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Incompatibility studies in sugarcane (*Saccharum* spp.)

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Abstract

The study was undertaken during the period 2001-2004 to investigate the behaviour of the pollen tubes in the stigmatic tract of pistil parents utilized in intervarietal hybridization. Among the 173 breeding material screened for occurrence of incompatibility, 36 parents were identified self incompatible and among them nine incompatible parents were further studied for their cross compatibility. Pollen germination was normal in Co 86011, Co 91004 and Co 62198 and slightly inhibited in a cross with Co 99002. Fertilization was accomplished within 4 hr of pollination in crosses Co 8371 x Co 86011, Co 88028 x Co 86011. The presence of Co 86011 and Co 91004 pollen tubes in the ovary 8h after pollination indicated the possibility of normal fertilization, while in cross with Co 94008, restricted pollen tube growth was observed. Pollen tube in the clone 2000-147 did not penetrate the ovary even 10 h after pollination. Self pollen did not support the development of pollen tubes in Co 88028, Co 91004, Co 99002, Co 62198 and Co 86011. In the crosses of Co 88028 x Co 91004, Co 88032 x Co 99002 and Co 88028 x Co 86011 pollen germination and entry of pollen tube was normal. The study indicated that there is no hurdle at the pollen germination stage and delayed/restricted growth of pollen tubes indicated that the barrier operates in the stylar region of the parent. Thus, from our study it could be inferred that i) cross-incompatibility systems are responsible for the failure to produce successful crosses among Co 88028 x Co 91004, Co 88028 x Co 99002.ii) self incompatible parents could be used as safe pistil (Co 837, Co 8371, Co 88028, Co 88025) and pollen parents (Co 62198, Co 86011, Co 94008, Co 91004).

Key words : Sugarcane, incompatibility, pollen, stigma

Introduction

Incompatibility is of widespread occurrence in plants and, self incompatibility occurs in greater frequency among the members of gramineae which is controlled by two loci system. The system is also of special interest to breeders since it encourages outcrossing and prohibits selfing and contributes to propagation of species. The success of fertilization depends on interaction between the male gametophyte and the sporophytic tissues it comes in contact with at different stages of development. Interaction becomes even more apparent during pollen pistil interaction (germination and tube growth). These interactions involve the effect of style on pollen which results in incompatibility [1, 2]. Enzymes have been detected in pollen and among them esterase isozyme has been shown to be of both sporophytic and gametophytic origin and provide a source of markers for studying development of pollen pistil interaction. Sugarcane as any other polyploid crop with high level of heterozygosity exhibits poor seed set to varying degrees. Wide variation in the setting of seeds among different sugarcane crosses is frequently noted. Certain genetic and environmental factors, acting independently or in combination with each other, are known to affect the production of true seed. In a number of crosses incompatibility exists because of genetic, cytoplasmic and nutrient (matrix) conditions in pistil parents and these appear to exert an influence on the viability of hybrid seeds. However, factors contributing to these phenomena are not properly sorted out so far. The earlier reports [3, 4] related to this aspect indicates that self incompatibility and cross compatibility occur in certain parental combinations which are to be identified to ensure proper selection of parents to evolve superior hybrids and understand the pollen-pistil interaction in order to identify safe pollen and pistil parents,obtain higher seed set in crosses and ensure the hybridity of the progenies .

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Materials and methods

Sugarcane forms an open panicle type of inflorescence, whose shape, degree of branching and size are highly genotype specific. The inflorescence or arrow consists of a main axis and first, second and third order branches. Attached to the branches are spikelets arranged in pairs that contain individual flowers. The sugarcane flower consists of three stamens (male) and a single carpel with a feathery stigma (female) typical of wind pollinated flowers. Sugarcane pollen is very small, hairy and wind dispersed. For male gametophytic studies, fresh pollen grains were collected from different cultivars after anthesis and pollen viability tests were carried out by aceto carmine-glycerine mixture. *In vitro* [5] and *in vivo* pollen germination and pollen tube growth were recorded from 25 pollen grains. One hundred and seventy three clones were screened for incompatible reaction and 36 were identified to be self incompatible. Nine self incompatible clones (Co 8371, Co 88028, Co 88025, Co 91004, Co 99002, Co 62198, Co 86010, Co 2000 10 , Co 837) were used for further studies. Crosses were made in the field grown plants for three seasons (2001-2004). Proper care was taken to avoid contamination from stray pollen. Arrows (inflorescences) were covered with cloth bags as soon they start emerging to obtain selfed seeds. Each pistil was pollinated with an abundant amount of pollen and seeds /0.1g of fluff was determined. The self and pollinated spikelets were tagged accordingly and the material fixed after 2, 3, 4, 8, and 16 and 24 hours in fixative containing methanol 60 ml, chloroform 30 ml, distilled water and stored at 4-10^{o}C for 24 hrs. Then these were transferred to 70% ethyl alcohol and stored in refrigerator till further use. For *in vivo* studies, two or three hours after pollen dusting, a few spikelets were removed from the arrows, fixed in Carnoy's fluid, passed through alcohol series and the spikelets dissected, hydrolysed and stained. The pollen tube growth was measured in three different pollinated spikelets with each taken as a replication. In each replication, 5 pistils were measured for pollen tube growth. The styles were treated for 20-25 minutes in 1N NaOH and stained with 1 % aniline blue.

Results and discussion

The parents under study were classified as pistil (strong females) and pollen (strong males) based on viable pollen produced measured as pollen fertility. The pistil parents had very low counts of fertile pollen (4 to 18 percent only) and the male parents had normal and healthy pollen grains (50 to 95 percent). The pollen grains were viable for one hour and under low humidity they dried quickly. The pollen tube growth was good under lighted condition. Under microscopic examination, the mature pollen grains stained completely black while partially mature ones incompletely black. The maturity of grains was highly variable with about one third showing above 75 % maturity. The starch filled grains germinate on landing receptive stigma shortly after anthesis.

The size of pollen grains varied greatly among the clones. Large size pollen grains harbour nutrients for autonomous growth length of upto 2 cm in grasses. Among the material studied, *S. spontaneum* clones had pollen grains with diameter ranging from 35.1µ microns to 50.2µ microns. Genetic stocks and commercial varieties had relatively larger and more viable grains (37.6-53.9µ microns) with a coefficient of variation of 14.3 % and pollen germination of 70%. Pollen grains appeared in groups which varied among the clones (10-50) and was maximum in Co 86011 and minimum in Co 837. They were also observed in singles in parents 2000-147 and CoJ 65 which might have resulted in low pollen germination in them.

In vitro studies

In vitro test is based on the germination of pollen in artificial medium and a viable tool for determining viability of pollen grains in intervarietal crossing programme. Observations on *in vitro* pollen grain germination showed overall high rates of pollen germination in artificial media (Brewbaker's media). Pollen germination varied - the slowest being in *S.officinarum* and the fastest in commercials [3]. In our study, the highest pollen germination recorded was 25.0 % in Co 1148 (Fig. 1), Co 99008 and Co 86011 and 23.38 % in Co 62198 and Co 89003 had minimum germination of 11.11 %. All other clones recorded germination within this range of

Fig. 1. *In vitro* pollen germination

11.11% to 25.0%. The clones Co 88032 was male sterile and Co 87023 showed fertility upto 20 % with no pollen germination. The pollen tube grew upto a length of 1-3mm in Brewbaker's media which varied among the clones and was 20 times the diameter of the pollen grains. Pollen germination and elongation appeared to be associated with pollen size and pollen tube growth and was positively correlated with pollen germination. Another important difference was noted for lengths of stigma and style in the parents. The parent's *viz.*, Co 837, Co 8371, Co 86010, 2000-147 with longer pistil had higher proportion of stigma projected outside the flower which would have been efficient in pollen catching.

In vivo studies (Pollen –pistil interaction)

Among the 173 breeding material screened for the occurrence of self incompatibility, thirty six clones were self-incompatible which was confirmed through fluorescent microscopy, kryotome and seed set studies. Nine self-incompatible clones were utilized for crossing studies.

Selfing

In the selfs of the incompatible parents, there were no normal pollen grains and the incompatible pollen was inhibited in the transmitting tracts of the style which agrees with nature of self incompatibile reaction in grasses [1]. The nature of self incompatibility detected in the selfed clones is given in Table 1. After 4 hours, 6 hours and 12 hours of selfing there were no normal pollen grains but were broken at the tips and appeared in bulged condition. Nil seed set (three years)in these clones also supported the possibility of occurrence of self incompatiblilty.

Open pollination

The pollen in the open pollinated clones showed moderate to high pollen tube growth and all styles had pollen grains in bulged condition or with some growth only. This was observed upto 94.7 % in the clone Co 837, 93.8 % in Co 2000 10 and 96.2 % in 2000 147. Incompatible pollen tubes grow through the style more slowly than compatible tubes and are thus prevented from fertilizing the ovules because they fail to reach the ovary.

Crosses

In pollinations involving nine self incompatible parents, pollen germination and tube growth were studied *in vivo* by observing the pollinated pistils with fluorescence microscopy after 2, 4 and 6 hours. No evidence of pollinations could be observed in any of the fixations made in after 24 hours of pollination which might be due to the shedding off of the germinated pollen grain or sufficient loss of pollen viability of pollen grains as time lapsed. Among the clones Co 88025 pollen exhibited long growth of 3405µ and a pistil size of 1800µ and Co 88028 had the short pollen tube growth of 990µ. MS 6847 had the largest pistil size of 3400µ (Table 2).

In 40 cross combinations involving the above combined parents, pollen germination was immediate after pollination with an overall germination ranging from 77 to 88% for the different crosses. The pollen tube growth rate was high at initial period (within 4 hrs) which decreased gradually (within 24 hrs) Table. Though under natural conditions sugarcane pollen is viable for only 30 minutes after which it drops, in our study it extended upto 8 hours. Three crosses were identified as incompatible crosses (Table 3). The cross Co 8371 x

Table 1. Nature of incompatibility reaction in selfed sugarcane clones

S.No	Clones	Response of pollen grains on styles of selfed clones	Compatibility reaction
1.	Co 62198, Co 62387, Co 7915, Co 8209, Co 90018, Co 86011,Co 91004, Co 92013, Co 93009, Co 94008, Co 94019, Co 97009, Co 98010, Co 99002, Co 94019, CoH 110 and CoJ 65, Co 87023	Pollen grains on the styles of selfed pollen showed slow pollen germination with interruption in growth within two hours	Self incompatible
2.	Co 88032, Co 837 and Co 2000 10	Immediate germination with interruption in growth within 2 hrs	Self incompatible
3.	Co 7201, Co 88028, Co 8371, Co 98006, Co 94005, Co 85002, Co 88025, Co 89024,Co 95005, Co 85246, Co 97007, MS 6847, Co 86010, 95-90	Immediate germination with subsequent inhibition in growth within 3 hours	Self incompatible
4.	2000 -147	No penetrance into the ovary even after 10 hrs	Self incompatible

Table 2. Pollen and pistil parameters in parents (after 4 hrs)

S.No	Entries	Pollen germination	Length of tube (μ)	Average pistil size (μ)	Length of pollen tube after germination(μ)	Self incompatibility
1	Co 88028	3.0 -16.23	168	2250+72.41	990	+
2	Co 86011	5.83 -25.63	298	3316 +107.19	2350	+
3	Co 91004	2.10-12.5	225	3126+140.15	2000	+
4	Co 88025	4.68 -21.0	318	1800+333.46	3405	+
5	Co 94008	4.0 -18.36	121	2333+137.32	1100	+
6	Co 1148	2.38-21.38	185	3215+124.16	2000	+
7	Co 93009	2.31-15.76	105	3168+110.27	2004	+
8	MS 6847	1.86-18.2	168	3400+103.64	2095	+
9	Co 99002	2.12-11.11	104	3010+119.79	2010	+

Co 86011 and Co 88028 x Co 86011 were identified as most compatible crosses from the study.

In pollination involving Co 8371 as pistil and Co 86011 as male, the germinated pollen tube reached the micropylar end within eight hours. The pollen tube failed to reach ovule in cross combination involving Co 99002 as male parent. The pollen tube grains germinated and tube growth was normal when parents were crossed with pollen parent Co 62198. Co 86011 pollen was cross compatible with all the pistil parents utilized in the study. In all cross combinations involving Co 86011 as male parent, pollen tube reached the ovule within 4hrs. The pollen tube length varied between 1025 μ microns in Co 86010 x Co 86011 to 1181μ microns in Co 8371 x Co 86011. Histological studies in the fixed material of Co 8371 x Co 86011 also confirmed the normal growth with endosperm as free nucleus surrounding the proembryo which later becomes cellular with wall formation confirming the successful fertilization in the cross.

The stigmas of Co 88028 showed greater response to the pollen of Co 86011 with high pollen germination, faster pollen tube growth and good seed set in comparison with Co 91004. This may be probably due to the inherent closeness of Co 88028 (Co 7201 x Co 775-32) with Co 91004 (Co 7201 x Co 62174) that has restricted the entry of Co 91004 pollen in the style of Co 88028. Pollen tube elongation also appeared to be associated with pollen size. The clone Co 86011 showed larger grains and longer pollen tubes than Co 93009, Co 94019, Co 91004, 2000 147, Co 62387 with a ratio of tube length to grain diameter at 22:1. However Co 90018 had smaller grains with tube extended to 540m or about the half the length with a ratio of tube

Table 3. Nature of incompatibility reaction in sugarcane crosses

S.No.	Cross	Response of pollen grains on styles	Compatibility reaction
1.	Co 88032 x Co 91004	Pollen tube growth was arrested shortly after penetration with limited number of tube growth to different locations	incompatible
	within the stigma		
2.	Co 88028 x Co 91004	Cessated/ shortened pollen tube growth. Two - three pollen tubes reached the upper part of the style	incompatible
3.	Co 88028 x Co 99002	Pollen tubes grew into the style but were arrested before entering the ovary	incompatible
4.	Co 88028 x Co 86011	Pollen grains entered through the stigma indicating compatibility at stigmatic surface. Normal growth of pollen tube, fertilization and subsequent development of the embryo and endosperm and tissues were observed within 4-8 hrs of pollination	compatible
5.	Co 8371 x Co 86011		
6.	Co 88028 x Co 86011		
7.	Co 86010 x Co 86011		
8.	Co 88025 x Co 99002		
9.	Co 88025 x Co 91004		
10.	Co 837 x Co 62198		
11.	Co 2000 10 x Co 62198		

length to grain diameter of 10:1.

Several abnormalities were observed on germination studies [4] of maize pollen on sugarcane stigmas in several crosses indicating the presence of strong incompatible barrier between the genera. Incompatibility reaction was observed in *Saccharum* species [3] which was attributed due to shortness of the pollen tube of *S. officinarum* and its failure to travel the style and reach the ovule of bamboo. [6] Clearly indicated that the pollen tube growth is vigorous for first 4 hrs, reach the stem of style and reach micropyle within 32 to 60 hrs depending on the variety.

In incompatible crosses, growth of incompatible pollen tubes is inhibited either close to the stigmatic surface or towards the top of the style. This may be due to the inhibitory substances secreted at the time of pollination or activated in the style. It is well established that certain plant species are naturally furnished with auxin adequate for their germination. Abnormal pollen tube growth (PTG) was observed in grasses [1] suggesting the less of PTG orientation with random growth and failure of the tubes to locate the microphyle as one of the causes of pollen tube rejection causing cross incompatibility.

The slow growth of pollen tube *in vivo* is one of the major forms of parental incompatibility. In the present study though many pollen grains of Co 94008 germinated on Co 88025 stigma, most of the pollen tubes stopped half way in various parts of the pistil, only one tube was found to reach ovary/ fertilize the egg . The failure of pollen tube germination and growth, delayed pollen tube growth may be also due to incongruent reaction [2]. Extensive study in biology of pollen [7] also suggest that the expression of self incompatibility in the style is restricted to members of gramineae with gametophytic incompatibility system which also exhibits 50-75 % cross compatibility. Our study also confirms the occurrence of self incompatibility in the screened sugarcane clones which expressed cross compatibility to an extent of 55 %. Studies on incompatibility system in *Narenga and Sclerostachya* [8] also indicated that both genera were self-incompatible and partially cross compatible and the incompatibility was attributed to reaction that occurs in stigmatic papillae which hindered with pollen tube growth.

In the present investigation also the expression of pollen was restricted within the style resulting either in compatible or incompatible reaction and thereby suggesting the possibility of occurrence of gametophytic incompatibility system in sugarcane as depicted below:

Scheme of incompatibility in Sugarcane

Pollen germination

↓

Incompatible	Compatible
Incompatible : Bulging	Compatible: Normal growth

Stigma Invasion

↓

Incompatible	Compatible
Bulged pollen at stigmatic surface	Pollen tube gains entry Into the style

Style (Transmitting Tract)

↓

Incompatible	Compatible
Hindered and rejected in the stylar tract	Accepted in the stylar matrix

Growth in Ovary

↓

Incompatible	Compatible
No growth	Compatible pollen grows unhindered towards ovary and the micropylar region

Pollen Pistil interaction

Incompatible	Compatible
No interaction with embryo degeneration	Normal embryo development

Seed Set

Practically, it is likely that the varieties with 30 % mature pollen make good male parents and those with more than 60% are expected to produce highly satisfactory seed sets under good conditions. The most desirable female parents are those with low pollen maturity (to minimize selfing) and largely exposed stigmatic surfaces (to enhance crossing). Low viability reflects low seed sets. On comparison of seed set data in the selfs, open pollinated progenies and crosses effected it was observed that some of good pollen parents Co 90018, Co 62198, Co 7915, Co 62387, Co 62382, Co 86011, Co 99002 , ISH 69, Co 91004,Co 90010, 95-251, 95-465, 95-90 and pistil parents *viz*., Co 87023, Co 94005, Co 88025, Co 95005, Co 85246,Co 97007 did not set seed on selfing but the crosses produced good amount of seed set indicating their use as safe pistil parents in hybridization purpose. Analysis of seed derived from the crosses showed that seed set varied between 1-2.0% in selfs, 30-65% in crosses and 50-75% in open pollinations.

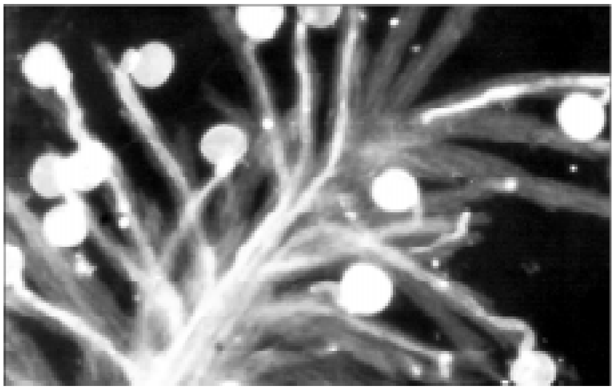

Fig. 2. ***In vivo*** **pollen germination on stylar tissue**

Fig. 3. Pollen germination inside the ovule

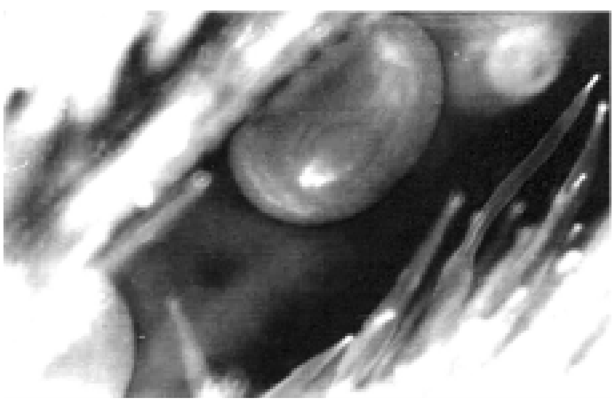

Fig. 4. Failure of pollen to germinate in stylar tissue

Thus, it was concluded from our study that cross or parental incompatibility systems are responsible for the failure to produce successful crosses among the parental clones. The self-incompatible clones identified from the study can be safely utilized as pollen and pistil parents in hybridization programmes as they ensure true hybridity of progenies. Self incompatibility has been known for half a century to be controlled gametophytically by two multiallelic and independent loci, *S* and *Z* in grasses. But still none of the gene products for *S* and *Z* is known and only limited information on related biochemical responses is available. The investigation also envisages further studies on genetics of incompatibility in sugarcane.

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