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Research Article

Pollen–pistil interaction in self-pollinated and sib pollinated flowers of sunnhemp (*Crotalaria juncea* L.)

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Abstract
Pollen pistil interaction studies were carried out in six sunnhemp genotypes. Pollen viability percentage of the six genotypes ranged from 0.0 to 93.5% and pollen germination ranged from 0.0 to 84.0% at four different floral stages starting from bud to opening of a flower. The viable pollen grains were present at the later three stages of flower for globose type anthers, while the pollen grains were viable at the later two floral stages for heart shaped anthers. Stigma receptivity was high to very high during S3 and S4 stages. Artificial self-pollination and sib pollination with a mixture of pollen grains were carried out and *in vivo* pollen tube growth in the style was investigated using fluorescence microscopy. In self-pollinated pistils, eight hours after pollination, callose wall development was seen around the nucellus cell. However, under sib pollinated pistil, eight hours after pollination, pollen tube penetrated into embryo sac of an ovule. The formation of callose in self-fertilized pistil prevented fertilization and seed set. But sib pollinated flowers were fertilized normally and a seed set could be observed. The investigation proved the existence of self-incompatibility.

Keywords: Sunnhemp, *Crotalaria*, Pollination, Self-incompatibility, Callose

INTRODUCTION

Sunnhemp (*Crotalaria juncea* L.) is grown as a fibre crop, cover crop and green manure or fodder crop cultivated in almost all Indian states. This crop is mostly grown for fibre in the states of Bihar, Madhya Pradesh, Maharashtra, Rajasthan, Orissa, and Uttar Pradesh. These states account for nearly 87 per cent of the entire area under sunnhemp cultivation (Chaudhury *et al*., 1978). The fibre has a high cellulose content, a low lignin content and a negligible ash content (Tripathi *et al*., 2013), making it appropriate for the manufacturing of valuable tissue paper, cigarette paper and currency paper. It improves soil physical and biological properties on incorporation as a green manure crop while reducing pollutants, making the agricultural system more sustainable. It has a high photosynthetic rate, allowing it to assimilate carbon dioxide from the atmosphere and thereby minimizing the greenhouse gas (GHG) effect. Aside from that, it is highly valued for its weed-controlling abilities (Rupper, 1987). Sunnhemp is an annual herbaceous shrub with many branches and has a terminal open raceme flower and lobbed nodules. Its stamen is dimorphic consisting of two types of anthers, the first five outer whorl anthers are globose shaped and the second five inner whorl anthers are heart shaped anthers.

Sunnhemp is considered to be a species that is incompatible with itself. Because pollination in sunnhemp is regulated due to self-incompatibility, studies on this aspect will help to exploit heterosis in sunnhemp (Ribeiro *et al.,* 1977). In the absence of insect visitors, self-pollination is delayed in the genus *Crotalaria*, hence it is considered as facultative xenogamy (Etcheverry *et al.,* 2003). According to Shivanna (2003), pollen viability combined with stigma receptivity at different stages is crucial for the effective initiation of pollen pistil interaction.

Studies on pollen pistil interaction are essential for understanding the reproduction behaviour and it helps to formulate further breeding programs. Therefore, the aim of the current research was to study pollen viability and pollen germination, stigma receptivity, pollen pistil interaction and understand the self-incompatibility mechanism of selected genotypes of sunnhemp.

MATERIALS AND METHODS

The study was conducted at the Department of Genetics and Plant Breeding, Tamil Nadu Agricultural University, Coimbatore, during the year 2021-2022, with six sunnhemp genotypes *viz.,* SUIN 053, SUIN 37, JRJ 610, K12 Yellow*,* CO 1 and CCJ 1. The seeds of SUIN 053, SUIN 37, JRJ 610, and K12 Yellow were obtained from the Central Research Institute for Jute and Allied Fibres, Barrackpore, West Bengal, while CO 1 and CCJ 1 genotypes were obtained from Tamil Nadu Agricultural University, Coimbatore. The crop was raised with a spacing of 40 x 20 cm in four replications. All genotypes were covered separately by insect-proof nylon netted cage before flowering to keep out all pollinators and cross pollination with other genotypes.

Based on the time of standard petal openings, the floral stages of sunnhemp can be classified into four stages *viz.,*1) Matured closed bud (S1), 2) Standard petal starting to break out of calyx (S2), 3) Full emerging of standard petal (S3), 4) Complete opening of flowers (S4), (Chandrashekar *et al*., 2013). Pollen viability was examined using acetocarmine staining methods (Dafni *et al*., 2005). Pollen grains at four floral stages were collected and dusted on a glass slide, which was subsequently stained with a drop of staining solution and covered with a glass cover slip. A 10 x magnification light microscope was used to analyse pollen grains. Pollen grains with normal shape and staining were considered to be viable and pollen grains with poor staining or irregular shapes were considered non-viable. Pollen fertility was calculated as a percentage of the total number of stained pollen grains divided by the total number of stained and unstained pollen grains. Germination of pollen grains in different floral stages of sunnhemp genotypes was evaluated under *in vitro* conditions. When the pollen tube length was longer than the pollen grain diameter, the pollen grain was considered as germinated (Tuinstra and Wedel, 2000). Pollen grains of anther collected from each genotype were studied for their *in vitro* germination using the cavity slide technique. A drop of Brewbaker and Kwack liquid media (1963) (10 % sucrose, 100 mg of boric acid, 300 mg of calcium nitrate, 200 mg of magnesium sulphate, and 100 mg of potassium nitrate) was placed in the cavity, and pollens were dusted and covered with a coverslip (Shivanna and Rangaswamy, 1992). Pollen germination was studied in different time intervals of incubation.

The slides were examined under a light microscope at 10 x magnification and the germination percentage was calculated. The number of germinated pollen grains per field view was divided by the total number of pollen grains per field view, and the result was expressed as a percentage (Kearns and Inouye, 1993). Around 100 numbers of pollen grains obtained from flowers at different floral stages were subjected to *in vitro* germination study with five replications. Statistical analysis was done using the AGRES software tool. Results of pollen viability and pollen germination rates were analyzed using the t test, and different means were compared using Least Square Difference (LSD) at ($p < 0.05$). Correlation analysis was performed to determine the relationship between pollen viability and pollen germination.

Stigma receptivity was investigated using a 3% hydrogen peroxide solution. Four to six flowers were collected at various stages and all sepals and petals were removed. Stigmas were dipped in a petri-plate containing a 3% H_2O_2 solution for 2-3 minutes. The stigma receptivity was assessed based on the bubbles developed on the stigmatic surface. The extent of stigma receptivity was categorized as low, medium or high based on the extent of bubbles developed.

Healthy plants were selected at random and subjected to various modes of pollination as follows: 1) Selfing with artificial hand pollination of the same flower, 2) Sib mating (pollen from flowers of different plants of the same genotype), 3) Control (allowed to self-pollination naturally).

Fluorescent microscopy was used to observe pollen tube development in the stigma, style and ovary at various time intervals (Kho and Baer, 1968) in the sunnhemp genotype SUIN 053 with self and sib pollination. To better understand pollen pistil interaction, 20 flowers were artificially self-pollinated and another 20 flowers were artificially sib pollinated with the pollen mixture of same genotype and for control, the flowers were untouched either by insects or human (plants are grown under insect proof cage). Pollen grains were collected at different time intervals *viz*. 1, 2, 3, 4, 6, 8 and 10 hrs after pollination. Sepals and petals were removed from each bud and the pistil from each bud was separated and immersed in fixative solution (Acetic acid glacial: absolute ethanol, 1:3) for overnight, then transferred to 70% ethanol and kept under refrigeration 4℃ until further processing of the gynoecium (Sogo and Tobe, 2006). Pistils were rinsed 2-3 times in distilled water and then treated with 8M NaOH for one hour at 60℃ to soften them. Afterwards for two hours, pistil samples were immersed in a solution of 0.1 per cent of aniline blue dissolved in 0.1N potassium phosphate. To avoid drying of the samples, they were put in a drop of staining solution and observed using a fluorescent microscope (Nikon Eclipse Ni-U, Japan) with a Nikon filter (330-380 nm excitation filter, 410 nm barrier filter). Images were captured with a Nikon DS- Fi3 camera and processed using the NIS- Elements F v.4.60.00 image

processing platform. The style was squashed, and the ovary was sliced longitudinally with a razor blade, allowing for a clear view of the micropylar end. The numbers of pollen tubes growing in the stylar and ovary regions were recorded.

RESULTS AND DISCUSSION

The results of acetocarmine staining test in various floral stages of different genotypes are given in **Table 1**. The viability of two types of anthers was studied at various time intervals throughout the anthesis stage. At stage S1 no pollen viability was observed in globose and heart-shaped anthers. At stage S2 (88.6%), globose anthers exhibited production of viable pollen grains which showed an increasing trend in stage S3 (92.1%), which started declining at stage S4 (88.5%). Viability of heart shaped anthers was first observed as S3 stage (88.5%) only and was maximum at stage S4 (90.9%) **(Fig. 1).** A similar result was reported earlier by Thimmaiah *et al*. (2018).

Table 1. Pollen viability of dimorphic anthers in six sunnhemp genotypes at different floral stages

Genotypes	% of pollen viability of globose shaped anthers at different floral stages				% of pollen viability of heart shaped anthers at different floral stages			
	S1	S2	S3	S4	S1	S2	S3	S4
SUIN 053	0.0 (0.25)	90.0 (71.56)	92.7 (74.32)	89.0 (70.63)	0.0 (0.25)	0.0 (0.25)	89.0 (70.63)	91.3 (72.84)
SUIN 37	0.0 (0.25)	88.5 (70.17)	93.0 (74.65)	88.7 (70.35)	0.0 (0.25)	0.0 (0.25)	87.3 (69.12)	90.0 (71.56)
JRJ 610	0.0 (0.25)	88.0 (69.73)	91.3 (72.84)	88.0 (69.73)	0.0 (0.25)	0.0 (0.25)	88.0 (69.73)	90.0 (71.56)
K12 Yellow	0.0 (0.25)	87.7 (69.46)	90.0 (71.56)	87.0 (68.86)	0.0 (0.25)	0.0 (0.25)	89.0 (70.63)	91.0 (72.54)
CO 1	0.0 (0.25)	89.0 (70.63)	93.5 (75.22)	89.7 (71.28)	0.0 (0.25)	0.0 (0.25)	89.3 (70.90)	92.7 (74.32)
CCJ 1	0.0 (0.25)	88.2 (69.90)	91.8 (73.36)	88.5 (70.17)	0.0 (0.25)	0.0 (0.25)	88.5 (70.17)	90.5 (72.04)
Mean	0.0	88.6	92.1	88.5	0.0	0.0	88.5	90.9

CD ($p < 0.05$) =3.14 (NS) & 3.82 (NS) for globose and heart shaped anther, respectively. Values in parenthesis are arcsine transformed values

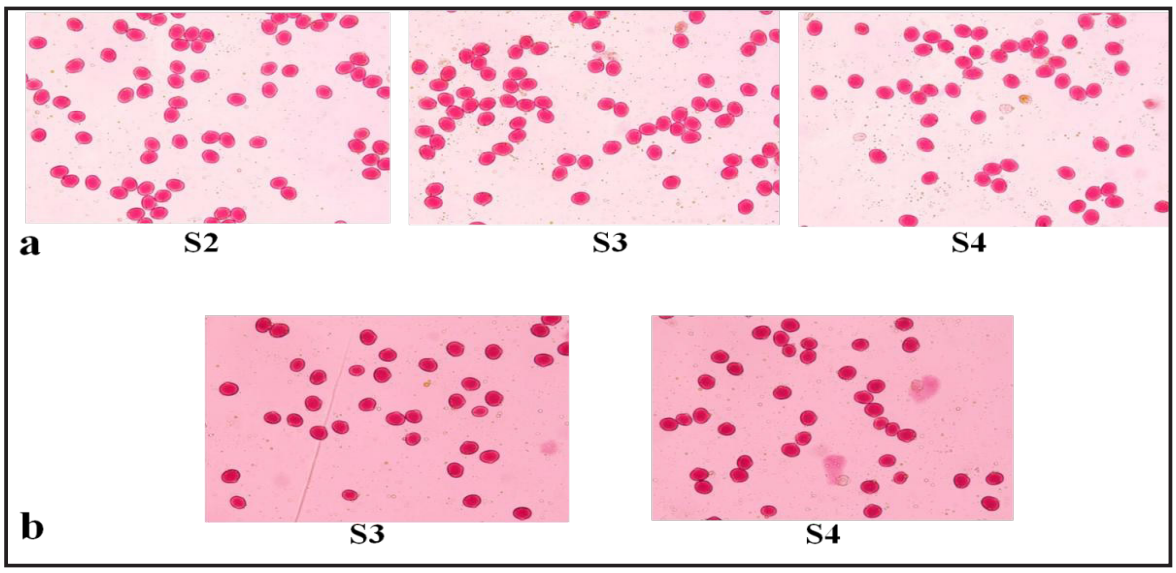


Fig. 1. Pollen viability of dimorphic anthers in different stages of sunnhemp
a) globose shape, b) heart shape, S- stage.

Among the two types of anthers within same flower, the maximum pollen viability percentage was almost similar for both types of anthers (S3 stage for globose anthers 93.5% and S4 stage for heart shaped anthers 92.7%). This might be due to the late maturity of heart shaped anthers, which started growing faster after S3 stage and reached full growth at S4 stage. No significant differences between genotypes were noticed at each stage in the case of both globose shaped anthers as well as heart shaped anthers. Thus, viable pollen grains were present at the later three stages of flower for globose type anthers while the pollens were viable at the later two floral stages for heart shaped anthers.

Germination of pollen grains collected from globose and heart shaped anthers at different floral stages of six different genotypes was studied and the results obtained are presented in **Table 2**. An increase or decrease in per cent pollen germination followed a trend similar to that of per cent pollen viability between different genotypes and different floral stages of the six sunnhemp genotypes studied.

The maximum per cent germination of pollen grains was 83.0% in both SUIN 37 and CO1 sunnhemp genotypes at S3 stage in case of globose anthers and was 82.3% in K12 Yellow and CO1 genotypes at S4 stage in case of

Table 2. Pollen germination of dimorphic anthers in six sunnhemp genotypes at different floral stages

Genotypes	% of pollen germination of globose shaped anthers at different floral stages				% of pollen germination of heart shaped anthers at different floral stages			
	S1	S2	S3	S4	S1	S2	S3	S4
SUIN 053	0.0 (0.25)	81.0 (64.15)	84.0 (66.42)	79.0 (62.72)	0.0 (0.25)	0.0 (0.25)	80.0 (63.43)	82.0 (64.89)
SUIN 37	0.0 (0.25)	80.0 (63.43)	83.0 (65.64)	79.0 (62.72)	0.0 (0.25)	0.0 (0.25)	80.3 (63.65)	81.0 (64.15)
JRJ 610	0.0 (0.25)	79.3 (62.93)	82.0 (64.89)	79.7 (63.22)	0.0 (0.25)	0.0 (0.25)	79.0 (62.72)	81.0 (64.15)
K12 Yellow	0.0 (0.25)	80.3 (63.65)	82.3 (65.12)	79.3 (62.93)	0.0 (0.25)	0.0 (0.25)	80.0 (63.43)	82.3 (65.12)
CO 1	0.0 (0.25)	81.3 (64.37)	83.0 (65.64)	80.0 (63.43)	0.0 (0.25)	0.0 (0.25)	80.3 (63.65)	82.3 (65.12)
CCJ 1	0.0 (0.25)	80.1 (63.50)	82.5 (65.27)	79.2 (62.86)	0.0 (0.25)	0.0 (0.25)	79.7 (63.22)	81.4 (64.45)
Mean	0.0	80.3	82.8	79.4	0.0	0.0	79.9	81.7

CD ($p < 0.05$) = 4.7 (NS) & 5.2 (NS) for globose and heart shaped anther, respectively. Values in parenthesis are arcsine transformed values

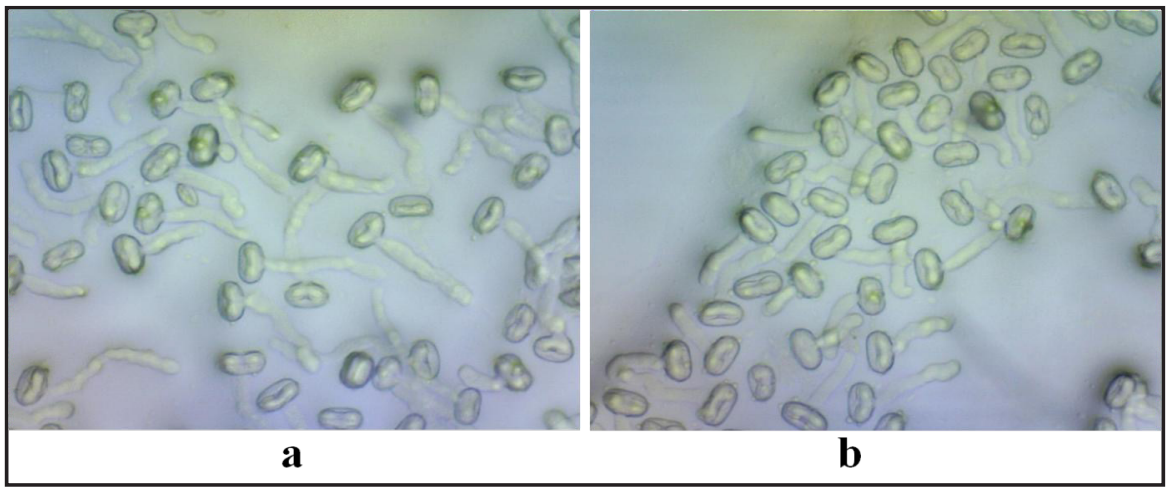


Fig. 2. *In vitro* germination of pollen grains collected from two types of anthers of the sunnhemp genotype SUIN 053, (10 X magnification). a) Pollen grain from globose shaped anthers at two hours after inoculation. b) Pollen grain from heart shaped anthers at two hours after inoculation.

heart shaped anthers **(Fig. 2).** There was no significant difference in per cent *in vitro* germination of pollen grains. The simple correlation between pollen viability percentage and pollen germination percentage of globose shaped anther ranged from (-0.18 to 0.95) and heart shaped anther ranged from (0.22 to 0.92) at different floral stages (**Table 3**).

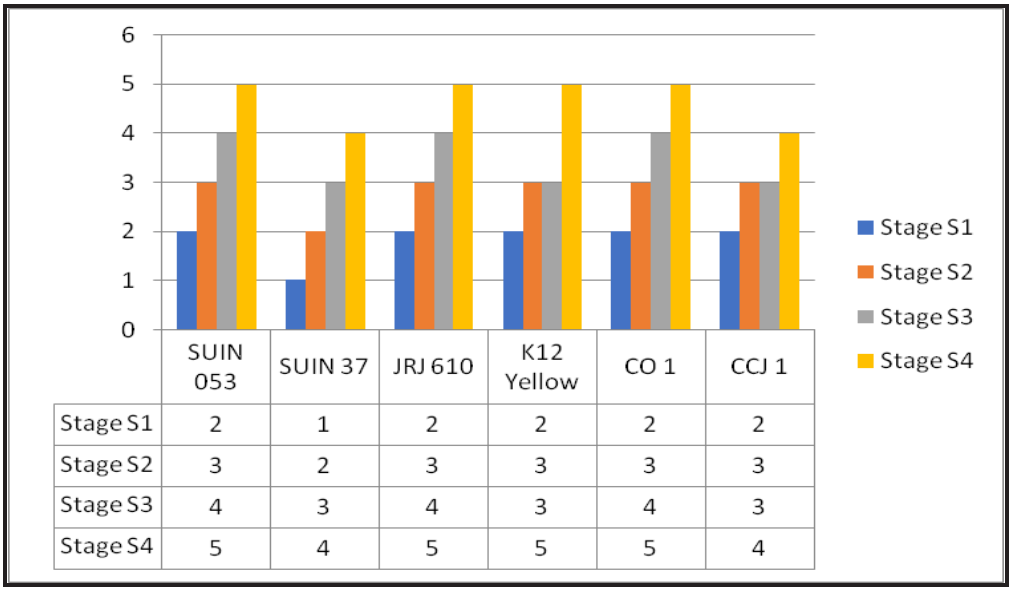
During various floral stages, stigma receptivity was examined in different genotypes **(Fig. 3)**. In the present study, stigma receptivity of six sunnhemp genotypes was observed to be very low or low in stage S1, low or medium in stage S2, medium or high in stage S3, and high or very high in stage S4 (**Fig. 4**). Similar results were obtained by Rajagopal *et al.* (2021). The peak pollen viability of globose anthers and heart shaped anthers was at S3 stage and S4 stage of the flower respectively. However, at S2 stage the pollen viability of globose anthers was moderate, while it was not observed in heart shaped anthers. Since the stigma receptivity in general was medium and high at S3 and S4 stages, coinciding with the pollen viability of both the dimorphic anthers, it can be concluded that there is good synchrony of pollen viability and stigma receptivity in the sunnhemp genotypes studied. However, the dehiscence of globose anthers was approximately six hours earlier than the anthesis of sunnhemp according to the report of Nirmalaruban *et al.* (2020).

Table 3. Simple correlation between pollen viability percentage and pollen germination percentage of globose and heart shaped anthers on six sunnhemp genotypes at different floral stages

Particulars	Viability				
	Stages	Anther shape	S2	S3	S4
Germination	S2	I	0.70	0.54	0.61
		II	0	0	0
	S3	I	0.95*	0.65	0.62
		II	0	0.22	0.52
	S4	I	-0.18	0.11	0.25
		II	0	0.92*	0.84*

* Significant at 5 % level,
I- Globose shape, II- Heart shape

Fig. 3. Stigma receptivity of different floral stages in sunnhemp genotype, S- stage.



	SUIN 053	SUIN 37	JRJ 610	K12 Yellow	CO 1	CCJ 1
Stage S1	2	1	2	2	2	2
Stage S2	3	2	3	3	3	3
Stage S3	4	3	4	3	4	3
Stage S4	5	4	5	5	5	4

Fig. 4. Stigma receptivity of different floral stages in six genotypes of sunnhemp.
1- Very low, 2- Low, 3- Medium, 4- High, 5-Veryhigh

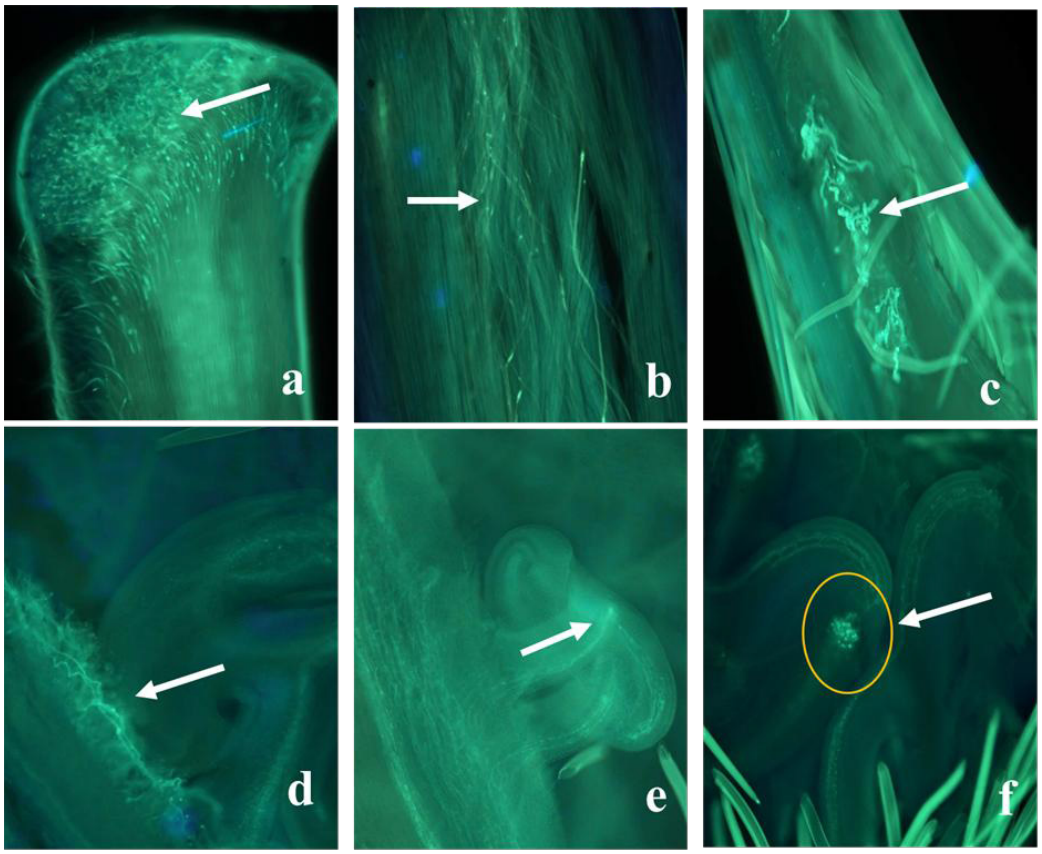


Fig. 5. Pollen tube growth after different hours of self pollination a) One hour after pollination, majority of pollen grains germinated in papillary region of style. b) Two hours after pollination, very few pollen tubes reaches the mid stylar region. c) Three hours after pollination, very few pollen tubes nearing towards ovary. d) Six hours after pollination, pollen tubes approaching the ovules. e) Eight hours after pollination callose wall development at and around nucellus cells. f) 12 hours after pollination; increased size in callose wall development.

Pollination studies were carried out in one of the genotypes SUIN 053 by following forced artificial self-pollination and sib pollination by using needles in a netted cage, preventing the entry of insect pollinators. Observations were recorded after different hours of (self/sib) pollination (1, 2, 3, 4, 6, 8, and 10 hours).Fluorescence microscopic observations were made in the stigmatic, stylar and ovarial regions of the flower in both self and sib mated pistils. The data was recorded on the number of pollen tubes reaching half of the length of style, 3/4th of the length of style and the number of pollen grains reaching the ovary after different hours of pollination by self and sib pollination in the sunnhemp genotype of SUIN 053 is presented in **Table 4**.

In self-pollinated pistils, majority of pollen grains germinated in the stigmatic region of the style two hours after pollination, but the elongation of pollen tube was restricted and a small number of pollen tubes reached mid stylar region. Only a very few pollen tubes approached its ovule, but did not enter into it. Callose wall formation was detected after eight and 12 hours of pollination and it showed an increasing trend thereafter, obstructing further pollen tube growth and preventing fertilization resulting in self-incompatibility (**Fig. 5**). Germination of pollen grains in the papillary region of the pistil was similar in the case of both artificial self -pollination and sib pollination.

In sib mated pistils, the majority of the pollen grains germinated in the papillary region of the style and pollen tubes grow towards the ovary in the style. The significant number of pollen tubes advanced towards the ovary, approaching the ovule and penetrated into the embryo sac of the ovule and for resulted seed set (**Fig. 6**).

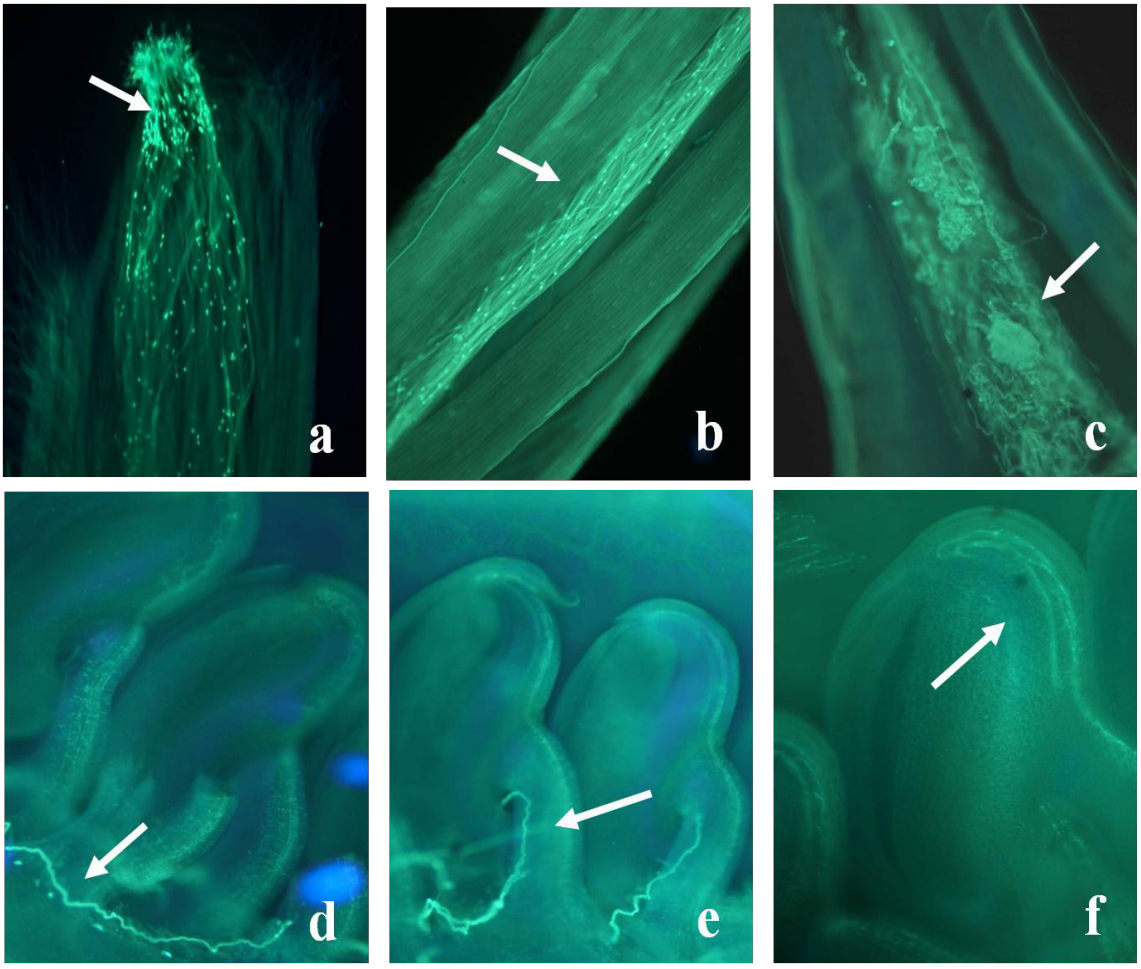


Fig.6. Pollen tube growth after different hours of sib pollination a) One hour after pollination, majority of pollen grains germinated in papillary region of style. b) Two hours after pollination, pollen tubes reach the mid stylar region. c) Four hours after pollination, pollen tubes nearing ovary. d) Six hours after pollination, pollen tubes approaching the ovules. e) Eight hours after pollination, pollen tube penetrated in embryo sac of ovule. f) 10 hours after pollination; normal growth of ovule in sib mated pistils.

Table 4. Pollen tube growth in the style and ovary regions of sunnhemp genotype SUIN 053 by self and sib pollination

Hours after pollination	Artificial self-pollination					Sib pollination				
	Number of pollen tubes reaching					Number of pollen tubes reaching				
	1/4th length of style (a)	1/2th length of style (b)	3/4th length of style (c)	Full length of style (d)	Total (a+b+c+d)	1/4th length of style (a)	1/2th length of style (b)	3/4th length of style (c)	Full length of style (d)	Total (a+b+c+d)
1	3.0	0.0	0.0	0.0	3.0	8.0	0.0	0.0	0.0	8.0
2	4.1	2.5	0.0	0.0	6.6	10.5	8.2	0.0	0.0	18.7
3	4.6	2.2	1.5	0.0	8.3	11.8	9.8	8.2	0.0	29.8
4	6.2	3.8	2.4	1.2	13.6	15.0	11.2	10.0	7.8	44.0
6	8.5	4.4	3.7	2.0	18.6	16.5	14.5	11.5	10.6	53.1
8	0.0	5.2	0.0	3.2	8.4	20.4	17.4	13.4	12.2	63.4
10	0.0	0.0	0.0	0.0	0.0	23.0	19.2	16.0	14.5	72.7

Thus, the study on pollen viability, pollen germination, stigma receptivity, pollen pistil interaction and *in vivo* pollen tube growth after artificial self and sib pollination in sunnhemp indicated the existence of self-incompatibility.

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