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An insight into the reproductive success in an interspecific cross of *Sesamum*

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Abstract
The study deals with pollen morphology, pollen fertility assessment and pollen pistil interaction in two species of Sesame *viz*., *Sesamum indicum* (CO 1) and *Sesamum alatum*. The wild species are the potential source for biotic and abiotic stress-resistant genes and therefore high crossability with the cultivated variety is very essential for the transfer of genes through a conventional breeding programme. The pollen images obtained and the number of colpi counted from Light Microscope and Scanning Electron Microscope assisted in differentiating the pollen of *Sesamum indicum* from *Sesamum alatum*. The pollen fertility assessment by Acetocarmine test proved that *Sesamum indicum* (CO 1) was more fertile than *Sesamum alatum* and hence the former was presumed to be the better pollen-producing parent in terms of fertility. The pollen pistil interaction by aniline blue technique between direct and reciprocal crosses of two species of *Sesamum* reported higher and lower germination per cent at 2 HAP and 8 HAP, respectively. The mean pollen germination per cent of the direct and reciprocal cross was observed to be 69.99 and 58.59, respectively. In the direct cross, the pollen tube managed to reach the ovary but did not set seeds and hence is claimed to possess a post-zygotic fertilization barrier. In the reciprocal cross, the growth of the pollen tube stopped before reaching the ovary apparently, no seed set was observed and therefore designated to have pre zygotic fertilization barriers. Direct and reciprocal crosses were compared and ways to mitigate fertilization barriers are discussed.

Key words Sesame, interspecific cross, pollen pistil interactions, aniline blue technique, fertilization barriers

INTRODUCTION

Sesame is one of the oldest oilseed crops known to human beings, and its cultivation dates back to 3050–3500 BC (Bedigian and Harlan, 1986). It is a member of the family Pedaliaceae. Despite Sesame being claimed as "Queen of Oilseeds", it was considered as an orphan crop because of the very little attention it received from science. Sesame is a drought-tolerant crop that comes up with a good yield in lesser inputs and hence is well suited for rainfed cultivation (Ariharasutharsan *et al*., 2019). Biotic and abiotic stresses are the major determinants that curb the yield potential of Sesame (Arpitha *et al*., 2019). With the uncovering of candidate genes and quantitative trait loci for various agronomic traits, Sesame has reformed from an "orphan crop" to a "genomic rich crop" (Dossa *et al*., 2017).

Pollen grains manifest the male accessories in sexual reproduction. As the seed is the economic product of most of the crop plants, investigations on pollen biology are indispensable to expand the yield (Knox *et* *al.,* 1986). *Sesamum alatum* is reported to possess desirable attributes like resistance to phyllody (Srinivasalu, 1991), shoot webber (Thangavelu,1994), powdery mildew (Thangavelu, 1994), *Phytophthora* blight, *Fusarium* wilt, leaf blight and seedling blight (Lee *et al*., 1991) and reported a good plant type (Ram *et al*., 2006).

The prerequisite in germplasm enhancement scheme lies in successful hybridization rather than the widespread occurrence of wild relatives of cultivated forms (Ram *et al*., 2006). Previous attempts to incorporate potential genes from wild relatives into cultivated species and *vice versa* have been less successful or even unsuccessful due to low crossability and reproductive barriers acting at both pre and post-zygotic levels in the interspecific crosses (Amirthadevarathnam, 1965; Ram *et al*., 2006; Kumari *et al*., 2015). An insight into the pollen pistil interaction, that directs wide hybridization and seed development would instigate the production of interspecific hybrids in *Sesamum*. This study was carried out to observe the pollen germination and pollen tube growth of *Sesamum alatum* and *Sesamum indicum* through direct and reciprocal crosses to assess whether pollen–pistil interactions act as a reproductive barrier in the interspecific cross.

MATERIALS AND METHODS

The seeds of *Sesamum indicum* (CO 1) and *Sesamum alatum* were collected from the Department of Oilseeds, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore and were raised in individual rings filled with red soil at Wild Species Garden, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during the first week of February 2021 **(Table 1)**. Recommended package of practices was followed for raising the crop. The pollen studies were done by collecting the anthers from ten randomly matured, newly opened flowers around 7.00 to 8.00 AM and taken to the laboratory immediately for microscopic observations. The pollen sterility analysis was done using the Acetocarmine test (Dafni *et al.,* 2005). Pollens were suspended from the anther and mounted on a glass slide with a drop of two per cent acetocarmine solution and covered with coverslip which was observed under a fluorescent microscope (Nikon Eclipse Ni-U, Japan) using Nikon filter (330–380 nm excitation filter, 410 nm barrier filter) and counted for five microscopic fields. Images were recorded with the help of a Nikon DS-Fi3 camera using NIS Elements F v.4.60.00 image processing platform. Deeply stained pollen grains were counted as fertile, while the unstained pollen grains were counted as sterile. Pollen fertility per cent was calculated by the per cent ratio of the number of stained pollen grains to the total number of pollen grains.

The pollen was released in the microscopic slide with a drop of water and covered with a coverslip and viewed under a light microscope and the images were captured for studying pollen characters using Image view software. The suspended pollen was mounted on double-sided conductive carbon tape fixed on the stub, sputter-coated with gold alloy for 15 seconds in an EMITECH SC7620 sputter coater (Quorum Technologies Ltd., Laughton, East Sussex, England) and placed on the sample chamber of Scanning Electron Microscope (SEM) (Quanta 250; FEI Company, Eindhoven, Netherlands) with a high energy beam of electrons. After attaining a high vacuum, the filament was switched on and adjusted for various parameters like electron beam, intensity, spot size, voltage, emission current and the images were captured by SEM.

The flowers from pollen acceptor plants (taken as female) were hand emasculated by pulling out epipetalous corolla in the previous evening, between 4 and 5 PM and covered with butter paper covers, to avoid contamination from foreign pollen and were tagged. Cross-pollination was done by dusting desired pollen grains collected at the time of anthesis (7.30 to 9 AM) directly onto the stigmas of emasculated flowers. Immediately following pollination all pollinated flowers were again covered with wax paper bags (Thangavelu and Nalathambi, 1982).

The pistils from the tagged cross and self-pollinated flowers were collected at 1, 2, 4 and 8 hours after pollination (HAP) and fixed in acetic acid: alcohol (1:3 v/v) for 12 hours. The maximum time interval for collection of pollinated pistils was restricted to 8 because under natural conditions most of the crossed flowers will wither within 10. For every collection time, ten each of crossed and self-pollinated

Table 1. *Sesamum* species used in the study

Species	Chromosome number	Desirable attributes
Sesamum indicum (CO 1)	2n = 26	High yielder, black seeded, high quality seed oil and high oil content
Sesamum alatum	2n = 26	Resistant to phyllody, shoot webber, Powdery mildew, *Phytophthora* blight, *Fusarium* wilt, leaf blight and seedling blight , Less crossability with other species of *Sesamum*

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pistils from five tagged plants of *Sesamum* were used for microscopic observations. The pistils can be softened in 0.8N NaOH overnight, stained with 0.1% (w/v) aniline blue in 0.1M K3PO for four hours and mounted on microscopic slides in 50% glycerol (Sitch, 1990). The slides were observed under a fluorescent microscope (Nikon Eclipse Ni-U, Japan) using a Nikon filter (330–380 nm excitation filter, 410 nm barrier filter). Images were recorded with the help of a Nikon DS-Fi3 camera using NIS Elements F v.4.60.00 image processing platform.

Pollen germination per cent was recorded by the per cent ratio of the number of pollen grains germinated to the total number of pollen grains. Pollen tube growth was observed based on the rate at which it reaches varying positions *viz.,* stigmatic surface, mid-style region and ovary.

The data were analyzed using the statistical tool package STAR 2.0 (IRRI, Philippines). Pollen characters and pollen fertility per cent were studied using a t-test for determining the significant difference between two species. The mean pollen germination per cent of the selfed and crossed pistil of wild and cultivated species was subjected to square root transformation and Least Square Difference (LSD) for comparison of significant differences among them.

RESULTS AND DISCUSSION

The polar and equatorial views of pollen images obtained from Light Microscope (LM) and Scanning Electron Microscope (SEM) and the number of colpi of two species were detailed **(Table 2 and Fig. 1 to 5).** Colpi may be defined as the elongated apertures that are equally spaced on the equatorial plane or over the general surface of a pollen grain. The number of colpi in *Sesamum indicum* (CO1) were found to be 12 (**Table 2 and Fig. 1a, 2a and 3a**), whereas in *Sesamum alatum* it ranged from 6 to 8 (**Table 2 and Fig.1b, 2b and 3b**). Although the number of colpi in both the species were more than three in equatorial position and were denoted to be stephanocolpate, the difference in the number of colpi between both species can be used to distinguish them as they are significantly different at the five per cent level of significance. Akhila *et al*. (2015) reported that escalation in the number of colpi designates that the species has evolved and hence can be deduced that the higher the colpi number, the more the evolution has taken place in the species. As the number of colpi in cultivated species is higher than the wild species, the former can be meant to possess higher evolution than the latter. Zhigila *et al*. (2014) established a close relationship in the pollen morphology of *Sesamum* accessions collected from Northern Nigeria. According to Perveen and Qaiser (2010), the pollen grains of Pedaliaceae were similar to those of Acanthaceae. Pfahler *et al*. (1996) elucidated genotype and environmental influence on pollen characters in Sesame. Kordofani *et al*. (2013) reported that the pollen characters did not show significant variation in the genus *Sesamum*. Ultimately, it might be implied that the number of colpi observed can be helpful in the identification of species rather than establishing any relation with fertility status or *in vivo* pollen-pistil interaction of interspecific crosses.

The pollen fertility per cent and images of fertile and sterile pollen grains of two species were detailed (**Table 2 and Fig. 4 and 5**). The pollen fertility per cent of *Sesamum indicum* (CO 1) was 98.75 (**Table 2 and Fig. 4a**), against *Sesamum alatum* which was 82.85 (**Table 2 and Fig. 4b**). Contrarily, pollen fertility per cent in *Sesamum indicum* (TMV 7) and *Sesamum alatum* was reported to be 94.23 and 96.02, respectively by Ram *et al*. (2006). The fertile pollen was found to be larger than the sterile pollen which was smaller and shrivelled (**Fig.5**). From the observations made, it can be concluded that both are significantly different at a five per cent level of significance for pollen fertility and hence the pollen fertility per cent of *Sesamum indicum* was greater than *Sesamum alatum***.** Therefore, *Sesamum indicum* (CO 1) can be regarded as a better pollen-producing parent than *Sesamum alatum* in terms of fertility.

The present study was done to understand the pollen-pistil interaction between *Sesamum indicum* and *Sesamum alatum* and hence selfing, direct and reciprocal crosses were made. The per cent pollen germination at different time intervals was observed in selfed and crossed pistils of *Sesamum* species (**Table 3 and**

Table 2. Pollen characters and Pollen fertility of species of *Sesamum*

Particulars	Number of colpi		Pollen fertility (%)	
	Sesamum indicum	***Sesamum alatum***	***Sesamum indicum***	***Sesamum alatum***
Mean	12*	7.25*	98.7525*	82.85*
Variance	0.141067	0.916667	0.061425	3.892847
t Stat	9.237097		15.99418505	
P value	0.000764		0.000531504	
t Critical value	2.776445		3.182446305	

*Significant at 0.05 level.

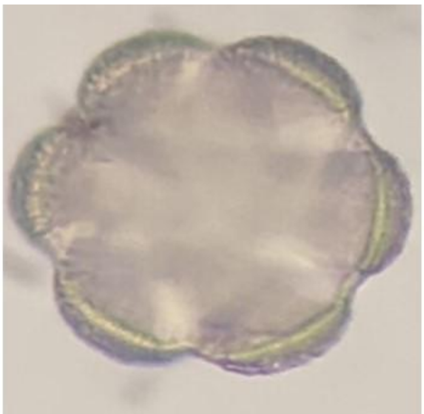

Fig. 1a. LM view of Sesamum indicum pollen (Scale bar 20µm)

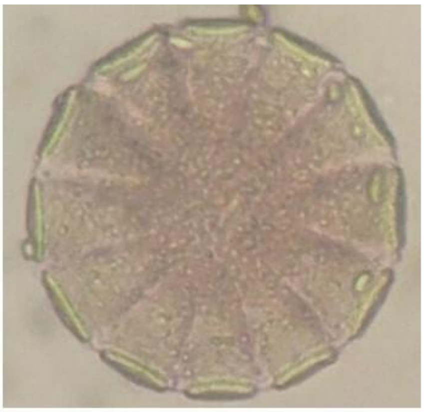

Fig. 2a. LM view of Sesamum alatum pollen (Scale bar 20µm)

LM- Light Microscope

Fig.2a. Polar view

Fig.2b. Equatorial view

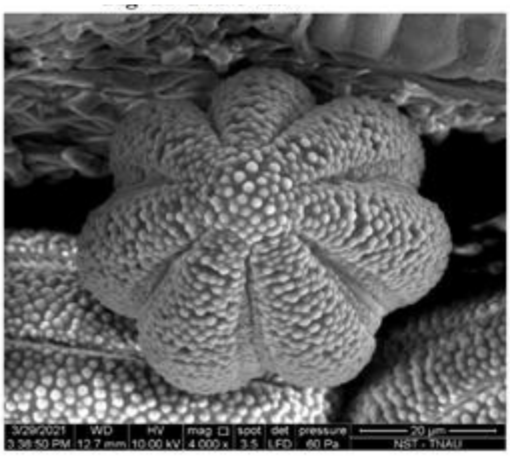

Fig.3a. Polar view

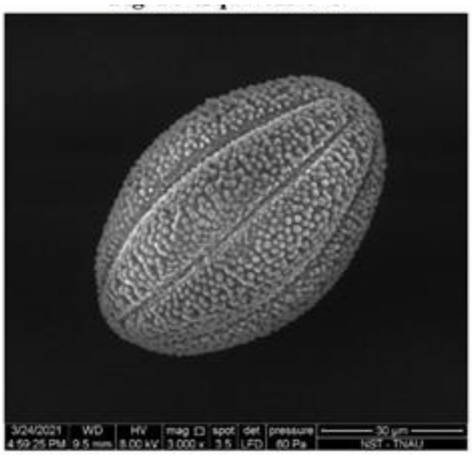

Fig.3b. Equatorial view

Fig. 2 and 3 SEM images of Sesamum indicum and Sesamum alatum
SEM – Scanning Electron Microscope

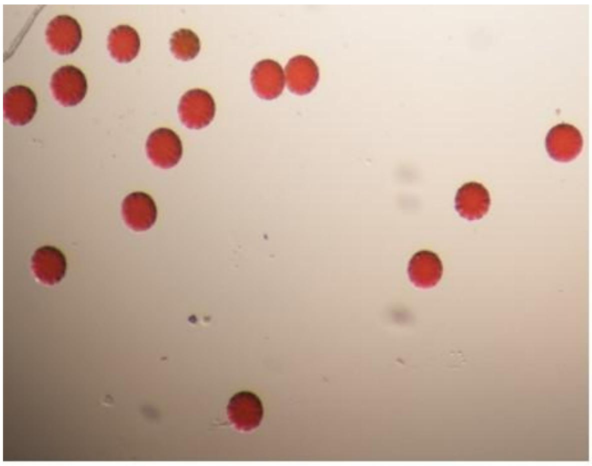

Fig.4a. Pollen fertility assessment of Sesamum indicum – CO 1 (Magnification 10X)

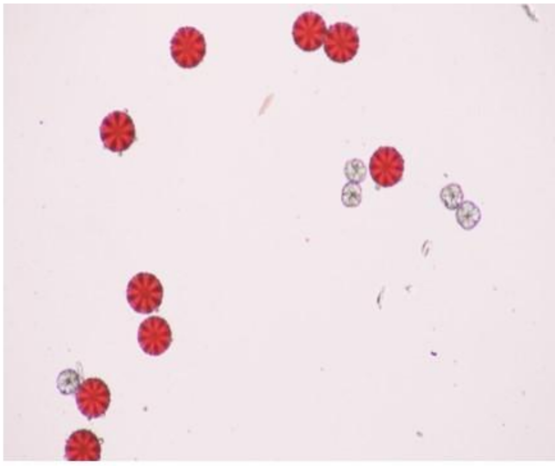

Fig.4b. Pollen fertility assessment of Sesamum alatum (Magnification 10X)

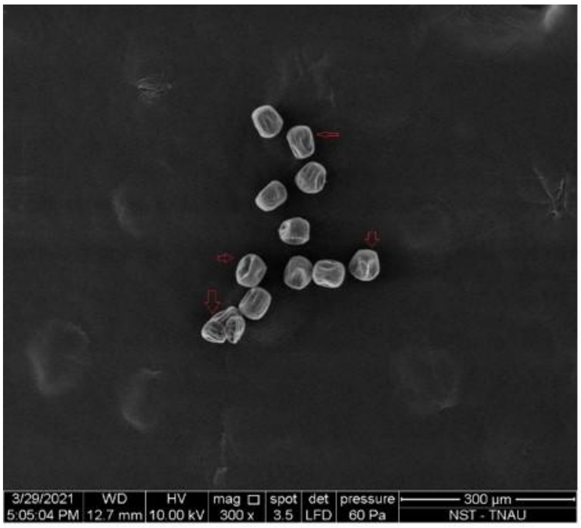

Fig 5a. SEM images of sterile pollen grains of Sesamum indicum

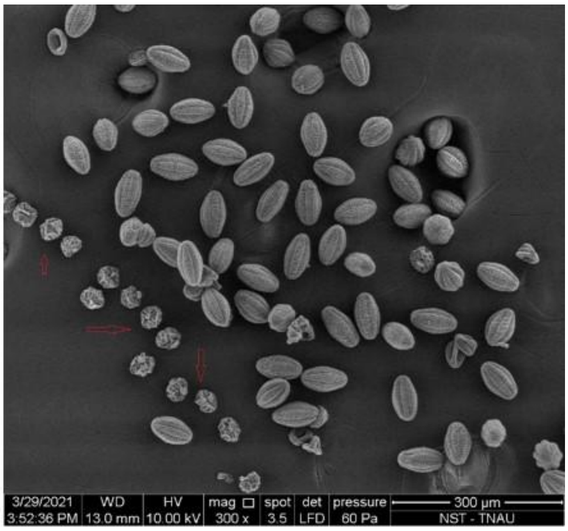

Fig 5b. SEM images of sterile pollen grains of Sesamum alatum

SEM – Scanning Electron Microscope

Fig.6 and 7). The pollen germination per cent was recorded at a maximum of 95.20 **(Table 3 and Fig. 6a)** and 91.40 (**Table 3 and Fig. 6b**) at 1HAP and minimum of 85.71 (**Table 3 and Fig. 6a**) and 74.28 (**Table 3 and Fig. 6b**) at 8 HAP in selfed *Sesamum indicum* and *Sesamum alatum,* respectively. The mean per cent of pollen germination in *Sesamum indicum* (CO1) and *Sesamum alatum* upon self-pollination were found to be 91.04 and 84.07, respectively (**Table 3**). The mean germination per cent in *Sesamum indicum* was investigated to be higher than *Sesamum alatum*.

In the case of the direct cross between *Sesamum indicum* (CO1) and *Sesamum alatum,* a maximum per cent of 85.71 (**Table 3 and Fig.7a**) during 2 HAP in mid style region and minimum germination per cent of 42.85 **(Table 3 and Fig.7a**) during 8 HAP while reaching ovary was recorded. In the reciprocal cross between *Sesamum alatum* and *Sesamum indicum* (CO1), a higher germination per cent of 84.37 (**Table 3 and Fig.7b**) was recorded at 2 HAP when pollen tubes were travelling from the stigmatic surface towards mid style region and lower germination per cent of 21.87 (**Table 3 and Fig.7b**) during 8 HAP where the pollen tube growth stopped just before reaching ovary. The mean germination per cent in direct and reciprocal crosses were recorded to be 69.99 and

58.59, respectively (**Table 3**). The mean germination per cent of the direct cross was found to be greater than the reciprocal cross. Hence it can be delineated that the mean germination per cent of selfed cultivated species was higher followed by selfed wild species, direct cross and reciprocal cross and all of them were significantly different from each other at five per cent level of significance.

Taking into account the pollen tube growth, it was similar in the selfed pistils of cultivated and wild species in such a way that the pollen grains germinated on the stigmatic surface at 1 HAP, pollen tube growth was observed in mid style region at 2 HAP, continued to travel towards ovary at 4 HAP and reached ovary at 8 HAP (**Fig.6**). The same pattern was followed in direct cross between *Sesamum indicum* and *Sesamum alatum* (**Fig.7a**). In the case of a reciprocal cross between *Sesamum alatum* and *Sesamum indicum*, the growth was slower when compared to selfed pistil and the direct cross because the pollen tube reached mid style region only during 4 HAP and did not reach the ovary even after 8 HAP (**Fig.7b**).

The pollen tubes germinated and travelled to the ovary and succeeded in reaching the ovary in selfed pistils and direct cross of Sesame, while the pollen tubes failed to enter the ovary in the reciprocal cross and hence no seed set was reported. However, the pollen tubes reached the ovary, the seed set was not observed in direct cross. In both direct and reciprocal crosses between *Sesamum indicum* and *Sesamum alatum* there was no seed set although the chromosome number (2n = 26) of both the species are the same. It can be concluded that the post-zygotic barrier was observed in the direct cross and the pre-zygotic barrier in the reciprocal cross. The above barriers may be due to inharmonic reactions such as callose formation and incompatible genetic interactions taking place between the pollen and pistil in wide hybridization which hindered the free entry of pollen tubes into style and thereby preventing its entry into the ovary and resulting in no seed set. Kedharnath (1954) made crosses between these two species and reported that, when *Sesamum indicum* was used as the female parent, fruit set was normal but seeds appeared shrivelled and nonviable and identified an early abortion of the young embryo and in the reciprocal cross, very few fruit set were observed with few healthy seeds but they were not viable. Similar results were obtained by Amirthadevarathnam (1965), Sundaram (1968), Arivudainambi (1990), and Kobayashi (1991). Parani *et al*. (1997) concluded that *in vivo* pollen germination and pollen tube growth study revealed a complete failure of direct cross due to pre-zygotic barriers posed by gametophytic incompatibility prevented the pollen tube to grow down the style and added that reduced capsule and seed set in the cross was found to be due to both partial pre-and post-zygotic barriers. The post-zygotic fertilization barrier was reported by Rajeswari *and Ramaswamy* (2004). Kumari *et al*. (2015) in both direct and reciprocal interspecific cross of *Sesamum* and observed no seed set and hence reported post-zygotic fertilization barrier. The use of irradiated mentor pollen by Sastri and Shivanna (1976) and *in vitro* fertilization can be done to promote pollen tube growth and effect fertilization. Suggestions were provided by Bhat and Sarla (2004) for the use of techniques such as bud pollination, stump pollination and application of growth hormones for mitigating pre-zygotic barriers. Rajeswari *et al.* (2010) developed an ovule culture method for obtaining interspecific hybrids.

The pollen images obtained and the number of colpi counted assist in the identification and differentiation of pollen grain of one species from another. The pollen fertility status studies revealed that *Sesamum indicum* (CO 1) proved to be more fertile than *Sesamum alatum* and hence the former can be contemplated as a better pollen producer in terms of fertility. From the study undertaken, it can be concluded that direct and reciprocal cross between *Sesamum indicum* (CO 1) and *Sesamum*

Table 3. Per cent of pollen germination at four different time intervals

Parents /cross	Time interval and pollen germination (%)				
	1 HAP	2 HAP	4 HAP	8 HAP	Mean
Sesamum indicum (CO1)	95.20	90.40	92.85	85.71	91.04 (9.53)[a]
Sesamum alatum	91.4	90.62	80.0	74.28	84.07 (9.16)[b]
S. *indicum* x *S. alatum*	80.00	85.71	71.42	42.85	69.99 (8.37)[c]
S. *alatum* x S. *indicum*	75.00	84.37	53.12	21.87	58.59 (7.66)[d]
					CV=1.18 CD (0.05) = 0.16

CV- Coefficient of Variation; CD - Critical Difference; Figures in parenthesis are square-root transformed using Least Square Design at 5% level of significance.

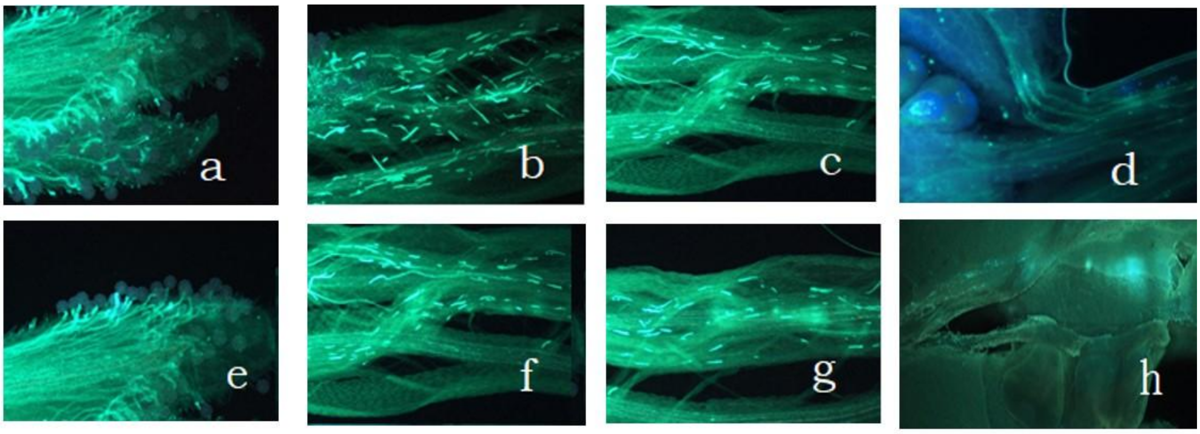

Fig. 6a. Selfed pistils of S. indicum (a. 1 HAP- Pollen tubes in stigma; b. 2 HAP- Pollen tubes grown in mid style region; c. 4 HAP- Pollen tubes on their way to reach ovary; d. 8HAP- Pollen tubes reaching ovary); Fig. 6b. Selfed pistils of S. alatum (e. 1 HAP- Pollen tubes in stigma; f. 2 HAP- Pollen tubes grown in mid style region; g. 4 HAP- Pollen tubes on their way to reach ovary; h. 8HAP- Pollen tubes reaching ovary)

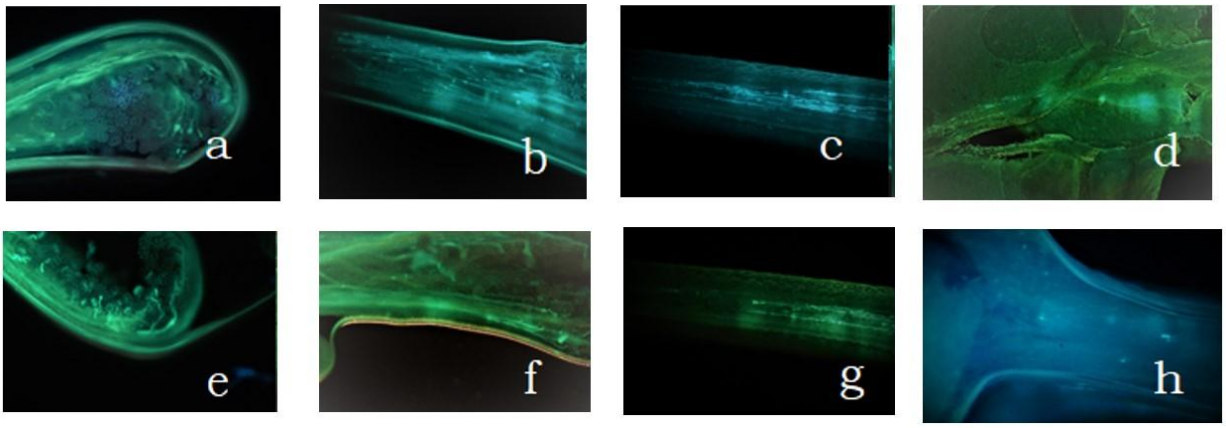

Fig. 7a. Direct cross of S. indicum × S. alatum cross (1 HAP- Pollen tubes in stigma; b. 2 HAP- Pollen tubes grown in mid style region; c. 4 HAP- Pollen tubes on their way to reach ovary); Fig. 7b. Reciprocal cross of S. alatum x S. indicum (e. 1 HAP- pollen tubes in stigma; f. 2 HAP- Pollen tubes travelling from stigmatic surface towards mid stylar region; g. 4 HAP-Pollen tube growth in mid stylar region; h. 8HAP- Pollen tube growth stopped just before reaching ovary)

alatum did not set seed and hence referred to possess post-fertilization barrier and pre fertilization barrier, respectively which may be due to complex reactions like callose formation and adverse genetic interaction between pollen and pistil undergoing interaction. As *Sesamum alatum* is claimed to embody candidate genes for biotic and abiotic stress, techniques like ovary-ovule culture, embryo culture, embryo rescue technique, bud pollination, stump pollination, use of irradiated mentor pollen and application of growth hormones may pave way for obtaining interspecific hybrids.

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