

Effects of seasons and hormones on pre- and postfertilization barriers of crossability and *in vitro* hybrid development between *Vigna unguiculata* and *V. mungo* crosses

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Abstract

Effect of two different seasons and hormones on pre- and postfertilization barriers in crossability were studied in *Vigna mungo*, *V. unguiculata* and their crosses. Significant differences between the two seasons for pollen fall, pollen germination, tube growth and pod set were observed. In kharif season with an average temperature of 26 to 30 °C and 75 to 85 % humidity all the characters showed higher values than in the spring season with a higher temperature of 32 to 36 °C and low humidity of 40 to 50 %. Application of gibberellic acid (GA) alone and a combination of gibberellic acid, naphthaleneacetic acid (NAA) and kinetin (KIN) significantly increased the pollen fall by 7 - 13 %, pollen germination by 10 - 12 % and pollen tube growth by 27 - 30 %. More pollen tube abnormalities in interspecific crosses only indicates partly incompatible reaction of pollen tube in the stylar tissue. Hormonal treatments, especially GA, significantly increased the pod set and pod harvest by 20 % and 34 %, respectively. Higher pod harvest was seen in crosses with *V. unguiculata* as female parent. GA treatment enhanced pollen germination and pollen tube growth, and by partially overcoming embryo abortion for 10 to 12 d, immature embryos were successfully rescued for *in vitro* production of hybrid plantlets.

Introduction

Interspecific hybridisation has been found to be complicated by the presence of barriers both at pre-fertilization and postfertilization levels. Wide crossing with wild or other cultivated species of *Vigna* are largely unsuccessful due to variation in pollen germination, inhibition of pollen tube growth (Chowdhury and Chowdhury

Received 9 October 1992, accepted 12 March 1993.

Abbreviations: BAP - benzylaminopurine, 2,4-D - 2,4 dichlorophenoxyacetic acid, GA - gibberellic acid, IAA - indoleacetic acid, KIN - kinetin, MS - medium of Murashige and Skoog (1962), NAA - naphthaleneacetic acid.

Acknowledgements: Thanks are due to Dr. S. Chandra for guidance on statistical analysis.

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1977), delay in pollen tube growth (Gopinathan *et al.* 1986) and slowness of pollen tube growth (Bassiri *et al.* 1987). Other problems encountered are early hybrid pod abscission (Al-Yasiri and Coyne 1964, Chen *et al.* 1990) and embryo abortion during embryogenesis (Sabja *et al.* 1990). This investigation relates to the study of crossability barriers and effect of different seasons and hormones in *Vigna* species.

Materials and methods

The material comprised one cultivar each of black gram (*Vigna mungo* L. Hepper cv. PU 19) and cowpea (*V. unguiculata* L. Walp. cv. IT 124). Studies were conducted in kharif (July to October) and spring (March to June) seasons. Average temperature and relative humidity during the flowering period were 26 to 30 °C and 75 to 85 % in kharif season and 32 to 36 °C and 40 to 50 % in spring season. The flower buds expected to blossom the following day were emasculated in the evening and pollinated on the next day morning. To study the effect of hormones on crossability barriers, three treatments were used. H₀ - control, H₁ - GA 100 mg l⁻¹ solution, H₂ - hormonal solution consisted of GA 100 mg l⁻¹, NAA 25 mg l⁻¹ and KIN 5 mg l⁻¹. Hormonal solutions were applied daily at the base of flower bud and inside the flower bud with the help of hypodermic syringe needle immediately after pollination till the pods were harvested.

Pre-fertilization studies: Pollen tube studies on different characters *viz.* pollen fall on stigma surface 24 h after anthesis, pollen germination (%) and pollen tube growth and pollen tube abnormalities at 10 min, 60 min, 6 h and 24 h after pollination were recorded. Flower buds at different time intervals were fixed in 1:2 lactoalcohol for 48 h and preserved in 70 % alcohol. Pistils were stained with cotton blue solution, destained in 40 % acetic acid and mounted in pure lactic acid.

Postfertilization studies: Pod set (%) at 48 h after pollination and pod harvest (%) at 20 d after pollination were recorded. Pod growth and development (cm) was recorded for 10 pods at 24 h interval for selfed (IT 124) and crossed pod of IT 124 × PU 19.

Embryo rescue: The developing pods of crosses were collected between 9 to 11 d after pollination. Pods were surface sterilized with 10 % commercial sodium hypochlorite solution. Immature hybrid embryos which were heart to cotyledonary stage of growth were excised from the aborted seeds and cultured on Murashige and Skoog's (1962) medium (MS) supplemented with various growth regulators and 500 mg l⁻¹ casein hydrolysate. MS media for embryo culture was prepared for two different purposes: 1) Direct hybrid plantlet formation - MS media was supplemented with IAA or NAA (0.2 to 0.4 mg l⁻¹) and BAP or KIN (1.0 mg l⁻¹); 2) Organogenesis - MS media supplemented with 2,4-D or IAA (1.0 to 2.0 mg l⁻¹) and BAP or KIN (0.5 mg l⁻¹) for callus induction. After callus induction, calli were transferred on media which were used for direct plantlet formation. Cultures were incubated in a well illuminated temperature controlled room at 25 ± 1 °C. Data were analysed using three factor completely randomised design. Data taken on percentage values were

subjected to angular transformation before analysis.

Results

Analysis of variance for different pre- and post-fertilization characters showed significant variation for all the parameters *viz.* crosses, seasons, hormones and various interactions except season $\times$ hormone and hormone $\times$ crosses interaction for average pollen fall and pollen germination.

Effect of season: All the pre- and postfertilization characters showed significantly higher values in kharif than in the spring season (Table 1). Parents showed significantly higher values for all the characters than crosses in both the seasons and characters showed better expression in kharif season. In crosses values were higher when cowpea (IT 124) was the female parent. The increase in pollen tube growth (62 %), pod set (34 %) and pod harvest (38 %) was seen in kharif season as compared to spring season. Pod harvest shows 16.8 % increase in IT 124 $\times$ PU 19 cross while increase was 30.2 % in PU 19 $\times$ IT 124 cross in kharif season when compared with spring season.

Table 1. Effect of seasons on different crossability characters of *Vigna mungo* and *V. unguiculata* crosses.

Crosses	Average pollen fall	Pollen germination [%]	Average pollen tube growth	Pod set [%]	Pod harvest [%]
Spring season					
PU 19	133.0	(32.6) 29.9	114.9	(61.1) 76.3	(61.1) 6.3
PU 19 $\times$ IT 124	75.4	(25.0) 18.1	86.2	(20.5) 17.6	(13.2) 8.6
IT 124 $\times$ PU 19	77.5	(29.1) 23.9	111.5	(37.3) 45.6	(20.9) 18.3
IT 124	137.8	(35.1) 33.1	168.6	(66.9) 84.3	(66.9) 84.3
Mean	105.9	(30.4) 26.1	127.8	(46.5) 56.0	(40.5) 46.9
Kharif season					
PU 19	146.7	(37.4) 37.0	219.2	(65.8) 82.6	(65.8) 82.6
PU 19 $\times$ IT 124	107.7	(31.4) 27.5	181.1	(46.5) 52.6	(30.8) 34.6
IT 124 $\times$ PU 19	119.2	(36.0) 34.7	194.2	(59.3) 72.3	(39.4) 49.0
IT 124	162.0	(38.4) 39.2	237.4	(77.9) 92.3	(77.9) 92.3
Mean	133.9	(35.9) 34.6	207.9	(62.4) 75.0	(53.5) 64.6
CD s	3.29	1.18	3.99	2.66	2.77
CD sc	8.06	2.92	9.80	4.66	4.81

Data recorded on percent values were converted to angular values (in parentheses) to make the data linear and normal distributed.

Effect of hormones: H₁ (GA) and H₂ (GA + NAA + KIN) hormonal applications showed significant increase in values of all the pre- and postfertilization characters

over the seasons and crosses (Table 2). The H_1 and H_2 hormonal applications increased pollen fall by 7 - 13 %, pollen germination by 10 - 12 %, pollen tube growth by 27 - 30 % while pod set and pod harvest by 20 - 30% over control. There were non- significant differences between the H_1 and H_2 hormonal treatments in case of pollen fall, pod set and pod harvest. H_1 application of GA was found to be better in partially overcoming of different crossability barriers. Hormonal application showed increase in pod set and pod harvest in crosses but on parents there was not significant effect of hormonal application. In control treatment (H_0) 15 to 24 % pods were formed in crosses but not a single pod could be harvested. However application of H_1 and H_2 treatments resulted in 33 to 52 % pod harvest. IT 124 $\times$ PU 19 cross in which IT 124 was the female parent, showed better response after H_1 treatment as compared to reciprocal cross of PU 19 $\times$ IT 124.

Table 2. Effect of hormonal treatments on different crossability characters of *Vigna mungo* and *V. unguiculata* crosses.

Crosses	Average pollen fall	Pollen germination [%]	Average pollen tube growth	Pod set [%]	Pod harvest [%]
H_0					
PU 19	129.2	(31.1) 27.0	159.4	(63.4) 79.5	(63.4) 79.5
PU 19 $\times$ IT 124	84.7	(24.2) 17.0	104.0	(16.5) 15.0	-
IT 124 $\times$ PU 19	93.4	(28.6) 23.4	123.6	(21.9) 24.0	-
IT 124	135.8	(33.5) 30.7	181.4	(71.9) 88.0	(71.9) 88.0
Mean	110.8	(29.4) 24.5	142.1	(43.5) 51.6	(33.8) 41.9
H_1					
PU 19	145.3	(37.5) 37.2	199.3	(63.1) 79.0	(63.1) 79.0
PU 19 $\times$ IT 124	95.0	(31.0) 26.8	153.7	(42.8) 46.5	(33.6) 33.5
IT 124 $\times$ PU 19	101.1	(24.7) 32.6	168.5	(62.6) 78.0	(46.3) 52.0
IT 124	157.5	(39.0) 39.7	219.3	(72.4) 88.5	(72.4) 88.5
Mean	124.7	(35.6) 34.1	185.2	(60.2) 73.0	(63.8) 63.2
H_2					
PU 19	145.0	(36.4) 35.3	187.5	(63.7) 80.0	(63.7) 80.0
PU 19 $\times$ IT 124	94.9	(29.4) 24.5	143.2	(41.2) 44.0	(32.3) 31.5
IT 124 $\times$ PU 19	100.6	(34.3) 31.9	166.4	(60.4) 75.0	(44.3) 49.0
IT 124	156.3	(38.1) 30.1	208.2	(72.9) 88.5	(72.9) 88.5
Mean	124.2	(34.5) 32.4	176.3	(59.5) 71.9	(63.3) 62.2
CD h	4.0	1.4	4.9	3.3	3.4
CD hc	6.6	2.4	8.0	6.6	6.8

H_0 - control, H_1 , GA, H_2 mixture of GA NAA and KIN. Data recorded on percent values were converted to angular values (in parentheses) to make the data linear and normal distributed.

Pollen tube abnormalities: These were variable and increased with the passage of time. Abnormalities were more in crosses (10.5 to 18 %) as compared to parents (1 to

4 %) irrespective of season and hormonal effect. Different abnormalities observed were swelling of pollen tube at the tip, pollen tube growing in the wrong direction, more than one pollen tube coming from the same pollen and branching of pollen tubes (Fig. 1).

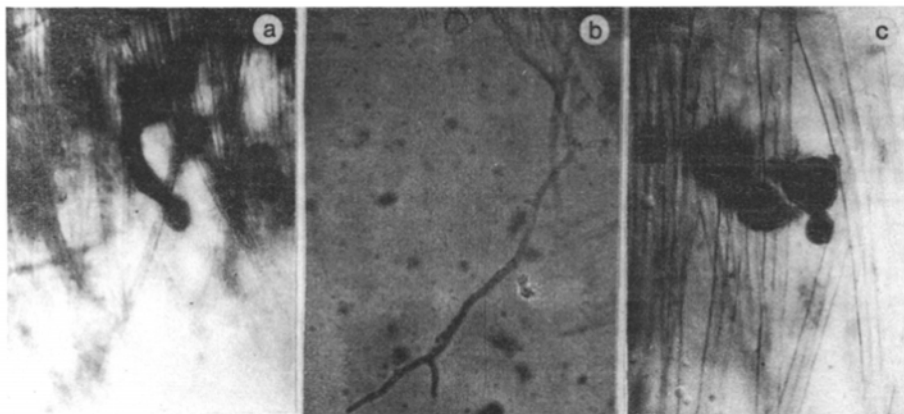


Fig. 1. Pollen tube abnormalities in *Vigna mungo* and *V. unguiculata* crosses. a - pollen tube in wrong direction and swelling at the tip, b - branched pollen tube in wrong direction, c - pollen tube with swollen tip.

Pod growth and development: Hybrid (IT 124 $\times$ PU 19) and selfed pod of IT 124 showed better pod growth and development in kharif season. The crossed pod without hormonal application reached maximum length of 5 cm in 4 to 5 d and then wilted in 10 to 11 d. GA treated hybrid pod and selfed pods grew at the same rate up to 72 h but after that growth rate became slow in hybrid pod and completely stopped 10 to 12 d after pollination, while selfed pod continued to grow (Fig. 2).

Statistical analysis revealed that pod set had a positive and significant correlation with pollen fall, pollen germination and pollen tube growth while negative and significant with pollen tube abnormalities.

Embryo rescue: It was seen that even after hormonal applications, hybrid pods stopped growing 10 - 12 d after pollination. Embryos in the shrunk and shrivelled seeds were in heart to cotyledonary stage of growth and abortion took place. Before the abortion, embryos were rescued and cultured. Seven plantlets of *V. mungo* $\times$ *V. unguiculata* and a plantlet of *V. unguiculata* $\times$ *V. mungo* were obtained directly. On best hormonal combination of 0.4 mg l⁻¹ NAA and 1.0 mg l⁻¹ BAP, 33 % and 2.5 % was the plantlet formation frequency in *V. mungo* $\times$ *V. unguiculata* and reciprocal crosses respectively (Fig. 3a). Small embryos were kept for callus induction and 40 to 65 % callusing frequency was obtained with high callusing on MS media supplemented with 2 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ BAP. Shoots were induced from these calluses with a low frequency of 5.5 % (Fig. 3b).

Discussion

Pollination of the pistil usually induces physiological and biochemical changes in the ovary before the pollen tube reaches the ovule. Thus, any change in temperature, humidity or hormonal balance in the styler tissue may change the whole complexion of interspecific cross and might result in hybrid formation. It was seen that during kharif season when temperature was low (26 to 30 °C) and relative humidity high (75 to 85 %) there was higher pollen fall, pollen germination and pollen tube growth as well as high pod set and pod harvest as compared to spring season when average

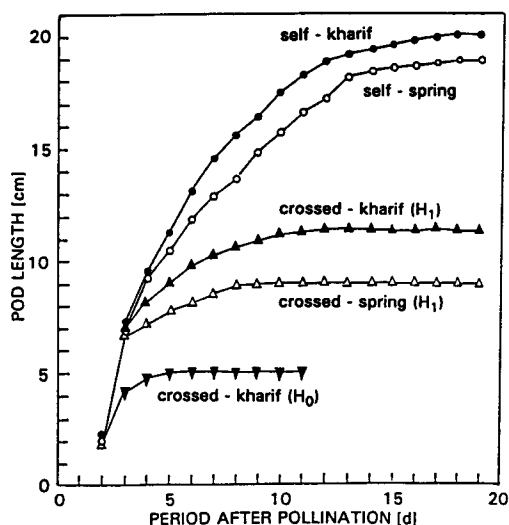


Fig. 2. Effect of hormones and seasons on average pod length [cm] in selfed and crossed pod of *Vigna mungo* and *V. unguiculata*.

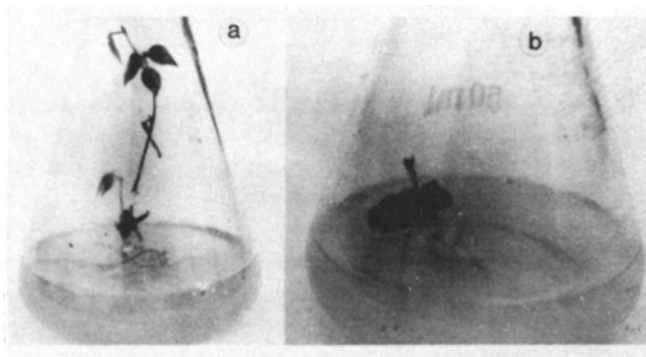


Fig. 3. *In vitro* culture of embryo for plant formation. a - direct hybrid plantlet formation in *V. mungo* × *V. unguiculata*, b - hybrid plantlet formation via organogenesis in *V. unguiculata* × *V. mungo*.

temperature was comparatively high (32 to 36 °C) and humidity low (40 to 50 %). implies that high temperature reduces the growth of pollen tube and affects pollen germination which results in less pod set and pod harvest. Hallerlein *et al.* (1980) though reported that temperature stress reduces pollen viability but found no effect on pollen tube growth.

Hormonal application especially gibberellic acid resulted in an increase in pollen characters and higher pod set and pod harvest. The increase in pollen fall might be due to increased stigma receptivity by hormonal application. Dumas and Gaude (1981) have reported that pollen adhesion on the stigma surface involves impaction and subsequent formation of attachment bonds. On initial contact, components could be contributed by either or both partners to enhance mutual adhesion (Clark *et al.* 1979). Pollen germination and pollen tube growth was found to be higher in parents as compared to crosses because species tend to prefer their own pollen. Reciprocal cross differences between different species have also been observed. In order to enhance pollen germination and pollen tube growth, so that fertilization should take place in interspecific crosses, gibberellic acid application was found to be effective. Gibberellic acid increased pollen germination and pollen tube growth in rye-barley cross (Larter and Chaubey 1965) and in *Vicia faba* (Gupta and Murty 1985).

Pollen tube abnormalities were less and hormonal solutions did not affect the frequency of abnormalities. Abnormalities were seen mainly in interspecific crosses and the most frequent abnormality being swelling of pollen tube at the tip. Pod set showed negative association with percentage of abnormal tubes. The occurrence of pollen tube abnormalities have been reported by Sangduen *et al.* (1983) and Singh and Khanna (1988) which points to incompatible reaction of pollen tube in the stylar tissue. Ahmad *et al.* (1988) suggested crossability barriers may be due to factors operating after fertilization. Pod set and pod harvest percentage increased significantly by hormonal applications in case of crosses while in parents hormones had no effect. Pod abscission and embryo abortion are the main postfertilization barriers. Various workers have reported the use of different hormones and compounds for delay of pod abscission (Al-Yasiri and Coyne 1964, Barker *et al.* 1975, Gosal and Bajaj 1983, Chen *et al.* 1990). Early pod abscission was found to be the main postfertilization barrier in interspecific crosses involving different species of *Vigna*. GA treatment in the present study has increased pod harvest by 22 to 34 % in crosses. But the main objective in such interspecific crosses was that ovules in the pods should also develop simultaneously. It was seen that pod growth could take place up to 10 to 12 d after pollination with the result that embryos could grow just beyond the heart stage. Seeds in 10 to 12 d old hybrid pods were comparable to 5 d old seed of developing selfed seed. Hybrid embryos with *V. mungo* as female could grow up to cotyledonary stage and gave direct plantlets as compared to reciprocal cross when *V. unguiculata* was female parent which generally gave rise to callus. Embryo derived callus responded to different hormonal combinations in culture media resulting in shoot formation. Chen *et al.* (1990) also obtained hybrid plantlets through somatic embryogenesis. Thus crossability barriers can be partially overcome and pod abscission checked to a reasonable time with gibberellic acid treatment so that hybrid embryos could be rescued for raising plantlets.

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Communicated by J. TUPÝ