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Pollen germination characteristics, pollen–pistil interaction and reproductive behaviour in interspecific crosses among *Abelmoschus esculentus* Moench and its wild relatives

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Abstract
Pollen–pistil interactions play a crucial role in successful introgressions for desirable traits and are often restricted by presence of pre- or post-fertilisation barriers in various crops. Importance of wild germplasm of *Abelmoschus* (Malvaceae) as source of genes for biotic and abiotic stress resistance in crop improvement programmes have long been recognised by breeders. In the present study, behaviour of pollen germination and pollen tube growth were evaluated with respect to the seed set among four species of *Abelmoschus*. Alien pollen tubes showed significantly high growth inhibition in okra (*A. esculentus*) pistils and seldom penetrated the stigma. Pollen tube growth was normal in *A. esculentus* × *A. caillei* and its reciprocal cross, indicating the potential for its use in genetic improvement. Delayed pollen tube growth along with other structural abnormalities like twisting, swelling, high branching, bi-furcated tip and variation in callose form (reverse orientation and irregularity in callose plugs along the pollen tube) were frequently noticed in the interspecific crosses. The results indicated that the crosses *A. manihot* subsp. *tetraphyllus* var. *pungens* × *A. esculentus* had high incompatibility, while *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus* × *A. esculentus* were partially compatible and *A. esculentus* × *A. caillei* were fully compatible.

Keywords: Abelmoschus, *callose plug, pollen viability, pollen-tube abnormalities, SEM*

The cultivated okra (*Abelmoschus esculentus* Moench, Malvaceae) is an important multipurpose vegetable grown throughout tropical and sub-tropical low altitude regions of Asia and Africa. It is a seed propagated crop grown during summer and rainy seasons and hence categorised as a 'warm season crop' sensitive to frost, low temperature, water-logging, drought conditions and biotic stresses. It is popularly known as 'bhindi' in India and easy to cultivate and is adapted to varied ecological conditions. Okra is cultivated for its immature, edible fruits, with rows of tiny seeds and slimy or sticky texture when cut open. Okra fruits are rich in valuable nutrients including calcium, potassium, carbohydrate and vitamin C (Gopalan et al., 2007). Fruits are low in calories, are fat-free and provide a valuable supplementary food in the tropical diet. In Indian cooking, it is sautéed or added to gravy-based preparations and is very popular in southern India. The leaves are also occasionally eaten raw in salads.

Over the past ten years, the average productivity of okra has increased by only 3.1 mt/ha (in 1991–1992, productivity was 8.5 mt/ha and in 2010–2011, it was 11.6 mt/ha; Kumar et al., 2011). The low productivity in okra is attributed to poor seed replacement due to the limited availability of quality seeds, high incidence of pests (jassids, white fly and borers) and yellow vein mosaic virus (YVMV; Jambhale & Nerkar, 1981; Nerkar, 1991). Genetic resistance in wild species has been utilised for YVMV and

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other abiotic stresses through interspecific crosses. Although several location specific cultivars have been developed in India by private sectors and government organisations, the stability and uniformity of these varieties is not yet known.

The Indian sub-continent has been considered as a centre of diversity for *Abelmoschus* species. The wild species of *Abelmoschus* have a wide geographical distribution in India and neighbouring countries (Paul, 1993; Bisht & Bhat, 2006). A number of wild relatives of *Abelmoschus* have been identified as potential source of resistance for jassids and white flies, *Fusarium* wilt, *Alternaria* blight, powdery mildew and YVMV as well as abiotic stresses (Sandhu et al., 1974; Arumugam et al., 1975; Dhankar et al., 2005). Though the procedure of emasculation and cross-pollination is less cumbersome in the gene pool of cultivated okra, it is more difficult to cross *A. esculentus* with its wild species. Earlier studies and efforts to bring useful genes from wild okra were only partially successful due to presence of unidentified pre- and post-fertilisation barriers to gene flow in this genus (Pal et al., 1952; Kuwada, 1957, 1966; Siemonsma, 1982). However, there are highly sporadic attempts aimed at interspecific hybridisations among *A. esculentus* and its wild relatives so far (Jambhale & Nerkar, 1981; Nerkar, 1991; Tyagi, 2002).

Pollen–pistil interaction includes the initial contacts between the male pollen and female stigma, which is specific and subsequently followed by rapid pollen-recognition, pollen hydration and pollen tube formation (Gregory & Daphne, 2000). This pathway is often restricted by some barrier factors in hybridisation between two different species of the same genus. Dumas and Knox (1983) and, more recently, Chen and Kim (2009) drew attention to the application of aniline blue to trace the callose response, which is specific and related to rejection phenomena. By using the Aniline Blue Florescent (ABF) method and fluorescence microscopy, influence of pollen–pistil interaction on the seed set has been studied in various crops such as sorghum, pearl millet (Heslop-Harrison, 2000), sorghum (Hodnett et al., 2005), cotton, sesame (Ganesh Ram et al., 2006, 2007), *Vigna* (Krishnasamy et al., 2008) and *Cucumis* (Yuichi et al., 2012). However, in okra, only a few attempts were made to detect the reasons for failure of seed set by means of pollen–pistil interactions (Abdullah et al., 2000; Tyagi, 2002).

It now is well recognised that successful hybridisation between cultivated and wild species of the same genus will be successful only if there is perfect co-ordination (compatibility) between gene complexes of pollen and the ovule parents (Kuboyama et al., 1994). An understanding of the biological nature of the incompatibility systems that prevent this compatibility of donor pollen and subsequent seed development is most essential for the successful hybridisation between okra and its wild relatives. Hence, the present study was undertaken with three wild *Abelmoschus* species that have greater potential as donors (*A. manihot* (L.) Medik. subsp. *tetraphyllus* (Roxb. *ex* Hornem.) Borss. var. *pungens* (Roxb.) Hochr., *A. moschatus* Medik., *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus* (Roxb. *ex* Hornem.) Borss. and two cultivated species (*A. caillei* (A. Chev.) J. M. C. Stevels, *A. esculentus*) to evaluate the extent and nature of reproductive isolation barriers operating at different stages of gynoecia.

Material and methods

Plant material

Two cultivated species, *Abelmoschus esculentus*, *A. caillei* and three wild species, *A. manihot* subsp. *tetraphyllus* var. *pungens*, *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus* and *A. moschatus* (AES, ACA, AMP, AMT and AMO, respectively; see Table I), were grown and maintained at the National Bureau of Plant Genetic Resource (NBPGR), Regional Station, Bhowali, as well as at NBPGR, New Delhi for this study (Table I). The Bhowali location at the foothills of the Himalaya was preferred due to prevalence of milder conditions during the cropping season so that the deleterious effects of climatic conditions are reduced.

Pollination technique

Emasculation of unopened flower buds was done by removing the petals and un-dehisced anthers with a sharp knife in the afternoon between 4:00–6:00 p.m. The flower buds were then covered with paper bags to prevent drying of stigmatic surface and cross-pollination due to insect activity. Cross-pollination was made by tapping the pollen grains directly over the stigmas of emasculated flowers in the following morning between 9:00–10:00 a.m. and the flowers were again covered with paper bags. For each cross, pollen grains were collected from a single tagged plant of the wild donor species. Self-pollination was done at the same time following similar procedures. The bags were removed carefully from all pollinated flowers one day after pollination. Some of the crossed flowers were retained to allow fruit set for the study of post-pollination barriers to gene-flow.

Crossing experiment

Crossing experiments were undertaken in August–September 2009 (at 25–30 °C day temperature) at

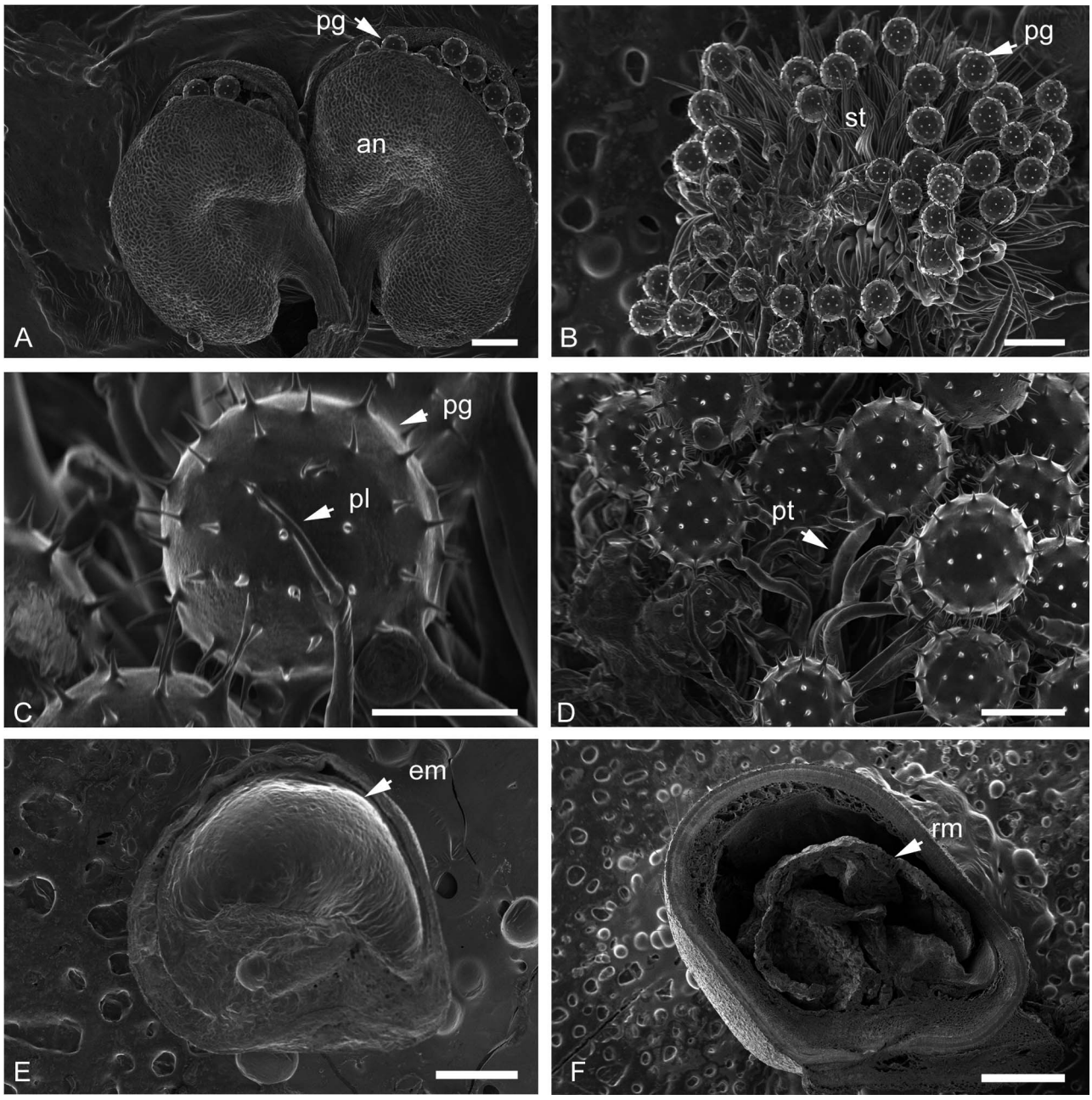


Figure 1. Scanning electron micrographs of pollen and its behaviour on the stigma of *Abelmoschus esculentus* (self). **A.** Dehiscence of anther. **B.** Pollen deposition on the stigma (*arrow*). **C.** Pollen adherence to stigmatic papillae (*arrow*). **D.** Pollen tube penetration to the stigma (*arrow*). **E.** Healthy embryo of AES seed. **F.** Ruptured embryo of seed in AES × AMO. an, anther; pg, pollen grain; pl, papillae; st, stigma; em, embryo; rm, ruptured embryo. Scale bars – 1 mm (E, F), 100 μm (A–D).

the NBPGR Regional Station, Bhowali, India. Four reciprocal crosses were made involving three wild and two cultivated species. For each cross, pollen from donor plant was transferred to receptive stigma of the recipient species. Details of pollinations made (P), fruit set (F), number of fruits with seeds (Fs), number of healthy seeds (S) and reproductive success (RS) were recorded. Crossing efficiency (CE) was calculated as reproductive success divided by number of pollinations made in each cross (Table II). The pistil from crossed and self-pollinated flowers were collected at 1, 2, 4, 8, 12 and 24 hours after pollination (HAP), fixed in FAA solution (5% formalin : 5% acetic acid : 90% ethanol) for 12 hours and then transferred to 70% ethanol for further processing. The maximum time interval for collection of pollinated pistils was restricted to 24 HAP because, under natural conditions, most of the crossed flowers

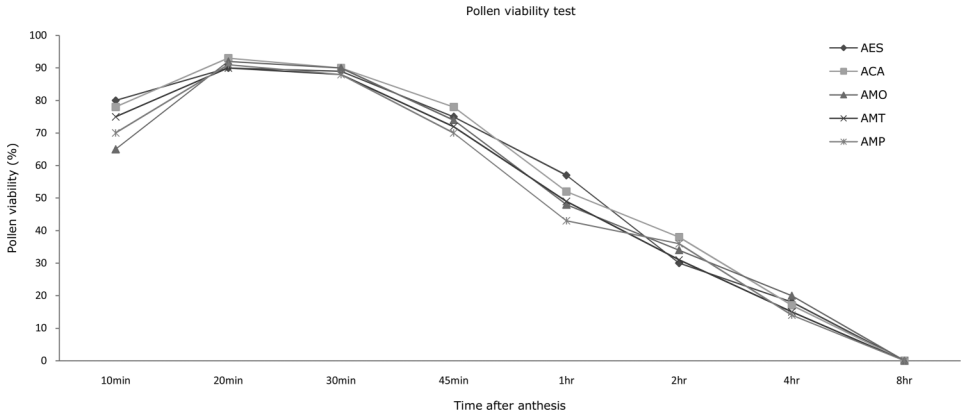


Figure 2. Pollen viability test by *in vitro* germination of AES, AMO, ACA, AMT and AMP at various time intervals after anther opening.

dropped-off within ten HAP. Ten of each crossed and self-pollinated flowers from five tagged plants were used for microscopic observations. Five pistils from each cross as well as from the controlled (self) plant were fixed to study the pollen–pistil interaction by microscopy.

Fluorescence microscopy

Alcohol preserved pistils of *Abelmoschus* species were gently rinsed in water and hydrolysed in 4N NaOH for 30 minutes. To remove the colour pigments from the stigma and stylar tissue, clearing was done with 50% sodium hypochlorite solution for 20 minutes. Pistils were stained with 0.001% decolourised aniline blue dissolved in 0.1% K_3PO_4 solution (Kho & Baer, 1968) for at least 15 minutes. The stigma lobes from each flower were carefully divided into two to three parts on a slide and squashed. The preparations were kept in dark until observation under a fluorescence microscope using ultraviolet light.

The observations were carried out with a Leica DM 5000B fluorescent microscope at 390–420 nm with a 450 nm emission filter. For callose, aniline blue stained tissues were observed at 350–400 nm light (Dumas & Knox, 1983). Images were captured with a mounted Leica DFC 420C zoom digital camera.

Table I. Details of plant material of *Abelmoschus* used in the study with their accession/collection number, biological status, chromosome number and potential source (source of information: Siemonsma, 1991).

No.	Taxon	Accession/ collection number	Code used in the study	Distribution range	Biological status	Chromosome number (2n)	Potential source	Crossability with cultivated species
1	*A. caillei*	IC587017	ACA	Northeast region	Cultivated	194	Shoot and fruit borer (*Earias* spp.) resistance/tolerance	Normal
2	*A. esculentus*	Pusa Sawani	AES	Cultivar (throughout India)	Cultivated	66–144	Tolerance to abiotic stress(salinity and photo-sensitivity)	Normal
3	*A. manihot* subsp. *tetraphyllus* var. *tetraphyllus*	IC090339	AMT	Gujrath, Karnataka, Maharashtra, Orissa, Chhattisgarh, Rajasthan	Wild	130	Promising source of resistance to YVMV Fusarium wilt (*Fusarium oxysporum*) resistance/tolerance	Medium
4	*A. manihot* subsp. *tetraphyllus* var. *pungens*	IC429939	AMP	Uttaranchal, Kerala, Karnataka, Rajasthan, Assam, Manipur, Sikkim, Tripura	Wild	138	Carries symptomless type of resistance to YVMV, Powdery mildew (*Erysiphe cichoracearium*) resistance/tolerance	Very low
5	*A. moschatus*	IC141068	AMO	Arunachal, Kerala, Western Ghats	Wild	72	Resistant to insect attack (Leaf hopper)	Very low (shriveled seed)

Pollen viability test

To study pollen viability un-dehisced anthers were collected from the field at the time of anthesis in all the five species. Anthers were dehisced in an incubator at 20–22 °C, which usually took 10–12 minutes. The viability test was immediately performed by *in vitro* pollen germination. The germination media consisted of 100 ppm boric acid, 15% sucrose, 10% PEG (polyethyleneglycol-6000 grade) and 1% agar dissolved in boiling water at pH 5.6. The medium was poured into 90 nm Petri dishes and pollen was distributed on the surface of the cooled but still somewhat fluid medium. The Petri dishes were then kept at 23–25 °C. A pollen grain was considered germinated when the tube had grown to a length of approximately twice the diameter of the pollen grain.

Scanning electron microscopy

The stigmatic features of un-pollinated and pollinated flowers at different stages as well as the development of the embryo inside the seeds were assessed by scanning electron microscopy (SEM). Flowers at different stages (30 minutes before anthesis; 10, 20, 30 and 60 minutes after pollination) were collected and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) followed by an ascending series of alcohol. At last, the samples were transferred to 100% hexamethyldisilazane (HMDS) through a graded series of ethanol-HMDS mixtures, then exchanged with new 100% HMDS and allowed to dry at room temperature (Zhang et al., 1990). The same procedure was followed for the mature, healthy as well as the aborted seeds to observe the micro-morphological difference in the development of the embryo in healthy and aborted seeds. Samples were fixed on the brass specimen stubs with a double adhesive carbon tape and gold coated using a JEOL JFC-1100 ion sputter. Each sample was uniformly coated with 20–30 nm thick gold film. Digital images were taken under a JEOL JSM-840A scanning electron microscope at accelerating voltage of 10 kV. Observations were made on anther opening, pollen adhesion to stigma papillae and pollen tube penetration to stigma in different samples.

Data analysis

Quantification of fertilisation barriers was done as pollen grains observed on the stigma (phase I), percentage of pollen germination and pollen tube growth subsequent to penetration of the stigma (phase II), style and up to the ovary (phase III) and inside the ovary (phase IV). Pollen germination percentage was calculated as the proportion of pollen grains germinated to the total number of pollen grains observed. Growth of pollen tube estimated as their per cent-occurrence in the various regions of gynoecia was calculated with reference to the percentage of total pollen grains germinated. Number, mean and standard error, percentage of pollen tubes entry in the style and their pathway during the various crosses were recorded (Tables III, IV). Data obtained for respective crosses were subjected to an analysis of variance (ANOVA, Table V) and significant differences between crosses and error against four phases (phases I–IV) were determined by Fisher's least significant difference (LSD) method using the statistical software package STATGRAPHICS Centurion, version 16.1.17.

Results

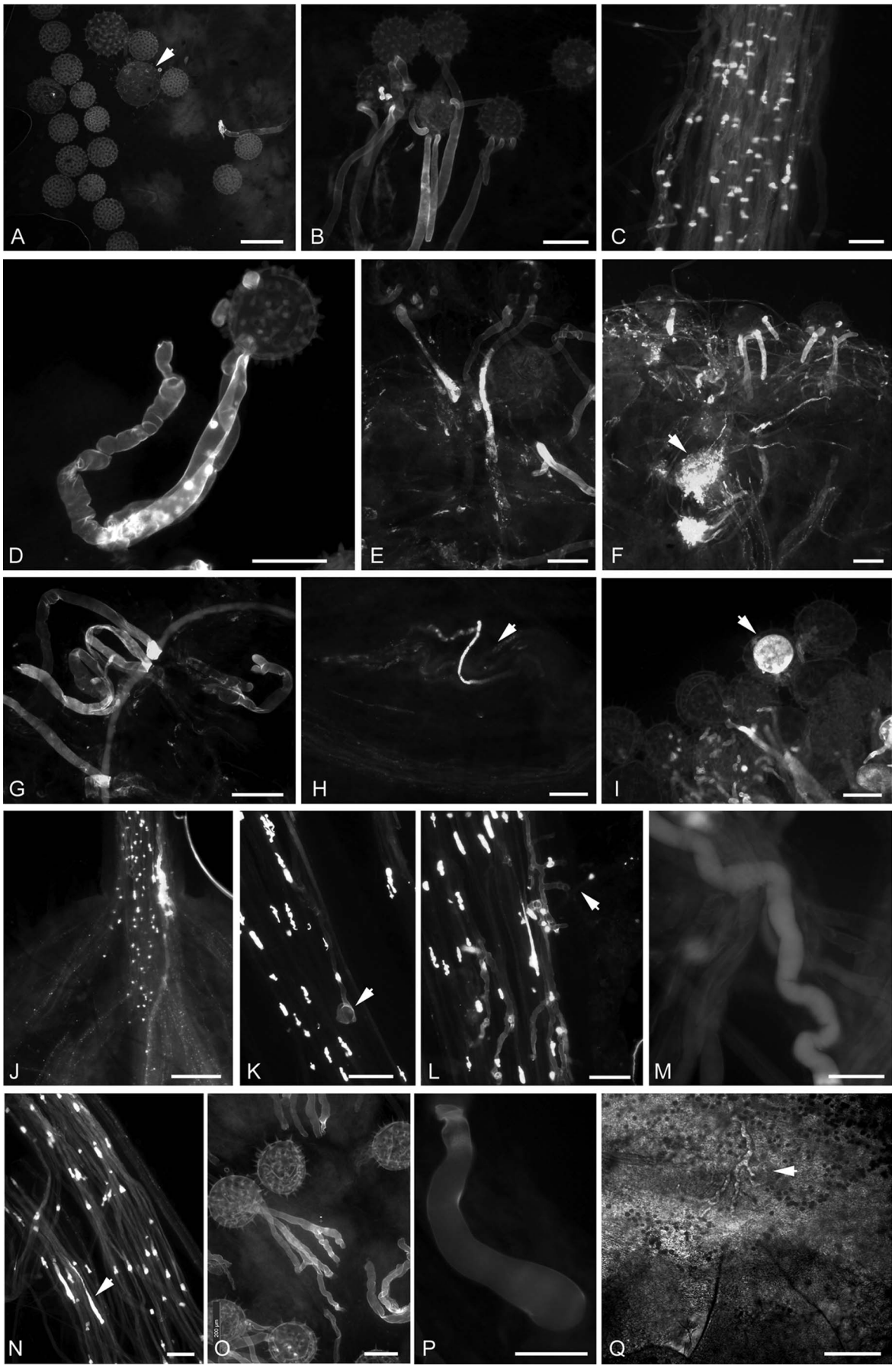
Anthesis, anther opening and pollen viability

The flowers present typical Malvaceous features in all the species studied here. The flowers are hermaphrodite, with terminal style and papillate stigma lobes ranging in number from five in *Abelmoschus manihot* subsp. *tetraphyllus* var. *pungens*, *A. moschatus* and *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus* to seven to eight in *A. esculentus* and *A. caillei*. In the present study, flowers fully opened at 9:00–10:00 a.m. and closed by 3:00–5:00 p.m. on the same day in August and September at temperatures ranging from 28 to 30 °C. However, the time of anthesis varied with species, cultivar, temperature and humidity.

Anthers were monothecous (Figure 1A). Dehiscence is transverse and occurs 15–30 minutes after anthesis. Pollen grains are spinescent and multiporate (Figure 1B, C). Ovules are bitegmic, crasssinucellate, slightly inclined and micropyles pointed towards the ovarian axis. Seed development was normal with a healthy embryo (Figure 1E). Pollen were found highly viable (80–90%) 20–30 minutes after anthesis while almost 50% viability was lost after one hour and gradually declined with time (Figure 2) in all species. After eight hours, pollen grains lost their viability fully. Flowers were self-compatible and showed varied levels of cross-fertilisation.

Fruit set, seed set and reproductive success from self- and cross-pollinations

From a total of 196 pollinations, fruit set was observed in all crosses except in AMO and AMP (Table II). Selfing of AES (control), fruit setting (nine out of ten pollinations), RS (31.11%) and CE (3.11%) were comparatively high in interspecific crosses made. In case of AES × AMT, 18 from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19 pollinations were successful with 0.76 CE, but when AES was used as pollen donor parent, CE was reduced to 0.55. Low CE was also observed in AMO × AES and AES × AMP, resulting only in 0.03% (from 14 pollinations) and 0.05% (from 45 pollinations), respectively. The highest CE was recorded in ACA × AES (1.31%) from 18 pollinations out of which 13 were successful with 23.54 reproductive successes, but when AES was used as seed parent, CE was only 1.11%. Shrivelled and immature seeds were produced in all cross combinations with varying frequencies.

Pathway of pollen tube guidance to the ovule

The pathway of the events, which contributed to the successful fertilisation in *Abelmoschus esculentus* (control) was traced with the help of the ABF method as well as by SEM. Pollen grains germinated quickly on the papillate stigma and produced pollen tubes in a polysiphonous manner (Figure 1D). They penetrated through the well-marked transmitting tissue of the style and entered the ovule in porogamous manner (Figure 3D). Pollen adhesion, hydration (Figure 3A), germination and penetration of the pollen tube into the stigma were found to be completed within an hour after deposition of the pollen on the stigma. During the next six hours, pollen tubes travelled through the transmitting tissue and after eight hours, reached the ovary. This event is followed by the dropping of stigma and style while only the ovary remained. In the next few hours, fruit development and seed setting took place. This reproductive pathway depicting pollen tube growth on stigma (phase I), in the style (phase II), entry into the ovary (phase III) and growth inside the ovary (phase IV).

Normal fertilisation showed ability for pollen germination and penetration of the pollen tube into the stigma and growing straightforward into the transmitting tract with a uniformly deposited callose plug at regular intervals and finally reached to the ovary without any barriers. However, in this study, the presence and extent of barriers in crosses with control as low percentage or no pollen grain germination, abnormalities in pollen tube structure and anomalies in callose deposition in pollen tube have been comparatively described so far (Figure 3, Tables III, IV).

Pollen germination and pollen tube behaviour in wide crosses among Abelmoschus *species*

All studied species of *Abelmoschus* have wet stigma, which open via a mucilaginous track into the ovary providing an ideal germination and growth system for pollen on the stigmatic surface. However, discrimination against alien pollen was observed in this mucilage track at a number of points. Pollen grain germination was scored as the number of pollen grains that had germinated on the stigma, with or without having penetrated in the stigma papillae.

Behaviour of Abelmoschus esculentus *pollen grains (♂) on the stigma of alien* Abelmoschus *species (♀)*

When AES was selfed, pollen germination was 87.6%, which was significantly ($P < 0.05$) higher than the interspecific crosses with AMT (55.1%), AMO (36.1%) and AMP (36.7%), whereas ACA (89.7%) was not significant. The differences among entries for pollen tube growth at different phases of gynoecia were more significant at initial phase than at phase IV (Table III). After pollen tube penetration to the stigma, the rate of pollen tube growth substantially reduced in the style of AMT (40.5%), AMO (28.5%) and AMP (31.5%), which was significantly ($P < 0.05$) lower than the control. In AMT × AES (34.2%), AMO × AES (26.5%), pollen tube required six hours to reach phase II, whereas in AMP × AES, it took 12 hours with very low frequencies (5.1%).

Expression of an incompatibility barrier was found to be more severe in phase II and phase III in the crosses made, which yielded very low CE. Very low fruit set (2.0) and RS (0.50) occurred from 14 pollinations in AMO × AES with 0.035 CE. Structural abnormalities were also evident like heavy callose accumulation at the entry point of the ovary (Figure 3P), bifurcation of the growing tip (Figure 3N) and the swollen tip (Figure 3O), twisting of pollen tubes was commonly observed in the AMO pistil, while the inhibition of the pollen tube

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Figure 3. Fluorescent microscopy of the pollen–pistil interactions in *Abelmoschus* interspecific crosses using the decolourised aniline blue method. **A.** Pollen hydration (*arrow*). **B.** Germinated pollen grain of ACA in phase II in an AES pistil. **C.** Germinated pollen grain of AMT growing in reversed direction. **D.** Pollen tube travelling normally through the transmitting tissue in AES × AES. **E.** Pollen tubes of AES that have not penetrated the stigma of AMP at 12 hours TAP. **F.** Callose accumulation in transmitting tissue of AMP × AES. **G.** Pollen tube growth inhibited in AES × AMP. **H.** Profuse branching of pollen tube in AES × AMT. **I.** Inhibited pollen tube in AMT × AES (*arrow*). **J.** Crooked pollen tube of AMO. **K.** Heavy callose deposition on pollen grain in AES × AMO. **L.** Balloon like tip in AES × ACA (*arrow*). **M.** Abnormal callose plug formation in AMT pollen tubes (*arrow*). **N, O.** Branched tip and swollen tip of AES. **P.** Callose accumulation in pollen tube at entry point of AMT ovary. **Q.** Pollen tube entering the ovule in AES × AMT (*arrow*). Scale bars – 100 μm.

Table II. Pollinations made, fruit set, seed set and crossing efficiency in interspecific crosses between *Abelmoschus esculentus* and its wild relatives (P, pollinations; F, fruit set; Fs, fruit with seed; S, number of healthy seeds; RS, reproductive success; CE, crossing efficiency).

Cross	P	F	Fs	S	RS (S/Fs)	CE (RS/P)
AES × AMO	12	5	3	8	2.66	0.22
AMO × AES	14	2	2	1	0.50	0.03
AMT × AES	20	17	17	189	11.11	0.55
AES × AMT	19	18	18	259	14.38	0.76
AES × AMP	45	10	3	8	2.66	0.05
AMP × AES	38	4	4	10	2.50	0.06
AES × ACA	20	18	14	310	22.14	1.11
ACA × AES	18	13	11	259	23.54	1.36
AES × AES	10	10	9	280	31.11	3.11

Abelmoschus esculentus (AES), *A. caillei* (ACA), *A. manihot* subsp. *tetraphyllus* var. *pungens* (AMP), *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus* (AMT), *A. moschatus* (AMO).

(Figure 3I) and the deformation of the callose plug in the pollen tube (Figure 3M) of AES was observed in the AMT style. Significantly ($P < 0.05$) higher (45.6%) pollen tubes successfully reached phase III in selfed AES at 12 HAP. In contrast, the lowest percentage of pollen tubes was observed in AMP (4.7%) and AMO (4.7%), while it was 25.5% in ACA, which was non-significant. With 38 pollinations of AMP × AES, only four pollinations were successful with 0.065 CE. Additionally, a severe occurrence of barriers like arrested pollen tubes (Figure 3E, P) was also observed in this cross.

Regarding AMP × AES, very few pollen tubes (2.2%) entered phase IV and reached the ovule though the overall pollen germination was observed to an extent of 36.7%. An interesting phenomenon noticed is that after 24 HAP, fruit dropping was the main obstacle to get fruits with viable seeds. Callose deposition in the transmitting tissue (Figure 3F) of AMP was a strong stylar resistance force functioning in the style. Comparatively, ACA showed significantly ($P < 0.05$) more compatible response to the *Abelmoschus esculentus* pollen than AMO, AMT and AMP. Though 89.7% pollen germination was observed in ACA × AES, substantial reduction of pollen tubes (20.51%) was evident at phase IV with 1.307 CE.

Behaviour of alien Abelmoschus *species pollen grains (♂) on the stigma of* A. esculentus *(♀)*

Reciprocal crosses were carried out to evaluate the occurrences of an incompatibility phenomenon between *Abelmoschus esculentus* and its wild relatives. When *A. esculentus* was used as female parent, significantly ($P < 0.05$) lower pollen germination percentage was observed with AMO (39.5%) and AMP (41.2%). Moderately high pollen germination percentage (77.1%) was recorded in AMT and the highest (89.3%) was recorded in ACA on AES stigma. After germination of the pollen grains, movement of pollen tube towards the various phases of the AES style were significantly ($P < 0.05$) higher than that observed in crosses, where AES was used as the male parent (Table IV).

In spite of high pollen germination (77.1%) in the initial phase in AES × AMT, occurrence of high branching (Figure 3H) and reverse direction of pollen tube growth (Figure 3C) resulted in reduced (36.2%) pollen tube growth (significant at $P < 0.05$) in phase II. However, in the crosses AES × AMO and AES × AMP, with germination percentages of 39.5 and 41.2, respectively, only 22.2% and 20.3% of pollen tubes entered in phase II. With pollen germination of ACA, 51.3% pollen tubes entered normally in phase II (Figure 3B). As a consequence, AES × ACA resulted in comparatively high CE (1.11%) from 20 pollinations. Lowest CE was observed in AES × AMP (0.05%) from 45 pollinations since germinated pollen grains could not travel further (Figure 3G). With 0.22% CE in AES × AMO, incidence of barriers like heavy callose deposition on pollen grains (Figure 3K), crooked pollen tubes (Figure 3J) were also common in phase II and phase III.

In phase III, the check point for pollen tubes to enter phase IV, only 27.1% (AES × AMT), 10.1% (AES × AMO) and 10.5% (AES × AMP) of the pollen tubes were able to enter (Figure 3Q). The occurrence of barriers was not so severe in AES × ACA as in the other interspecific crosses except for a few structural abnormalities in the pollen tubes such as balloon like pollen tube tips (Figure 3L). Comparatively less pollen tube growth, i.e. 3.7% (AES × AMP), 4.6% (AES × AMO) and 8.8% (AES × AMT), were observed in phase IV, which resulted in low RS in crosses attempted. However, an aborted embryo was also evidenced in AES × AMO (Figure 1F). A multiple range test revealed that AES × AMO and AES × AMP were significantly ($P < 0.05$) different to AES × AMT and AES × ACA at phase III.

Further statistical analysis (ANOVA) treated phases I–IV as 'groups' and crosses as 'treatments'. A one-way ANOVA analysis revealed significant variation in crossability among populations. Comparatively less success was observed in crosses such as AMT × AES, AMO × AES and AMP × AES (significant at $P < 0.05$), while AES × AES and ACA × AES were not significantly different. In case of reciprocal combinations, AES × AMO and AES × AMP were significantly different ($P < 0.05$), while AES × AES and AES × ACA were not

Table III. Pollen grain behaviour of *Abelmoschus esculentus* (♂) on stigma of alien *Abelmoschus* species (♀).

Pollen recipient (♀)	HAP (h)	Pollen observed (No.)	Pollen germination (%)	Number of pollen tubes travelling through transmitting tissue			
				Phase I	Phase II	Phase III	Phase IV
A. caillei	1	347	302 (87.1)	290 (83.5)	—	—	—
	2	327	290 (88.7)	278 (85.0)	250 (76.4)	—	—
	6	220	197 (89.5)	172 (78.1)	159 (72.3)	115 (52.3)	90 (40.9)
	12	340	318 (93.5)	290 (85.3)	254 (74.7)	170 (50.0)	140 (41.2)
	Total	1234	1107 (**89.7***)	1030 (**83.0***)	663 (**55.8***)	285 (**25.5**)	230 (**20.5**)
	Mean	308.5 ± 25.79	276.75 ± 23.55	257.5 ± 24.80	221 ± 25.32	142.5 ± 19.44	115 ± 17.67
A. esculentus	1	250	230 (92.1)	202 (80.1)	—	—	—
	2	310	285 (91.9)	264 (85.1)	247 (76.7)	196 (63.2)	—
	6	230	189 (82.2)	170 (73.9)	140 (60.8)	129 (56.1)	105 (45.6)
	12	290	245 (84.4)	228 (78.6)	201 (69.3)	178 (61.7)	140 (48.3)
	Total	1080	949 (**87.6***)	864 (**79.4***)	588 (**51.7***)	503 (**45.6**)	245 (**23.5**)
	Mean	270 ± 15.81	237.25 ± 17.17	216 ± 17.24	196 ± 25.30	167.66 ± 16.34	122.5 ± 12.4
A. manihot subsp. *tetraphyllus* var. *tetraphyllus*	1	300	170 (56.6)	135 (45.0)	—	—	—
	2	340	187 (55.0)	128 (37.6)	—	—	—
	6	260	148 (56.9)	100 (38.5)	89 (34.2)	60 (23.1)	—
	12	270	139 (51.5)	110 (40.7)	102 (37.7)	87 (32.2)	68 (25.2)
	Total	1170	644 (**55.1***)	473 (**40.5***)	191 (**18.0***)	147 (**13.8**)	68 (**6.3**)
	Mean	296.5 ± 20.44	105.5 ± 2.35	84.25 ± 5.20	66 ± 5.65	49 ± 0	34
A. manihot subsp. *tetraphyllus* var. *pungens*	1	357	123 (34.4)	103 (28.8)	—	—	—
	2	268	110 (41.0)	97 (36.2)	—	—	—
	6	270	107 (39.6)	95 (35.2)	—	—	—
	12	346	110 (31.7)	90 (26.1)	70 (20.2)	65 (18.7)	30 (8.7)
	Total	1241	450 (**36.7***)	385 (**31.5***)	70 (**5.1***)	65 (**4.7**)	30 (**2.2**)
	Mean	310.25 ± 20.7	112.5 ± 3.09	96.25 ± 2.32	70	65	30
A. moschatus	1	365	110 (30.1)	98 (26.8)	—	—	—
	2	285	105 (36.8)	80 (28.1)	—	—	—
	6	279	109 (39.6)	89 (31.8)	74 (26.5)	—	—
	12	257	98 (38.1)	70 (27.2)	58 (22.5)	49 (19.0)	34 (13.2)
	Total	1186	422 (**36.1***)	337 (**28.5***)	132 (**12.3***)	49 (**4.7**)	34 (**3.3**)
	Mean	365	110 (30.1)	98 (26.8)	—	—	—

Figures in parentheses denote percentage value and bold typeface indicate percentage of average value calculated on total pollen grains germinated; ±, standard error.
*Significant at $P \leq 0.05$.

significantly different. The *F*-ratio for Tables III and IV data was 3.98 and 3.62, and the *P*-value 0.0156 and 0.0225 of the *F*-test was less than 0.05, respectively (Table V), indicating statistically significant differences in the percentage of pollen tubes in the style between the crosses at the 95.0% confidence level.

Discussion

Among numerous obstacles to a successful seed set, the most significant feature observed was the presence of a physical barrier of multiple tissues between the male (pollen) and female (pistil) gametophytes leading to a low seed and fruit set in *Abelmoschus* crosses involving the wild relatives. Earlier studies reported that the hybridisation between *A. esculentus* and its wild species resulted in a low fruit and seed set along with sterile and shrivelled seeds (Ustinova, 1949; Pal et al., 1952; Jambhale & Nerkar, 1981). In the present study, field data substantiated with comparative pollen–pistil interaction studies were conducted at various intervals by means of fluorescence microscopy and SEM.

Preliminary studies indicate that bi-nucleate pollen generally germinates readily in *in vitro* condition (Mulcahy & Mulcahy, 1983). Shivana and Sharma (1985) reported that pollen germination in *Petunia* began within a few minutes after the release of the pollen to the media. This was also true for *Abelmoschus* spp. pollen, in which highest pollen germination (80–90%) was observed in pollen released at 9:00–9:30 a.m. (20–30 minutes) after anther opening. This indicates that pollen used for control as well as cross-pollination was highly viable. This concludes that the failure of pollen germination and pollen tube growth in most cases was not due to lack of pollen vigour and viability, but probably due to some barrier factors.

Table IV. Pollen grain behaviour of alien *Abelmoschus* species (♂) on the stigma of *A. esculentus* (♀).

Pollen donor (♂)	HAP (h)	Pollen observed (No.)	Pollen germination (%)	Number of pollen tubes travelling through transmitting tissue			
				Phase I	Phase II	Phase III	Phase IV
A. caillei	1	320	297 (92.8)	280 (87.5)	—	—	—
	2	290	259 (89.3)	245 (84.5)	215 (74.2)	190 (65.5)	—
	6	310	279 (90.1)	230 (74.2)	196 (63.2)	169 (54.5)	156 (50.3)
	12	265	225 (84.9)	202 (76.2)	180 (67.9)	164 (61.8)	139 (52.4)
	Total	1185	1060 (**89.3***)	957 (**80.6***)	591 (**51.3**)	523 (**45.5**)	295 (**25.7**)
	Mean	296.25 ± 10.5	265 ± 13.36	239.25 ± 14.1	197 ± 8.25	174.33 ± 6.50	147 ± 6.01
A. esculentus	1	310	287 (92.6)	254 (81.9)	—	—	—
	2	290	270 (93.1)	264 (91.1)	240 (82.7)	225 (77.6)	—
	6	258	237 (91.8)	215 (81.3)	195 (75.6)	180 (69.7)	150 (58.1)
	12	260	226 (86.9)	212 (81.5)	190 (73.1)	170 (65.4)	137 (52.7)
	Total	1118	1020 (**91.1***)	945 (**83.9***)	625 (**57.8**)	575 (**53.2**)	287 (**27.5**)
	Mean	279.5 ± 10.84	255 ± 12.28	236.25 ± 11.5	208.33 ± 12.9	191.66 ± 13.8	143.5 ± 4.59
A. manihot subsp.	1	267	190 (71.2)	160 (59.9)	—	—	—
tetraphyllus var.	2	280	219 (78.2)	190 (67.8)	125 (44.6)	90 (32.1)	—
tetraphyllus	6	315	250 (79.4)	210 (66.6)	145 (46.1)	110 (34.9)	—
	12	290	230 (79.3)	190 (65.5)	157 (54.1)	120 (41.4)	102 (35.1)
	Total	1152	889 (**77.1***)	750 (**64.9***)	427 (**36.2**)	320 (**27.1**)	102 (**8.8**)
	Mean	288 ± 8.74	222.25 ± 10.8	187.5 ± 8.92	142.33 ± 7.62	106.66 ± 7.2	102
A. manihot subsp.	1	280	129 (46.1)	105 (37.5)	—	—	—
tetraphyllus var.	2	295	119 (40.3)	98 (33.2)	75 (25.4)	—	—
pungens	6	290	110 (37.9)	90 (31.1)	79 (27.2)	65 (22.4)	—
	12	305	124 (40.6)	102 (33.4)	87 (28.5)	60 (19.7)	45 (14.7)
	Total	1170	482 (**41.2***)	395 (**33.8***)	241 (**20.3**)	125 (**10.5***)	45 (**3.7**)
	Mean	292.5 ± 4.50	120.5 ± 3.50	98.75 ± 2.81	80.33 ± 2.88	62.5 ± 1.76	45
A. moschatus	1	310	126 (40.6)	105 (33.8)	—	—	—
	2	270	165 (24.1)	102 (37.7)	89 (32.9)	—	—
	6	297	139 (46.8)	97 (32.6)	75 (25.2)	50 (16.8)	—
	12	300	140 (46.6)	106 (35.3)	92 (30.6)	70 (23.3)	56 (18.6)
	Total	1177	570 (**39.5***)	410 (**34.9***)	256 (**22.2**)	120 (**10.1***)	56 (**4.6**)
	Mean	294.25 ± 7.40	142.5 ± 7.05	102.5 ± 1.75	85.33 ± 4.27	60 ± 7.07	56

Figures in parentheses denote percentage value and bold typeface indicate percentage of average value calculated on total pollen grains germinated; ±, standard error.
*Significant at $P \leq 0.05$.

Adhesion is currently considered as one of the first interactions in pollen–stigma recognition (Lord & Russell, 2002). A species specific adhesion mechanism was observed in *Abelmoschus* species demonstrating the importance of adhesion in pollen–pistil interaction. Pollen grain adherence was very low in AMO × AES, AMT × AES and AMP × AES, which might be due to the comparatively large size of *A. esculentus* (♂) pollen that could not adhere to the smaller stigmatic papillae of former species preventing the primary interaction as discussed earlier for *Abelmoschus* species (Abdullah et al., 2000). In this study, occurrence of non-hydrated pollen grains indicated a possibility of rejection and depletion of the pollen of *Abelmoschus* species by stigmatic papillae.

Pollen–pistil recognition mechanism is characterised in the manner that adhesion/germination of pollen and growth of the pollen tubes of a species are restricted in the stigma of the other. This recognition mechanism operates in a number of incompatible crosses (Liedl et al., 1996; Vervaeke et al., 2001; Ganesh Ram et al., 2007) and may be responsible for the reduced percentage of pollen germination and growth towards the stylar region of AMT, AMP and AMO. Highest pollen germination percentage upon selfing of AES and comparatively lower percentage of pollen germination in AMO × AES, AMT × AES and AMP × AES indicated initially a surface retardation phenomenon operating in these crosses. However, a dramatically reduced percentage of pollen tubes in phase II showed that the existence of partial incompatibility for AES pollen in crosses attempted during our study. Low percentage of pollen germination may result in the reduction of pollen tube number growing towards the micropyle end, which reduces the chances of seed set in these species as revealed in *Vigna* species (Krishnasamy et al., 2008).

Normally, pollen tubes take six HAP to reach phase III, but in AMP × AES and AMO × AES, pollen tubes took 12 HAP to reach phase III. This considerable delay phenomenon possibly revealed

that a resistance force was predominant due to a high intensity of pollen tubes of AES in the stigmatic surface. As a consequence, growth of pollen tubes might have been prevented due to the inter-pollen-tube competition as reported earlier in wheat and rye (Jalani & Moss, 1980). Also, Hodnett et al. (2005) pointed out that a normal metabolism of the pollen tube was hindered in unfavourable conditions, and as a result, the pollen tube malformed, which reduced further growth to the micropyle. These unfavourable conditions might have been responsible for unexpected fruit dropping in AMP × AES, which lead to a low fruit set. This analysis can be explained for the reason of low fruit set, less number of seed set and ultimately reducing the crossing efficiency in *Abelmoschus* crosses.

Next to the pollen germination, pollen tubes are guided to the ovary by some unknown (biochemical) signals originating in the transmitting tissue, which may be interrupted in the interspecific crosses. This interruption can be assessed by callose response with the presence of incompatible pollen (Dumas & Knox, 1983; Heslop-Harrison, 1983; Chen & Kim, 2009). Heavy callose accumulation in the transmitting tissue of AMP, at the entry point of the AMT ovary, indicates that these species have strong incompatible response to AES pollen. This may also be due to the high interspecific morphological distinctness of these species (Borssum-Waalkes, 1966). Overall observation throughout our study demonstrated that the diversity in callose response in relation to the interspecific crosses indicates the temporal variation in process of pollen tube inhibition and its role in pollen–pistil interaction and reproductive isolation among *Abelmoschus* species.

Table V. Summary of the ANOVA test of the crossability data. Each main effect is tested against the error, *F*-ratio and *P*-value for interspecific crosses in *Abelmoschus*.

Source	Sum of squares	Df	Mean square	*F*-Ratio	*P*-Value
Table III					
Between group (crosses)	8271.48	4	2067.87	3.98	0.0156*
Within group (error)	10392.6	20	519.632		
Total (corrected)	18664.1	24			
Table IV					
Between group (crosses)	7475.2	4	1868.8	3.62	0.0225*
Within group (error)	10335.3	20	516.763		
Total (corrected)	17810.5	24			

Df, degree of freedom.
*Significant at $P \leq 0.05$.

In the present study, reciprocal direction of pollination was found to be more successful. Highest pollen adherence was noticed in reciprocal crosses of AMP, AMO and AMT with the AES stigma. It may be due to the small pollen size of AMP, AMO and AMT and the more receptive stigmatic surface (six to eight lobes) of AES. Pollen tubes entered phase II and III normally in AES × AMT, but the formation of shrivelled seeds and aborted embryo in AES × AMO indicated operation of post-zygotic barriers in this cross. It was also observed that being the female parent, the AES stigma, the short length and the broad style and ovary contribute to a high percentage of pollen tubes reaching phase IV, which resulted in a maximum seed set. This may be providing a furnished platform for the alien pollen tubes to travel along the styles as reported earlier in *Vigna* (Gopinathan et al., 1986; Barone et al., 1992), *Leucaena* (Sorensson & Brewbaker, 1994), *Lycopersicon* (Liedl et al., 1996) and *Aechmea* (Vervaeke et al., 2001). Less structural anomalies were noticed in reciprocal combination. Reverse orientation of pollen tubes in AES × AMT indicated that some biochemical signals originating in the style and embryo sac may be disturbed by incompatible factors (Lord & Russell, 2002) in the AES style. Field data showed that reproductive success was comparatively more when AES was used as a female parent. This analysis revealed that reciprocal direction of crosses would be more useful to get hybrids of desirable resistance/tolerance genes in cultivated okra.

Among the interspecific crosses attempted, ACA × AES as well as their reciprocal cross, were more successfully indicating comparatively high pollen germination, high percentage of pollen tubes at all phases and moderate occurrence of structural abnormality like crooked and huge branching of pollen tubes. Moreover, maximum fruit set and high CE in these crosses made this combination more compatible, which can be exploited for developing potential hybrids. The adequate success of crosses using these two species is probably designated to commonness of these cultivars from Africa and India. Morphological similarities especially of vegetative and reproductive characters may have further contributed to this success. This leads to perfect bonding of the gene pool between pollen and the ovule parents, which resulted in healthy hybrid seeds (Kuboyama et al., 1994). Therefore, the use of *Abelmoschus caillei* is recommended as potential bridging parent to transfer alien genes for deployment in okra breeding programmes.

However, in the present study, besides low percentage of pollen germination and slow rate of pollen tube growth, several other types of pollen

tube abnormalities were encountered. Reverse direction of pollen tube growth, lateral expanded or swollen pollen tubes, irregular callose deposition and an abnormal shape of the callose plug, branched pollen tubes and balloon like tips of pollen tubes were commonly observed. Such structural abnormalities were also reported in other interspecies crosses involving wheat and rye (Jalani & Moss, 1980), okra (Abdullah et al., 2000), pearl millet (Heslop-Harrison, 2000), sorghum (Hodnett et al., 2005), cotton and sesame (Ganesh Ram et al., 2006, 2007) and *Vigna* (Krishnasamy et al., 2008). In *Abelmoschus* interspecific crosses, it is mostly possible to obtain first-generation interspecific hybrids, but apparent occurrence of ruptured embryos in AES × AMO indicates that the ploidy level of parents affect the balance of meiotic events (Kuwada, 1974), which resulted in the malformation of the embryo. Just observing by naked eye, it is difficult to say, which seeds possess aborted embryos to produce a subsequent generation or even to carry out backcrosses with these seeds (Kuwada, 1957; Siemonsma, 1982).

Nevertheless, the earlier discussed reproductive barriers in interspecific crosses can be explained adequately by the concept of incongruity (Kuboyama et al., 1994). Incongruity includes a series of barriers (e.g. pollen germination on the stigma, penetration of the pollen tube into the transmitting tract, straightforward growth of the pollen tube towards the ovary), whereas incompatibility involves S-gene action (Pickersgill, 1993; Shivanna, 1996). However, it is not yet clear if the incompatibility system of *Abelmoschus* species is due to S-allele-action and consequently inhibiting the effective fertilisation. In the present study, normal growth of pollen tubes coupled with inhibition at the stylar end as well as at the stigma or the style top was reported. Also, for a better understanding of the incompatibility system, further genetic analysis is needed, since the S-allele status and the genetic distance among the studied *Abelmoschus* species/cultivars is not yet known. Therefore, in the present circumstances, it would be better to explain the existing reproductive barriers as due to incongruity rather than incompatibility.

Additional obstacles to hybridisation may include hybrid sterility due to the differences in chromosome number or limited homology between the genomes in the hybrids. Also, visual differences in stigma lobes and style length in the studied species would require further detailed investigation with respect to the timing and duration of the stigma receptivity, particularly in wild species. Altogether, potential utilisation of the germplasm of wild species in okra breeding programmes would require overcoming the hybridisation barriers. Recently, a range of techniques, such as bud pollination, stump pollination, use of mentor pollen, grafting of the style, ovule and embryo rescue, have been used successfully to overcome pre-fertilisation barriers (Bhat & Sarla, 2004). Previously, successful *in vitro* embryo rescue has been demonstrated for interspecific crosses between *Abelmoschus esculentus* and *A. moschatus* (resistant wild species) to transfer virus resistance in *A. esculentus* (Gadwal et al., 1968).

Additionally, the prolonged controversies surrounding the release of genetically modified crops make it difficult to develop new resistant varieties by advanced genetic engineering approaches. Therefore, deployment of useful alien genes from wild germplasm of *Abelmoschus* to existing varieties is only possible by traditional breeding approaches. Hence, the present study would be beneficial to the okra breeders to addresses the obstacles in interspecific hybridisation so far.

Conclusion

The present study on pollen viability indicates that effective use of pollen grains for pollinations by hand has only been successful up to one hour after anthesis. Further, the study reflects that pollen–pistil interaction plays an important role in interspecific hybridisation between *Abelmoschus esculentus* and *A. manihot* subsp. *tetraphyllus* var. *tetraphyllus*, *A. moschatus* and *A. manihot* subsp. *tetraphyllus* var. *pungens*, and this appears to be due to the predominant presence of pre-zygotic barriers operating at all the stages of reproductive pathway with limited fertilisation (low seed set) and embryo formation, followed by seed abortion (post-zygotic barriers). Utility of *A. caillei* as potential bridging parent to transfer alien genes for deployment in okra breeding programmes would be beneficial.

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