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Cytochemical analysis and stain development in pollen grains of parental lines and hybrids based on cytoplasmic genetic male sterility system in pigeonpea (*Cajanus cajan*)

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ABSTRACT

A study was conducted during 2006–08 to determine viability of pollen grains in cytoplasmic genetic male sterile lines of pigeonpea [*Cajanus Cajan* (L.) Millsp.]. Poor pollen grain formation and their degeneration in subsequent mitotic cell division were evident in male sterile lines. Application of various specific stains revealed the following: (i) carmine, which is used very often for assessing pollen viability may not be very reliable because it is non-specific to nucleus, cytoplasm and other organelles, (ii) iodine + potassium iodide (I_2 + KI) stain can precisely distinguish the fertile from sterile pollen grains and can also differentiate pollen grains in terms of chemical composition, and (iii) the mixed stain (carmine + ferrous ammonium sulphate + haematoxyline) is very specific to fertile vs sterile pollen grains and can identify various pollen grains with more precision. Staining technique can also be used to detect the distortions in respiratory mechanism and nucleic acid synthesis. Further, the under sized pollen grains and their frequency significantly varied among the 'A', 'B', 'R' lines and the hybrids synthesized there from.

Key words: *Cajanus cajan*, Cytoplasmic genetic male sterility, Pollen grains, Staining

The exploitation of hybrid vigour in food legumes offers optimism for a breakthrough in the yield potential as it has been witnessed in a number of field crops. Among the grain legumes pigeonpea [*Cajanus cajan* (L.) Millsp.] offers an opportunity for developing commercial hybrids as it meets the major pre-requisites, such as well established cytoplasmic genetic male sterility (CGMS) system, i e cytoplasmic genetic male sterile line ('A' line), maintainer line ('B' line) and restorer line ('R' line) and substantial outcrossing. Since the hybrid vigour and its utilization through heterosis breeding is an excellent tool to achieve the genetic enhancement of yield therefore, to achieve the yield advantage, the perfection of CGMS system, variability and desirable traits in parental lines ('A', 'B' and 'R' lines) and their stability in wider environment with economic advantages are most essential prerequisites. Pigeonpea being a long-duration crop is subjected to more stresses due to environmental fluctuations with respect to rainfall, temperature, photoperiod, wind direction, velocity etc. The CGMS system is an interaction of cytoplasm and nucleus leading to abnormal pollen grain development. The validity of pollen sterility associated with its viability has been questioned in recent years (King 1961), particularly when encountered with degradation of protoplast which occurs immediately after microsporogenesis during late ontogeny. Male sterile mutant with irregular or delayed enzyme synthesis of adenine phosphoribosyl transferase, alchohol dehydrogenase and esterase was reported by Regan and Moffatt (1990). Therefore, the present study was undertaken to know the process of distinct sterility characteristics of 'A' lines following cyto-chemical test and *in-vitro* pollen development to determine the number and viability of pollen grains with greater specificity at varying temperatures and size of pollen grains in parental lines and hybrids based on cytoplasmic genetic male sterility system in pigeonpea.

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MATERIALS AND METHODS

Experimental material for the present investigation consisted of 8 CGMS lines, viz 'UPAS 120 A', 'ICPL 84023 A', '67A', 'PA 163 A', 'GT 288A', 'GT 100A', 'GT 290A' and 'GT 33 A'; 8 maintainers, viz 'UPAS 120 B', 'ICPL 84023 B', '67 B', 'GT 288 B', 'PA 163 B', 'GT 100 B', 'GT 290 B' and 'GT 33 B'; 10 restorers, viz 'IPR 03-1', 'IPR 03-2', 'IPR 03-3', 'IPR 03-4', 'IPR 03-5', 'IPR 03-6', 'IPR 03-7', 'IPR 03-8', 'IPR 03-9' and 'IPR 03-10' and 10 hybrids, viz 'GT 288 A' × 'IPR 03-1', 'GT 288 A' × 'IPR 03-2', 'GT 288 A' ×

'IPR 03-3', 'GT 288 A' × 'IPR 03-4', 'GT 288 A' ×'IPR 03-5', 'GT 288 A' × 'IPR 03-6', 'GT 288 A' × 'IPR 03-7', 'GT 288 A' × 'IPR 03-8', '288 A' × 'IPR 03-9' and 'GT 288 A' ×'IPR 03-10'. Fresh and opened flowers and buds were collected from cytoplasmic male sterile lines, maintainers, restorers and hybrids in moist Petri-dishes and brought to the laboratory.

Single anther was pierced and allowed to grow on pollen growth media (Dundas 1990) in 3 different tubes of 10 ml separately in incubator at optimal temperature (25°C± 1°C) to simulate with field situation which was observed during February–March when temperature remained < 30°C and sub optimal temperature (10°C ± 1°C) in incubator to simulate with field situations which was observed during December–February when temperature remains near about 10°C and also the above optimal temperature (30°C ± 1°C) in incubator to simulate with field situation which is observed during April–May when temperature remained >30°C. For pollen grain count/anther, the dilution method was followed in which 0.1µ ml of pollen growth media containing pollen grains in the tube of 10 ml capacity was dropped on glass slide and subsequently, stained with iodine + potassium iodide (I_2 + KI) solution. Number of pollen grain/anther was calculated on the basis of number of pollens available in the area of count, and dilution factor, as observed on the liquate. Pollen size was recorded on 10 individual grains of all the lines, viz male sterile line ('A' line), maintainer line ('B' line), restorer line ('R' line) and hybrids during crop seasons of 2006–07 and 2007–08.

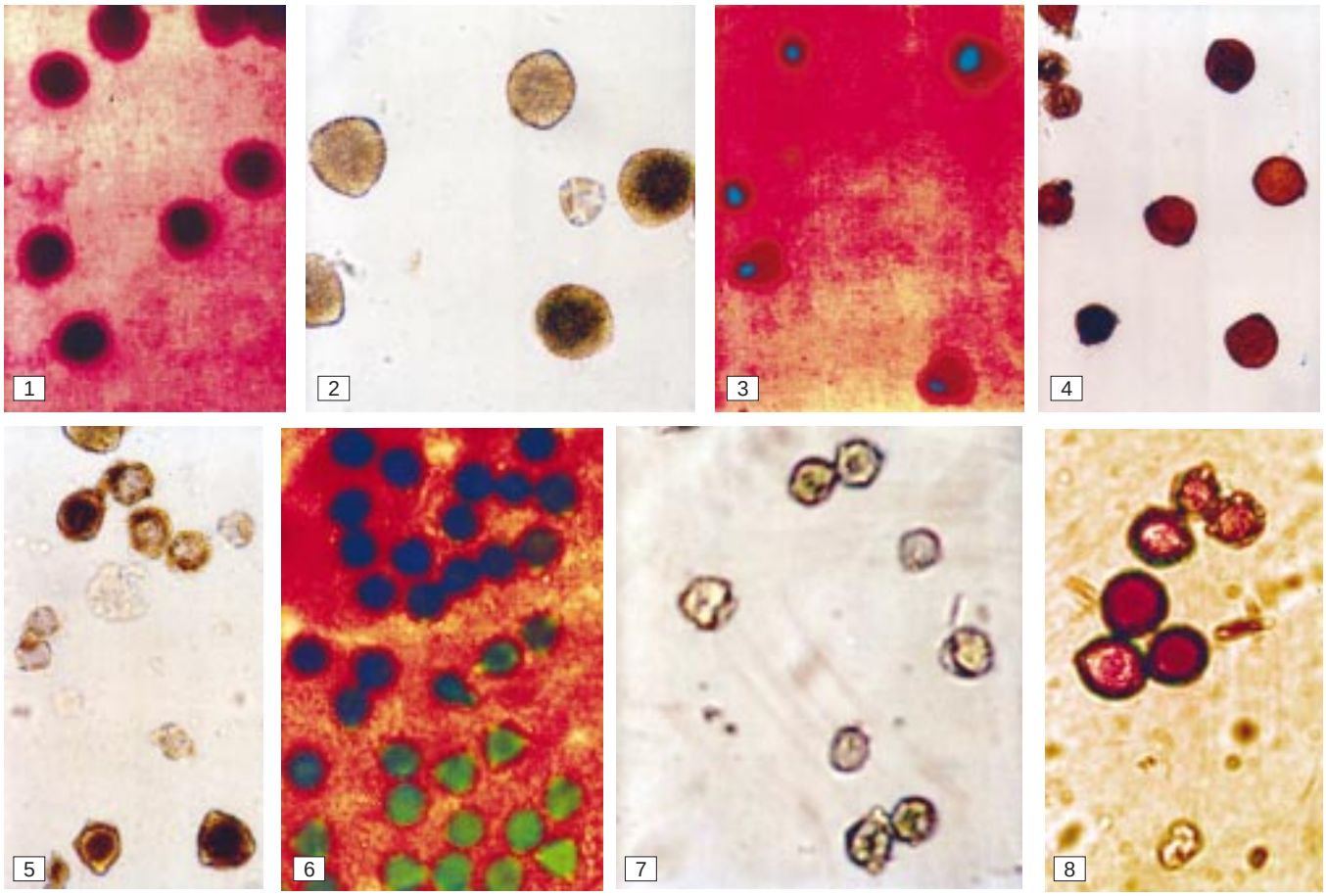
Pollen viability, its germination and extent of pollen tube growth was assessed for all the 3 lines (A, B and R lines) grown at three temperature regimes, ie optimal temperature (25°C ± 1), sub-optimal (10°C ± 1) and above optimal (30°C ± 1).

The micro chemical tests were done for various purposes using different stains:

(a) For pollen respiration and viability – staining with 2-3-5-tetrazolium chloride
(b) For carbohydrate (starch) and protein molecules on the pollen matrix – staining with I_2 + KI, (blue/black colour was observed for starch and reddish brown for protein bodies)
(c) Differential staining with methyl green and pyronin (1:1) to detect the level of DNA and RNA syntheses.

RESULTS AND DISCUSSION

During pollen grain formation, pollen mother sac was packed with very few pollen grains in 'A' lines as compared to 'B', 'R' and hybrids which, clearly showed that pollen grain formation was affected in CGMS lines. The mitotic cycle was affected and limited pollen grains were formed in the anther and further degeneration of pollen grains was also evidenced with 2-3-5 tetrozolium chloride staining (Edward *et al*. 1964). Kelly *et al.* (2002) reported that the colour intensity showed the active/weak respiration in pollen grains, more precisely in case of staining with aniline blue in combination with lacto phenol, probably due to its ability to stain callose, which was easily distinguishable from the aborted one and electronic particle. Carmine, a dibasic dye possesses anthraquinone group which provides chromophoric property (Gatenby and Beams 1950) due to chemical reaction and physical absorption and when carmine was dissolved in the acids, isoelectric point acquired positive charge. Carmine often stained the cytoplasm as well due to acidophilic nature and metallic hydroxide attached with the tissue to form a lake resulting into disrupted respiratory activity carrying out oxidative metabolism. The carmine stain, with crimson colour (Plate 1) cannot differentiate the entine and exine further and application of little more stain, often misleads the identification of sterile and fertile pollen grains while, I_2 + KI clearly differentiated both entine and exine by showing varying colour intensity and also differentiated (Plate 2) both the normal and abnormal pollen grains depending on the chemical composition (starch and protein bodies). This study indicated that as compared to 'B' and 'R' lines, 'A' line behaved differently with respect to carbohydrate and protein synthesis due to alteration in biochemical pathways with the involvement of CGMS related genetic control. Progressive staining with I_2 + KI (Plate 3/4) differentiated the pollen grains to varying sizes. On the contrary, stain prepared with mixing of ferrous ammonium sulphate, carmine and haematoxylin (Zichao *et al.* 1996 and Kato 1998) could easily differentiate (Plate 5) sterile and fertile pollen grains, and further differentiate parts of pollen with more precisions and specificity. Therefore, this stain could be used more reliably in routine identification of sterile and fertile pollen grains in parental lines involved in CGMS-based hybrid breeding programme. Pollen grains also varied in DNA and RNA synthesis (Plate 6) as evident with differential staining with green colour specific to DNA synthesis with methyl green and pink colour specific to RNA synthesis with pyronin. So, the pollen grains did vary with respect to DNA and RNA synthesis. Once pollen grains were stained with 2-3-5 tetrazolium chloride, both weak (plate 7) and active (Plate 8) respiration activity of the pollen could be recognized. Pollen grains had variety of starch and protein bodies, thus indicating the involvement of bio-chemical alteration (Plate 9), as it was evident in the sterile pollens which were deficient in protein and carbohydrate content. Therefore, the sluggish or disrupted respiratory mechanism, led to suspect the presence of mitochondrial activity, ie role of cytoplasm in creating sterility. While staining with I_2 + KI, presence of distinct colours, i e blue vs reddish-brown with varying intensity, distinguished between the anthers of male sterile lines and highly packed anther (anthers full of pollen grains) consisting of both starch granules and protein bodies at cellular level in maintainer, restorer and hybrids.



Plates 1–8 **1.** Carmine staining (no differentiation between entine and exine); **2.** I_2 + KI staining (clear differentiation between entine and exine); **3.** I_2 + KI (progressive staining in Plate 3 and 4 differentiates the pollen grains of varying sizes); **4.** I_2 + KI; **5.** Carmine + FAS + haematoxylin; **6.** Methyle green + pyronin; **7.** Tetrazolium (sterile); **8.** Tetrazolium (fertile)

Carmine staining with crimson colour is neither very specific to entine or exine nor it can differentiate the entine or exine very precisely. In comparison to carmine alone, I_2 + KI were better but combination of carmine with ferrous ammonium sulphate and haematoxylin produced better results. Iron ions intensify the staining but excess iron causes blackening in the cytoplasm. Iron ion gave progressive staining but on water wash it resulted regressive staining. Application of double stains of methyl green and pyronin which have specificity of green and pink colours, respectively, differentiated either presence of DNA and RNA or synthesis of both in varying degree.

CGMS lines ('A' line) were generally devoid of pollen grains (Plate 10a, b) but occasional presence of minute pollen grains with very poor fertility have been observed in both the years (Tables 1, 2). Increment in pollen size was more in a good hybrid while in poor hybrid, proportion of smaller pollens was more in both the years (Tables 3, 4). In general, reduction in pollen size was negligible in CGMS line, GT 288A but the restorer lines had variable pollen size. Number of large sized pollen grains was more in hybrids as compared to its male parent, i e respective R line (Tables 3, 4). Among the 10 hybrids all were better as compared to respective restorer parent, but 4 hybrids, viz 'GT 288 A' × 'IPR 03-3', 'GT 288 A' × 'IPR 03-9', 'GT 288' A × 'IPR 03-10' and 'GT 288' A × 'IPR 03-8' were very good with respect to retention of pollen size in both years (Tables 3, 4). Pollen size increased in hybrids because of increase in formation of large sized pollen grains at the expense of medium ones, whereas number of small size pollen almost remained unchanged. This study had practical utility in identification of better restorers and hybrids characterized with bigger pollens, that too with high proportion. The per cent plant fertility and pollen sterility varied to great extent among the hybrids which depended on its parents (A and R lines). Besides showing variability in pollen size and fertility, hybrids showed variability in pollen staining ability as well with differential and specific staining techniques.

In long duration pigeonpea which is mainly grown in north-east plains zone of India, formation of fertile pollen and its involvement in fertilization, pod formation and seed development was seriously affected when temperature approached around 10°C or less in winter season (December–January). An increase in temperature in spring season

Table 1 Plant and pollen fertility and variability in pollen size of 'A' (male sterile) and 'B' (maintainer) lines of pigeonpea in 2006–07

CGMS line	Plant fertility (%)	Pollen fertility (%)	Pollen size (µ)	Anther size and colour
'UPAS 120 A'	0	0		Small, shriveled, brownish
'UPAS 120 B'	100	100	45–50	Large, yellow
'ICPL 84023 A'	0	0.2	6–8	Small, pale-yellow
'ICPL 84023B'	100	100	40–45	Large, yellow
'67A'	0	0		Small, creamy-white
'67B'	100	100	40–45	Large, yellow
'PA 163A'	0	0.2	5–10	Small, shriveled, pale-yellow
'PA 163B'	100	100	40–45	Large, yellow
'GT 288A'	0	0		Small, creamy-white
'GT 288B'	100	100	45–50	Large, yellow
'GT 100A'	0	0.1	2–4	Small, pale-yellow
'GT 100B'	100	100	100	Large, yellow
'GT 290A'	0	1	5–10	Small, pale-yellow
'GT 290B'	100	100	45–50	Large yellow
'GT 33A'	0	1	5–10	Small, shriveled, brownish
'GT 33B'	100	100	45–50	Large, yellow

Table 2 Plant and pollen fertility and variability in pollen size of 'A' (male sterile) and 'B' (maintainer) lines of pigeonpea in 2007–08

CGMS line	Plant fertility (%)	Pollen fertility (%)	Pollen size (µ)	Anther size and colour
'UPAS 120 A'	0	0		Small, shriveled, brownish
'UPAS 120 B'	100	100	43–50	Large, yellow
'ICPL 84023 A'	0	0.15	3–5	Small, pale-yellow
'ICPL 84023B'	100	100	40–44	Large, yellow
'67A'	0	0		Small, creamy-white
'67B'	100	100	40–45	Large, yellow
'PA 163A'	0	0.1		Small, shriveled, pale-yellow
'PA 163B'	100	100	40–45	Large, yellow
'GT 288A'	0	0		Small, creamy-white
'GT 288B'	100	100	46–51	Large yellow
'GT 100A'	0	0.1	2–4	Small, pale-yellow
'GT 100B'	100	100	100	Large yellow
'GT 290A'	0	1	5–10	Small, pale-yellow
'GT 290B'	100	100	48–50	Large, yellow
'GT 33A'	0	1	5–6	Small, shriveled, brownish
'GT 33B'	100	100	45–52	Large, yellow

Table 3 Comparison of varied pollen size in hybrids and their respective restorers of pigeonpea in 2006–07

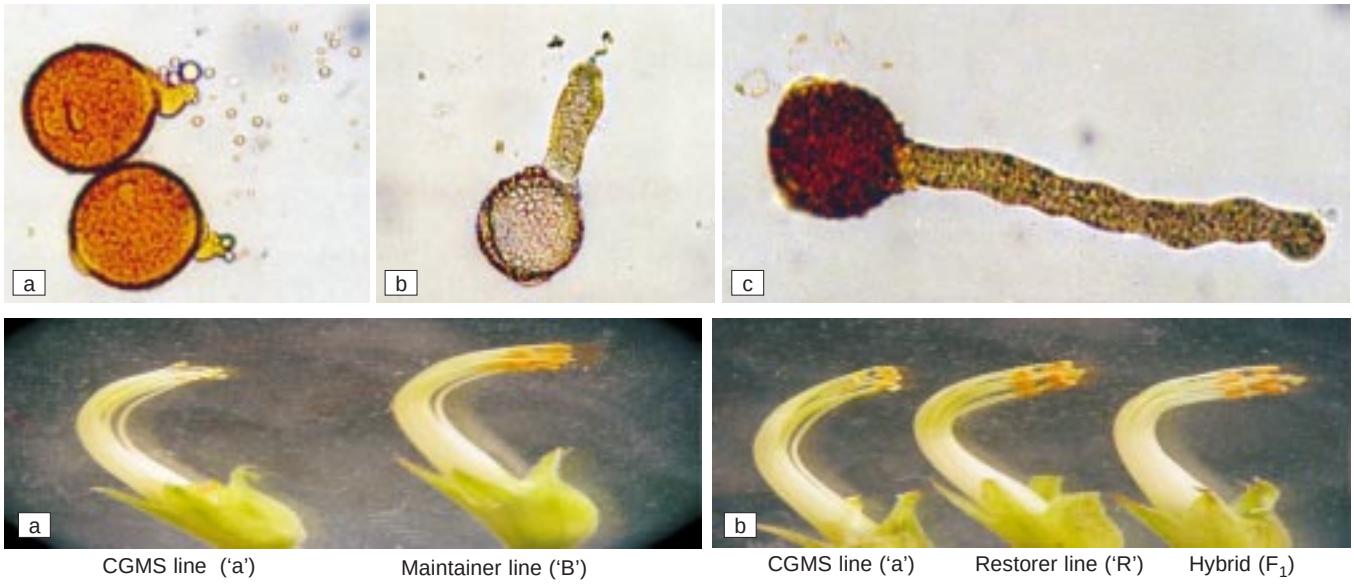
Hybrid (restorer)	Varied pollen size (µ)		
	Small (<20 µ)	Medium (>20–30 µ)	Large (>30–50 µ)
'GT 288 A' × 'IPR03-3' ('IPR 03-3')	23(56)	36(42)	41(2)
'GT 288 A' × 'IPR 03-9' ('IPR 03-9')	25(40)	28(59)	47(1)
'GT 288 A' × 'IPR 03-10' ('IPR 03-10')	20(39)	34(59)	46(2)
'GT 288 A' × 'IPR 03-8' ('IPR 03-8')	23(38)	38(61)	39(1)
'GT 288 A' × 'IPR 03-2' ('IPR 03-2')	43(39)	18(58)	39(3)
'GT 288 A' × 'IPR 03-5' ('IPR 03-5')	33(48)	42(50)	25(2)
'GT 288 A' × 'IPR 03-1' ('IPR 03-1')	38(48)	38(49)	24(3)
'GT 288 A' × 'IPR 03-4' ('IPR 03-4')	32(30)	40(66)	28(4)
'GT 288 A' × 'IPR 03-6' ('IPR 03-6')	34(29)	38(69)	28(2)
'GT 288 A' × 'IPR 03-7' ('IPR 03-7')	33(38)	37(61)	30(1)
Mean Hybrid	30.4	34.9	34.7
Restorer	(40.5)	(57.4)	(2.1)

Figures in parentheses belong to restorers

Table 4 Comparison of varied pollen size in hybrids and their respective restorers of pigeonpea in 2007–08

Hybrid (restorer)	Varied pollen size (µ)		
	Small (<20 µ)	Medium (>20–30 µ)	Large (>30–50 µ)
'GT 288 A' × 'IPR03-3' ('IPR 03-3')	27(58)	30(40)	43(2)
'GT 288 A' × 'IPR 03-9' ('IPR 03-9')	22(45)	30(53)	48(2)
'GT 288 A' × 'IPR 03-10' ('IPR 03-10')	20(36)	37(62)	43(2)
'GT 288 A' × 'IPR 03-8' ('IPR 03-8')	23(41)	42(58)	35(1)
'GT 288 A' × 'IPR 03-2' ('IPR 03-2')	39(37)	19(60)	42(3)
'GT 288 A' × 'IPR 03-5' ('IPR 03-5')	30(46)	45(52)	25(2)
'GT 288 A' × 'IPR 03-1' ('IPR 03-1')	34(45)	41(52)	25(3)
'GT 288 A' × 'IPR 03-4' ('IPR 03-4')	33(34)	40(62)	27(4)
'GT 288 A' × 'IPR 03-6' ('IPR 03-6')	32(39)	40(59)	28(2)
'GT 288 A' × 'IPR 03-7' ('IPR 03-7')	28(39)	42(60)	30(1)
Mean Hybrid	28.8	36.6	34.6
Restorer	(42)	(55.8)	(2.2)

Figures in parentheses belong to restorers



Plates 9–10 **9.** (a) Pollen germ ination *in-vitro* sub-optimal temperature: 10°C ± 1°C; 9 (b) Pollen germination *in-vitro* above optimal temperature: >30°C ± 1°C; 9 (c) Pollen germination *in-vitro* optimal temperatures: 25°C ± 1°C. **10.** (a) and (b) Pollen dehiscence in parental lines and hybrids

(February–March), however resulted in normal pod and seed development. Again in summer (April–May) fertilization, pod formation and seed development were seriously affected due to high temperature, i e >35°C. Under such circumstances pollen formation, pollen size, pollen viability (Khatun and Flowers 1995) and its *in-vitro* germination were verified under optimal (25°C ± 1), sub-optimal temperature (10°C ± 1) and above optimal temperature (>30°C). During the period of sub-optimal temperature (10°C ± 1), the number of pollen/anther reduced by 20% and pollen germination and pollen tube growth were arrested as evidenced from *in-vitro* experiments and per cent pollen germination was significantly low in both hybrids and R lines as compared to B lines (288B and 67B). There was no effect of varying temperature on pollen size but number and viability of pollen grain and pollen tube growth (Plates 9a, b, c) were adversely affected. In growing media, the pollen tube growth was reduced in hybrids as compared to its male parent (R line), and it varied in varying R lines at suboptimal (10°C ± 1) and above optimal (>30°C) temperatures. During the period of optimal (25°C ± 1) temperature number of pollen grains, pollen viability and its *in-vitro* germination were normal in all the parental lines and hybrids.

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