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Pollen storage in vegetable crops: A review

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Abstract
Pollen storage is one of the valuable tools for overcoming time as well as location isolation in flowering of germplasm lines in various breeding programmes, facilitating germplasm conservation and its exchange and assisting in supplementary pollinations in vegetable crops. There is need for pollen storage owing to short duration of pollen viability. Pollen viability greatly varies within cultivated species as well as between crop related species of different vegetable crops. It is also associated with cellular stage of pollen at time of its dispersal. Binucleate pollen grains have longer viability than those with trinucleate types. The duration of pollen storage can be increased by manipulating temperature, relative humidity and storage atmosphere. The use of organic solvents, refrigeration, freeze drying and cryopreservation are different methods of pollen storage. Storage at low temperature and low relative humidity is desirable for most of the crops. Collection of good quality pollen at appropriate stage and reduction of moisture to optimum level is required for prolonged storage. However, pollen should be stored at freezing temperature for seasonal hybridization and its cryopreservation becomes necessary for long term usage. Pollen storage improves breeding efficiency and hybrid seed production. Therefore, there is need to standardize the conditions for particular germplasm lines and species in different vegetable crops.

Keywords: Pollen storage, pollen viability, temperature, relative humidity

Introduction
Pollen can be defined as product of cellular division within male gametophyte which is naturally used for pollination and fertilization to produce seed of sexually propagated crops. Pollen is shed either at two celled (binucleate) or three celled (trinucleate) stage. Binucleate pollen grains have vegetative and generative cells at maturity and trinucleate pollen grains have vegetative cell and two sperm cells formed from mitotic division of generative nucleus. This developmental stage is important for pollen storability and longevity. Most of angiosperms (70%) shed pollen at binucleate stage (Brewbaker, 1959) [4]. Trinucleate pollens are present in Graminaeae, Umbelliferae, Cruciferae, Araceae, Caryophyllaceae and Chenopodiaceae.

After shedding pollen germination, tube growth and fertilization takes place. Mature pollen grain has small quantity of reserve food materials, so pollen remain viable only for short duration. Pollen viability is the ability of pollen deliver functional sperm cells to embryo sac following compatible pollination (Shivanna and Ram, 1993) [41]. Pollen viability greatly varies from species to species and with prevailing environmental conditions. Pollen viability is important for successful fertilization and seed set. The objective of pollen storage is to maintain the pollen in its viable condition to effect fertilization and seed set. Although pollen storage need is much greater for crops with long life cycle but it increases the breeding efficiency in crops having short life cycles. Purpose of this review is to summarize the recent studies in pollen storage in various vegetable crops.

On the basis of pollen longevity or viability pollen grains classified into three categories (Barbanas and Kovacs, 1997) [3].

Long longevities: Pinaceae, Palmae, Saxifragaceae, Rosaceae, Leguminosae, Anacrdiaceae, Vitaceae and Primulaceae.

Intermediate longevities: Liliaceae, Amaryllidaceae, Saliceae, Cruciferae, Ranunculaceae, Rutaceae, Solanaceae and Sccorphulariaceae.

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Short longevities: Alismataceae, Graminaceae, Cyeraceae, Compositae, Commelinaceae and Junceae.

Causes of loss of pollen viability

The various reasons for decrease in pollen viability are deficiency of respiratory substrates, inactivation of enzymes or growth substances (Stanley and Linskens, 1974) [43], irreversible loss of membrane permeability (Jain and Shivanna 1989) [18] and changes in oil deposits in the pollen exine (Pfeiffer, 1955) [39]. Nielsen (1956) [35] suggested decrease in pantothenic content during storage also leads to loss of pollen viability. Pantothenic acid is important constitute of Coenzyme A which is very important for respiration and metabolism of plant. Decrease in membrane permeability was due to leaching of phospholipids, sugar or amino acids during storage.

Need of pollen storage

To use over extended period there is need to store pollen to maintain its viability. Further the role of pollen storage becomes more relevant when cultivars used in crossing programmer differ in their flowering times or flower at different locations. So it avoids need for staggered or multiple planting and also eliminates the need to grow male parent continuously, thus land can be used more efficiently. It gives us constant supply of short lived pollen. Besides its role in wide hybridization it also facilitates germplasm conservation and exchange, physiological research and identification of self incompatible alleles. Objective of pollen storage is to collect the pollen and maintain it in viable condition.

Earlier reports on storage were available in Date palm. Male flowers were stored in dark and dry place and it was noticed that it did not lost its fertilization capacity (Kampfer, 1712) [19]. Simon (1911) [40] found pumpkin pollen can be stored for five weeks in sealed vessel containing anhydrous Calcium chloride. However systematic research started at end of 19th century.

Factors affecting pollen storage

There are different factors which influence the longevity of pollen. Among them temperature and moisture content of pollen are important (Stanley and Linskens, 1974) [43]. Other factors include storage atmosphere, cytology of pollen or pollen quality.

Relative humidity

Relative humidity during pollen collection and storage affects the pollen moisture content, thereby affecting its viability. Response to humidity is related to intrinsic hydration state of pollen at dehiscence (Nepi *et al.* 2001) [34]. Pollen which is partially hydrated at dehiscence (>30% water) not able to withstand adverse environmental conditions than partially dehydrated (<30%). Humidity needs to be controlled so that pollen should not become rehydrated during storage. Low relative humidity is required for storage of pollen grains except trinucleate which are dessication sensitive. Storage at high humidity (60% or above) results in attack of bacteria or fungi but at low relative humidity it remain viable for longer time. Nebel and Ruttle (1937) [33] observed growth of mould in pollen samples stored at 100% RH. Pollen cannot withstand extreme variations in environment, so relative humidity should be kept constant. Atmosphere with different RH can be produced by using silica gel or saturated salt solutions.

Temperature

Pollen storage at low temperature does not cause any phase change in water provided its moisture content is reduced to certain limit because water below critical level is in bound or unfreezable state (Vertucci, 1990) [48]. High temperature increases the metabolic activity of pollen degrading the reserve food material and at low temperature its metabolic activity is slowed down. Celery pollen stored well at temperature of -10°C than 4°C but viability decreased over period of 18 months (D'Antonio and Quiros, 1987) [9]. Potato pollen successfully store in liquid nitrogen (-196°C) up to 9 months whereas at -20°C germination reduced to 29% (Weatherhead *et al*. 1978) [50]. Lettuce pollen stored at -18°C with no significant decrease in pollen viability compared to control for 30 days as compared to 4°C (Eenink, 1983) [11].

Storage atmosphere

Storage atmosphere should be manipulated such that it should reduce the oxidative stress and improve pollen longevity. High concentration of carbon dioxide increase pollen longevity while oxygen shorten it (Knowlton, 1922) [27]. Modification in storage atmosphere is needed if temperature less than -20°C are not available. Akutsu (2018) [2] reported storage under nitrogen or carbon dioxide gave good germination after storage. Also storage under nitrogen resulted in greater fruit set than vacuum at 4°C, but noted little difference in two storage atmospheres when stored at -25°C.

Other factors

Diluents are fine powdered dry material mixed with pollen. Its effect in maintaining pollen longevity has not been extensively tested yet. Diluents are used to minimize the deterioration during pollen storage. It regulated the moisture and air around pollen. Most commonly used diluents are Lycopodium powder, egg albumin, talc and casein. Diluents can be effectively used with dry pollen since with sticky pollen they form large clumps. They are also used to increase bulk to reduce wastage of pollen during artificial pollination.

Cytology of pollen also influences longevity. Binucleate pollen grains have higher longevities than trinucleate. Binucleate pollen grains have thicker exine, dessication tolerant and has long longevities whereas Trinucleate pollen has thinner exine, sensitive to dessication, respire faster and shorter longevity. However, large bicellular pollen grains of cucurbits has short longevity (Digonnet- Kerhoas *et al.* 1989) [10] and tricellular pollen of sugar beet has long longevity (Hecker *et al*. 1986) [12]. Pollen quality like its initial viability or moisture content also effect storage.

Steps for pollen storage

Basic steps for pollen storage are pollen collection, Dehydration and then storage under various environment conditions.

Pollen collection

Collection of viable pollen in sufficient quantity is pre-requisite. Poor quality pollen does not store well quickly reducing its viability and vigour. Collection method varies with species. There are different methods for pollen collection. It can be done by shaking flower over folded sheet of paper or by using vibrator, whole anthers or flower can be used or by use of insects. Pollen collection time is also very

important because if initial viability is low it will not store well under any conditions. Pollen must be collected one day prior anthesis to avoid any contamination by foreign pollen and to assure genetic purity. In melon, pollen harvested from flowers at anthesis maintained its germination better after 30 days of storage than pollen harvested one day before anthesis (Agustin *et al.* 2014) [1]. Pollen collection method significantly influence berry set percent and seeds per berry in Potato (Thakur *et al.* 1994) [46]. It was reported that collection with vibrator produced more seeds per berry than pre dried sieved anthers but involves more time to extract pollen so it was recommended on limited scale and for commercial seed production sieving of pre dried anthers is recommended in Potato.

Dehydration

Moisture from field collected pollen is high, it needs to be reduced below critical level varying with species for better longevity. Pollen can be dried by different ways- air drying, oven drying or drying over dessicants such as $CaCl_2$. Dehydration is essential to prevent the risk of intracellular ice formation. Differences in viability loss when exposed to dehydrated conditions also related to type of carbohydrates stored in cytoplasm (Pacini *et al.* 2006) [36]. Pollen having more sucrose content can better maintain its viability due better retention of membrane permeability. Importance of dehydration has been reported by many workers. In cucumber pollen moisture reduced to 6.71% from 12.05% by exposing for 8 hours to magnesium chloride showed better fruit set (Palupi *et al.* 2017) [37]. Fresh field or glass house collected pollen with moisture content of 26-33% and 16-21% can be dried in closed chamber for 24 hours at 5°C lowering moisture content to 11-12% and 6-9% respectively to obtain higher longevities in Sugarbeet (Hecker *et al.* 1986) [12]. Moisture content before storage reduced to 5-10% (Hoekstra, 1995) [13].

After dehydration it is stored in various containers like capped screw vials, eppendorff tubes, cryovials, glassine bags or gelatin capsules. Mostly cryovials or eppendorff tubes are used. Type of container does not appear a critical factor if it is stored under air tight conditions. Cooling and warming rates are not critical for desiccation-tolerant pollens. Containers are placed directly at storage temperature. Containers are also transferred directly to room temperature and allowed to warm before opening.

Storage methods

Organic solvents

This method was first demonstrated by Iwanami (1972b) [16] in *Camellia* and Iwanami and Nakamura (1972) [15]. It avoids the problem of maintenance of low relative humidity. It is for short term storage only. It does not work well for all species. Non polar solvents (benzene and diethyl ether) gives better results than polar solvents (acetone) due to less leakage of electrolytes like sugars, phospholipids and amino acids (Jain and Shivanna, 1988) [17] Benzene shows good potential to store pollen of brinjal, okra (Khan and Perveen, 2006) [24] and watermelon (Khan and Perveen, 2006) [25] as compared to acetone and chloroform. Mishra and Shivanna (1982) [31] worked for storage of leguminous taxa in organic solvents and reported that cyclohexane and diethyl ether gives better results but for short duration only.

Refrigeration

It is one of the easiest way to store pollen at low temperature with suitable dessicator to control relative humidity. Pollen can be store in sealed vials with suitable dessicator and placed in refrigerator. Cabbage pollen can be stored at 4°C for 50 days with 18% germination (Chiang, 1974) [6]. Asparagus pollen can be stored at 4°C with dessication over silica gel for 30 days (Marcellan and Camadro, 1996) [30]. Nascimento *et al*. (2003) [32] stored pollen of 'Cica' hybrid for 60 days at 5°C and concluded that pollen can be used for seed production up to 20 days because after 50 days seed yield was reduced and after 30 days fertilization rate decreased. Patta *et al.* (2016) [38] stored pollen of Pusa-120 at refrigerated (9°C-10°C) and ambient (25°C-26°C) for 9 days and observed no significant reduction in fruit set, fruit weight and seeds per fruit upto 4 days pollen stored under refrigeration as compared to control. No effect was noticed on seedling parameters like seedling length and seedling dry weight but EC increase after four days of pollen storage. Pollen can be stored for 7 days under refrigeration.

Freezing temperature

Storage is done at temperature of -10°C to -34°C. Pollen can survive for 1-3 years depending on species. It is suitable for bi-nucleate pollen grains. Cabbage and Radish pollen can be stored at -20°C with silica gel as dessicant for 2 years (Huihong *et al.* 1995) [14]. It is suitable for crossing programmes but not for germplasm preservation. Punjab Neelam, male parent of brinjal hybrid BH-2 can be successfully stored upto 7 weeks at -16°C without any significant difference to get high fertilization rate, seed yield and quality followed by storage in refrigeration at 5°C with dessicant like calcium chloride for 35 days (Singh, 2010) [44].

Cabbage pollen can be stored at -20°C for 78 weeks (Cunninghum, 1981) [8]. Asparagus pollen can be stored at -20°C for 60 weeks without any significant loss in pollen germination (Snope and Ellison, 1963) [42]. Cabbage pollen successfully stored for 175 weeks at -20°C (Brown and Dyer, 1991) [5].

Freeze drying or vacuum drying

It is also known as lyophilization. Lyophilization means 'to make solvent loving'. Freeze drying involves freezing of sample to sub zero temperature followed by sublimation to remove water, but vacuum drying involves direct exposure to vacuum removing water by evaporative cooling. Viability under this method is influence by freezing temperature, thawing, rate of warming and cooling, moisture content or pressure of gas (Snope and Ellison, 1963) [42]. King (1960) [23] examined the length of drying for 12 species and found 0.5 to 3 hours of drying was optimum but sweet potato pollen even after drying for 30 hours gave high in vitro germination. Results of vacuum or freeze drying were encouraging in pea (Layne and Hagedorn, 1963) [28].

Cryopreservation

It involves storing the sample at -196°C in liquid nitrogen. It was first used in *Antirhinum majus* (Knowlton, 1922) [27]. In cryogenic preservation vessel containing dried pollen immersed in bath of desired temperature. *Solanum* pollen survived cryogenic condition provided its moisture content adjusted between 35% to 40% (Towill, 1981) [47]. Pollen of

tomato remain viable for 1062 days when exposed to -190°C (Visser, 1955) [49] but only one month in *Vicia faba* (Telaye *et al.* 1990) [45]. Cryopreserved pollen show low germination (43%) 2 months after storage with control (97%) in Broccoli and found no genetic changes in progenies (Crisp and Grout, 1984)

Karipidis *et al.* (2007) [20] was able to store tomato pollen in liquid nitrogen upto 22 months provided its moisture content adjusted to 6.8-9.3% with results comparable to fresh pollen in terms of seed number per fruit and seed germination. After determining the optimum moisture content Karipidis and Douma (2011) [21] extended their experiment to determine the suitability of two storage temperatures (-20°C and -196°C) upto 18 months. Decline in number of seeds per fruit was observed after 10 months of storage at -20°C but at -196°C results were at par with fresh pollen. Hot pepper pollen can be stored under ambient condition (25°C) for 3 days, 6 days under deep refrigeration(-20°C) and for 37 days under liquid nitrogen (-196°) with moisture content less than 6% to get economic seed setting of 20 seeds per fruit. However seed germination showed no difference with respect to storage method (Mathad *et al.* 2015) [29]. Sugar beet pollen dessicated with anhydrous calcium chloride for 24 hours with moisture content reduced to 12% from 32% stored for 1 year under three conditions (5°C,-18°C and -196°C). Seed set reduced 26 days after storage in case of 5°C and -18°C however no further decline has been reported under -196°C, stored upto 1 year. However storage condition does not influence seed quality (Hecker *et al.* 1986) [12].

Retrieval and usage

For dessicant tolerant pollen warming rates are not important and pollen can be directly taken to room temperature. Pollen dessicated to moisture level of below 20% fresh weight basis should be rehydrated by exposing to 95 to 100% for 3 hours before germination test but may not require for pollinations.

Limitations

Erratic germination of stored samples (Nebel and Ruttle, 1937) [33] is often noticed. Sometimes pollen does not germinate well in one or two test but show adequate germination in another test. Also sometimes stored pollen for few days show more germination than fresh. It may be due to lack of uniformity in pollen sample or other unknown factors. Failure of stored sample to germinate in vitro may be due to deficiencies caused during pollen storage and germinates in vivo due to compensation by stigmatic or stylar tissues. Potato pollen stored at -13°C for 7, 12 or 13 months showed no in vitro germination but give satisfactory fruit set (King, 1955) [22]. Pollen grains showing 30-60% germination can give normal fruit set.

No information is available on cytoplasmic effects when exotic germplasm is used directly. Genetic selection during pollen storage is also issue which needs to be addressed. It is possible that gamate selection could take place altering gene frequencies but it would be problem in heterogenous population. Conceptually it is not problem in inbred line. Species and genotypic differences are higher when stored at higher temperature or RH. In case of germplasm exchange there is risk of pathogen transfer.

Conclusion

Low temperature near freezing and low relative humidity (25-35%) are ideal for storage of most of species. Storage at -20°C is beneficial for seasonal crossing but for long term pollen preservation cryopreservation is beneficial. Collection of good quality pollen is essential to obtain useful longevities. Moisture content needs to be reduced before storage to get useful longevities. However, pollen storage can improve efficiency of breeding programme and hybrid seed production. There is need to standardize the storage technique for each species. Pollen from potential parents can be stored for continuous supply for crossing desired parents, it would increase hybrid seed production overcoming seasonal and geographical barriers. Our knowledge is still lacking how different changes during pollen storage affect pollen viability and response of pollen grain to artificial conditions during storage. More research is required to test use of cryo protectants and need of more biophysical studies regarding determination of optimum moisture content, cooling rates, warming rates to increase efficiency of stored pollen.

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