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Optimized Protocol for *in vitro* Pollen Germination in Brinjal (*Solanum melongena* L.)

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ABSTRACT

Background: Understanding floral and pollen biology is essential for efficient artificial hybridization in hybrid seed production. This knowledge helps ensure proper fertilization of the ovary and optimal fruit and seed set. Selecting suitable pollen parents and preserving pollen for future use allows for better control and optimization of hybridization practices in brinjal breeding programs. To achieve this, a robust protocol for pollen collection and viability testing is necessary to monitor pollen quality in the field and during storage. This information is crucial for maintaining desired characteristics and genetic traits in the resulting hybrid seeds.

Methods: This study; aimed at optimizing the pollen germination assessment protocol for brinjal. The step wise modification standard medium composition was done, the optimal growth condition was tested and *in vivo* predictions were made. So, the study aimed to standardization of media for *in vitro* pollen germination evaluate and viability in brinjal in ambient as well as cold storage conditions. The pollen germination was tested *in vitro* as well *in vivo*, by incubating the pollen grains for 3 hours at 25°C in different concentrations of sucrose, boron and agar media and distilled water at interval of 12 hours.

Result: The most favorable germination of fresh pollen was achieved with a treatment consisting of 1% Agar, 12% Sucrose and 300 ppm Boron. Comparing the two storage conditions, pollen stored in the refrigerator exhibited higher *in vitro* germination percentages compared to ambient storage conditions. The highest *in vitro* pollen germination and viability percentages were observed in fresh pollen collected within 12 hours (95.00%). As the duration of pollen storage increased in both storage conditions, the *in vitro* pollen germination decreased. This decline in *in vitro* pollen germination was reflected in the *in vivo* fertilization fruit set percentage. The highest fruit set percentage was observed when pollen was stored in the refrigerator (64.52%) and when female flowers were pollinated with pollen stored for less than 12 hours (84.66%).

Key words: Brinjal, Germination, *In vitro*, *In vivo*, Pollen, Viability.

INTRODUCTION

According to Howlett (1936), the evaluation of pollen viability holds significant importance in the hybridization process. Numerous endogenous factors, such as the nutritional status of the plant, agricultural pesticides and chemicals, can affect the viability of pollen grains in brinjal (Mac Daniels and Hildebrand, 1939; Dubey and Mall, 1972). Pal and Singh (1943) observed that the maximum pollen fertility in brinjal occurs on the second day after flower opening. Popova (1958) further reported that brinjal pollen grains can remain viable for approximately 7-10 days.

The success of *in vitro* pollen germination is influenced by multiple factors, including plant species, nutritional status, timing of harvest, photoperiod, temperature, harvest method, incubation period and the presence of micro and macronutrients in the culture medium (Soares *et al.,* 2008). Adjusting the composition of the culture media for each species is necessary (Chagas *et al*., 2010; Sinimbu *et al*., 2011). Carbon sources, boron and other nutrients are essential for the germination of angiosperm pollen (Galleta, 1983). Thompson and Batjer (1950) highlighted that the inclusion of boron in the medium significantly enhances the germination percentage and pollen tube length. Moreover, sugar is commonly used in the culture medium to regulate osmotic balance and provide energy for pollen tube development (Miranda and Clement, 1990).

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Pollen storage is vital for maintaining viability over time, especially in crossing programs with cultivars having different flowering times. It enables streamlined plantings, eliminates the need for continuous male parent cultivation and maximizes land efficiency. Adjusting temperature, humidity and storage atmosphere extends pollen storage duration. Low temperatures and relative humidity enhance pollen viability. In brinjal hybrid seed production, assessing pollen viability and *in vitro* germination is crucial. However,

designing plant tissue culture media for this purpose is challenging due to complex interactions among factors like sucrose, agar and boron concentrations. The lack of predictability in certain response parameters is often influenced by these key media components and their impact on pollen germination in brinjal.

MATERIALS AND METHODS

Plant material

The study was conducted in field at Sagdividi Farm, Department of Seed Science and Technology, College of Agriculture, Junagadh Agricultural University, Junagadh during *kharif* 2020-2021 using randomized block design (Factorial) using parents of brinjal hybrid GJBH 4 and the lab experiment was carried out at Laboratory of Department of Genetics and Plant Breeding, College of Agriculture, Junagadh Agricultural University, Junagadh, with Male parent (JBR 03-16) of hybrid brinjal GJBH 4.

Media preparation

For *in vitro* pollen germination, in different eleven solid and liquid media containing different concentration of sucrose, boron ($H_3BO_{3)}$ and Agar media [1) 2% Sucrose + 4 ppm Boron; 2) 2% Sucrose + 8 ppm Boron; 3) 5% Sucrose + 4 ppm Boron; 4) 5% Sucrose + 8 ppm Boron; 5) 10% Sucrose + 4 ppm Boron; 6) 10% Sucrose + 8 ppm Boron 7) 0.5% Agar + 10% Sucrose + 100 ppm Boron; 8) 1% Agar + 10% Sucrose + 300 ppm Boron 9) 0.5% Agar + 12% Sucrose + 100 ppm Boron; 10) 1% Agar + 12% Sucrose + 300 ppm Boron 11) Distilled water (Control)] with pH value is 5.8. The range of boron concentration is determined by the physical state of the media. For the first six media, which are in a liquid state, the boron concentration is set within the range of 4 and 8 ppm, while for the remaining four, which are in a solid state, the boron concentration is higher, ranging from 100 and 300 ppm. Basis of fixation of boron concentration depends on solubility and uptake of boron. In liquid media, boron is more readily soluble and accessible to plants or organisms. Therefore, a lower concentration range of 4 and 8 ppm may provide adequate boron for their growth and development. In contrast, solid media may retain boron less efficiently, necessitating a higher concentration range of 100 and 300 ppm to ensure that an adequate amount is available for plant growth. As for the previous steps, solutions were autoclaved for 15 min (121°C), cooled for 30 min, pipetted into 60 mm plastic Petri dishes and stored at 4°C in a refrigerator for use by the next days. The Petri dishes containing pollen were sealed prior and during incubation using a parafilm tape. The pollen germination was in dark for all experiments (used incubators had light bulb but which automatically switched off upon closure of the incubator solid outer door).

Flower collection

Mature male flowers collected at 8:30-11:00 a.m. Collected flower keep at room ambient storage condition (~25°C) as well as cold storage condition (refrigerator temp ~3°C).

Collection of pollen by extracting anther

In vitro germination was carried out immediately followed by twelve hours interval by using of flowers stored under two different condition. Anthers were carefully removed using forceps; the collected anthers are dehisced under sunlight for about 5-10 min, depending upon the intensity of light. Dehisced pollens were subjected to *in vitro* pollen germination, in different eleven media. A total of 50 anthers were deposited in each Petri dish to ensure availability of sufficient pollen grains for scoring.

Pollen incubation

The dehisced pollen dusted on petri plate which contain a solidify media and seal the petri dishes with parafilm tape and for liquid media cavity slides (sitting drop technique) is used. Cavity slides are favored for *in vitro* pollen germination because they provide a controlled environment, simplifying the observation of germination under a microscope. They prevent contamination, ensuring accurate results and are efficient in their use of small sample volumes. The sitting drop culture method is simpler than the hanging drop method. It involves culturing of pollen grains in a drop of culture medium placed on a microslide. The culture is then maintained in a humid chamber to prevent evaporation (Shivanna and Rangaswamy, 1992). This makes them ideal for quick and reliable pollen germination studies. After that incubating the pollen for 3 hours at 25°C.

Pollen visualization and count

The pollen germination percentage was calculated under stereo-microscope (10 × magnification of Leica Laborlux K microscope with dedicated camera of model Leica EC3) and image capture by MagVision software. Outer growth of the pollen tube beyond the diameter of the pollen was assumed as germinated.

Validation of result

Female flowers bagged 4 days before hand pollination transfer of anther (stored in two condition) to the female flower using a brush and compare lab germination and fruit setting rate two weeks after pollination. Female parent flowers were enclosed in bags four days before the transfer of anthers of male parent (which were stored in two different conditions). Anthers of male parent were applied to the female flowers using a brush. Two critical outcomes were observed and compared in this experiment. Firstly, the lab germination rate was examined, which measures the success of pollen germination on the stigma and the subsequent growth of pollen tubes to reach the ovules within the female flower. Secondly, the fruit setting rate, or the rate at which successfully pollinated flowers develop into fruits, was assessed. This rate provides insight into the effectiveness of the pollination process in terms of fruit production. The observations and data collection took place over a two-week period following the pollination process. This timeframe was chosen to allow sufficient time for the progression of pollen germination and fruit development.

The experiment aims to provide insights into how the storage conditions of anthers and the method of hand pollination using a brush affect the germination and fruit setting rates in female flowers. The percentage fruit setting rate was then calculated as follows:

Fruit setting rate (%) =

$$\frac{\text{Number of fruit set}}{\text{Total number of flowers pollinated}} \times 100$$

Data analysis

The field experiment was conducted using randomized block design (Factorial) repeated thrice as per the method suggested by Cochran and Cox (1957) using the parents of Gujarat Junagadh Brinjal Hybrid 4 (GJBH 4). The observations were recorded on Fruit setting rate (%). Standard germinating medium optimize with Male parent (JBR 03-16) of hybrid brinjal GJBH 4 using completely randomized design with three replications. *In vitro* pollen germination was carried out with Male parent (JBR 03-16) of hybrid brinjal GJBH 4 at different twelve hours interval of stored pollen. Data on pollen germination were recorded from an average of three repetitions of each treatment. Mean data for pollen germination percentage were analyzed in FCRD (Cochran and Cox, 1957). The observations were recorded on pollen germination percentage.

$$\text{Pollen germination (\%)} = \frac{\text{Number of germinated pollen}}{\text{Total number of pollen}} \times 100$$

Table 1: Effects of different solid and liquid media on brinjal pollen germination.

Treatment	Pollen germination (%)
T_1 - 2% Sucrose + 4 ppm Boron	8.33
T_2 - 2% Sucrose + 8 ppm Boron	11.33
T_3 - 5% Sucrose + 4 ppm Boron	23.67
T_4 - 5% Sucrose + 8 ppm Boron	29.00
T_5 - 10% Sucrose + 4 ppm Boron	16.67
T_6 - 10% Sucrose + 8 ppm Boron	20.33
T_7 - 0.5% Agar + 10% Sucrose + 100 ppm Boron	63.67
T_8 - 1% Agar + 10% Sucrose + 300 ppm Boron	77.33
T_9 - 0.5% Agar + 12% Sucrose + 100 ppm Boron	67.33
T_{10} - 1% Agar + 12% Sucrose + 300 ppm Boron	95.00
T_{11} - Distilled water (Control)	0.00
S. Em. ±	0.67
CD	1.97
CV	3.08

RESULTS AND DISCUSSION

Effects of different solid and liquid media on brinjal pollen germination

The results presented in Table 1 and Fig 1 and 2 clearly showed that among eleven different treatments (10 media with varied concentrations of sucrose, boron and agar and one control/distilled water), the high germination of fresh pollen was recorded in media containing agar, sucrose and boron (treatment T_7 to T_{10}), of which the best germination of fresh pollen (95.00%) was recorded in treatment T_{10} (1% Agar + 12% Sucrose + 300 ppm Boron) as shown in Fig 2. A close perusal of the results of *in vitro* pollen germination (Table 1) showed that the lowest germination percentage was recorded in media with different concentration of sucrose and boron, but not containing agar and there is no pollen germination of taken place in distilled water. The result clearly indicates the importance of sucrose, boron and agar for *in vitro* pollen germination of brinjal.

The results are in accordance with the reports of Guler *et al.* (1995) and Rathod *et al.* (2018). Guler *et al.* (1995) reported that most convenient germination medium was 1 per cent agar, 12 per cent sucrose, 300 ppm H_3BO_3 and 300 ppm $Ca(NO_3)_2$ for *in vitro* pollen germination of eggplant. Rathod *et al.* (2018) compared three *in vitro* pollen germination media in *Momordica* spp. and their interspecific hybrids reported that sucrose @ 15% + Boric acid @ 0.25% + Calcium nitrite @ 300mg solution showed the maximum per cent of pollen germination and pollen tube growth. Silva *et al.* (2020) showed that the agar plays several roles in the germination medium: It promotes the solidification and the osmotic equilibrium of the culture medium, ensures a constant relative humidity and facilitates the incorporation of nutrients, aiding the formation of the pollen tube.

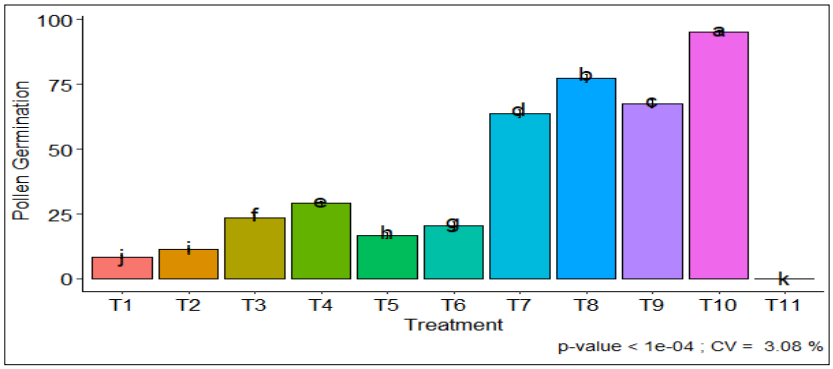

Fig 1: *In vitro* pollen germination in different media.

Influence of method and period of pollen storage on *in vitro* pollen germination of brinjal

The results presented in Table 2 and Fig 3 revealed that different method of pollen storage (M) exerted significant effect on Pollen germination (%). Significantly the highest Pollen germination (%) (74.09%) was obtained in the pollen stored in refrigerator compare to ambient condition. These results may be attributed to sufficient loss of pollen viability due to diurnal fluctuations in ambient temperature and relative humidity. Significantly the highest *in vitro* pollen germination percentage was recorded in <12 hour old pollen (93.50%) and as the duration of pollen storage increased in both the storage conditions, the *in vitro* pollen germination decreased. These results are in accordance with the findings of Islam and Khan (1998). They collected Bitter gourd pollen

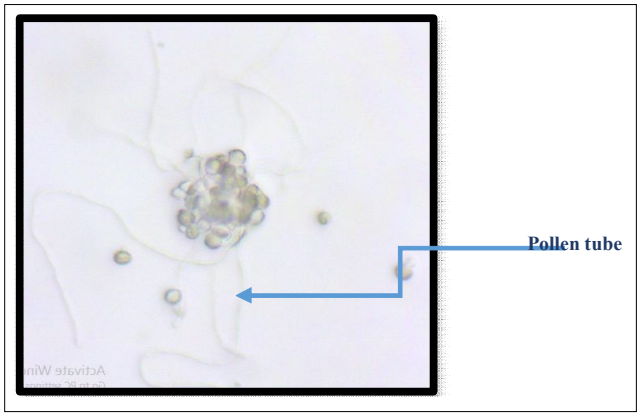

Fig 2: *In vitro* pollen germination in 1% agar + 12% Sucrose + 300 ppm boron.

Table 2: Influence of method and period of pollen storage on *in vitro* pollen germination and fruit set (%) of brinjal.

Treatments	Pollen germination (%)	Fruit set (%)
Method of pollen storage (M)		
M_1 = Pollen stored in refrigerator	74.09	64.52
M_2 = Pollen stored in ambient condition	63.19	54.81
S. Em. ±	0.30	1.02
C.D. at 5%	0.88	2.98
Period of pollen storage (P)		
P_1 = <12 hour old pollen	93.50	84.66
P_2 = 12 hour old pollen	86.83	78.17
P_3 = 24 hour old pollen	80.50	70.00
P_4 = 36 hour old pollen	69.83	61.67
P_5 = 48 hour old pollen	58.50	48.67
P_6 = 60 hour old pollen	49.16	41.33
P_7 = 72 hour old pollen	42.16	33.16
S. Em.±	0.566	1.91
C.D. at 5%	1.64	5.59
Method of pollen storage (M) × Period of pollen storage (P)		
M_1P_1	95.00	84.33
M_1P_2	92.67	80.33
M_1P_3	85.00	75.00
M_1P_4	77.00	68.00
M_1P_5	66.00	55.33
M_1P_6	56.67	47.00
M_1P_7	46.33	41.67
M_2P_1	92.00	85..00
M_2P_2	81.00	76.00
M_2P_3	76.00	65.00
M_2P_4	62.67	55.33
M_2P_5	51.00	42.00
M_2P_6	41.67	35.67
M_2P_7	38.00	24.67
S. Em.±	0.797	2.70
C.D. at 5%	2.32	NS
C.V. %	2.01	7.84

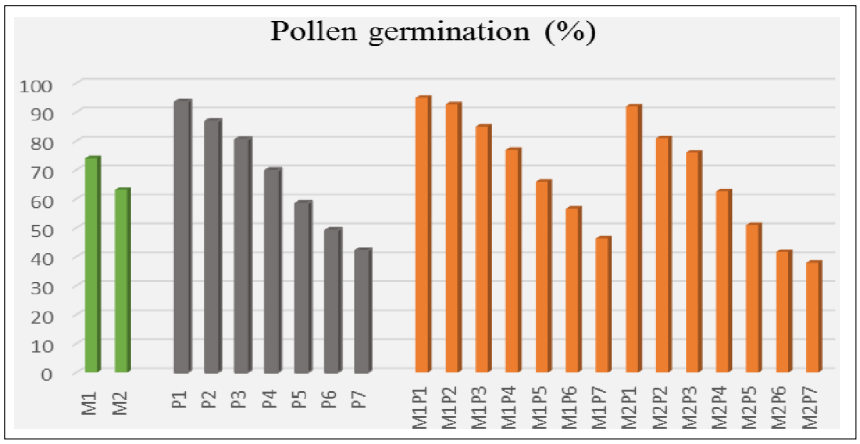

Fig 3: Influence of method and period of pollen storage on *in vitro* pollen germination of brinjal.

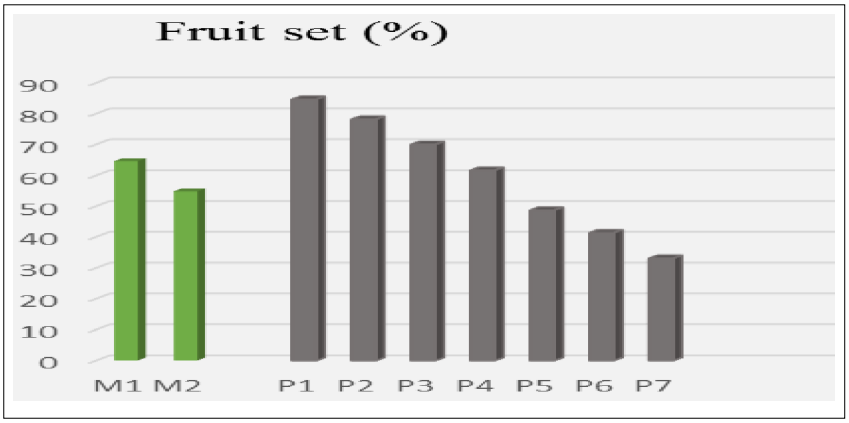

Fig 4: Influence of method and period of pollen storage on fruit set (%) of brinjal.

samples and stored at four different temperature conditions [room temperature (ranging from 27-30°C), 10°C, 0°C and -5°C for various time periods (0, 3, 6, 9, 12, 15, 25, 35, 45 and 55 days). They recommended that maintaining kakrol pollen grains at low temperatures between 0 and 10 degrees Celsius is effective for preserving them for approximately two months. Khan and Anjum (2006) noticed germination and viability of Solanum melongena L. brinjal pollen for up to 48 weeks. They tested pollen germination using different sucrose and boric acid solutions (ranging from 10% to 100%) and various storage conditions, including refrigeration, freezing, vacuum over silica gel and organic solvents (Acetone, Benzene and Chloroform). They concluded pollen stored at low temperatures (-30°C, -20°C) and freeze-dried pollen (-60°C) exhibited better germination compared to +4°C storage and fresh pollen.

Interaction effect of Method of pollen storage (M) × Period of pollen storage (P) on *in vitro pollen* germination found significant. Highest pollen germination was observed in case of pollen culture carried out at <12 hour old pollen which was pollen store in refrigerator under lower temperature (95%). Increasing the time interval between pollen collection and culture negatively affected the pollen germination ability.

Influence of method and period of pollen storage on in fruit set (%) of brinjal

To assess the accuracy of the *in vitro* pollen germination in predicting fruit set, male parent (JBR 03-16) of hybrid brinjal GJBH 4 hand pollinated at different twelve hours interval with stored pollen. The Fruit set (%) exactly followed the same declining trend with the decrease in *in vitro* pollen germination percentage. Significantly maximum fruit set percentage observed in pollen stored in refrigerator (64.52%) and female flower pollinated with <12 hour old pollen store (84.66%). And the interaction effect found non-significant on fruit set (%) which show in Table 2 and Fig 4.

Our study revealed a positive correlation between the percentage of pollen germination and the fruit set observed in the field after hand pollination. The positive relationship between *in vitro* germination and *in vivo* fruit set was also reported by a study on yam (Ng and Daniel, 2000) and other plant species (Volk, 2011).

CONCLUSION

This study showed that the brinjal pollen germination is primarily influenced by the growing conditions such as the incubation temperature, the medium viscosity and time to

use. The best germination of fresh pollen was recorded in treatment of 1% Agar + 12% Sucrose + 300 ppm Boron. A close perusal of the results of *in vitro* pollen germination showed that the lowest germination percentage was recorded in media with different concentration of sucrose and boron, not containing agar and there is no germination of pollen takes place in distilled water. Significantly the highest *in vitro* pollen germination and viability percentage was recorded in fresh pollen and as the duration of pollen storage increased in both the storage conditions, the *in vitro* pollen germination and pollen viability decreased. Of the two storage conditions of pollen, the higher *in vitro* germination percentage and viability of pollen found in pollen stored in refrigerator compared to ambient storage condition.

Conflict of interest

All authors declared that there is no conflict of interest.

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