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Overcoming Interspecific Hybridization Barriers in Cyamopsis Species

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Abstract

Three species of Cyamopsis were studied to find out barriers to interspecific crosses between C. tetragonoloba x C. serrata and C. tetragonoloba x C. senegalensis which serve as a stepping stone for guar improvement.Quantitative production of pollen were identical in all the species. Pollen grains of C. tetragonoloba and C. senegalensis showed more than 95% of viability and C. serrata have 87% viability. Nutritive requirement for in vitro germination of pollen revealed that C. tetragonoloba required 25% sucrose + 100 ppm boric acid + 300 ppm calcium nitrate and C. senegalensis needed 35% sucrose with same basal medium.While C. serrata required 35% maltose + 6% PEG 6000 along with above dose of boric acid and calcium nitrate. Moreover, pollen germination in C. serrata was initiated after 30 h of incubation and its pollen tubes were slow growing attaining 174.7 µm length in 48h. The length of style of C. tetragonoloba and C. serrata was nearly identical (2.6 mm) while C. senegalensis possess longest style (3.8 mm). Protein content of stigma + style was nearly identical in all the species and total soluble carbohydrate content in C. tetragonoloba and C. serrata was nearly identical (5-6mg/100mg FW) but lower content was in C. senegalensis (2.4mg/100mg FW). It was observed that interspecific hybridization between C. tetragonoloba x C. serrata was successful by use of stub smeared with PGM and 10.43% of pod setting.Colour and shape of hybrid seeds was akin to the female parent (C. tetragonoloba),hybrid plants showed early flowering just like male parent (C. serrata) whereas the plant height was intermediate between the two parents.

Keywords: Interspecific hybrids, pollen, in vitro germination, stub pollination, in vivo tube growth.

1. Introduction

Cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.) (2n = 2 x = 14) commonly called as guar is a drought tolerant commercial crop. Guar cultivation has been an attractive subject due to availability of varieties with high guar gum (30-35% of whole seed) content (galactomannans) in its endosperm which has great value as an enhancer of viscosity in food industry. It is widely used from paper and cosmetic manufacture to mining and explosive (Whistler and Hymowitz, 1979). Its uses in tissue culture media as a gelling agent has been reported (Jain *et al.*, 2005).Being a leguminous plant, its roles in improving the soil fertility is undebatable. Hence, the crop is important for multipurpose industrial uses, nutritional adequacy and sustainable desert agriculture. A critical requirement for crop improvement in general, is the introduction of new genetic material in the cultivated lines of interest, whether through conventional or non-conventional breeding or plant tissue culture technologies. *Cyamopsis tetragonoloba* ,an erect herb with indeterminate growth and broad trifoliate leaves, is a late maturing species (>90 days). However, one of its wild relatives i.e. *C. serrata* is an early maturing (40-50 days), slow growing with narrow trifoliate leaves while the other wild species i.e. *C. senegalensis* is also slow growing with narrow pentafoliate leaves and matures in 120-130 days (Menon,1973).Both these wild relatives possess some desirable attributes like drought resistance (Menon,1973),photo-and thermo-insensitivity (Anonymous,1982) and disease resistance (Orellana, 1966). The conventional breeding technique has so far failed to yield desired results (Sandhu,1988; Virender,2008).Such a failure may be due to presence of pre-and/or post-fertilization barriers.To combat such barriers, it is essential to have detailed knowledge of reproductive biology of all the species of *Cyamopsis* in question. It was therefore, contemplated to follow a systematic approach to identify pre- as well as post- fertilization barriers in interspecific crossing of *Cyamopsis*. Supplementing conventional breeding with unconventional less popular methods along with plant biotechnological techniques is anticipated to go headway in resolving the issue. Present investigation was thus undertaken to study some relevant reproductive characters in three different species of *Cyamopsis* and work out cross ability barriers for production of interspecific hybrids.

2. Materials and Methods

Plants of three species of *Cyamopsis* viz. *C. tetragonoloba* cv. HG 563, *C. serrata* and *C. senegalensis* were raised in cemented pots in the screen house Department of Botany & Plant Physiology. At flowering,reproductive characters of every species were recorded as following.

Number of pollen/flower: Flower buds from each species were collected a day before anther dehiscence. For this,twenty anthers were suspended in 2 ml of 50 per cent glycerine containing a few drops of safranine.Anthers were crushed with the help of glass rod and the suspension was passed through a brass sieve with a mesh of 48 sq/cm^2(Kapoor and Nair 1974).Number of pollen grains per flower drop was counted by haemocytometer.

Pollen viability and *In vitro* pollen germination:Viability of pollen grains was assessed by 2, 3, 5 triphenyl tetrazolium chloride (TTC) test (Hauser and Morrison 1964). *In vitro* germination on the semi solid medium contained in petri dishes. Preliminary studies revealed that sugar type and its concentration and other adjuvants required for pollen germination varied with species. After preliminary trials,germination medium consisting of 25% sucrose (for *C. tetragonoloba*),35% sucrose (for *C. senegalensis*),35% maltose +6% PEG 6000 (for *C. serrata*) alongwith 100ppm boric acid, 300ppm calcium nitrate and 0.8% agar were used for *in vitro* germination and tube growth. After pollen inoculation, petriplates were incubated at 30±2ºC for 4h in dark in a BOD incubator with three replicates per treatment.However, inoculated petriplates of *C. senegalensis* and *C. serrata* were incubated for 30 and 48 h respectively.After pollen germination, the pollen activity was terminated by flooding the surfaces of the media with killing and fixing solution (Sass, 1951).Pollen producing a tube length of a size greater than double of its diameter was designated as germinated. Twenty readings for pollen germination and thirty for tube length from different microscopic fields of each petriplate were made from area with uniform distribution of pollen and fairly good population.

Pistil and yield related characters: The collected flower buds were used to record shape of the stigma and length of the ovary and style by micrometry. Pistils were cut open under a stereoscopic microscope and number of ovules per pistil from at least twenty pistils was recorded. At maturity, thirty pods from each species were collected randomly and used to measure length and breadth of pods, number of seeds per pod and test weight of 100 healthy, uniform sized seeds from each species was recorded and three replicates per species were used.

***In vivo* tube growth:**Self pollinated pistils from flowers of different species of *Cyamopsis*, were collected at 24, 48 and 72h of anther dehiscence and fixed in acetic alcohol (Acetic acid :Ethanol ::1:3) for 4h and processed for aniline blue test (Dumas and Knox, 1983).The observations for germination of pollen grains on stigmatic surface and extent of tube growth in stylar tissue and penetration of the ovule by tube were made under florescent microscope. Fifteen random pistils for each species were used for such studies.

Interspecific hybridization: In the study three species of *Cyamopsis* were employed differed in their flowering schedule,these were grown in a staggered manner to synchronize their flowering. The flowers of the female designate parent were emasculated in the evening (between 1600-1800h) prior to their anther dehiscence.Generally only two floral buds were emasculated on a raceme to permit their proper development.The crossing of *C. tetragonoloba* was carried out on the

following morning between 0700 - 0830 h with pollen grains of *C. serrata* or C. *Senegalensis.*Pollinated flowers were harvested after 24, 48 and 72 h fixed in acetic alcohol and processed for *in vivo* germination and tube growth by aniline blue method.The allogamous pistils left *in situ* were allowed to set pods. Per cent pod set and number of seeds/pod was recorded.

In addition to the conventional breeding method, non-conventional methods like stub pollination with or without smearing with pollen germination medium (PGM), in vivo placental pollination and placental pollination followed by *in vitro* pistil culture on MS medium supplemented with IAA, NAA and BAP were attempted.

Stub pollination: The stigma of emasculated pistils of *C. tetragonoloba* was excised and smeared with cool molten pollen germination medium (PGM) with the help of camel hair brush and then pollen of *C. serrata* and *C. senegalensis* were applied separately on the stigma of emasculated flowers. Pollinated pistils were collected after 24, 48 and 72 h and processed in the same way as explained for study *in vivo* pollen germination and tube growth. All self pollinated flowers below the selected buds were removed thereby ensuring that all the lowest buds on the raceme are always emasculated ones. To avoid damage to the raceme,upper buds were not removed until 3 day after emasculation; however, upper buds blooming during this period were removed immediately. The whole inflorescence was bagged to check any undesired pollination.

Pollination through perforation in the basal part of style: With the help of sterilized syringe needle, a hole was made in the upper part of ovary. Another hole opposite to the hole was also made opposite to this to allow release of air. Pollen suspension of *C. serrata* and *C. senegalensis* in the liquid PGM was injected separately into the ovary through a hole. The hole was plugged with petroleum jelly and pistils were collected after 24 and 48 h and processed them.

Placental pollination: With the help of sharp needle a cut was made along the ventral suture of the pistil to open up the pistil and expose the placenta. Pollen grains were dusted on the placenta and pistil was rolled back into their normal configuration under *in situ* conditions. Pistils were collected after 24 and 48h of placental pollination and processed as explained earlier. In other set of experiment, pistils were excised from the plant,sterilized with 95% ethanol on the hood of laminar flow,cut open to expose placenta, pollinated with the desired pollen viz *C. serrata* or *C. senegalensis* and inoculated on MS basal medium adjunct with BAP, IAA, Kn, NAA, adenine sulphate and casein hydrolysate (CH).

3. Results and Discussion

3.1 Reproductive characters and *in vivo* pollen germination

The flowers of all species of *Cyamopsis* studied produced nearly identical number (nearly 4500) of pollen grains (Table 1). Among different species,pollen diameter of *C.senegalensis* is maximum (54.46 ±1.10µm) whereas the value was least in *C. tetragonoloba* cv. HG 563 (34.90 ±0.63µm).The diameter of *C. serrata* pollen grains

in 38.47 ±0.80µm. Pollen viability as assessed by TTC test was more than 95% in *C. senegalensis* and *C. tetraganoloba* whereas the value was comparatively lower (87.01%) in *C. serrata*. Pollen grains of *C. tetragonoloba* showed maximum germination (94.42%) whereas pollen grains of *C. senegalensis* yielded 70.01% germination. After 48 h of incubation the pollen grains of *C. serrata* germinated and value was only 25.59%.Among the tested species, pollen grains of C. tetragonoloba cv. HG 563 produced the longest tube (1204.9 µm) after 4 h of incubation while those of *C. serrata* produced the smallest pollen tube (174.7µm) after 48 h of incubation.Tube length of *C. senegalensis* pollen was 949.2 µm after 30 h of incubation. (Fig 3). Standardization of *in vitro* germination would be helpful in interspecific hybridization in a number of ways.It is pre-requisite for stub pollination smeared with PGM, *in vitro* pollination and fertilization etc.Interestingly, nutritive requirement and lag period for *in vitro* germination varied significantly in the three species.Pollen grains of *C. tetragonoloba* require 25% sucrose + 100 ppm boric acid + 300 ppm calcium nitrate + 0.8% agar. Tyagi (1974) observed that 69.3% pollen germination with 60.5 µm tube length in *C. tetragonoloba* in medium containing 20% sucrose solution. Addition of 0.015% boric acid to 20% sucrose enhanced pollen germination to 84.7% and tube length to 208.4 µm.Sandhu (1988) employed germination medium containing 25% sucrose +100 ppm boric acid +300 ppm calcium nitrate + 0.5% agar and achieved 89.6% germination and 184.3 µm long tubes in *C. tetragonoloba.*

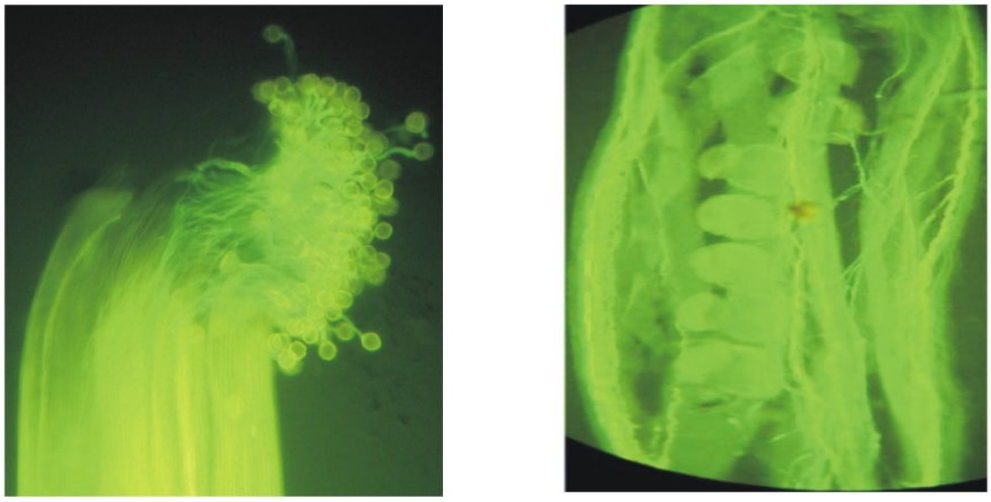

Fig. 3: L-R In vivo germination of C. serrata pollen on the pistil stub of C. tetragonoloba smeared with pollen germination medium.
Pollen tube entering the ovule.

Table 1: Comparison of floral and male reproductive characters in three different species of *Cyamopsis.*

S. No.	Parameters	C. tetragonoloba	C. serrata	C. senegalensis
1.	No. of pollen/flower	4765.94 ± 241.23	4684.26 ± 506.55	4582.66 ± 453.49
2.	Pollen size (□m)	34.90 ± 0.23	38.47 ± 0.30	54.46 ± 0.52
3.	Shape of pollen grain (P/E X 100)	Sub-oblate	Sub-prolate	Prolate spheroidal
4.	Pollen viability (%)	95.77	87.01	98.12
5.	In vitro pollen germination (%)	94.42	25.59	70.01
6.	Pollen tube length (□m)	1204.9	174.7	949.2
7.	Pistil length (cm)	0.67 ±0.02	0.53 ±0.01	0.77 ±0.02
8.	Shape of stigma	Capitate	Subapical crescent	capitate
9.	Number of ovules/pistil	7-8	8-9	7-8
10.	Number of pods/ cluster	6.75±0.23	12.00±0.10	9.50±0.52
11	Length x breadth of pod (cm)	5.95 x 0.54	4.51 x 0.36	5.1 0x 0.40
12.	Number of seeds/pod	7.58±0.19	8.75±0.12	7.45±0.35
13.	Test weight of 100 seeds (g)	2.78±0.21	1.81±0.19	1.84±0.30

The pistil plays a crucial role in the reproductive biology of flowering plants. Studies on the pollen-pistil interaction in leguminous taxa are limited inspite importance of legumes in agricultural production. Stigma of guar is most receptive during 7:30-9:00 am while pollen grains are reported to remain viable throughout the day (Anonymous, 1984). Among three species of *Cyamopsis*,the pistil and style length was maximum (77, 38 mm) in *C.senegalensis* and minimum in (53,25mm).*C.serrata.C.tetragonoloba* and *C. senegalensis* possess capitate type of stigma whereas *C. serrata* is characterized by subapical crescent shaped stigma (Fig 4).Proteins,constitute one of the main constituent of stigma + styles of angiosperms was nearly identical quantitatively in all three species of *Cyamopsis* studied (8 mg/100mg FW).Total soluble carbohydrate content of Stigma + styles of *C. tetragonoloba* and *C. serrata* was nearly identical (5-6 mg/100mg FW),whereas *C. senegalensis* consisted of minimum quantity (2.4 mg/100mg FW) of soluble carbohydrates.Similar Cruden (2009) observed in Fabaceae, pollen size was not correlated with style length in different species of *Cyamopsis.* The pistil of *C. tetragonoloba* and *C. senegalensis* possess nearly identical number of ovules (7-8) while *C. serrata* is characterized by 8-9 ovules per pistil. Number of seeds per pod ranged between 7-9 and did not reveal any significant difference in the wild and

cultivated species of *Cyamopsis (*table 1*)*. Among different species, 100 seed weight of *C. tetragonoloba* was maximum (2.78 g) whereas the value was nearly identical in *C. serrata* and *C. senegalenis* (1.80g).

Selfing in *C. tetraganoloba* resulted a good percentage of *in vivo* pollen germination and pollen tubes could be traced upto the base of the ovary.Micropylar entry of pollen tube in the ovule was evident.

3.2 Interspecific Hybridization

Interspecific hybridization between *C. tetragonoloba* x *C. serrata* and *C. tetragonoloba* x *C. senegalensis* was attempted using conventional and non-conventional breeding methods.The conventional method of breeding revealed that no pod setting in the above said crosses. Among an array of non conventional breeding methods tried smearing of stub of *C. tetragonoloba* with agarified pollen germination medium (PGM) prior to pollination was successful (Fig.1).Among 792 crosses attempted, 83 pods were recovered for the cross *C. tetragonoloba* x *C. serrata* which amounted to 10.47% pod set. The hybrid pods were nearly 1.70 cm long which contained 2.28 seeds per pod .Seed colour and shape of F1 hybrid was akin to the female parent i.e. *C. tetragonoloba (Fig 2).*However,no success was achieved in 429 crosses attempted between *C. tetragonoloba* x *C. senegalensis* even with stub pollination combined with PGM application, although number of pollen grains sticking on the stub increased.

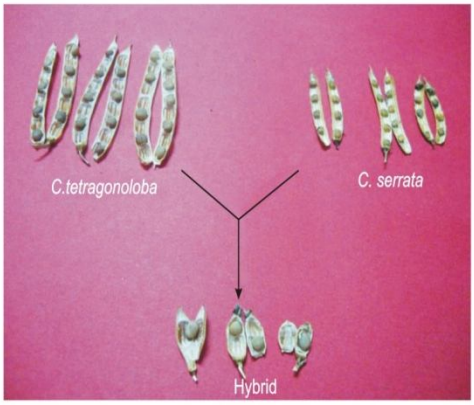

Fig. 1: Comparison of morphological features of pods of Cyamopsis tetragonoloba, C. Serrata and their hybrid

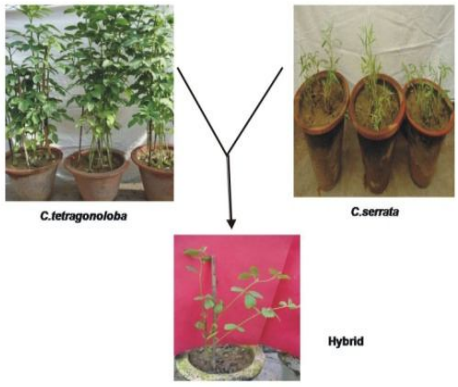

Fig. 2: Comparison of plant morphological features of Cyamopsis tetragonoloba, C. Serrata and their hybrid

Other methods tried viz. pollination through perforation in the basal part of style, *in vivo* placental pollination and placental pollination followed by *in vitro* pistil culture did not reveal any pod set; the pistils turned brown and abscised after about 2-3 days of pollination from the plant.Failure of placental pollination may be ascribed to withering

of ovules after pollen application as has been described for Fabaceae genera (Zenkteler, 1980).In case of placental pollination followed by *in vitro* pistil culture, callusing was observed in all growth combinations tried.

The F1 seeds obtained from crossing (2008-09) *C. tetragonoloba* × *C. serrata* were sown during the year 2009-10.Morphological and phenological features revealed that the plants showed flowering 21 days after sowing.Flowers were pinkish in colour like the female parent (*C. tetragonoloba)* but the shape of pods was akin to the male parent (*C. serrata*).Height of the plant and pod size was 45.5 cm and 4.32 × 0.50 cm, respectively which was intermediate between the two parents. The hybrid plants produced 14.8 pods per plant, 3-4 pods per cluster, 6.47 seeds per pod and 3^{rd} or 4^{th} leaf turned out to be the first trifoliate leaf in contrast to 5^{th}-7^{th} leaf in the parent plants. All these morphological and phenological characters were suggestive of the hybrid nature of the plants (Fig.3). Inheritance of seed size in this cross revealed its association with the female parent due to its large size over *C. serrata*. Yield potential of F1 hybrid was low.Thus, evident differences in the nutritive requirements and wide variations in lag period during pollen germination of three species of Cyamopsis was the potent pre-fertilization barriers in rearing interspecific hybrids by conventional breeding methods.Smearing of pistil stub of *C.tetragonoloba* with molten and cool pollen germination mediumfollwed by manual pollination with C.serrata pollen induced germination and subsequent tube growth culminating in seed set.Since hybrid plant showed early flowering over *C. tetragonoloba*, the transfer of earliness trait from *C serrata* in the cultivated background is possible by the above method.

4. Conclusion

In conclusion, the main objective of this study to transfer earliness gene(s) from wild species (*C. serrata*) in the background of cultivated species could be achieved by smearing pistil stub of *C. tetragonoloba* with pollen germination medium followed by application of pollen grain repeatedly for three successive days and obtained a pod set of 10.47%.The hybrid pods were smaller in size with 2-3 seeds. The seed colour and shape was akin to *C. tetragonoloba.* Morphological and phenological features revealed that the hybrid plant showed flowering after 21 days of sowing which is even earlier than *C. serrata*. The flowers were pinkish in colour like the female parent (*C. tetragonoloba)* but the shape of pods was akin to the male parent (*C. serrata*). The height of the hybrid plant and length of pods was intermediate between the two parent plants. All these morphological and phenological characters are suggestive of the hybrid origin of these plants. This is supported by *in vivo* germination and tube growth. Failure of hybrid seed formation by conventional breeding method may be ascribed to the incompatibility factor that resides in the stigma. This is so because smearing of stigma with PGM, which is otherwise effective on stub, did not support any *in vivo* pollen germination. Furthermore, differences in the nutritive requirement of pollen of *Cyamopsis* species involved for germination which are also evident by the difference in total soluble carbohydrate content of stigma + style, long lag phase for pollen

germination in both the wild species and duration of stigma receptivity and style length of *C. tetragonoloba* are the other possible hinderances in achieving the goal. Failure of *C. tetragonoloba x C. senegalensis* cross may be ascribed to lack of *in vivo* germination on the stub smeared with PGM. Since in the study, only one PGM supporting germination of *C. serrata* pollen was employed, the other PGM supporting germination of *C. senegalensis* pollen can be tried in future. In this cross, style length of *C. tetragonoloba* may not be a limiting factor.

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