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Pre-fertilization incompatibility barriers to interspecific hybridizations in *Pennisetum* species

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SUMMARY

The study was undertaken at Punjab Agricultural University, Ludhiana, in 1991–1992 to investigate the behaviour of the pollen tubes of four wild *Pennisetum* species in the stigmatic tract of *P. typhoides* and to identify stages of pre-fertilization impediments to interspecific hybridization. Pollen germination was normal in crosses with *P. violaceum*, *P. squamulatum* and *P. orientale* and slightly inhibited in a cross with *P. setaceum*. Fertilization was accomplished within 3 h of pollination in a control cross within *P. typhoides*. *P. typhoides* × *P. violaceum* was a compatible cross and the pollen tubes of *P. violaceum* reached the ovary within 4 h of pollination. The presence of *P. squamulatum* and *P. orientale* pollen tubes in the ovary 10–15 h after pollination indicated the possibility of normal fertilization, while in crosses with *P. setaceum*, pollen tubes did not penetrate the ovary even 15 h after pollination. The study indicated that there is no impediment at the pollen germination stage or in the stigmatic hairy region. Delayed/restricted growth of pollen tubes indicated that the barrier operates in the styllar hairless region for the crosses with *P. squamulatum* and *P. orientale*, and at the ovarian level for the *P. typhoides* × *P. setaceum* cross.

INTRODUCTION

A number of cultivated species of *Pennisetum* L. Rich (family Poaceae, tribe Paniceae) are grown both for grain and fodder purposes. *P. typhoides* (*P. glaucum*), commonly known as pearl millet, is grown on > 20 million hectares worldwide. In the USA, it is primarily grown as a fodder crop, while it is an important food and fodder crop in the semi-arid tropics. Many desirable genes are found within the primary and secondary gene pools of *P. typhoides* which, potentially, could be used for pearl millet improvement (Hanna 1986). Attempts have been made to transfer traits such as resistance against ergot (Kannaiyan *et al.* 1972) and apomixis (Dujardin & Hanna 1986; Hanna *et al.* 1993) into the cultivated gene pool. Although a number of reports are available on the production of interspecific hybrids involving pearl millet as one of the parents, the crossability in the genus is very low and depends largely on the genotype and ploidy levels of the parents (Burson & Young 1983; Dujardin & Hanna 1989; Marchais & Tostain 1997). Heslop-Harrison (1982) reported that in the family Graminae, difficulty in obtaining the first generation hybrid is due to pre-fertilization barriers operating between pollen reception and the entry of the pollen tube into the embryo sac. Stigmatic as well as styllar incompatibilities were reported to be the reasons for failure of seed set in some *Pennisetum* interspecific hybrids (Mohindra & Minocha 1991; Chaix & Marchais 1996). The present study was undertaken to investigate further the stages of pre-fertilization impediments to interspecific hybridization in this genus.

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MATERIALS AND METHODS

A male sterile line (L-111 A) of pearl millet was used as the female parent and four wild species (*P. violaceum*, *P. squamulatum*, *P. setaceum* and *P. orientale*) as male parents. Plants were grown at the experimental area of the Department of Genetics, Punjab Agricultural University, Ludhiana, India. The crosses were attempted during September–October. The pollinated spikelets of *P. typhoides* were harvested immediately and then subsequently every hour after pollination and fixed in FAA (formalin 1, 80% alcohol 8, acetic acid 1). Martin's method (1959) as modified by Shivanna & Johri (1985) was used for observations of pollen tubes under a fluorescent

Table 1. *Pollen germination and pollen tube growth in interspecific crosses of* P. typhoides *with wild species*

S. no.	Hours after pollination	No. of pollen grains		Germination (%)	No. of pollen tubes growing up to:			
		Total	Germinated		Stigmatic hair	Hairy region of style	Hairless region of style	Ovary
1.	*P. typhoides* (L-111 A) × *P. typhoides* (L-111 B)							
	1 h	520	353	67·88	230 (65·15)	15 (4·25)	—	—
	2 h	634	412	64·98	301 (73·06)	61 (14·80)	20 (4·85)	—
	3 h	496	387	78·02	270 (69·77)	77 (19·90)	43 (11·11)	8 (2·07)
	5 h	721	520	72·12	383 (73·65)	114 (21·92)	62 (11·92)	26 (5·00)
	7 h	496	411	82·86	311 (75·67)	74 (18·00)	37 (9·00)	25 (6·08)
	10 h	616	527	85·55	416 (78·94)	116 (22·01)	53 (10·01)	36 (6·83)
	Total	3483	2610	75·23*	1911 (72·21)	457 (16·81)	215 (7·81)	95 (3·33)
2.	*P. typhoides* × *P. violaceum*							
	1 h	593	373	62·90	90 (24·13)	2 (0·54)	—	—
	2 h	465	320	68·81	83 (25·94)	28 (8·75)	1 (0·31)	—
	3 h	514	370	71·98	118 (31·89)	40 (10·81)	12 (3·24)	—
	4 h	683	512	74·96	150 (29·29)	49 (9·57)	28 (5·47)	18 (3·51)
	5 h	444	331	74·55	106 (32·02)	39 (11·78)	20 (6·04)	8 (2·42)
	7 h	397	273	68·76	90 (32·97)	33 (12·09)	19 (6·96)	7 (2·56)
	Total	3096	2179	70·33	637 (29·37)	191 (8·92)	80 (3·67)	33 (1·41)
3.	*P. typhoides* × *P. squamulatum*							
	1 h	422	202	48·00	67 (33·17)	—	—	—
	3 h	360	190	52·80	68 (35·79)	3 (1·58)	—	—
	5 h	380	203	53·40	78 (38·42)	15 (7·39)	1 (0·49)	—
	7 h	296	172	58·10	67 38·95)	25 (14·53)	3 (1·74)	—
	10 h	287	172	60·00	68 (39·53)	28 (16·28)	3 (1·74)	—
	15 h	418	241	57·60	91 (37·76)	38 (15·77)	4 (1·66)	1 (0·41)
	Total	2163	1180	55·00	439 (37·27)	109 (9·26)	11 (0·94)	1 (0·07)
4.	*P. typhoides* × *P. orientale*							
	1 h	240	140	58·33	67 (47·85)	—	—	—
	3 h	221	118	53·40	64 (54·23)	4 (3·38)	—	—
	5 h	228	142	62·28	89 (62·67)	8 (5·63)	1 (0·70)	—
	7 h	125	75	60·00	43 57·33)	4 (5·33)	2 (2·67)	—
	10 h	237	132	55·69	54 (40·90)	7 (5·30)	3 (2·27)	1 (0·76)
	Total	1051	607	57·94	317 (52·59)	23 (3·92)	6 (1·12)	1 (0·15)
5.	*P. typhoides* × *P. setaceum*							
	1 h	180	69	38·33	20 (28·98)	—	—	—
	3 h	220	88	40·00	33 (37·50)	2 (2·27)	—	—
	5 h	142	64	45·07	25 (39·06)	2 (3·12)	—	—
	7 h	197	91	46·19	38 (41·75)	3 (3·30)	2 (2·20)	—
	10 h	211	98	46·44	41 (41·84)	5 (5·10)	2 (2·04)	—
	15 h	173	75	43·33	33 (44·00)	4 (5·33)	4 (5·33)	—
	Total	1123	485	43·23	190 (38·85)	16 (3·19)	8 (1·59)	0 (0·00)

* mean value; figures in parentheses denote percentage values calculated on total number of pollen tubes germinated.

microscope. Fixed styles were rinsed thoroughly in tap water and treated with 4N NaOH for 3–4 h at 60 °C to soften the tissues and to permit adequate penetration of the stain. The softened styles were transferred to a small beaker containing tap-water for 2–4 h to remove excess sodium hydroxide. Staining was carried out using 0·005% decolourized aniline blue (water-soluble) dye in 0·05% Na_2HPO_4 at pH 8·2, for 2–5 min on a covered glass slide. The styles were crushed by gently pressing the coverslip over the style. Slides were illuminated with UV rays of wavelength 330–380 nm in a darkened room.

Observations were taken of the percentage pollen germination and number of pollen tubes entering in the stigmatic hairs, the style, and the ovary following Reger & Sprague (1982). The various regions of *P. typhoides* gynoecium have been described by Mohindra & Minocha (1991). Percentage pollen germination was calculated as the proportion of pollen grains germinated to the total number of pollen grains observed, while pollen tube presence in various regions was calculated on the percentage of total pollen grains germinated. Average number of pollen tubes within a specific region of gynoecia was

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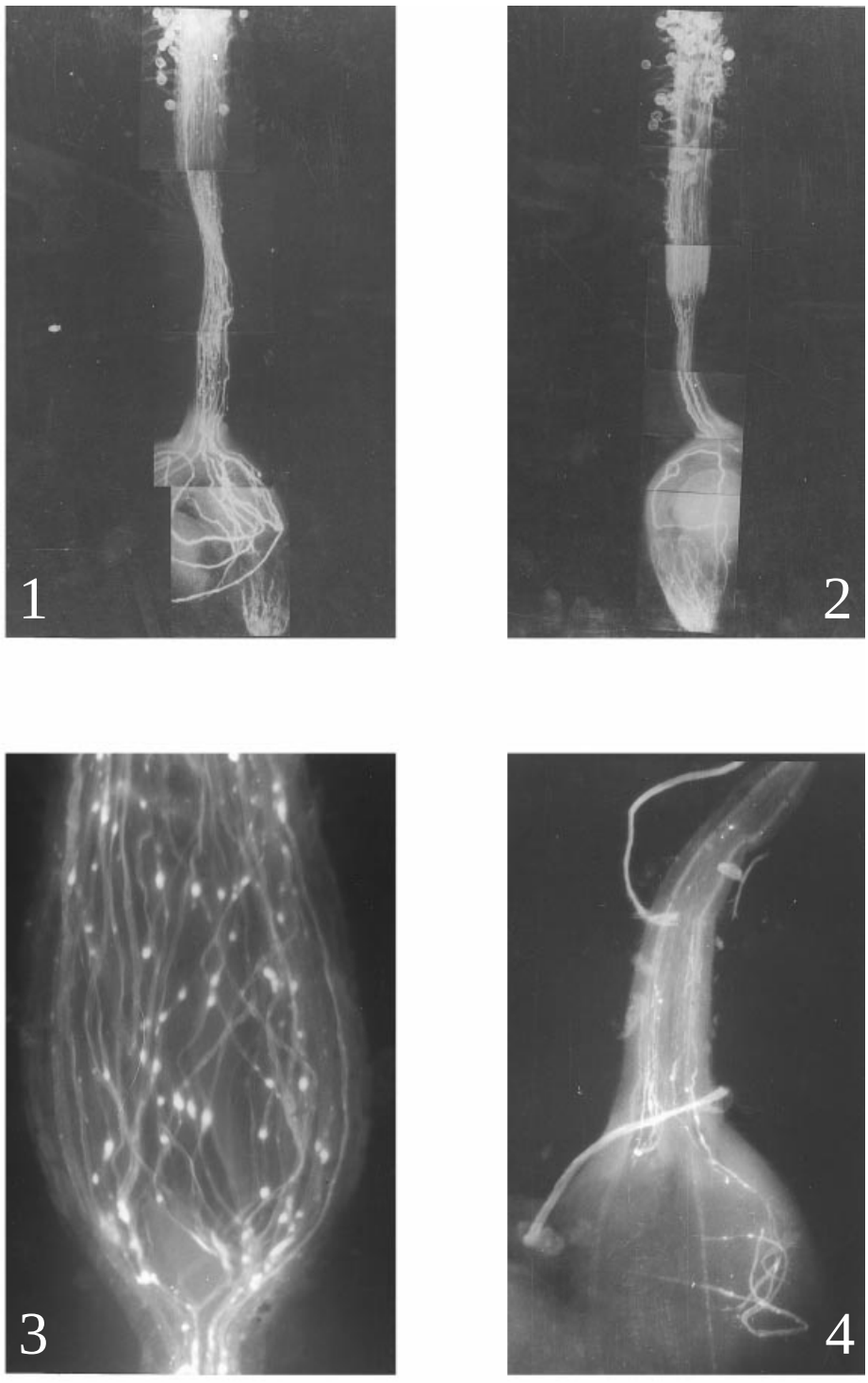

Fig. 1. Pollen tubes of *P. violaceum* in the gynoecia of *P. typhoides* representing compatible cross. Fig. 2. Growth of *P. squamulatum* pollen tubes in *P. typhoides* gynoecia (12 h after pollination). Fig. 3. Incompatible reaction as revealed by formation of callose plugs in hairless region of the style. Fig. 4. Pollen tube growth in *P. typhoides* × *P. orientale* cross.

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also calculated by taking the mean total number of pollen tubes observed in that region during the investigation. Pollen tubes were observed up to the ovary till micropyle and at that stage they were presumed to have caused fertilization, as suggested by Chaix & Marchais (1996).

Crosses between L-111 A and its maintainer L-111 B were used as controls (*P. typhoides* × *P. typhoides*) in comparisons including *t*-tests.

RESULTS AND DISCUSSION

Control crosses

In the control cross (*P. typhoides* L-111 A × L-111 B), pollen grains germinated within 20–25 min of their contact with the stigma. On average, the germination percentage was 75·2% (Table 1). At the end of the first hour after pollination, pollen tubes had traversed the stigmatic hairy region and 4·3% had reached the hairy region of the style. By the end of the third hour, some pollen tubes had reached the ovary, showing that a minimum of 3 h is required for fertilization. The percentage of pollen tubes reaching the ovary increased with time up to 10 h after pollination. There were markedly fewer pollen tubes in the hairy region than in the stigmatic hairs. This could be attributed to the high density of pollen tubes which could reduce pollen tube growth beyond this region due to inter-pollen-tube competition (Jalani & Moss 1980; Heslop-Harrison 1982).

Interspecific crosses

P. typhoides × *P. violaceum*

The pollen grains germinated within 30–45 min of pollination with an average of 70·3% germination. The growth of pollen tubes was as rapid as in the control cross in the initial stages as they were observed in the hairy region by the end of the first hour and in the hairless region by the end of the second hour (Fig. 1). They had penetrated the ovary by the end of the fourth hour, and it is presumed that fertilization would have occurred. Success in obtaining viable seeds from this cross demonstrated that it was a compatible cross.

P. typhoides × *P. squamulatum*

Fifty-five per cent of pollen germinated within 1 h of pollination. The growth of the tubes was, however, comparable to the compatible cross of *P. typhoides* × *P. violaceum*, indicating the absence of an incompatibility barrier in the stigmatic hairy region. A substantial reduction in pollen tube growth was observed beyond this point and only a few pollen tubes reached the hairless portion even 5 h after pollination (see Fig. 6), indicating a strong styllar incompatibility. Abnormalities in the pollen tubes, including coiling, non-oriented growth and swollen tips (Fig. 2) were observed in this region. Large callose plugs viewed as densely illuminated regions were observed at irregular intervals along the pollen tubes (Fig. 3). Only one pollen tube was observed in the ovary within 15 h indicating the possibility of fertilization. Embryo abortion could thus be the reason for failure to get seed from this cross.

P. typhoides × *P. orientale*

P. orientale pollen germinated on *P. typhoides* stigmas within 1 h of pollination, with an average rate of 57·9%. Compared to other wild species *P. orientale* pollen tubes showed the highest growth rate in the first hour; 47·9% of pollen tubes were seen in the stigmatic hairy region. Growth slowed down drastically in later stages and only 1·1% of germinated pollen tubes reached the hairless portion of the style (see Fig. 6). The incompatibility reaction was characterized by abnormalities, including coiling and un-oriented growth of pollen tubes (Fig. 4). At the end of the tenth hour, however, one pollen tube was seen to have reached the ovary, indicating a possibility for normal fertilization to occur. Embryo abortion could be the cause for failure to obtain any viable seeds.

In the crosses between *P. typhoides* with *P. squamulatum* and *P. orientale*, the incompatibility barrier appeared to operate in the hairless region of the style, as expressed by retarded pollen tube growth and other abnormalities (Heslop-Harrison 1982).

P. typhoides × *P. setaceum*

P. setaceum showed the least pollen germination (43·2%), although germination was evident within an hour of their contact with the stigma. The pollen tubes reached the hairy portion of the style within 3 h and the hairless portion within 7 h of pollination. None of the pollen tubes reached the ovary within an 18 h period, even when quite a high number of pollen tubes (5·33%) accumulated at the hairless region (see Fig. 6). Failure of pollen tubes to locate the ovary, and loss of tube orientation indicated strong styllar as well as ovarian incompatibility (Heslop-Harrison 1982; Burson & Young 1983; Chaix & Marchais 1996). A comparatively greater number of pollen tubes in this cross were present in the hairless region than the crosses with *P. squamulatum* and *P. orientale* (5·33 as compared to 1·66 and 2·27%), but none reached the ovary. Thus in the cross with *P. setaceum* the intensity of the incompatibility barrier was greatest at the ovarian level.

A novel abnormality – clumping of pollen tubes to form plasmodium – was observed in this cross. It is reported that in incompatible crosses of grasses, gametes and vegetative nuclei pass into the tip without massive accumulation of callose and may either burst

Table 2. *Level of significance for* t*-tests comparing the pollen tube growth in different crosses with the control cross* (P. typhoides × P. typhoides)

Cross	Pollen germination	Up to stigmatic hairs	Up to hairy region of style	Up to hairless region of style	Up to ovary
P. typhoides × *P. violaceum*	NS	***	NS	NS	NS
P. typhoides × *P. squamulatum*	***	***	*	**	**
P. typhoides × *P. orientale*	***	***	**	**	**
P. typhoides × *P. setaceum*	***	***	**	**	**

NS, non-significant; *, significant at $P \leqslant 0{\cdot}05$; **, significant at $P \leqslant 0{\cdot}01$; ***, significant at $P \leqslant 0{\cdot}001$.

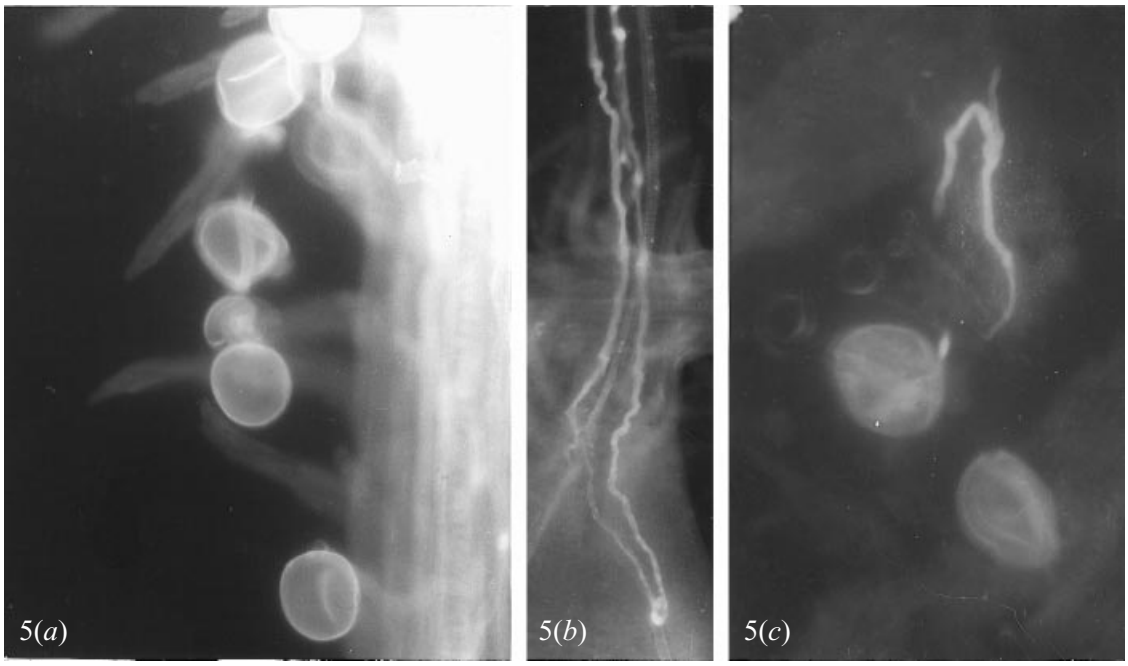

Fig. 5. Abnormalities in the pollen tube growth. (*a*) coiling of pollen tube around pollen grain; (*b*) clumping of pollen tubes near ovary; (*c*) non-oriented growth of pollen tube.

or may form plasmodium if many pollen tubes are present (Jalani & Moss 1980; Heslop-Harrison 1982).

Germination of pollen and pollen tube growth in interspecific crosses was compared in the stigmatic tract of *P. typhoides* at various intervals (Table 2, Fig. 6). Pollen from wild *Pennisetum* species behaved differently in compatible and incompatible cross(es). Pollen germination in crosses with *P. squamulatum*, *P. orientale* and *P. setaceum* was significantly different ($P \leqslant 0{\cdot}001$) compared to the control cross while in *P. violaceum* it was statistically equivalent to the *P. typhoides* pollen. A similar situation was found for pollen tube growth at different regions of the stigmatic tract where the pollen tube growth was significantly different ($P \leqslant 0{\cdot}05$) from the control cross, except in the case of the cross with *P. violaceum*. This study showed that there exists a partial incompatibility for the *P. violaceum* pollen tube in the stigmatic hairy region. However, pollen tubes appear able to overcome it, ultimately fertilizing the ovule and yielding viable hybrids (Robert *et al*. 1991). Comparisons of pollen tube growth in various regions of the stigmatic tract of *P. typhoides* is shown in Fig. 6. Pollen tube growth in three incompatible crosses are also compared among themselves. Their growth in the hairy region, the hairless region and in the ovary were statistically equivalent. In the stigmatic hairy region the pollen tube growth in the *P. typhoides* × *P.*

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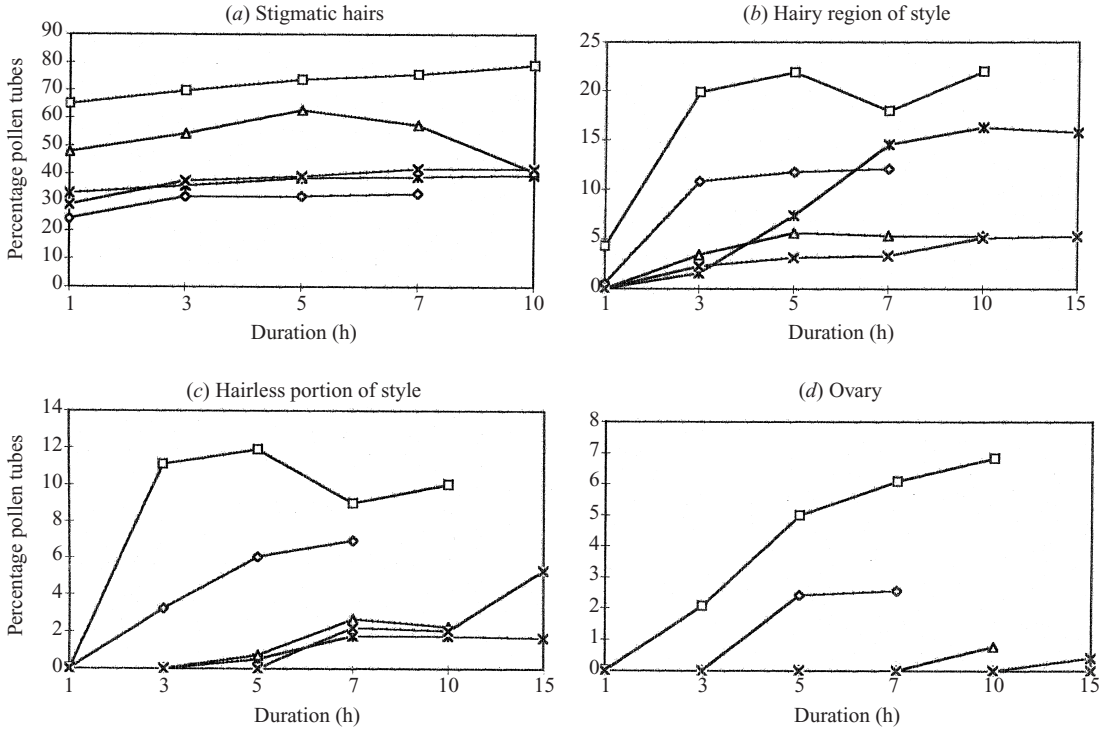

Fig. 6. Comparison of pollen tube growth in the stigmatic tract of *Pennisetum typhoides* in interspecific crosses. –□– Pt × Pt; –◇– Pt × Pv; –⁎– Pt × Psq; –△– Pt × Po; –×– Pt × Pset.

orientale cross was significantly better ($P < 0{\cdot}05$) than in the crosses with *P. squamulatum* or *P. setaceum* showing the acceptance of *P. orientale* pollen (tubes) at least in the stigmatic hairy region.

Many interspecific crosses exhibit normal pollen germination (Zenkteler 1980), such as the crosses involving *Paspalum* (Burson 1987), *Festuca* and *Lolium* (Matzk 1976), *Panicum* (Burson & Young 1983), *Oryza* (Sitch & Romero 1990) and *Pennisetum* (Robert *et al.* 1991). Nevertheless, strong incompatibility inhibits germination for certain specific combinations (Chaix & Marchais 1996). Knox *et al.* (1972) suggested that at pollination, proteins are released from the pollen wall, namely enzymes essential for germination and pollen tube growth and 'recognition substances' concerned with compatibility reactions. Incompatible pollen–pistil interactions prevent germination. This reaction can be partly inhibited by the simultaneous presence of proteins from compatible pollen. Such self-recognition systems operate in a number of incompatible reactions in plant hybridizations (Burnet 1971) and may be responsible for the reduced germination of *P. setaceum* pollen. A second reason could be the inability of the tube tip at an exine pore to penetrate the stigmatic cuticle (Shivanna *et al.* 1978).

Pollen tubes of *P. squamulatum*, *P. orientale* and *P. setaceum* showed coiling, clumping and other pollen tube abnormalities before their growth ceased (Fig. 5). A similar phenomenon was associated with self-incompatibility in *Brassica oleracea* L. var. gemmifera (Kho & Baer 1968) and *Brachiaria ruziziensis* (Coppens D'Eeckenbrugge *et al.* 1985). In several grasses such as perennial rye grass (*Lolium perenne*), callose reaction occurred after the contact of an incompatible pollen tube with the stigma which either prevented further pollen tube growth (Lundquist 1961; Elgersma *et al.* 1989) or the tubes tended to get arrested in the transmitting tracts of the stylodium or ovary walls, accompanied by an enlargement of the tube tips or bursting (Heslop-Harrison 1982). The cuticle layer is responsible for inhibition of germination and pollen tube growth on the stigma surface in crosses involving *Raphanus sativus* (Matsubara 1984), *Hordeum* and *Gaudina* (Shivanna *et al.* 1978) and in several other grasses (Heslop-Harrison 1982). Arrest of incompatible tube occasionally occurs at the stigma surface, but more commonly the tube penetrates the stigma cuticle and gets arrested during passage through the intercellular material in the hairy or hairless region of style and is followed by callose occlusion (Shivanna *et al.* 1978). A similar

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phenomenon may inhibit *P. squamulatum*, *P. orientale* and *P. setaceum* pollen tube growth.

The interspecific incompatibilities in crosses with *P. typhoides*, were thus associated with retarded growth of pollen tubes at different regions of the pistil, as well as morphological abnormalities of the pollen tubes. Mohindra & Minocha (1991) reported the absence of fertilization as the cause of failure of seed set in some interspecific crosses since the pollen tubes had not reached the ovary 6 h after pollination. However, our studies reveal that the ovary penetration was delayed due to the slower growth of pollen tubes and occurred 10–15 h after pollination in the *P. typhoides* × *P. squamulatum* and *P. typhoides* × *P. orientale* crosses. In crosses where poor pollen tube growth was observed, the application of growth regulators have the potential to enhance the opportunity for fertilization (Sitch & Romero 1990; Brar 1991). The absence of seed setting in the above crosses, even after presumed fertilization, may have been due either to the failure of gametes to unite (Burson 1987) or to post-zygotic embryo abortion due to endosperm genetic imbalance which is characteristic of interspecific crosses (Johnston *et al*. 1980) and some of the intraspecific crosses (Amoukou & Marchais 1993). Use of an embryo rescue technique is suggested to obtain hybrid seedlings in these crosses.

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