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In vitro germination and cryopreservation technique for long-term pollen conservation of underutilized legume: Grasspea (*Lathyrus sativus*)

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Grasspea (*Lathyrus sativus* L.) is a miraculous pulse crop, which needs less care and is commonly known as chickling pea, khesari, teora, kesari etc. There are 160 species in the genus *Lathyrus*, 16 of which are grown for food, feed, fodder and ornamentation purposes and have a high protein content of 28% (Hammer *et al.* 2019). Because of its extraordinary resistance to drought and flooding, grass pea is a legume crop with significant promise for ensuring global food security. Due to the presence of neurotoxin L-ODAP (L-oxalyl-2,3-diaminopropionic acid.) in seeds, prolonged consumption is known to cause neurolathyrism. Hence, the crop still remains an underutilized legume with high nutritional value. A wild relative of grasspea, *L. cicera* L., commonly known as chickling vetch, red vetchling, has low β-ODAP content (0.09–0.49%) and has resistance to drought, powdery mildew, rust or crenate broomrape etc. (Ferreres *et al*. 2017). Lack of synchrony in flowering in different accessions of *L. sativus* throughout the hybridization programme results in insufficient pollen availability, which further impacts number of crossings that can be attempted. Pollen cryoconservation circumvents this problem, especially during wide hybridization programs. However, *Lathyrus sativus* pollen cryoconservation has not yet been attempted so far. Thus, the present study was undertaken in *Lathyrus sativus* to (a) standardize *in vitro* pollen germination medium; (b) study the different storage regimes for pollen conservation; (c) standardize pollen cryoconservation protocol and its applicability in different accessions; (d) assess the fertilizing ability of cryoconserved pollen *in vitro* and *in vivo*.

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A total of 20 indigenous (IC) and exotic collections (EC) of *Lathyrus sativus* (Ratan, Prateek, Mahateora, Narayangaon, EC1068328, EC1068342, EC1068343, EC1068344, EC1068345, EC1068346, EC1068347, EC1068352, EC1068353, EC1068354, EC1068355, EC1073173, EC1073175, IFLA1, IFLA242, GP7) were used as source of pollen. Seeds of these germplasm were obtained from National Genebank at ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi and experiments were conducted at the net house and research farm of ICAR-NBPGR, New Delhi (28º35'N, 70º18'E, altitude 226 m amsl) during 2021–22. Standard package of practices was followed to raise a healthy crop, except for spraying of pesticides during the flowering period.

Freshly opened flower buds from all 20 accessions were collected between 9.00–9.30 A.M. in butter paper bags. In laboratory, freshly dehisced anthers were tapped on a filter paper in a petri plate (90 mm × 15 mm) to collect pollen. For each accession, pollen from 20–30 fresh and fully open flowers from 2–3 plants were combined to create aliquots for experiments. Initially four accessions (Ratan, Prateek, Mahateora and Narayangaon) were used to standardize *in vitro* pollen germination and pollen cryopreservation protocol. *In vitro* pollen germination medium was standardized using the sitting drop culture method (Shivanna and Tandon 2014). Fresh pollen was germinated on Brewbaker and Kwack medium (BK medium) with different sucrose concentrations (1, 5, 10, 15 and 20%) at *p*H 5.84 (Brewbaker and Kwack 1963). Fresh pollen was dusted on a micro slide (75 mm × 25 mm) with a drop (2 µl) of BK medium in a covered petri plate with moist filter paper to maintain high humidity (70–80%), covered with coverslips (18 mm × 18 mm) and incubated for 2 h at room temperature (22 ± 2ºC). After 2 h, slides were viewed under 10X magnification of a compound microscope (Carl Zeiss AX10 Microscope). Pollen grains with tube length exceeding twice the diameter of pollen grain were considered germinated and viable (Shivanna and Tandon 2014). The pollen

germination percentage was determined by dividing the number of pollen grains germinated by the total number of pollen.

To assess, pollen moisture content (MC) and desiccation period for storage, fresh pollen grains were placed in an aluminium foil cup and weighed gravimetrically. Next pollen were oven-dried using a high constant temperature method, by drying at 130ºC in a hot air oven for 2 h (ISTA 1985), and re-weighed. The difference between fresh weight (FW) and dry weight (DW) was calculated and expressed as g H_2O/g DW in percentage. The MC(%) was calculated using the formula, (FW−DW)/(DW−TW), where TW is the tare weight, i.e. the weight of the aluminium foil cup. For determination of the desiccation period, aluminium foil cups with fresh pollen were placed in a laminar airflow cabinet for air drying at 10, 20, 30, 40, 50 and 60 min. The MC was determined by the above equation and pollen viability through *in* vitro pollen germination was monitored at regular intervals.

Fresh and desiccated pollen grains were stored at four temperature regimes (25, 4, -20 and -196ºC), in dark. For storage at 25ºC, pollen were placed in aluminium pouches and placed in cryovials (1.8 ml) and kept in the laboratory. For storage at 4ºC, -20ºC and -196ºC, a refrigerator, deep freezer, liquid nitrogen (LN) cryotanks (Air Liquide, France) were used, respectively. The viability of the stored pollen grains was monitored at 1, 3, 5, 7, 9, 24 h, 1 week, 2 wks and 6 months through *in vitro* pollen germination. The standardized cryopreservation protocol was tested in other 16 accessions (except Ratan, Prateek, Mahateora and Narayangaon) after storage for one day in cryogenic temperature.

Fertilizing ability of fresh and cryopreserved pollen (*in vivo* pollen germination and field pollinations) was assessed in eight (Ratan, Prateek, Mahateora, Narayangaon, EC1068345, EC1068346, EC1068353, and EC1068354) collections. Pollen were dusted on the stigmas of emasculated flowers of respective accessions (5–10 flowers) and pistils were fixed in acetic alcohol solution (3:1) after 24 h of pollination and stored in 70% alcohol. The stored pistil was transferred to 1N NaOH for 2–4 h for clearing in a hot air oven. The cleared pistils were stored in 1% aniline blue solution overnight followed by mounting in glass slide using 1:1 mixture of aniline blue and 10% glycerine and observed under fluorescent microscopic with UV filter combination (Shivanna and Tandon 2014). The samples were observed for pollen germination on stigma, and pollen tube entry to the embryo sac. Pod and seed set after maturity was also observed. Germination of seeds obtained through crossing of cryoconserved pollen was done using petri plate method (10 seeds/replication). Seeds were placed on moist filter paper in petri dishes and watered frequently with distilled water and incubated in BOD (Biological Oxygen Demand) chamber (iGene Labserve, India) at 22–25ºC, 16 h light/8 h night photoperiod with the light intensity of 55 ± 2 µmol/m^2/s (ISTA 1985). After 1 week the seed germination (%) (radicle extension ≥ 2 mm), shoot length (cm), root length (cm) and seed vigour index (SVI) were recorded. The SVI was determined as:

SVI = standard germination (%) × seedling length (cm)

where, seedling length, comprised of shoot and root length. All the experiments were performed in Completely Randomized Design (CRD) and repeated thrice for each

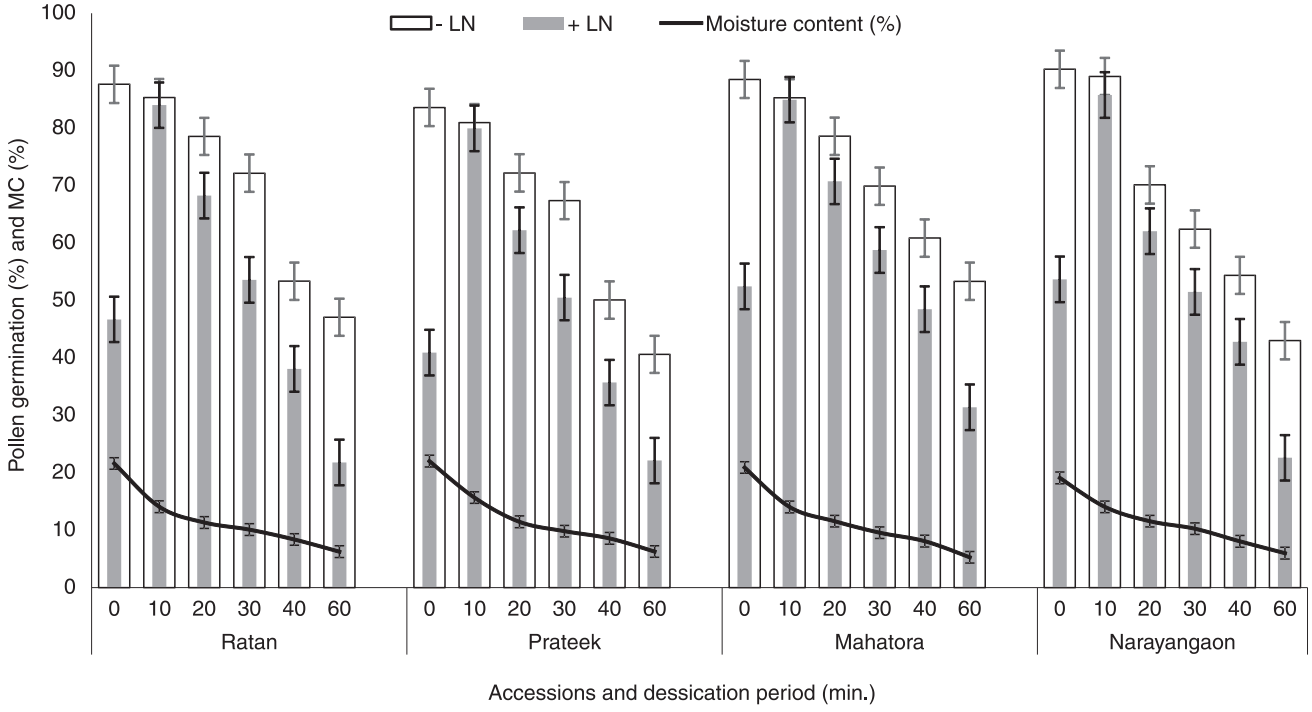

Fig 1 Determination of MC and desiccation period for cryopreservation of fresh pollen grains of 4 accessions of *Lathyrus sativus* (Values representing mean ± SE). MC, pollen moisture content; LN, liquid nitrogen.

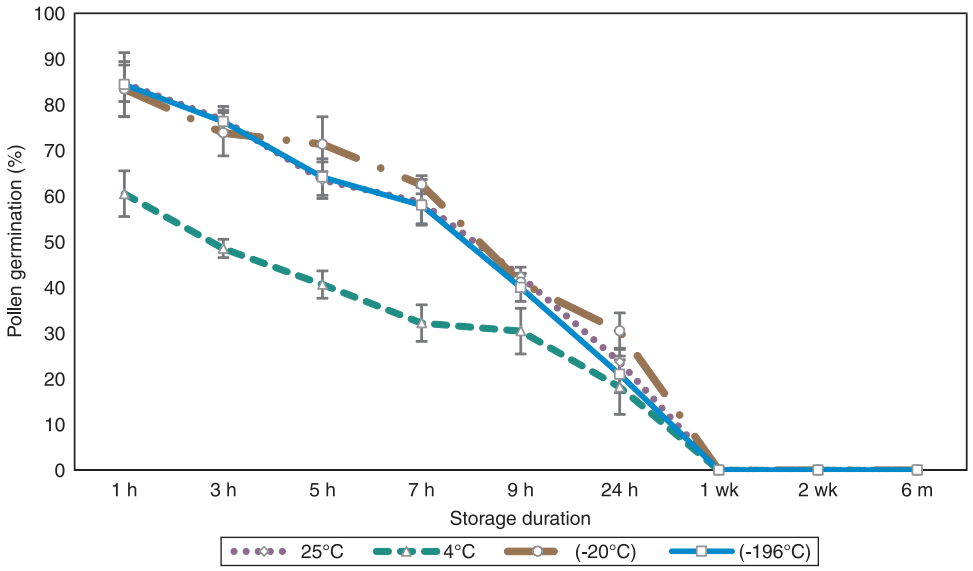

Fig 2 Effect of different storage temperatures and duration on storage of fresh pollen at LAF for 10 min (Ratan). LAF, Laminar air flow,

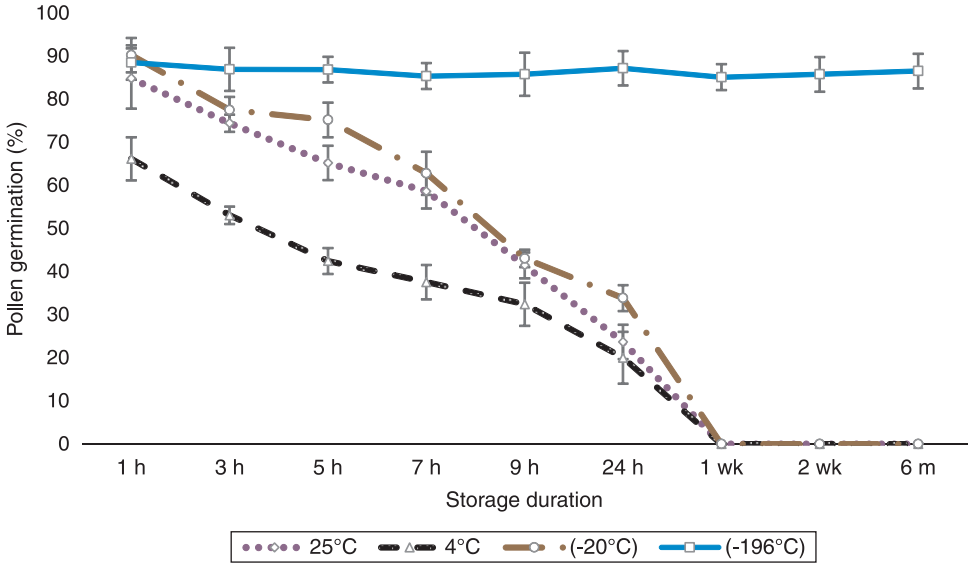

Fig 3 Effect of different storage temperatures and duration on storage of desiccated pollen at LAF for 10 min (Ratan). LAF, Laminar air flow,

accession. For pollen viability assessment, in each replication 10 microscopic field views were taken with a minimum of 500–600 pollen grains. The data are presented as mean ± standard error. Arcsine root square transformation was used for data in percentage and one-way ANOVA analysis and DMRT ($P \leq 0.05$) were used to compare differences between treatments. All data were analyzed using the SPSS version 22.0 statistical software package. Graphs were generated using GraphPad Prism 9.1.0 software.

For *in vitro* germination of *L. sativus* pollen, BK medium with 15% sucrose gave the highest mean pollen germination (87.5 ± 2.8%) as compared to 1% (22.4 ± 1.4%), 5% (36.3 ± 1.9%), 10% (46.6 ± 2.2%), 20% (71.8 ± 6.8%) sucrose concentrations, of the four accessions tested. A significant increase in pollen germination percentage was observed up to 15% sucrose which reduced thereafter at 20% sucrose (Supplementary Fig 1A). The result findings are in agreement with previous reports on *in vitro* pollen germination of different accessions of *Abelmoschus* species (Gowthami *et al.* 2021). Fig 1 gives the results of effect of dessication on cryopreservation of pollen grains. When fresh pollen with 19–21% MC were subjected cryopreservation, a ~ 40–50% reduction in pollen viability was observed. In contrast, amongst all desiccation duration, least reduction (~ 0.3–1.5%) was observed at 10 min desiccation (~14–16% MC) (Fig 1). These results are in line with previous reports, where pollen with highly reduced MC gave poor results after cryopreservation, possibly due to loss of osmotic and structural integrity of cells, resulting in injury and viability loss (Xu *et al.* 2014). From the present results, it is concluded that the ideal MC of 14–16% was effective in pollen cryopreservation which was attained after 10 min of desiccation in a laminar airflow chamber. Hence, for subsequent experiments to assess the effect of temperature and duration on pollen conservation, desiccated pollen with MC of 14–16% were used. Fig 2 and 3 represents the combined effect of different storage conditions (25, 4, -20 and -196ºC) at different time duration [1, 3, 5, 7, 9, 24 h, 1 week (wk), 2 wks and 6 months (m)] on fresh and dessicated pollen viability, respectively. Pollen stored at -196˚C was significantly different from those stored at 25, 4 and -20˚C. The viability of fresh pollen follows the same trend as in 25, 4 and -20˚C storage conditions. In contrast, the desiccated pollen does not lose its viability over time (up to 6 months) and the pollen viability was maintained throughout the storage period (Supplementary Fig 1). Compared to all other storage conditions, pollen stored for a long time at -196°C was on par with fresh pollen viability. Hence, cryopreservation can be most effectively used for the long-term conservation of *L. sativus* pollen.

The cryopreservation protocol standardized above

Table 1 Application of standardized pollen cryoconservation protocol to different *Lathyrus sativus* accessions

Accession	Fresh Germination % (-LN)	Desiccation (10 min LAF)	
		(-LN)	(+LN)
Ratan	87.61±1.74[a]	85.31±0.87[a]	83.99±1.28[a]
Prateek	83.58±1.04[a]	80.94±2.39[a]	79.96±2.39[a]
Mahateora	88.45±2.17[a]	85.26±1.53[a]	84.94±1.07[a]
Narayangaon	90.23±1.86[a]	88.99±3.84[a]	88.74±2.49[a]
EC1068342	90.73±0.96[a]	89.85±1.32[a]	88.98±2.66[a]
EC1068328	86.59±0.58[a]	85.31±2.40[a]	84.86±2.29[a]
EC1068343	83.88±1.03[a]	82.36±2.21[a]	80.80±1.57[a]
EC1068344	85.59±0.87[a]	84.79±2.47[a]	83.75±3.61[a]
EC1068345	82.50±1.58[a]	80.46±3.34[a]	79.34±2.11[a]
EC1068346	81.50±1.48[a]	79.90±2.11[a]	82.08±3.13[a]
EC1068347	90.60±1.19[b]	91.69±1.81[b]	95.34±0.89[a]
EC1068352	89.91±1.18[a]	87.80±1.51[b]	86.92±1.72[b]
EC1068353	80.37±2.20[a]	79.59±2.37[a]	78.67±1.19[a]
EC1068354	76.75±4.83[a]	75.58±1.14[a]	74.53±2.23[a]
EC1068355	77.47±3.67[a]	76.55±3.50[a]	75.97±2.48[a]
EC1073173	80.31±1.96[a]	79.46±4.60[a]	76.92±3.46[b]
EC1073175	90.41±0.98[b]	90.52±0.91[b]	94.82 ±1.67[a]
IFLA1	73.76±2.79[a]	71.92±1.03[a]	70.98±2.45[a]
IFLA242	93.08±1.67[a]	92.84±0.95[b]	93.95±1.57[a]
GP7	85.50±1.63[a]	83.14±2.21[a]	82.16±1.25[a]

Values representing Mean ± SE; values following same letter within row have no significant (P≤0.05) difference. LAF, Laminar air flow.

for four accessions was applied to another 16 accessions and results of all 20 accessions are shown in Table 1, wherein all accessions show >70% of pollen germination. The viability of the corresponding fresh pollen was not significantly altered by cryopreservation, except it significantly decreased in two accessions (EC1068352, EC1073173) and significantly increased in two accessions (EC1068347 and EC1073175). Similar increase/decrease in pollen viability after cryopreservation are reported in genotypes of other species also (da Silva *et al.* 2017, Ren *et al.* 2019, Gowthami *et al.* 2021).

Using fluorescent microscopy, pollen germination and pollen tube development on receptive stigma evaluated in fresh (control) and cryoconserved pollen showed identical levels and patterns of pollen germination and pollen tube development. The pollen germinated on the stigmatic surface in 2–4 h after pollination (HAP), and pollen tube entered into the embryo sac and fertilized the ovule within 24 HAP. No detrimental effect of cryopreservation on pollen germination, ability to fertilize, seed setting, seed germination, and seed vigour index was recorded, as compared to respective controls (Table 2), which is in accordance to previous reports in other crops (Tyagi and Hymowitz 2003). Interestingly significant increase in the mean performance for shoot length (Ratan and EC1068345), root length (Prateek), seedling length (Ratan) and SVI (Ratan and Prateek) was observed.

The establishment of a simple and reproducible protocol for long-term pollen storage is essential in grasspea hybrid breeding programs that use cultivars with an asynchronous

Table 2 Seeds obtained using fresh and cryoconserved pollen and its characteristics

Parameter	Treatment	Accessions							
		Ratan	Prateek	Mahateora	Narayangaon	EC1068345	EC1068346	EC1068353	EC1068354
Seed germination (%)	-LN	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	96.66 ± 3.33[a]	98.33 ± 1.66[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	+LN	100.00 ± 0.00[a]	100.00 ± 0.00[a]	99.00 ± 2.88[a]	99.58 ± 2.88[a]	95.00 ± 2.88[a]	97.66 ± 1.45[a]	99.55 ± 1.45[a]	100.00 ± 0.00[a]
Shoot length (cm)	-LN	8.07 ± 0.12[b]	8.66 ± 0.16[b]	9.96 ± 0.22[a]	8.07 ± 0.12[a]	8.51 ± 0.28[b]	10.00 ± 0.28[a]	8.53 ± 0.29[a]	8.10 ± 0.20[a]
	+LN	11.44 ± 0.44[a]	8.71 ± 0.41[a]	8.69 ± 0.28[a]	8.30 ± 0.14[a]	9.83 ± 0.16[a]	9.50 ± 0.28[a]	7.00 ± 0.28[b]	7.90 ± 0.05[a]
Root length (cm)	-LN	5.00 ± 0.57[a]	3.15 ± 0.12[b]	3.11 ± 0.06[a]	2.27 ± 0.14[a]	3.66 ± 0.16[a]	3.99 ± 0.16[a]	2.50 ± 0.28[b]	2.33 ± 0.16[a]
	+LN	4.47 ± 0.20[a]	4.55 ± 0.28[a]	2.89 ± 0.19[a]	2.27 ± 0.14[a]	2.83 ± 0.16[a]	2.66 ± 0.33[b]	3.00 ± 0.57[a]	1.93 ± 0.06[a]
Seedling length (cm)	-LN	13.07 ± 0.70[b]	11.81 ± 0.27[a]	12.07 ± 0.29[a]	10.35 ± 0.19[a]	12.18 ± 0.34[a]	13.16 ± 0.44[a]	11.03 ± 0.33[a]	10.43 ± 0.06[a]
	+LN	15.91 ± 0.61[a]	13.27 ± 0.16[a]	10.08 ± 0.18[a]	10.58 ± 0.29[a]	12.66 ± 0.16[a]	12.16 ± 0.60[a]	10.78 ± 0.50[a]	9.83 ± 0.12[a]
SVI	-LN	1307.66 ± 4.25[b]	1181.66 ± 2.27[b]	1307.00 ± 2.19[a]	1035.33 ± 3.19[a]	1176.66 ± 3.82[a]	1295.83 ± 6.37[a]	1103.33 ± 3.33[a]	1043.33 ± 6.66[a]
	+LN	1507.11 ± 3.74[a]	1327.00 ± 6.28[a]	1285.08 ± 4.07[a]	988.55 ± 5.08[a]	1140.50 ± 4.28[a]	1188.50 ± 4.08[a]	990.83 ± 6.63[a]	999.33 ± 2.01[a]

Values representing Mean ± SE; values following the same letter within row of the parameters have no significant difference (P≤0.05).

flowering time, temporal isolation, inadequate male parent population etc. To the best of our knowledge, this is the first report on the effect of pollen stored at different temperatures for six months in grasspea.

SUMMARY

Grasspea (*Lathyrus sativus* L.) is an underutilized pulse crop which can withstand adverse environmental conditions such as drought, heat, salinity, waterlogging, pest and diseases. To facilitate hybridization studies, pollen cryoconservation was attempted in *L. sativus*. Among the different media combination tested for *in vitro* pollen germination, BK medium with 15% sucrose was optimal. Pollen viability was tested at different storage regimes, viz. 25, 4, -20 and -196 ºC at different storage duration (1, 3, 5, 7, 9, 24 h, 1 week, 2 week and 6 months). For long-term pollen cryopreservation, an ideal MC of 14–16% was achieved by desiccating the pollen for 10 min in a laminar air flow (LAF) chamber. Negative correlation was found between pollen viability, storage duration and temperature (25, 4 and -20°C). Cryopreserved (-196°C) pollen showed significantly higher viability compared to all the other storage conditions. The standardized cryoconservation protocol was applied to 20 accessions of *Lathyrus sativus* for conservation up to six months without loss of pollen viability. Successful fertilization, fruit and seed set was observed in cross combinations attempted using cryoconserved pollen without any incompatibility barriers. Hence, the present protocol can be used for long-term cryoconservation of *L. sativus* pollen. The development of an effective long-term storage method of *Lathyrus sativus* pollen ensures the availability of pollen for grasspea breeding throughout the year.

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