
ASRET-7	69.95 ± 0.05^{b}	43.00 ± 0.40^{c}	6.25 ± 0.45^{c}	73.15 ± 0.35^{c}	59.10 ± 0.10^{b}	32.70 ± 0.40^{b}	4.40 ± 0.10^{b}
ASRET-9	54.95 ± 0.05^{e}	30.45 ± 0.45^{f}	0.00 ± 0.00^{f}	57.85 ± 0.05^{f}	42.85 ± 0.05^{e}	24.85 ± 0.45^{d}	0.0 ± 0.0^{e}
ASRET-17	40.35 ± 0.55^{i}	20.50 ± 0.50^{j}	0.00 ± 0.00^{f}	45.25 ± 0.35^{i}	35.90 ± 0.30^{g}	18.30 ± 0.40^{f}	0.0 ± 0.0^{e}

ASRET-18	49.05±0.45^{g}	27.95±0.04^{g}	0.00±0.00^{f}	52.45±0.15^{g}	47.20±0.30^{d}	18.40±0.20^{ef}	0.0±0.0^{e}
ASRET-19	37.15±0.04^{j}	13.75±0.35^{k}	0.00±0.00^{f}	62.34±0.56^{e}	56.95±0.25^{c}	28.85±0.65^{c}	2.45±0.05^{cd}
ASRET-20	42.25±0.15^{h}	21.80±0.20^{i}	0.00±0.00^{f}	62.55±0.05^{e}	27.00±0.30^{i}	18.40±0.20^{ef}	0.0±0.0^{e}
ASRET-23	57.75±0.15^{d}	37.35±0.25^{e}	2.80±0.20^{e}	49.75±0.25^{h}	31.05±0.15^{h}	14.20±0.30^{g}	0.0±0.0^{e}
ASRET-24	69.65±0.45^{b}	40.85±0.15^{d}	4.90±0.10^{d}	67.3±0.50^{d}	57.25±0.55^{c}	32.75±0.15^{b}	1.85±0.05^{d}
S.Em. (±)	0.05	0.39	0.05	0.35	0.37	0.35	0.04
C.D. @ 1%	0.21	1.70	0.21	1.51	1.62	1.49	0.18

3.4 Effect of cryopreserved pollen (-196°C) on viability *in vitro*

In vitro germination profiles of cryopreserved pollen of 12 different accessions of *Oroxylum* were compared with fresh pollen germination profiles. The data revealed that there was not much significant difference among viability profiles of fresh pollen and cryopreserved pollens of different accessions. Maximum per cent of pollen germination retained after storage of 6 months was in Accession ASRET-2 with 86.65±0.45 per cent where fresh pollen exhibited germination percentage of 98.25±0.25 which was followed by Accession ASRET-1 with 85.20±0.20 percentage when compared with fresh pollen germination of 96.90±0.40 percentage.

Table 3: Effect of storage temperature -196 ℃ on pollen germination of different accessions of *O. indicum*

Accessions	Months after storage (%) = -196°C					
	1	2	3	4	5	6
ASRET-1	94.50±0.30^{b}	92.27±0.12^{a}	89.50±0.30^{a}	88.17±0.52^{bc}	87.35±0.44^{b}	85.20±0.20^{b}
ASRET-2	95.90±0.20^{a}	91.85±0.44^{a}	90.72±0.42^{a}	89.50±0.20^{a}	88.95±0.25^{a}	86.65±0.45^{a}
ASRET-3	72.70±0.20^{h}	68.20±0.30^{i}	65.30±0.60^{g}	64.04±0.45^{h}	63.80±0.30^{h}	59.10±0.40^{h}
ASRET-4	80.85±0.35^{g}	77.70±0.20^{h}	75.85±0.65^{f}	75.37±0.22^{g}	75.10±0.10^{g}	73.75±0.05^{f}
ASRET-7	95.35±0.15^{ab}	92.45±0.25^{a}	90.03±0.13^{a}	88.88±0.32^{ab}	87.09±0.50^{bc}	85.55±0.25^{ab}
ASRET-9	87.80±0.09^{e}	84.67±0.52^{e}	84.98±0.08^{c}	84.75±0.25^{d}	83.49±0.09^{e}	80.20±0.40^{d}
ASRET-17	81.05±0.44^{g}	80.25±0.25^{f}	77.99±0.43^{e}	75.82±0.82^{g}	75.60±0.60^{fg}	71.25±0.65^{g}
ASRET-18	89.45±0.45^{d}	87.23±0.53^{c}	86.05±0.14^{c}	85.04±0.15^{d}	84.70±0.20^{d}	81.60±0.70^{c}
ASRET-19	87.53±0.03^{e}	84.25±0.25^{e}	83.14±0.25^{d}	82.87±0.33^{e}	82.55±0.34^{e}	81.00±0.40^{cd}
ASRET-20	84.25±0.65^{f}	78.92±0.22^{g}	78.25±0.65^{e}	77.20±0.30^{f}	76.10±0.10^{f}	75.25±0.25^{e}
ASRET-23	88.35±0.15^{e}	85.90±0.10^{d}	84.90±0.30^{c}	83.86±0.33^{de}	83.10±0.10^{e}	80.20±0.20^{d}
ASRET-24	91.90±0.20^{c}	89.75±0.25^{b}	88.15±0.55^{b}	87.12±0.53^{c}	86.25±0.25^{c}	84.85±0.35^{b}
S.Em. (±)	0.32	0.31	0.42	0.41	0.32	0.40
C.D. @ 1%	1.38	1.33	1.83	1.78	1.39	1.73

4. Discussion

The shelf life of pollen grains under different storage temperatures was evaluated and the findings of the investigation are discussed below.

4.1 Pollen growth media (PGM)

The composition of pollen media and the type of media employed varies with different species. Pollen of *O. indicum* germinated in liquid media containing 15 per cent sucrose using hanging droplet technique. Sucrose acts as an osmo-regulant that regulates the diffusion rate of water from the medium into the pollen grains. The sucrose concentration was found to be efficient in Aconitum heterophyllum at 5 per cent (Nautiyal *et al*., 2009) [5] whereas in *Gloriosa superba* at 10 per cent (Mamatha *et al*., 1993) [4] and 15 per cent in RET medicinal plant species such as *O. indicum, D. hamiltonii, H. ada-kodien, C. paniculatus* and *C. pedata* (Rajasekharan *et al*., 2012) [9].

4.2 Initial viability assessment

Initial pollen viability assessment was carried out to compare the germination per cent of fresh pollen and pollens stored at different temperature. The differences in the germination per cent of different accessions studied are attributed due to difference in the genetic make-up of the species. Pollen viability not only depends on the accessions, pollen maturity, pollen media composition, and the plant physiological status, but also it depends on the internal ongoing processes and factors within the pollen such as internal moisture content, reserve material and also with respect to the environmental issues such as relative humidity, air temperature and their interactions.

4.3 Effect of 5°C storage on viability *in vitro*

The effect of storage temperature and duration of storage on viability and germination percentage of different accessions of *O. indicum* pollen were studied and were found statistically significant. ANOVA revealed a significant decrease in pollen germination percentage when pollen was stored at 5°C. This is due to the insufficient chilling temperature to extend the window of pollen viability.

4.4 Effect of -20 °C storage on viability *in vitro*

A steep decrease in per cent of pollen germination was observed when pollen was stored at -20 °C. Pollen viability was reduced by 50 per cent after two months of storage and failed to germinate after four months of conservation at -20 °C. Due to insufficient freezing, the *Oroxylum* pollens were unable to maintain the cytoplasmic water content resulting in the cell death. It may also due to production of cellular lesions leading to cell crisis which causes significant changes in pollen properties and consequent reduction in the pollen viability.

4.5 Effect of cryopreserved pollen on viability *in vitro*

The results conforms that pollen cryopreservation is promising for conservation of endangered medicinal plants (Kasagana and Karumuri, 2011) [3]. Pollens stored in liquid nitrogen exhibited a slight reduction in pollen germination profiles upon retrieval. However after thawing, pollen retained the same characteristics as it had before cryopreservation and did not affect pollen viability

(Vishwakarma *et al*., 2020) [13]. The viability profiles of fresh pollens were on par with the viability profiles of cryopreserved pollen. Pollens once cryopreserved in liquid nitrogen can be preserved for infinite periods and thus enabling pollen availability within no time frame (Rajasekharan *et al*., 2015) [8]. Pollen cryopreservation in RET medicinal plants of Indian origin was investigated by Rajasekharan *et al*. (2015) [8] for 3 months.

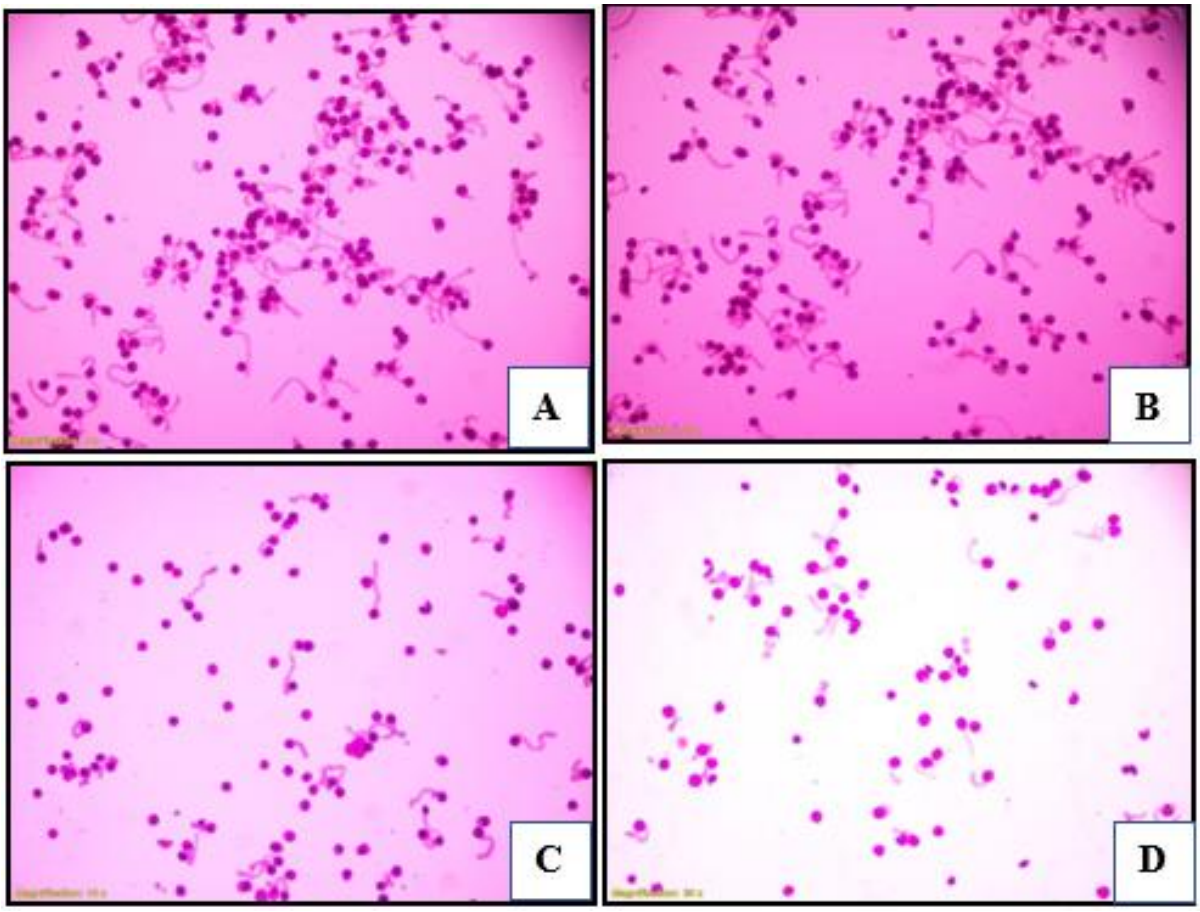
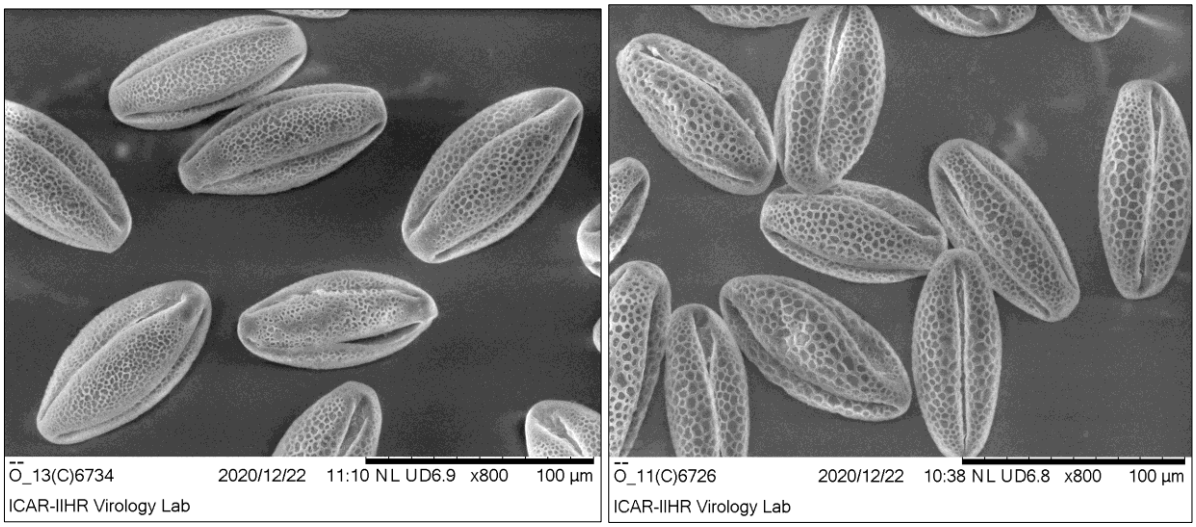
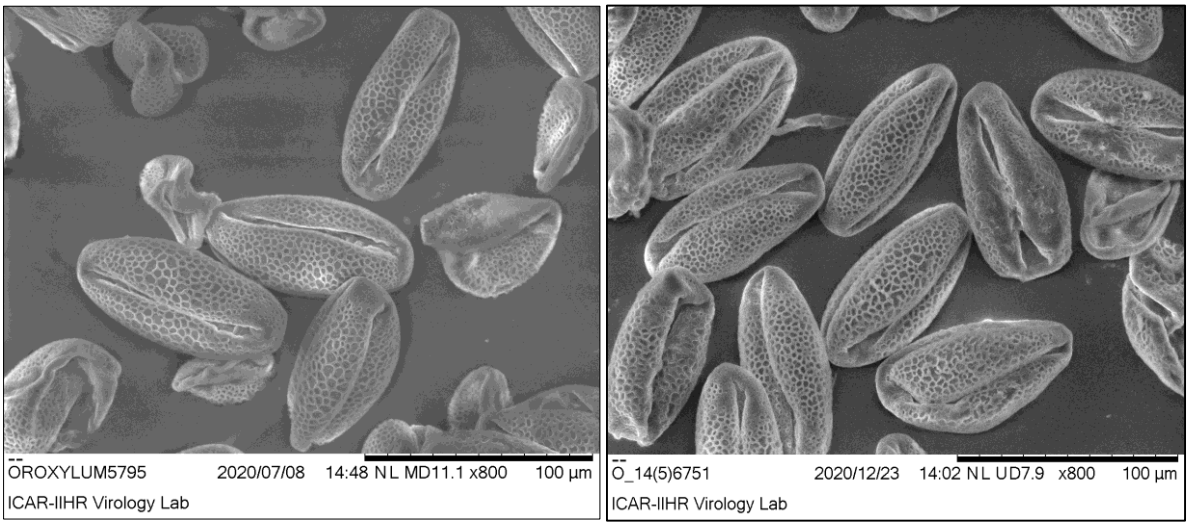


Fig 1: *In vitro* pollen germinated captured through Cellsens standard - Imaging software [A. Fresh pollen, B. Cryopreserved pollen (–196 °C), C. Pollen stored at –5 °C, D. Pollen stored at –20 °C]

Morphological measurements such as pollen length, pollen width and pollen perimeter were recorded with the help of a scanning electron microscope (SEM) for both fresh and pollens stored at different temperatures (when they lose viability). Though cryopreserved pollen exhibited a slight reduction in per cent pollen germination upon retrieval, results revealed that there was no significant difference among the accessions studied and also between the storage temperatures. However, it did not affect the pollen viability and showed the adaptability of pollen cryopreservation which serves as a backup for germplasm conservation.



A. B.



C. D.

Fig 2: Pollen visualized through Scanning Electron Microscope (SEM) at 800 µm magnification. [A. Fresh pollen, B. Cryopreserved pollen (–196 °C), C. Pollen stored at –5 °C, D. Pollen stored at –20 °C]

5. Conclusion

Pollen storage studies in liquid nitrogen (-196℃) is a relatively more effective conservation method in comparison with 5 ℃ and -20 ℃. Among the different accessions tested, Accession ASRET-2 elicited the highest germination percentage of 98.25±0.25 and after 6 months of successful cryopreservation it was 86.65±0.45 per cent. The pollen cryo-bank can be established with phenotypic and genetic stability which helps in the easy assessment of genetic resources within a lapse of time.

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