



Optimization and Development of Pollen Germination Medium for Cultivated and Wild *Cajanus* Pollen *in vitro*

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ABSTRACT

Background: The germination of pollen and the growth of pollen tubes are preconditions for fertilization and seed development. A complete knowledge of pollen viability will help in a proper understanding of pollination and fertilization in plants. Pollen from different cultivated and wild species of *Cajanus* were used to study the influence of different medium components on *Cajanus* pollen germination. This study established a reliable medium for pollen germination in *Cajanus cajan* by modifying the Brewbacker and Kwack (BK) medium to enhance understanding of pollination and fertilization dynamics.

Methods: The *in vitro* pollen germination studies were carried out in Pre-breeding department at the International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India. We developed 108 distinct media formulations by systematically varying concentrations of key constituents: sucrose (10%, 30%, 40%), boric acid (0, 100, 250 ppm), calcium nitrate (100, 300 ppm), magnesium sulfate (100, 200, 300 ppm), potassium nitrate (100 ppm) and polyethylene glycol 6000 (15%).

Result: Optimal results were achieved with medium G, which contained 30% sucrose, 100 mg/l boric acid, 100 mg/l calcium nitrate, 100 mg/l potassium nitrate, 100 mg/l magnesium sulfate and 15% PEG, yielding 69.5% pollen germination. When tested on wild relatives (*Rhynchosia bracteata*, *Cajanus platycarpus* and *Cajanus scarabaeoides*), this optimized medium demonstrated over 70% germination efficiency with robust pollen tube development. The improved medium shows promise for facilitating inter-specific and inter-generic hybridization where biological barriers exist, potentially enabling *in vitro* pollination/fertilization approaches to introgress valuable traits for pigeonpea variety development.

Key words: Pollen germination, Pollen viability, Wild species.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] holds a distinguished position among these high-protein crops, offering numerous nutritional and agronomic advantages over other pulse crops (Saxena *et al.*, 2010). This perennial shrub belongs to the Leguminosae family, is indigenous to India and possesses a diploid chromosome number of $2n = 22$ (van der Maesen, 1990).

A distinctive feature of pigeonpea among legumes is its unique floral morphology, which facilitates both self-pollination and cross-pollination, with varying degrees of outcrossing (5-40%) observed across different environments and locations (Saxena *et al.*, 2016). This reproductive flexibility has significant implications for genetic diversity maintenance and breeding approaches in pigeonpea improvement programs (Bohra *et al.*, 2017).

The growing demand for pigeonpea necessitates substantial increases in production capacity, requiring intensive efforts to enhance the crop's yield potential (Saxena, 2008). Hybridization, including wide hybridization involving wild *Cajanus* species, is a cornerstone of these improvement efforts. Wild relatives of pigeonpea, such as *C. scarabaeoides*, serve as a valuable reservoir of genes for desirable traits like disease resistance and drought tolerance (Odeny *et al.*, 2009). Among various approaches to productivity improvement, optimizing pollination efficiency represents a promising strategy (Saxena *et al.*, 2016). Effective pollination constitutes a fundamental prerequisite

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for successful fertilization and seed set in most plant species, including pigeonpea (*Cajanus cajan*) (Saxena *et al.*, 2024). To develop rational strategies for improving pigeonpea productivity through enhanced pollination, a comprehensive understanding of pollen biology is essential, including critical aspects such as pollen viability, germination capability and pollen tube growth dynamics (Dalvi *et al.*, 2008).

Pollen and pollination studies help breeders identify cultivars that are compatible with each other because incompatibility has an impact on plant yield (Kamrani, 2012). The time it takes for pollen grains to remain viable after being released can vary greatly; in ideal conditions,

most pollen remains viable for a few days to a week, but in dry conditions, some species of pollen are extremely vulnerable to drying out, losing their viability within an hour (Shivanna and Ram 1993). Choudhary *et al.* (2011) reported that native pollen tubes have been observed to grow below the stigma relatively slowly, indicating the presence of a self-incompatibility barrier.

The germination of pollen and the growth of pollen tubes are preconditions for fertilization and seed development. *In vitro*, pollen germination is a reliable method to test the viability of pollen. Additionally, *in vitro* pollen tube growth is necessary to assess pollen grain germinability. *In vitro*, pollen germination and pollen tube growth experiments are very insightful for clarifying the absence of fertility. Therefore, a pollen germination study is needed to diagnose the crossability barriers. The most widely used basal medium for such studies is the one developed by Brewbaker and Kwack (1963), hereafter referred to as BK medium. However, the nutritional and osmotic requirements for optimal pollen germination are highly species-specific and often genotype-dependent (Soriano and Boutilier, 2013). For pigeonpea, previous studies have reported significant challenges, most notably the profuse bursting of pollen grains and tubes in standard liquid media, which necessitates specific modifications (Reddy *et al.*, 2025).

These studies have received significant knowledge on the physiology and biochemistry of pollen germination and pollen tube growth. Particularly involving specific species or genera, the evaluation of pollen viability is very significant in artificial pollination. Pollen germination medium varies from species to species and even for different varieties of the crop. Linskens (1970) used basic sucrose / boric acid media and other complex media were later reported by adding polyethylene glycol and various amino acids. A commonly used medium and considered suitable for over 86 species comprising 39 plant families have been developed (BrewBaker and Kwack, 1964). The cultivated pigeonpea has a variety of diverse wild species in the genus *Cajanus* which includes 32 species. Pigeonpea *in vitro* pollen germination has been previously documented and a cumulative germination rate of 48% has been recorded (James *et al.*, 1987 and Singh *et al.*, 1992) which is quite low.

Therefore, a suitable medium for improving the pollen germination frequency and the pollen tubes' consistency that grow *in vitro* must be established. This study aimed to develop an appropriate medium for the *in vitro* germination of pollen in cultivated and wild pigeonpea.

MATERIALS AND METHODS

Cajanus cajan var ICPL 85010 which is popular cultivated species has been used to germinate pollen in present study. Further two wild species, *C. scarabaeoides* from secondary genepool and *C. platycapus* from tertiary genepool has been used. The seed material of wild species were procured from gene bank unit of International Crops

Research Institute for the Semi-Arid Tropics, Hyderabad, India during 2021. The *in vitro* pollen germination studies were carried out in Pre-breeding Department at the International Crops Research Institute for the Semi-arid Tropics, Hyderabad, India. Pollen grain was regarded as germinated if the pollen tube's size exceeded the pollen grain diameter (Tunistra and Wedel, 2000).

Two drops of media were placed on a glass slide. Pollen grains extracted from freshly dehiscent anthers was dusted on the germination medium and covered with cover slips. Pollen grains were recorded in the medium, which had germinated and produced pollen tubes. The length of the pollen tubes in the medium was measured 12 hours after germination began and the percentage of pollen germination was calculated and the average pollen tube length was recorded. Germination percentage was estimated by dividing the number of germinated pollen grains per field of view by the total number of pollens per field of view and expressed as a percentage (Kearns and Inouye, 1993). Pollen tube lengths were measured using an ocular micrometer fitted to the eyepiece of an Olympus microscope. Mean pollen tube length was calculated from each slide as the average of 10 pollen tubes' length. For statistical analysis of data, we used one-way ANOVA analysis and Tukey's tests ($P < 0.05$) to compare differences between treatments.

Brewbaker and Kwack's medium

Brewbaker and Kwack medium (Brewbaker and Kwack, 1964) was used (10% sucrose, 100 mg/l, boric acid, 300 mg/l calcium nitrate, 200 mg/l magnesium sulfate and 100 mg/l potassium nitrate) as the base medium for pollen germination experiments. One hundred eight key media was developed by altering the concentration of the media constituents. The media consists of 10%, 30%, 40% sucrose, 0 ppm, 100 ppm, 250 ppm boric acid, 100 ppm and 300 ppm calcium nitrate, 100 ppm, 200 ppm and 300 ppm magnesium sulfate, 100 ppm potassium nitrate and 15% polyethylene glycol 6000. Out of which 18 (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q and R) medium shows pollen germination of 7 to 69.5%. The best three medium was then used in subsequent studies and further improved by altering sucrose concentrations. Since the liquid medium gave inconsistent results, 1% agar was added in all the media used.

The percentage of pollen germination was calculated by the following method

% pollen germination =

$$\frac{\text{No. of germinated pollen grains}}{\text{No. of germinated and ungerminated pollen grains}} \times 100$$

Statistical analysis

The experiments were conducted in a completely randomized design. Data were subjected to Analysis of Variance (ANOVA) and treatment means were compared using Tukey Test at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of studies on *in vitro* pollen germination of *Cajanus cajan* are summarized in following headings.

In vitro pollen germination and pollen tube growth

The impact of various media on pollen germination and pollen tube growth was accomplished in *Cajanus cajan*. Pollen germination study was examined at different concentrations of sucrose and Brewbaker and Kwack's medium.

On changing sucrose concentration, boric acid, calcium nitrate and magnesium sulfate showed 7-69.5% improved pollen germination. However, pollen and pollen tube

bursting was a common feature in all these media used. Pollen germinated significantly $p < 0.001$ better in medium G compared to other media used. Pollen from medium C, H, I, L, N, P, Q and R had lower germination percentage without significant differences in germination among media (Fig 1). The agarified sucrose medium increased the germination percentage than the liquid medium.

A maximum of 69.5% pollen germination was observed in a medium (G) containing 30% sucrose, 100 mg/l boric acid, 100 mg/l calcium nitrate, 100 mg/l potassium nitrate and 100 mg/l magnesium sulfate and 15% PEG. The other combinations showed lesser pollen germination and more bursting. The addition of PEG improved pollen germination.

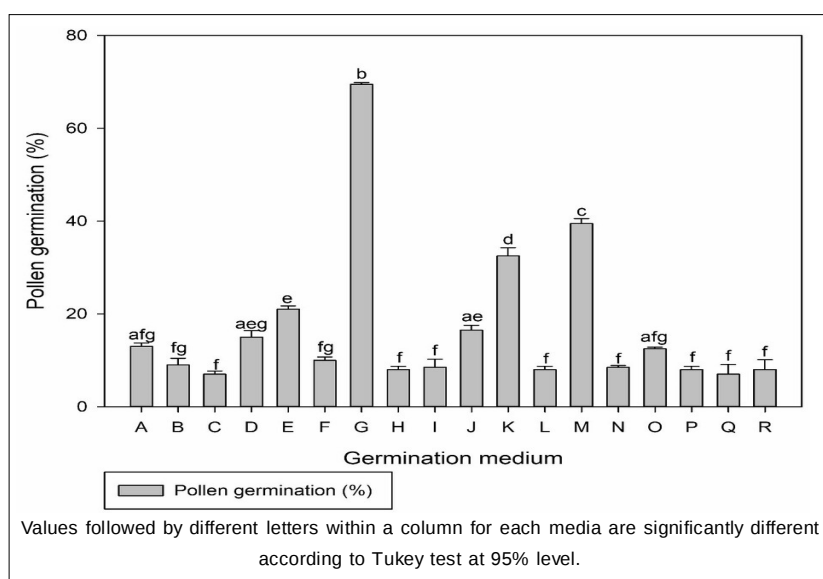


Fig 1: Pollen germination % shown by ICPL 85010 under different media.

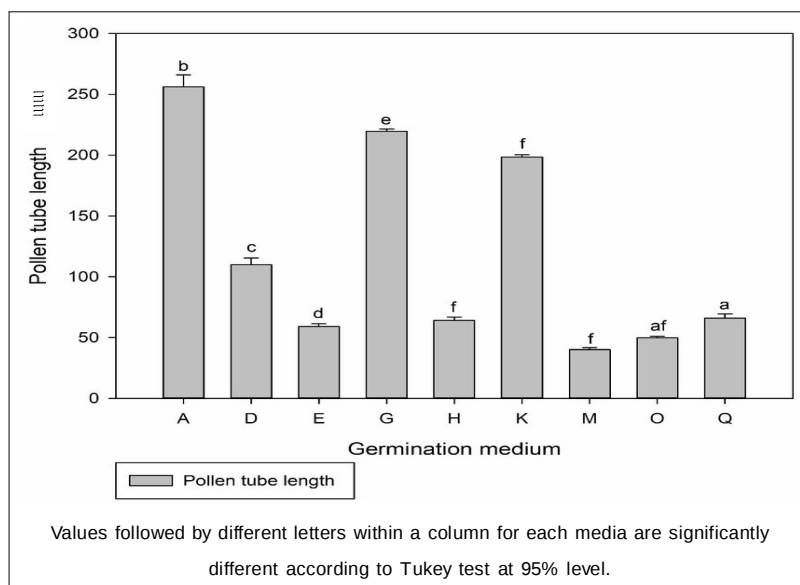


Fig 2: Pollen tube length (mm) observed in ICPL 85010 under different media.

Pollen tube growth

Pollen tubes from medium A were significantly ($p < 0.001$) longer than those in the other mediums. Pollen from medium H, I, L, N, O and Q had shorter pollen tubes than those from other media without significant pollen tube lengths among treatments (Fig 2 and 3).

The higher pollen tube length was observed in solid sucrose media than that of liquid media. A smooth, slender pollen tube was observed under solid sucrose media. Pollen tube was busted as soon as when it starts to germinate in liquid media. Similarly, budding and irregular pollen tube was also observed in case media devoid of boric acid. At higher concentration of sucrose also showed lesser germination percentage and rough pollen tube.

At 25°C, the medium G (30% sucrose, 100 mg/l calcium nitrate, 100 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 100 mg/l boric acid and 15% PEG) showed the maximum pollen germination and less pollen tube bursting. The pollen of *Cajanus cajan* exhibited maximum germination over 69% and a mean pollen tube length of 219.7 μm in a 1% agar medium (Table 1).

Hence this key medium was selected for further modification. Different combinations of the constituent's sucrose, boric acid and calcium nitrate were essayed to enhance the pollen germination.

At 25°C, the medium A2 (37% sucrose, 100 mg/l calcium nitrate, 100 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 100 mg/l boric acid and 15% PEG) showed maximum germination of 48.1 % and less pollen tube bursting with mean pollen tube length 123.4 μm (Table 2).

At 25°C, the medium B1 (30% sucrose, 100 mg/l calcium nitrate, 100 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 250 mg/l boric acid and 15% PEG) showed maximum germination of 39.5% and less pollen tube bursting with mean pollen tube length 40 μm (Table 3).

At 25°C, the medium B2 (37% sucrose, 100 mg/l calcium nitrate, 100 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 250 mg/l boric acid and 15% PEG) showed maximum germination of 38% and less pollen tube bursting with mean pollen tube length 49.8 μm (Table 4).

At 25°C, the medium C1 (30% sucrose, 300 mg/l calcium nitrate, 200 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 100 mg/l boric acid and 15% PEG) showed maximum germination 32.5% and less pollen tube bursting with mean pollen tube length 198.5 μm (Table 5).

At 25°C, the medium C2 (37% sucrose, 300 mg/l calcium nitrate, 200 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 100 mg/l boric acid and 15% PEG) showed

Table 1: Germination percentage and pollen tube length in the medium G.

Optimal media composition	<i>C. cajan</i> vars ICPL 85010
Sucrose	30
Boric acid (ppm)	100
Calcium nitrate (ppm)	100
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	100
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25
% pollen germination	69.5
Pollen tube length (μm)	219.7 $\pm$ 1.88

Table 2: Germination percentage and pollen tube length in the medium A2.

Optimal media composition	<i>C. cajan</i> vars ICPL 85010
Sucrose	37
Boric acid (ppm)	100
Calcium nitrate (ppm)	100
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	100
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25
% pollen germination	48.1
Pollen tube length (μm)	123.4 $\pm$ 2.8

Table 3: Germination percentage and pollen tube length in the medium B1.

Optimal media composition	<i>C. cajan</i> vars ICPL 85010
Sucrose	30
Boric acid (ppm)	250
Calcium nitrate (ppm)	100
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	100
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25
% pollen germination	39.5
Pollen tube length (μm)	40 $\pm$ 1.55

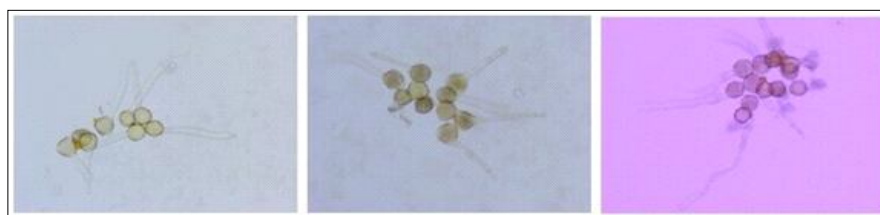


Fig 3: ICPL 85010 showing pollen germination under different media.

maximum germination of 33% and less pollen tube bursting with mean pollen tube length 59 μm (Table 6).

Bursting of pollen was observed at 10%, 30%, 40% sucrose solution devoid of boric acid. Pollen tubes exhibited irregularities such as coiling and bursting, in the absence of boron. Related findings were reported by previous workers (Shivanna *et al.*, 1997). An increase in the concentration of sucrose caused a corresponding decline in pollen germination. The addition of boric acid facilitates the pollen germination. Pollen germination was also checked at different sucrose concentrations combined with 100 and 250 ppm boric acid, germination percentage increased.

Pollen tube growth

Pollen tube length was more or less seen compared to the pollen germination level. At 30% sucrose + 100 mg/l boric acid, the pollen tube's length was recorded to be 219.7 μm , while a 40 μm long pollen tube was observed at 30% sucrose + 250 mg/l boric acid (Table 7).

Standardization of pollen germination media for Wild *Cajanus* Species

The above best media was further tested in wild *Cajanus* viz *Rhynchosia bracteata*. At 25°C, the medium G (30% sucrose, 100 mg/l calcium nitrate, 100 mg/l magnesium sulfate, 100 mg/l potassium nitrate, 100 mg/l boric acid and 15% PEG) showed maximum germination 74% and less pollen tube bursting with mean pollen tube length 198.16 μm (Table 8 and Fig 4, 5 and 6).

Brewbaker and Kwack's medium (Brewbaker and Kwack, 1964) were used in the present study with some mild modifications for the *in vitro* pollen germination of pigeon pea. PEG 6000 and agar were also used to complement the pollen germination media. Good germination was obtained in agar solidified media G (Jayaprakash, 2018). This media was further tested in wild *Cajanus* species and found effective. Among the wild *Cajanus* species, *Rhynchosia bracteata*, a tertiary gene pool species showed good germination with more pollen tube length. Studies on *in vitro* pollen germination in pigeonpea (*Cajanus cajan*) have demonstrated that agar-solidified media promote the development of straighter and more slender pollen tubes compared to liquid media alternatives (Brewbaker and Kwack, 1963; Jayaprakash and Sarla, 2001). This morphological characteristic is significant for assessing pollen vigor and predicting potential fertilization success.

In pollen germinability assays conducted *in vitro*, sucrose commonly serves as an energy source across numerous plant species, as it typically stimulates both germination initiation and subsequent tube elongation (Taylor and Hepler, 1997). Sucrose functions not only as an energy substrate but also contributes to maintaining appropriate osmotic balance in the germination medium (Shivanna and Rangaswamy, 1992).

A key finding from comparative media studies is that sucrose-containing solid germination medium (agar-

Table 4: Germination percentage and pollen tube length in the medium B2.

Optimal media composition	<i>C.cajan</i> vars ICPL 85010
Sucrose	37
Boric acid (ppm)	250
Calcium nitrate (ppm)	100
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	100
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25.8
% pollen germination	38
Pollen tube length (μm)	49.8 $\pm$ 1.15

Table 5: Germination percentage and pollen tube length in the medium C1.

Optimal media composition	<i>C.cajan</i> vars ICPL85010
Sucrose	30
Boric acid (ppm)	100
Calcium nitrate (ppm)	300
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	200
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25
% pollen germination	32.5
Pollen tube length (μm)	198.5 $\pm$ 1.89

Table 6: Germination percentage and pollen tube length in the medium C2.

Optimal media composition	<i>C.cajan</i> vars ICPL 85010
Sucrose	37
Boric acid (ppm)	100
Calcium nitrate (ppm)	300
Potassium nitrate (ppm)	100
Magnesium sulfate (ppm)	200
PEG 6000 (%)	15
Agar (%)	1
Incubation temperature (°C)	25
% pollen germination	33
Pollen tube length (μm)	59 $\pm$ 2.36

Table 7: Mean pollen tube length (μm) of *Cajanus cajan* var. ICPL 85010 in different media.

Concentration of	Boric acid	Calcium nitrate
Mean pollen tube length ($\pm$ SE)	(mg/l)	(mg/l)
100	100	219.7 $\pm$ 1.88
100	300	198.5 $\pm$ 1.89
250	100	40 $\pm$ 1.55
250	300	40.3 $\pm$ 0.97

30% sucrose + 15 % PEG + 1 % agar constant for all media.

based) appears to be optimal for evaluating both pollen germinability and vigor (as measured by pollen tube length) in pigeonpea compared to sucrose-containing liquid media formulations (Saxena *et al.*, 2018). This optimization of

germination conditions enables more reliable assessment of pollen quality parameters, which is essential for breeding programs aimed at improving pollination efficiency and seed set in pigeonpea (Saxena *et al.*, 2000).

Table 8: Optimal media composition for wild *Cajanus* species.

Optimal media composition	<i>Rhynchosia bracteata</i>	<i>Cajanus platycarpus</i>	<i>Cajanus scarabaeoides</i> (ICPW 281)
Sucrose	30	30	20
Boric acid (ppm)	100	100	100
Calcium nitrate (ppm)	100	100	100
Potassium nitrate (ppm)	100	100	100
Magnesium sulfate (ppm)	100	100	100
PEG 6000 (%)	15	15	15
Agar (%)	1	1	1
Incubation temperature (°C)	25	25	25
% pollen germination	74%	72%	76%
Pollen tube length (μm)	198.16 ±6.14	76.45 ±5.79	112 ±1.86

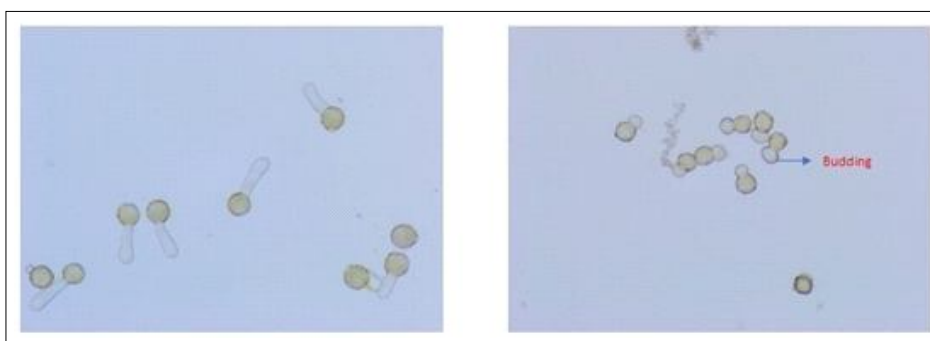


Fig 4: *C. platycarpus* showing germination in different media.

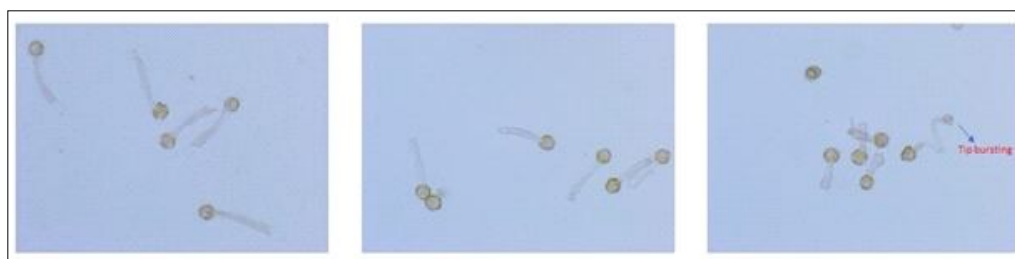


Fig 5: *Cajanus scarabaeoides* showing pollen germination.

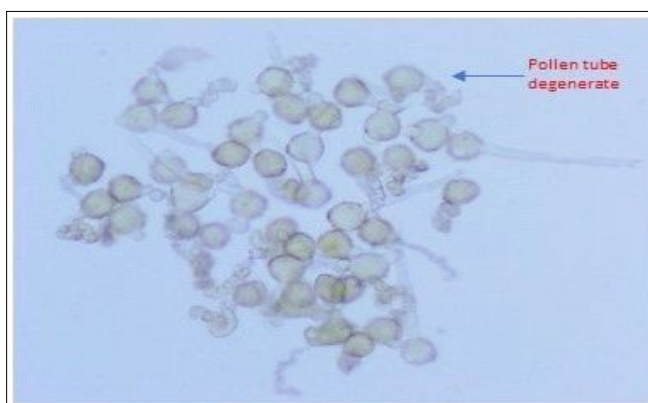


Fig 6: *Rhynchosia bracteata* showing pollen germination.

This is in agreement with the result obtained by Stadler *et al.* (1999) who claimed that sucrose was the only carbohydrate that supports *Arabidopsis* pollen's growth. Sucrose has the function in maintaining the osmotic pressure of the germination medium to provide enough moisture for enhance germination without rupturing pollen tubes and acting as a substrate for pollen metabolism. In conclusion, there was a significant influence of the germination medium on the pollen germinability and vigor. The liquid medium showed profuse pollen bursting due to quick hydration (Tushabe and Rosbakh, 2021).

Tip bursting was a common phenomenon observed in different media. Some pollen also showed budding, which is considered as ungerminated. The germination percentage tends to be decreased at higher concentration of sucrose (Shi *et al.*, 2023). Previous liquid medium usage to germinate pigeonpea pollen has been reported for *C. cajan*, but only 48.7% pollen germinated (James *et al.*, 1987). Singh *et al.* modified the medium by adding agar and achieved overall pollen germination of 43.1% (Singh *et al.*, 1992; Ginoya *et al.*, 2025). A set of media with PEG and agar were tested in the present study. In these media, germination with profuse pollen and pollen tube bursting was observed at 32.5-69.5 per cent. Medium G showed less pollen tube bursting. PEG addition has improved the germination percentage in pigeonpea. At higher concentrations of boric acid pollen tube length was shortened (Johnston *et al.*, 2005; Shi *et al.*, 2023). Another legume, chickpea, has also found it useful (Shivanna *et al.*, 1997). Medium G showed less pollen tube bursting. This study serves as a valuable reference for breeders seeking to create variability in pigeonpea through wide crossing and developing superior cultivar with higher productivity and resistance to diseases and pests.

CONCLUSION

To meet the germination necessity of pollen, a simple or complex medium can be developed. The proposed protocol would provide better guidance in developing a pollen germination medium. The author has standardized the pollen germination medium for cultivated *Cajanus cajan* vars. ICPL 85010. Further, the medium was standardized in wild species, used to test the pollen germination in wild *Cajanus*. Besides the addition of B.K. salts, these media have been reinforced with polyethylene glycol and agar. A lot of intraspecific variabilities occurs in the entire pigeonpea gene pool. This PGM can be applied in many different ways. Intra-specific heterogeneity based on a pollen germination medium can help to select appropriate hybridization accessions. It can aid *in vitro* pollination/fertilization attempts to introgress useful traits and subsequently developing suitable variety.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

Authors do not have any conflict of interests to declare.

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