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Pollen - pistil interaction study in interspecific crosses of *Medicago sativa* x *Medicago scutellata* for stem weevil resistance

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Abstract
Studies were carried out for the pollen tube growth, failure of pollen tubes to effect fertilization and post-fertilization ovule abortion to hybridize Indian tetraploid (2n = 32) *Medicago sativa* L. cultivar RL 88 with diploid (2n=30) exotic annual shield medics / snail medic genotypes *Medicago scutellata* L. (Mill.), for the transfer of stem weevil resistance. Pollen fertility of *M. scutellata* was found to be 89%. In the selfing study of *M. scutellata*, the pollen grain germinated and reached ovule by 28 hours after pollination (HAP). In cultivar RL 88, the pollen grains reached ovule by 48 HAP after selfing. However, very few pollen grains reached the ovule upon selfing even after 5 days after pollination (DAP). In *M. sativa* x *M. scutellata* crosses, the growth of the pollen grains on the stigma and stylar tissues were very slow, during the study period. However, it was observed that after 96 HAP to 120 HAP, hardly few pollen grains reached ovule in the interspecific crosses in *M. sativa* x *M. scutellata*, with many callose plugs resulting in putative hybrid embryos. Out of 1287 florets attempted for interspecific crosses between RL 88 x *M. scutellata* (EC 541685), only five normal appearing pods developed till maturity and containing one seed each. Reciprocal crosses involving *M. scutellata* as female parent did not yielded any capsule set. Obvious indicators of fertilization as well as the initiation of the embryonic growth in *M. sativa* are the persistence of the flower on the raceme and increase in the diameter of the ovary. However, the putative hybrids need further confirmation with cytological and molecular studies. Our results demonstrate that interspecific hybrid plants with *M. sativa* x *M. scutellata* can be successfully obtained by adapting to suitable tissue culture-based breeding methodologies and embryo rescue.

Keywords: Interspecific hybridization, *In-vitro* germination, *Medicago sativa*, *Medicago scutellata*, Pollen pistil interaction, Stem weevil resistance

Introduction

In lucerne (*Medicago sativa* L.) special pollination mechanism "tripping" occurs which lead to cross fertilization and good seed set. The plant being highly heterogeneous in nature, self incompatibility mechanism operates within and development of selfed seeds will be of varied degree. The mere presence of pollen on the stigma of a lucerne flower does not ensure fertilization and seed development, regardless favorable environmental conditions. Slow pollen tube growth, failure of pollen tubes to effect fertilization, and post fertilization ovule abortion have been shown to be important in causing low seed set after self-pollination (Sangduen *et al*., 1983). However, little has been reported on the extent to which these factors account for the very low seed set found in some plants after cross pollination. Upon pollination by compatible pollen, a series of events are set in motion which culminates in the delivery of sperm cells to the female gametophyte and double fertilization. Pollen germination and pollen tube growth are essential to the fertilization process. The critical events in this post-pollination process relate to the establishment of a polarized growth pattern within the pollen grain, the elaboration of a pollen tube that extends by tip growth, the invasion of the tube into tissues of the pistil, and its directed growth towards the ovary as it homes in on its ovule targets. Earlier studies on post fertilization ovule abortion (Cooper *et al*., 1937), frequency of post fertilization abortion by (Cooper and Brink,1940), embryo development by pollinating pollen of *Medicago* and other species (Fridriksson and Bolton, 1963); perennial x annual *Medicago* cross as well as, pollen germination and pollen tube growth after self- and intra- and interspecific pollinations of annual and perennial *Medicago* species respectively (Sangduen *et al*., 1982 and 1983) revealed the possibilities of obtaining viable seeds in lucerne by different pollination methods. Fluorescent microscopy has been used to study pollination problems and to determine the growth of pollen tubes in stylar tissues to differentiate compatible and incompatible crosses in several

plants (Martin, 1959).Through this study we intend to determine i. pollen-pistil interaction in self of *Medicago sativa* (L.) (2n=32), annual *Medicago scutellata* (L.) Mill. (2n=30) and the factors contributing to failure of perennial *Medicago sativa* (L.) (2n=32) x annual *Medicago scutellata* (L.) Mill. (2n=30) (Bauchan and Elgin, 1984) crosses, ii. Visible indicators of successful pollination and fertilization iii. Identify factors responsible for failures in interspecific crosses and towards development of interspecific hybrids for lucerne stem weevil (*Hypera postica* Gyll.) resistance.

Materials and Methods

Pollen fertility studies: Pollen fertility was obtained by staining the fresh pollen in acetocarmine solution (1%) and atleast 100 pollen grains per slide were counted. Plants from *Medicago sativa* var. RL 88 and *Medicago scutellata* exotic collection (EC 547739, 547741, 541685 & 541686) from the germplasm were used (Table 1).

Emasculation and pollination: Young buds were selected from the raceme in healthy branches and mostly from the middle raceme, top 6-10 young buds about to be matured in one or two days were selected for bud pollination. Emasculation was done using a sterile pointed forcep after removing the standard petal by cutting it at its base. Pollen from *M. scutellata* accessions were collected from the flowers about to open during 9.45 a.m. to 10.00 a.m. Crosses were effected between *M. sativa* (RL 88) x *M. scutellata* accessions during 10.00 a.m. to 11.00 a.m. Both direct and reciprocal crosses were attempted in *M. sativa* (RL 88) x *M. scutellata* (EC 541685) and only direct crosses were attempted in other accessions of *M. scutellata* (Table 2) with *M. sativa* (RL 88) as female parent.

Pollen-pistil interaction: Two racemes with about 8-10 florets were collected at 12, 24, 48, 60, 72, 96 and 120 hours after pollination (HAP). Selfing in both the parents were also carried out in fourty buds. Pollen fertility in all the *M. scutellata* accessions was carried out using acetocarmine staining and pollen counts were recorded using E - 12 Nikon microscope. Fluorescence technique (Martin, 1959) was used to study the pollen tube growth and fertilization in selfed pistils of *M. sativa* and *M. scutellata* as well as in *M. sativa* x *M. scutellata* pistils and observations were made using Olympus microscope with fluorescence attachment. Pistil clearing treatment with modification in 1 N NaOH at 60º C varied between species *viz*., 2 h for *M. scutellata* and 3 h for *M. sativa*. The concentration of aniline blue dye was 0.1% and the staining of the pistil was for 12 to 18 h. Whole pistil observations were carried out in an Olympus microscope with fluorescence filter attachment and image capturing facility.

Results and Discussion

Pollen fertility: Pollen fertility in *Medicago scutellata*'s was high and it was observed to be 93 per cent in EC 541685; 90, 88 and 84 per cent EC 541686, EC 547739 and EC 547741, respectively. *Medicago sativa* cv. RL 88 had 89 per cent pollen fertility when compared to cv. Anand 2 with 90.5 per cent. High percentage of pollen grains germinated and penetrated the stigmatic surface following self pollination of the self fertile *M. scutellata* accessions (Fig. 1). However within *M. scutellata* accessions, EC 541686 and EC 541685 the pollen germination was faster than that of EC 547739 and EC 547741. Pollen tubes of the self fertile *M. scutellata* have reached the ovules, 24 to 28 hours (h) after pollination (HAP). No evidence of self-incompatibility was noticed in *M. scutellata* accessions (Table 2).

In *M. sativa* cv. RL 88 and Anand-2 self's, there was delay in pollen tube reaching the ovule as compared to *M. scutellata* accessions. The pollen tube reached the ovule by 42 h to 48 h after selfing (Figs. 4 and 5) in cvs. RL 88 and Anand 2. However the time delay was noticed because of the *M. sativa* cultivars belongs to a composite population and individual plant genotypes under observation may have difference in fertility status. Growth of thread like tubes, with well defined contours was rapid and uniform through out the length of the pistils in *M. scutellata* accessions. The growth of the pollen tubes in the pistils were easily identified since the tubes were lined by callose and callose plugs formed at irregular intervals

Table 1. Source of *Medicago* materials used for interspecific hybridization

Genotype	Cultivar / Accession No.	Source	Remarks
Medicago sativa	Anand 2	AAU, Gujarat	Perennial; High biomass; Susceptible to stem weevil
Medicago sativa	RL-88	MPKV, Rahuri, Maharastra	Perennial; High biomass; Susceptible to stem weevil
Medicago scutellata	EC 547739	IGFRI, Jhansi	Annual; low biomass; resistant to stem weevil
Medicago scutellata	EC 547741	IGFRI, Jhansi	Annual; low biomass; resistant to stem weevil
Medicago scutellata	EC 541685	IGFRI, Jhansi	Annual; low biomass; resistant to stem weevil
Medicago scutellata	EC 541686	IGFRI, Jhansi	Annual; low biomass; resistant to stem weevil

in the tubes (Fig. 2). The callose fluoresced bright yellow against the pale grey / black background of the surrounding tissue (Fig. 3).

Table 2. List of self's and crosses attempted in *M. sativa* and *M. scutellata* accessions and capsule set recorded

Cross / self details	No. of buds pollinated	No. of capsules set
M. scutellata self (EC 541685)	50	32
M. sativa self (RL 88)	50	12
M. sativa self (Anand 2)	50	14
M. scutellata (EC 541685) x *M. sativa* (RL 88)	25	0
M. sativa (RL 88) x *M. scutellata* (EC 547739)	50	0
M. sativa (RL 88) x *M. scutellata* (EC 547741)	50	0
M. sativa (RL 88) x *M. scutellata* (EC 541685)	1287	5
M. sativa (RL 88) x *M. scutellata* (EC 541686)	250	1

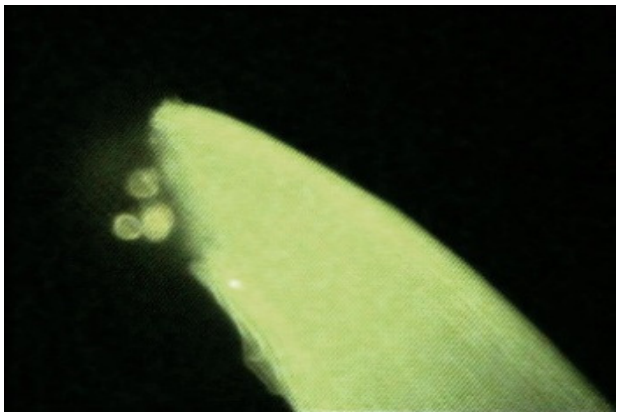

Fig 1. Self -fertile *M. scutellata* pollen on stigmatic surface

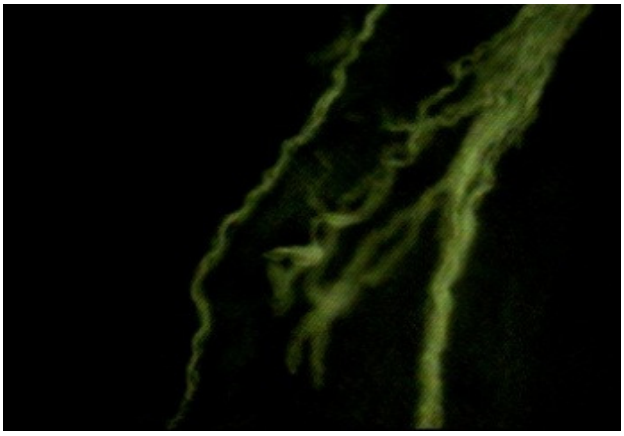

Fig 2. Pollen tubes growth in self – fertile *M. scutellata* stylar tissue

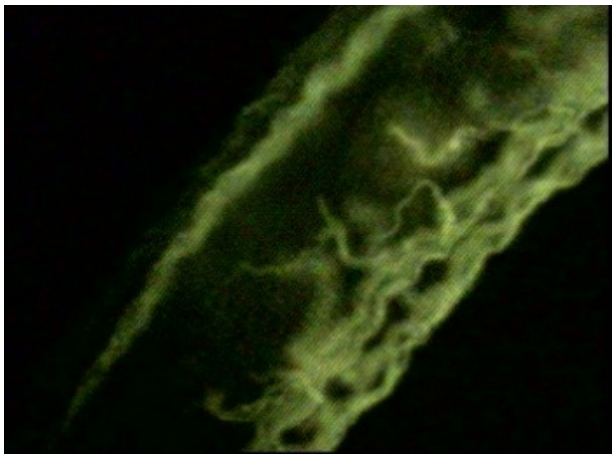

Fig 3. Pollen tubes fluorescing bright yellow in self – fertile *M. scutellata* ovule

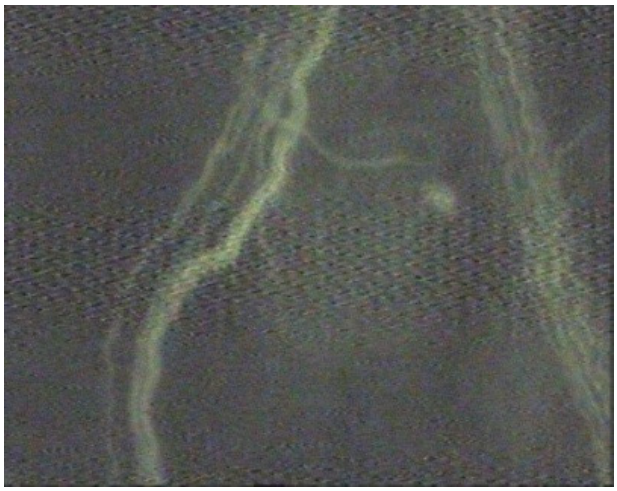

Fig 4. Pollen tubes growth in self – fertile *M. sativa* stylar tissue

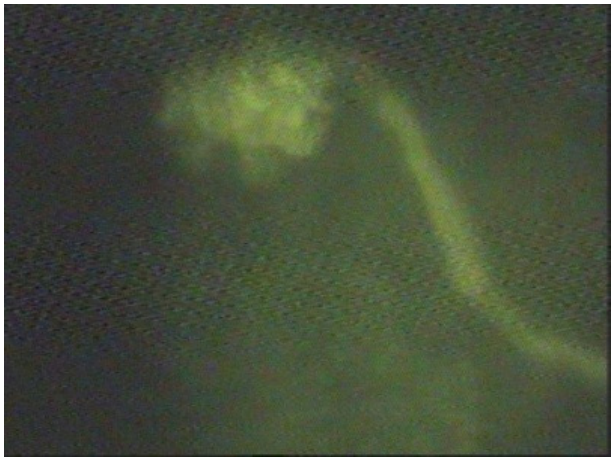

Fig 5. Close up view of pollen tubes growth in self – fertile *M. sativa* ovule

In *M. sativa* x *M. scutellata* crosses, very few pollen grains germinated on the stigmatic surface and grew down the styles was observed. It was noticed that the pollen tube growth was very slow and even after 120 HAP, hardly very few pollen grains reached the ovule (Figs. 6 and 7). Many callose plugs and irregular growth of the pollen tubes were also observed in these crosses. The same degree or slightly lesser effect were also seen when different cultivar of *M. sativa* were pollinated with its own pollen or upon selfing.

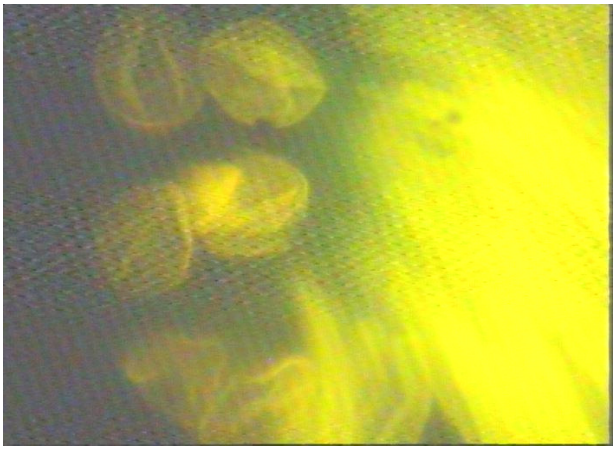

Fig 6. Close up view of pollen grains of *M. scutellata* on *M. sativa* stigmatic surface at 72 HAP

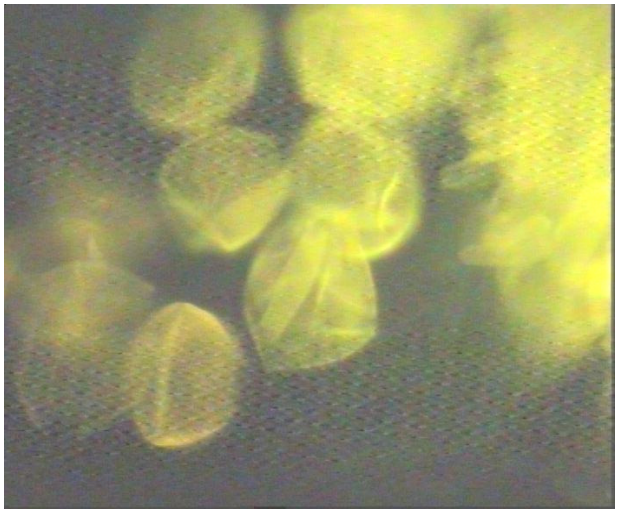

Fig 7. Close up view of pollen grains of *M. scutellata* on *M. sativa* stigmatic surface at 120 HAP

Induction of successful pollination: Many immature flower drop was noticed during 4 DAP to 7 DAP. The stigma and style becomes yellow, the wings and keel petals start drying, the calyx and pedicel will be yellow, and under these circumstances the buds will drop from the raceme of the crossed plant. Visible indication of successful fertilization and embryo formation is the development process initiation followed by stigma tip drying but the stylar tissue and ovary becomes green. Later the green ovary started protruding out from the staminal column with a slight bulging and twisting of the style.

Fig 8. Crossed pod development in *M. sativa* x *M. scutellata* in pots

Fig 9. Crossed pods at maturity in *M. sativa* x *M. scutellata*

Fig 10. Putative hybrid seeds obtained in *M. sativa* x *M. scutellata* cross

Out of the four *M. scutellata* accessions used for crossing with *M. sativa* cv. RL 88, it was observed that in *M. sativa* (RL 88) x *M. scutellata* (EC 541685) and *M. sativa* (RL 88) x *M. scutellata* (EC 541686) retention of the flowers for more than four to five days after crossing followed by few pod formation was noticed. In crosses with *M. sativa* (RL 88) x *M. scutellata* (EC 547739) and *M. sativa* (RL 88) x *M. Scutellata* (EC 547741), the flower drop was noticed on the third day itself. However in *M. sativa* (RL 88) x *M. scutellata* (EC 541686) cross, one green pod with good bulging was noticed and upon drying one shriveled seed with fully developed seed coat was noticed. Maximum number of crosses was attempted in *M. sativa* (RL 88) x *M. scutellata* (EC 541685) (Table 3). Out of 1287 florets attempted in this cross, only five normal appearing pods developed till maturity and contained only one seed each per pod while abortive seeds were found in two pods (Figs. 8, 9 and 10). All the three seeds were germinated *in-vitro* aseptically in tissue culture medium containing half strength of MS medium without hormonal treatment under dark condition, after proper scarification and pretreatment. Out of three seeds inoculated, germination was observed in two of the seeds. One seed did not germinate even after one month period (Fig. 11). Among the two seedlings, one seedling was with normal shoot and root development. The second seedling did not have proper root but showed stunted growth with thick shoot and no root development was noticed for about four months period. In lucerne, the embryogenic response is modulated by the interaction of several factors such as chemical composition of the media, the source of tissue explant and its genetic potential (McKersie and Brown, 1997). It is worth mentioning that in our studies tissue culture protocols as well as the explant source were different from those used in the genetic studies previously cited. The first hybrid plant after chemical analysis and morphological data with mixoploid chromosome number was reported by Sangduen *et al*. (1982), in a perennial x annual *Medicago* crosses. However, the hybrid nature of the plant obtained and its genetic composition is to be assessed with morphological, cytological, and molecular analyses.

Fig 11. Putative hybrid seeds of *M. sativa* x *M. scutellata* cross under *in-vitro* germination

Table 3. Details of self's and direct and reciprocal crosses attempted in *M. sativa* and *M. scutellata* accessions

Cross / self details	**Capsule set**	**Seedlings obtained**	**Seeds sown in pots / *in vitro* condition**	**Seeds obtained**
M. scutellata self (EC 541685)	22	56	25	19
M. sativa self (RL 88)	12	18	18	12
M. sativa self (Anand 2)	14	20	20	14
M. scutellata (EC 541685) x *M. sativa* (RL 88)	0	0	0	0
M. sativa (RL 88) x *M. scutellata* (EC 547739)	0	0	0	0
M. sativa (RL 88) x *M. scutellata* (EC 547741)	0	0	0	0
M. sativa (RL 88) x *M. scutellata* (EC 541685)	5	3	3	2
M. sativa (RL 88) x *M. scutellata* (EC 541686)	1	0	0	0

Conclusion

The differences between number of chromosomes in *Medicago sativa* (L.) with 2n=32 and annual *Medicago scutellata* (L.) Mill. with 2n=30 (Bauchan and Elgin, 1984), chromosome rearrangement and recombination between parental genomes during interspecific hybridization might be the cause of poor development of the putative hybrid plant. In conclusion, our results indicate that the use of *M. scutellata* as one of the parent in wide hybridization to improve the resistance against stem weevil in *M. sativa* is affected by the chromosomal differences *per se*. Embryo rescue and tissue culture based breeding methodologies *viz*., somatic hybridization could also be considered as a suitable method for transferring the desirable traits of interest.

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