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Research Article

Cytological investigation on pre and post fertilization barriers in interspecific cross of pigeonpea

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Abstract
The research deals with cytological investigation on pre and post-fertilization barriers, pollen fertility assessment and *in vitro* pollen germination in *Cajanus cajan* (CO 8) and *C. cajanifolius*. To determine the fertility of the species potassium iodide test was done. *Cajanus cajan* (CO 8) and *Cajanus cajanifolius*, were found to have fertility more than 97 per cent. Through *in vitro* pollen germination, it was ascertained that *Cajanus cajan* (CO 8) and *Cajanus cajanifolius* were found to be equally viable. Pollen-pistil interaction in wide crosses was analysed by fluorescent microscopy. At 2 HAP, higher pollen germination percentage was found in direct (83.6%) and reciprocal crosses (81.2%). At 24 HAP, lower pollen tube growth percentage was found in direct (46.7%) and reciprocal crosses (26.8%). In direct cross, pod set per cent was found to be less, indicating post-fertilization barrier. In reciprocal cross, there was no pod set, which indicates pre-fertilization barrier.

Keywords: Fluorescence microscopy, *C. cajan, C. cajanifolius,* Aniline blue technique, pollen pistil interactions

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp] is one of the most crucial pulse crops grown under rainfed, tropical and subtropical climate conditions. It belongs to the Leguminosae family, Papilionidae subfamily, Phaseolae tribe, and Cajaninae subtribe (Ghadge *et al.,* 2008). Pigeonpea has 32 species, with *Cajanus cajan* being the sole cultivated one (van der Maesen, 1986). India is the largest producer of pulses, of which pigeonpea is the most widely grown legume after chickpea, covering an area of 47.24 lakh hectares, with an average annual production of 43.16 lakh tonnes and productivity of 914 kg/ha (*www.indiastat.com*). The area, production and productivity of pigeonpea in Tamil Nadu is 0.47 lakh ha, 0.50 lakh metric tonnes and 1049 kg/ha, respectively during 2020-21 (*www.indiastat.com*). According to Van der Maesen (1990), the pigeonpea gene pool is partitioned into three groups. Most of the cultivars are in the primary gene pool, closely related wild species are in the secondary gene pool, and non-crossable wild species are in the tertiary gene pool. Despite great progress in pigeonpea breeding for yield and other agronomic qualities, more work needs to be done in terms of resistance to pod borer, fusarium wilt, phytophthora blight, and abiotic stress such as salinity, water logging and drought. *Cajanus cajan*, a cultivated species, has a lot of margins for genetic improvement by introducing desirable genes from wild species (Balarama and Padmaja, 2002). Distant or wide hybridization is the principal method

of expanding genetic variability (Thiruvengadam and Muthiah, 2007). Realizing that *C. cajan* is the sole cultivated species, it makes sense to look for more variability in the wild and related *Cajanus* species. Several genes from different wild relatives were successfully introgressed into *C. cajan* (Pundir and Singh, 1985; Kumar *et al.,* 1990; Mallikarjuna and Moss, 1995). *C. cajanifolius* has been revealed as a substantial resistance source against pod fly (Sithanantham *et al*., 1981) and Alternaria leaf spot disease (Singh *et al.,* 1984).

Crossability of wild pigeonpea with cultivated one is limited because of difficulties in creating viable hybrids, different degrees of compatibility and hybrid sterility. These difficulties have to be overcome for the successful hybridization and pod set to occur. The focus of this research is to assess the pollen fertility and role of pollen-pistil interactions as a reproductive barrier in direct and reciprocal crosses of *Cajanus cajanifolius* and *Cajanus cajan* (CO 8).

MATERIALS AND METHODS

Seed material of *Cajanus cajan* (CO 8) and *Cajanus cajanifolius* (ICP 15629) were collected from the Department of Pulses, Tamil Nadu Agricultural University, Coimbatore and raised in specific rings filled with red soil at the wild species garden, Tamil Nadu Agricultural University, Coimbatore (**Table 1**). The crop was grown by recommended package of practices.

Pollen fertility assessment was done mainly to analyse the pollen-pistil interaction's role in reproductive isolation of cultivated and wild species. The pollen fertility study was done by collecting the anthers from ten freshly opened flowers around 8.00 to 10.00 AM and taken to the laboratory immediately for microscopic observations. Potassium Iodide test (0.1%) (Baker and Baker, 1979) was performed to assess the pollen fertility. Pollen grains from anthers were suspended and positioned on a glass slide with a drop of 0.1% potassium iodide solution. Then it was visualized under a fluorescent microscope (Nikon Eclipse Ni-U, Japan) with a Nikon filter (330–380 nm excitation filter, 410 nm barrier filter). The score was taken from five microscopic fields. Images were recorded with a Nikon DS-Fi3 camera and processed using the NIS Elements F v.4.60.00 image processing platform with 10 X magnification. The deeply stained were considered as fertile, pollen which were not stained considered as sterile. Pollen fertility percentage was calculated by the below formula:

$$\text{Pollen fertility \%} = \frac{Number\ of\ pollen\ stained}{Total\ number\ of\ pollen\ obsereved} \times 100$$

In vitro pollen germination is extensively used technique to test the viability of pollen grains. Brewbaker and Kwack (1963) medium was employed with some modifications **(Table 2).** Cavity slide technique is used for studying the pollen germination of the selected species. Anthers with well-developed pollen grains were collected from the two species. A droplet of pollen germination media, with a drop of aniline blue stain (to differentiate between germinated and non-germinated pollen) were taken in a cavity slide. Pollen grains were dusted on the media and cover slip was kept on the top and sealed with vaseline. The slide was placed inversely on petriplate containing distilled water and left for incubation. The observations were recorded at 10 minutes interval after incubation (Jayaprakash, 2018).

Pigeonpea is an often-cross pollinated crop. Anthers encircle the stigma in a fully grown bud and dehisce a day before the flower opens. The peak anthesis time was recorded between 9:00 -10:00 hours (Sharma *et al*., 1980). During crossing, flower buds from tagged plants of wild donor species were collected and pollen grains were dusted on female parent. To identify artificially pollinated buds a small, bright coloured, thin nylon thread was tied to the pedicel.

For microscopic study, ten pistils were collected, in an interval of 2, 4, 6, 10, and 24 hours after pollination (HAP) from both selfed and cross-pollinated flowers of tagged plants. The pistils were isolated under laboratory condition. The isolated pistils were kept in a fixative of glacial acetic acid: alcohol (1:3 v/v) for 12 hours. Then transferred to 70% ethanol and preserved under refrigerated (4 °C) condition until further processing of the samples (Sogo and Tobe, 2006). The pistils were softened overnight in 10 N NaOH, followed by staining them for 3 hours in 0.1 per cent (w/v) aniline blue containing 0.1 M K_3PO_4. The stained pistil was mounted on microscope slides using 50 % glycerol (Sitch, 1990) for the proper vision. The slides were examined using a Nikon filter (330–380 nm excitation

Table 1. Cajanus species used in study

S. No	Species	Chromosome number	Desirable attributes
1.	*Cajanus cajan* (CO 8)	2n=22	High yielding, long duration, resistant to sterility mosaic disease, resistant to root rot and moderately tolerant to pod borer complex.
2.	*Cajanus cajanifolius*	2n=22	Donor for Cytoplasmic male sterility, high seed protein (> 30%), resistant to alternaria blight

Table 2. Different media used for *in vitro* pollen germination study

Media	Sucrose per cent used	Boric acid (mgl^{-1})	Calcium nitrate (mgl^{-1})	magnesium sulphate (mgl^{-1})	potassium nitrate (mgl^{-1})
A	10	100	300	200	100
B	20	100	300	200	100
C	30	100	300	200	100

filter, 410 nm barrier filter) and a fluorescent microscope (Nikon Eclipse Ni-U, Japan). Images were taken with a Nikon DS-Fi3 camera and processed using the NIS Elements F v.4.60.00 image processing platform.

A statistical tool package, STAR 2.0 was used to analyse the data, which is developed by IRRI, Philippines. By examining pollen features and pollen fertility per cent, t-test was used to assess the significant difference between two species. Arc-sine transformation and Least Square Difference (LSD) were used to compare significant variation in pollen germination percentage of selfed, crossed pistils and *in vitro* pollen germination of *Cajanus cajan* (CO 8) and *C. cajanifolius*.

RESULTS AND DISCUSSION

On the basis of potassium iodide test, *Cajanus cajan* (CO 8) showed a pollen fertility of 97.9 per cent (**Table 3, Fig.1a and 1b**) and *Cajanus cajanifolius* showed 97.6 per cent of pollen fertility. The pollen showed maximum fertility only when the standard petal is fully emerged (Rajagopal *et al*., 2021). By calculating t-test, there was no significant difference observed between wild and cultivated species (**Table 3**). Hence, both the species showed a high fertility of more than 97 per cent and utilized for *in vitro* pollen germination.

To study pollen germination, flower buds were taken in a fully matured state and analysed. To standardize,

Table 3. Assessment of pollen fertility in pigeonpea species

Particulars	Pollen fertility by Potassium Iodide test (%)	
	Cajanus cajanifolius	*Cajanus cajan* (CO 8)
Mean	97.64	97.99
Variance	3.00	0.28
t-stat	0.38*	
P value	0.36	
t-critical value	2.44	

*Significant at 0.05 level.

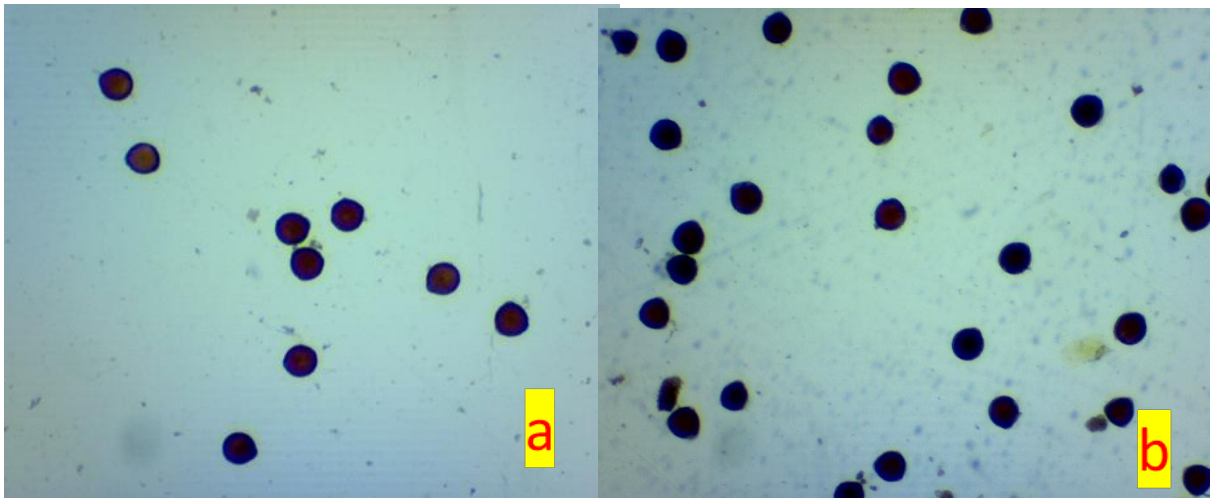

Fig.1a. Pollen fertility assessment of *Cajanus cajanifolius* (Magnification 10X) by Potassium Iodide test

Fig.1b.Pollen fertility assessment of *Cajanus cajan*-CO 8 (Magnification 10X) by Potassium Iodide test

different concentration of sucrose were employed, while the remaining components kept constant. At 10% sucrose (medium A), *Cajanus cajanifolius* showed initiation of pollen germination with high level of pollen bursting. When increased to 20% (medium B) less pollen bursting was observed, with good pollen germination and higher mean pollen tube length. Similarly, *Cajanus cajan* (CO 8) was first tested with 20% sucrose, which showed the commencement of pollen tube growth with high pollen bursting. Then increased to 30% (medium C), resulted in good pollen germination and higher mean pollen tube length (**Table 4 and Fig.2**). Finally, *Cajanus cajanifolius* responded well in medium B and *Cajanus cajan* (CO 8) in medium C. Similar results were observed by Jayaprakash (2018) in pigeonpea.

Table 4. *In vitro* pollen germination studies in *Cajanus cajanifolius* and *Cajanus cajan* (CO 8)

Species under study	Media	Concentration of sucrose (%)	Concentration of Boric acid (mg1^{-1})	Concentration of Calcium nitrate (mg1^{-1})	Pollen germination (%)	Mean pollen tube length (µm)
Cajanus cajanifolius	A	10	100	300	46.79±3.66 $(43.1)^c$	32.76±1.41 $(34.9)^b$
Cajanus cajanifolius	B	20	100	300	96.63±2.33 $(79.6)^a$	43.21±2.56 $(41.0)^a$
Cajanus cajan (CO 8)	C	30	100	300	60.89±1.96 $(51.2)^b$	15.76 ± 1.35 $(23.3)^c$
					CV=2.714	CV=1.428
					CD (0.05) =2.522	CD (0.05) = 0.757

CV- Coefficient of Variation; CD - Critical Difference; Figures in parenthesis are arc-sine transformed using Least Square Difference at 5% level of significance

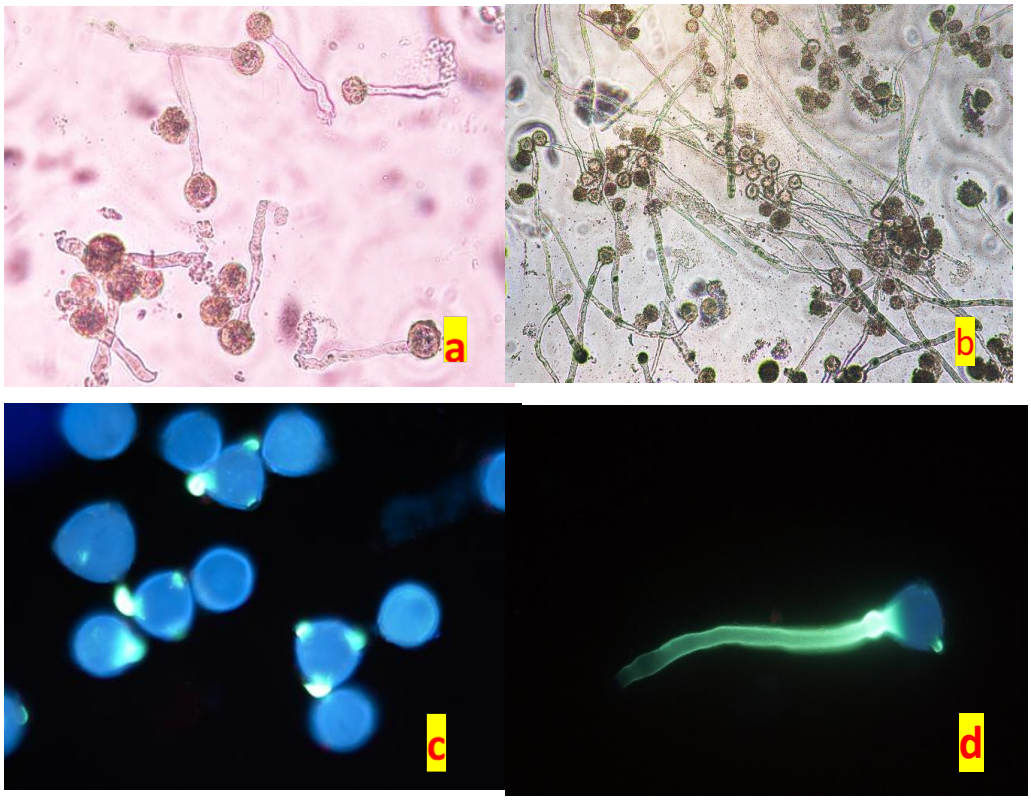

Fig. 2. Photomicrograph showing *in vitro* pollen germination of *Cajanus cajan* (CO 8) and *Cajanus cajanifolius*. a. Pollen showing initiation of germination b. Medium B with long irregular pollen tubes showing pollen tube bursting at tips c. Pollen germination in medium A by using 0.1% aniline blue staining after 10 minutes d. Pollen with good pollen tube growth by using 0.1% aniline blue staining after 30 minutes in *Cajanus cajan* (CO 8) in medium

Duration of exposure of pollen grains to the media also plays a key role in determining the viability. In general, pollen germination was detected only after 10 minutes of incubation. Pollen tube growth and minimal pollen bursting were seen after 20 minutes of incubation. At 30 minutes of incubation, the pollen tube reached its maximum length and stops its growth, with very little pollen burst. Therefore, 30 minutes of incubation was found to be ideal to determine the maximum length of pollen germination. Based on the above study, there was a significant difference (5% level of significance) between different pollen germination media and mean pollen tube growth. It was concluded that, pollen grains of both *Cajanus cajanifolius* and *Cajanus cajan* (CO 8) were viable at different media concentration.

Pollen-pistil interaction between *Cajanus cajan* (CO 8) and *Cajanus cajanifolius* was studied by performing direct and reciprocal crosses (**Table 5**). In the selfed flowers of *Cajanus cajan* (CO 8) and *Cajanus cajanifolius*, a large number of pollen grains were germinated on the stigmatic surface at 2 HAP (**Table 6**). At 24 HAP, pollen tubes reached the ovule through the stylar region with germination percentage of 86.7 and 75.5 per cent for *Cajanus cajan* (CO 8) and *C. cajanifolius,* respectively, (**Fig.3**). Based on the data presented above, it can be stated that *Cajanus cajan* (CO 8) had a higher pollen tube growth percentage than *Cajanus cajanifolius*. In the direct cross between *Cajanus cajan* (CO 8) and *Cajanus cajanifolius*, the maximum pollen germination percentage was observed on the stigmatic surface at 2 HAP (**Fig.4**). Enough pollen tubes reached ¼ th part of stylar region, indicating 88.5 per cent pollen tube growth at 6 HAP. At 24 HAP, only few pollen tubes reached the ovule with pollen tube growth percentage of 46.7 per cent (**Table 6**). In this cross, 0 to 8.6 per cent of pod set was obtained.

In the reciprocal cross between *Cajanus cajanifolius* and *Cajanus cajan* (CO 8), there was an evidence of pollen germination on stigmatic surface at the early stage, with the greater number of pollen tubes reached upto ¼ th part of stylar region (81.20%) in 6 HAP (**Table 6**). However, pollen tube growth was halted because it took 10 HAP to reach the mid stylar region. There was no further development in the stylar tissues and the pollen tubes stopped in the same position even after 24 HAP (**Fig.4**). It never reached the ovule in any of the pistils studied. Reciprocal cross incompatibility between the species might be the reason for pollen tube not reaching the ovule. Hence, no pod set was observed despite the fact that both species had the same chromosomal number (2n = 22). Similar results were reported in inter specific crosses between wild and cultivated species of sesame (Sruthi *et al*., 2021).

In wide crosses, the successful pollination and fertilization include complicated and harmonious interactions between the microgametophyte and the sporophyte of the pistil parent. Correct pollen tube growth, adequate identification of protein substances, pollen tube penetration on the

Table 5. Pollen-pistil interaction in direct and reciprocal crosses of pigeonpea

Cross combinations	Numberof buds pollinated	Numberof pods set	Pod set (%)
Direct			
CO 8 x *C. cajanifolius*	132	12	8.6
Reciprocal			
C. cajanifolius x *C. cajan*	87	-	-

Table 6. *In vivo* pollen germination studies at different time intervals

Parents /cross	Time interval and pollen germination (%)				
	2 HAP	6 HAP	10 HAP	24 HAP	Mean
Cajanus cajan (CO 8)	96.4	92.6	93.5	86.7	92.3 $(73.9)^a$
Cajanus cajanifolius	92.3	91.1	82.4	75.5	85.3 $(67.4)^b$
C. cajan x *C. cajanifolius*	83.6	88.5	75.8	46.7	73.6 $(59.0)^c$
C. cajanifolius x *C. cajan*	81.2	76.4	57.3	26.8	60.4 $(51.0)^d$
					CV=1.495
					CD (0.05) =1.449

CV- Coefficient of Variation; CD - Critical Difference; Figures in parenthesis are arc-sine transformed using Least Square Difference at 5% level of significance

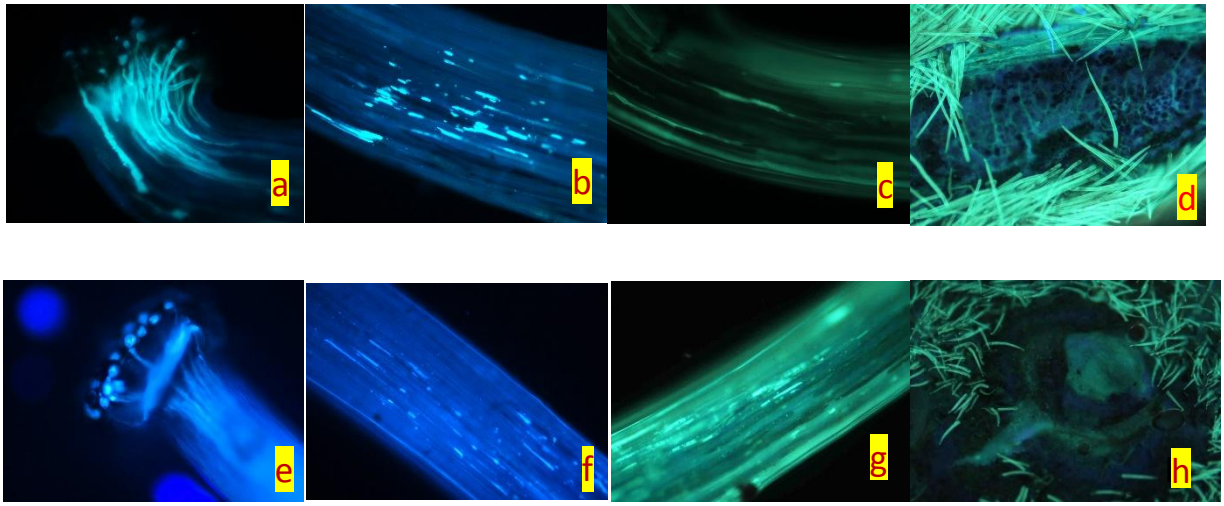

Fig. 3. Selfed pistils of *Cajanus cajan* (CO 8) (a. 2 HAP- Pollen germination on the stigma; b. 6 HAP- Pollen tube growth in stylar region; c. 10 HAP- Pollen tube growth in mid stylar region; d. 24 HAP- Pollen tubes reached ovule); Selfed pistils of *Cajanus cajanifolius* (e. 2 HAP- Pollen germination on stigma; f. 6 HAP- Pollen tubes growth in stylar region; g. 10 HAP- Pollen tube growth in mid stylar region; h. 24 HAP- Pollen tubes reached ovule)

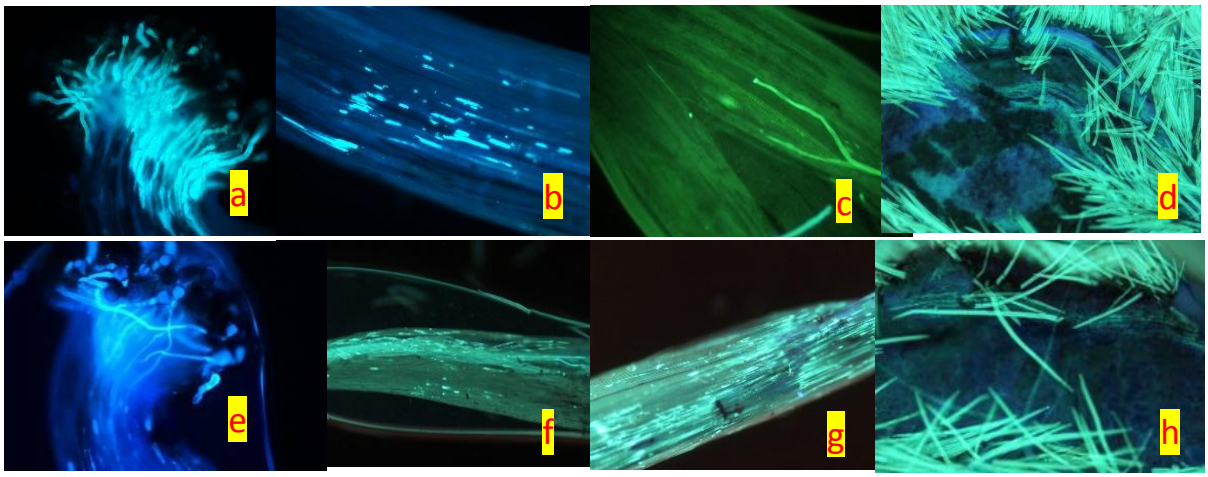

Fig. 4. (1) Direct cross of *Cajanus cajan* (CO 8) x *Cajanus cajanifolius* (a. 2 HAP- Pollen germination on stigma; b. 6 HAP- Pollen tubes growth in stylar region; c. 10 HAP- Pollen tube growth in mid stylar region; d. 24HAP- Pollen tubes reached ovule). (2) Reciprocal cross of *Cajanus cajanifolius* x *Cajanus cajan* (CO 8) (e. 2 HAP- Pollen germination on stigma; f. 6 HAP- Pollen tubes travelling from stigmatic surface towards mid stylar region; g. 10 HAP- Pollen tube growth in mid stylar region; h. 24HAP- Pollen tube growth stopped just before reaching ovule)

stigma and coordination between pollen and pistil proteins are required for successful hybridization. The signals from the style and embryo sac guide the pollen tubes to the micropylar end (Lord and Russell, 2002). During incompatible conditions, the pollen tubes normal metabolism was inhibited and the pollen tube growth collapsed, which limits future growth to the micropylar end. According to Johnston *et al*. (2005), undesirable pistil-pollen interactions lead to pollen tube growth inhibition in wide crosses. Which may result in inharmonious genetic interactions caused by genetic divergence of the species involved.

In direct crosses, the post fertilization barriers were significant, this observation is consistent with Kumar *et al.* (1990) and Pundir and Singh (1985) in pigeonpea. This one-way success could be attributed due to incompatibility of the cytoplasmic genome with the nuclear genome of the male parent (Pundir and Singh, 1985). Only one F_1 hybrid, (ICPL 87 x *C. cajanifolius*) survived out of four hybrids obtained (Thiruvengadam and Muthiah, 2007). The inviability of hybrids may be due to genetic imbalance or cytoplasmic incompatibility between two parental species.

In reciprocal crosses, the pre-fertilization barriers were significant, as evidenced by moderate pollen tube penetration and development. Pollen-pistil incompatibility is found to be the prominent pre-fertilization barrier encountered in many wide crosses. The incompatibility is mainly due to delayed pollen germination and pollen tube formation of one species on the stigmas of another species (Johnston *et al*., 2005). It was proposed to use techniques such as bud pollination, growth hormone application, *in vitro* fertilization, and protoplast fusion to promote pollen germination and pollen tube penetration in stylar and ovular tissues and to effect fertilization in wide crosses.

The present investigation on pollen pistil interaction in interspecific crosses of *Cajanus cajan* (CO 8) and *Cajanus cajanifolius* demonstrated that, post-fertilization barrier occurred in direct crosses. As an outcome, only a small percentage of pod set was obtained. Since *Cajanus cajan* has desirable characteristics such as high yield, resistance to sterility mosaic disease, resistance to root rot and tolerance to pod borer complex, techniques such as ovary culture, ovule culture, embryo culture, and embryo rescue will open the way for the development of interspecific hybrids. While in reciprocal crosses, pre-fertilization barrier was observed. In this instance, *in vitro* fertilisation may be advised to ensure a successful cross in future breeding programmes.

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