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Role Of Interspecific Hybridization In Production Of Hybrid Plants In *Cyamopsis* Species

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ABSRACT

Different reproductive characters of 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 may serve as a stepping stone for development of extra early varieties of guar.Pollen grains of *C.tetragonoloba* and *C.senegalensis* showed more than 95% of viability while those of *C. serrata* had 87% viability.Nutritive requirement for *in vitro* germination of pollen revealed that pollen of *C.tetragonoloba* required 25% sucrose + 100 ppm boric acid + 300 ppm calcium nitrate while *C senegalensis* pollen needed 35% sucrose with same basal medium.On the otherhand,*C.serrata* pollen 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 hrs 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* possessed longest style (3.8 mm).Interspecific hybridization between *C.tetragonoloba* x *C.serrata* was successful through the use of stub smeared with PGM and as a consequence 10.43% of pod setting was observed.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.

Key words: Interspecific hybrids,pollen,*in vitro* germination,stub pollination,*in vivo* tube growth

INTRODUCTION

India and Pakistan are the main producers of cluster bean, accounting for 80% production of the world's total, while Thar, Punjab Dry Areas in Pakistan and Rajasthan occupies the largest area (82.1%) under guar cultivation in India. In addition to its cultivation in India and Pakistan, the crop is also grown as a cash crop in other parts of the world (Pathak et al. 2010).In India,3.34 million hectares of the farmable land was under guar cultivation during the year 2006/07 (Ministry of Agri. and Co-op GOI,2010).It is cultivated in arid zones of Rajasthan,some parts of Gujarat,Haryana and Madhya Pradesh.The productivity of guar ranges from 474 kg/ha in Rajasthan to 1200 kg/ha in Haryana.The most important growing area centres on Jodhpur in Rajasthan,India where demand for guar for fracking produced an agricultural boom as of 2012 (Gardiner 2012).Guar responds well to irrigation during dry periods but does not tolerate waterlogging. Excessive rainfall and humidity affect fertilization, pod development and seed quality.In high rainfall areas, guar is more leafy and more suitable as a green manure and fodder crop.Guar grows well under a wide range of soil conditions and is tolerant of low fertiliy, soil salinity and alkalinity.It performs best on fertile, medium-textured and sandy loam alluvial soils but does not tolerate heavy black soils (Wong et al., 1997). Like other legumes,guar improves nitrogen availability in soils and crop residues plowed under have been shown to increase significantly the yields of succeeding crops (Wong et al.,1997).

In the recent past,guar cultivation has become an attractive option with the farmers due to availability of high yielding varieties with high gum (30-35% of whole seed) content (galactomannans) in its endosperm which has great value as an enhancer of viscosity in food industry,like stiffner in soft ice-cream,a stabilizer for cheese,instant pudding and whipped cream substitutes and as a metal binder.It is widely used from paper and cosmetic to mining and explosive industry (Whistler and Hymowitz, 1979).Al-Hafedh and Siddiqui (1998) reported that the C. *tetragonoloba* L. beans had 32.81% crude proteins,3.18% crude fats,4.19% ash and 10.87% crude fibers.Guar meal contains about 12 % gum residue (7 % in the germ fraction and 13% in the hulls) (Lee et al.,2005),which increases viscosity in the intestine, resulting in lower digestibilities and growth performance (Lee et al.,2009).Guar meal contains other types of antinutritional factors: trypsin inhibitors, saponin, haemagglutinins, hydrocyanic acid and polyphenols have been identified (Gutierrez et al.,2007).However,anti-trypsic activity was found to be lower than in heat-treated soybean meal and therefore not the main cause of antinutritional effects in poultry (Lee et al., 2004).The large saponin content of guar seed (up to 13% DM) could have both antinutritionals effect and a positive antimicrobial activity (Hassan et al.,2010).The sweet and tender young pods are consumed as a vegetable or snacks in north-western and southern India and the mature seeds can be eaten during food shortages. Young pods, fresh or dry forage are used as livestock feeds.The plant is also used as a green manure and cover crop. Guar yields up to 45 t/ha of green fodder, 6-9 t/ha of green pods and 0.7-3 t/ha of seeds (Wong et al.,1997).Guar meal is the main by-product of guar gum production. It is a mixture of germs and hulls at an approximate ratio of 25 % germ to 75 % hull (Lee et al., 2004).Its uses in tissue culture media as a gelling agent has also been reported (Jain *et al*., 2005).*C. tetragonoloba* L. is a well-known traditional plant used in folklore medicine. It acts as an appetizer, cooling agent, digestive aid, laxative, and is useful in dyspepsiaandanorexia Anti-ulcer,anti-secretory,cytoprotective,hypoglycemic, hypolipidemic and anti-hyperglycemic effects (Mukhtar et al.,2006).Hence,the crop is important for multipurpose industrial uses,nutritional adequacy and sustainable desert agriculture. Seventy five per cent of the guar gums or their derivatives produced in India are exported mainly to USA and European countries enabling its export to 65 countries.Increase demand of guar seeds and guar gum at both domestic and export front,necessitates an increase in production of this crop. In 2008, India accounted for 80% of the world trade of guar gum and guar seed was among the top three traded agricultural commodity on Indian bourses (Mishra, 2008).

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, matures in 80-120 days. However,one of its wild relatives i.e.*C.serrata* is an extra 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,photo-and thermo-insensitivity and disease resistanc.Interspecific hybridization among the *Cyamopsis tetragonoloba* and its wild relatives is anticipated to produce hybrid with trait of early maturity, disease resistance and photo-and thermo-insensitivity.According to Harlaen and Dewet (1971) gene pool concept, *C.tetragonoloba* is included in primary gene pool (GP-1) while *C. serrata* and *C. senegalensis* are included in secondary gene pool (GP-2).According to them species included in GP-2 can be crossed with GP-1 with some fertility in F1's and thus gene transfer is feasible. Unfortunately, conventional plant breeding technique has so far failed to yield desired results (Mathiyazhagan, 2009).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 plant 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 among these by conventional and unconventional less popular methods.

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 of the Department of Botany and Plant Physiology.Pots were filled with mixture of soil and farm yard manure. Before sowing, the seeds of uniform size were soaked in liquid broth of Rhizobium strain 1305 for 10 min. Five seeds were sown in each pot at uniform depth and distance.After 25 days of sowing thinning was done to leave three plants of uniform size in each pot. Irrigation with canal water was given as and when required.At flowering, following reproductive characters of each species were recorded.

Number of pollen/flower

Flower buds from each species were collected a day before anther dehiscence and employed for quantification of pollen grains produced per flower.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).Flower buds were collected from 3 randomly selected plants in the early morning (6.30 a.m.) on the day of anthesis and pollen of these floral buds was mixed thoroughly on glazed paper and used immediately for viability test and *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 incubatorwith 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 petri plate were made from area with uniform distribution of pollen and fairly good population.

Pistil and yield related characters

The above 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 in three replicates per species were used.

***In vivo* tube growth**

Self pollinated pistils from flowers of three 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 these studies.

Interspecific hybridization

Since the three species of *Cyamopsis* employed in the present study 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 72h fixed in acetic alcohol and processed for *in vivo* germination and tube growth by aniline blue method as described earlier.The allogamous pistils left *in situ* were allowed to set pods. Per cent pod set and number of seeds/pod were 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 also were attempted.

Stub pollination

The stigma of emasculated pistils of *C. tetragonoloba* was excised and was smeared with cool and 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 72h and processed in the same way as explained above 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 3day 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 this was also made 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 48h and processed as explained earlier.

Placental pollination

A cut with the help of sharp needle 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, Kinetin, NAA, adenine sulphate and casein hydrolysate (CH).

RESULTS AND DISCUSSION

Reproductive characters and *in vivo* pollen germinatio

Table 1 clearly evinces that flowers of all species of *Cyamopsis* studied produced nearly identical number (nearly 4500) of pollen grains.Inbreeding species, like legumes in general,are known to produce few small pollen grains in small flowers (Diaz and Macnair 1999) and such a reduced male allocation in the species studies is reflective of cost effective allocation to male function (Berlin 1988). Among different species studied, pollen diameter of *C. senegalensis* was maximum (54.46 ±1.10m) whereas the value was least in *C. tetragonoloba* cv. HG 563 (34.90 ±0.63m).The diameter of *C.serrata* pollen grains was 38.47 ±0.80m.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. On the other hand, 25.59% pollen grains of *C. serrata* germinated after 48 h of incubation. Among the tested species, pollen grains of C. tetragonoloba cv. HG 563 produced the longest tube (1204.9 m) after 4h of incubation while those of *C. serrata* produced the smallest pollen tube (174.7m) after 48h of incubation. Tube length of *C. senegalensis* pollen was 949.2 m after 30h 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* required 25% sucrose +100 ppm boric acid+300ppmcalcium nitrate+0.8% agar.

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.Among the three species of *Cyamopsis* studied, 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* possessed capitate type of stigma whereas *C.serrata* is characterized by subapical crescent shaped stigma (Fig 4).Proteins,which constitute one of the main constituent of stigma +styles of angiosperms was nearly identical quantitatively in all the 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 those of *C.senegalensis* consisted of minimum quantity (2.4 mg/100mg FW) of soluble carbohydrates. Similar to the observations of Cruden (2009) in Fabaceae, pollen size was not correlated with style length in different species of *Cyamopsis* studied. The pistil of *C. tetragonoloba* and *C.senegalensis* possessed nearly identical number of ovules (7-8) while *C.serrata* is characterized by 8-9 ovules per pistil.Number of seeds per pod ranged from 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 in 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. Interestingly, in *C. senegalensis* and *C. serrata* no pollen germination was evident after 1 day of anthesis. Pollen germination and tubes became evident on/after two days of anthesis and grew until the 3d after the anthesis.

Interspecific Hybridization

Interspecific hybridization holds great promise in broadening the genetic base of domesticated plant species and success in interspecific hybridization depends upon the extent cross compatibility between the cultigen and its wild relative. Interspecific hybridization between *C.tetragonoloba* x *C.serrata* and *C. tetragonoloba* x *C. senegalensis* was attempted using conventional and non-conventional breeding methods. Studies deploying conventional method of plant breeding revealed no pod setting in the above said crosses. Among an array of non conventional plant 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 F_1 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.

Among the 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 F_1 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 after 21 days of sowing as that of *C. Serrata*, the wild parent. 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 plant and pod size was 45.5 cm and 4.32 × 0.50 cm,respectively which is 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 3rd or 4th leaf turned out to be the first trifoliate leaf in contrast to 5th-7th leaf in the parent plants. All these morphological and phenological characters are 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* however, yield potential of F_1 hybrid was low.

It is thus evident that the differences in the nutritive requirements and wide variations in lag period during pollen germination of three species of *Cyamopsis* are 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 medium followed 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. Further, the attempts can be made to test the fidelity of the interspecific hybrid using molecular markers which may further be tested in the field and transgressive segregants can be selected which may help in identifying extra early varieties of guar. This may prove to be a stepping stone for raising two crops of guar in one year under north Indian conditions which ultimately help in increasing production and productivity of guar.

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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 (μm)	34.90 ± 0.23	38.47 ± 0.30	54.46 ± 0.52
3.	Pollen viability (%)	95.77	87.01	98.12
4.	*In vitro* pollen germination (%)	94.42	25.59	70.01
5.	Pollen tube length (μm)	1204.9	174.7	949.2
6.	Pistil length (cm)	0.67 ±0.02	0.53 ±0.01	0.77 ±0.02
7.	Shape of stigma	Capitate	Subapical crescent	Capitate
8.	Number of ovules/pistil	7-8	8-9	7-8
9.	Number of pods/ cluster	6.75±0.23	12.00±0.10	9.50±0.52
10.	Length x breadth of pod (cm)	5.95 x 0.54	4.51 x 0.36	5.1 0x 0.40
11.	Number of seeds/pod	7.58±0.19	8.75±0.12	7.45±0.35
12.	Test weight of 100 seeds (g)	2.78±0.21	1.81±0.19	1.84±0.30

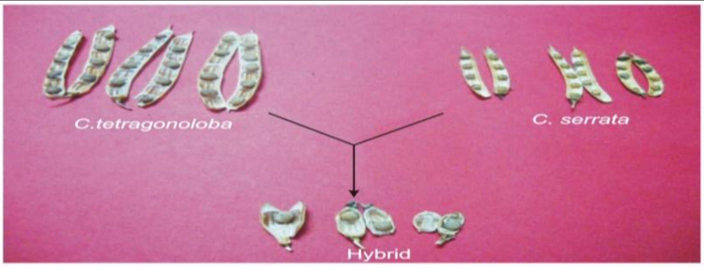

Fig.1:Comparison of morphological features of pods of *Cyamopsis tetragonoloba, C. Serrata* and their hybrid

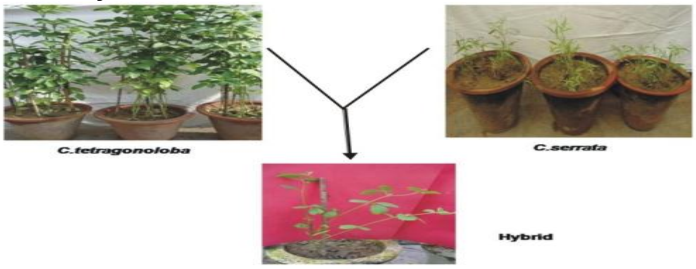

Fig.2. Comparison of plant morphological features of *Cyamopsis tetragonoloba, C. Serrata* and their hybrid

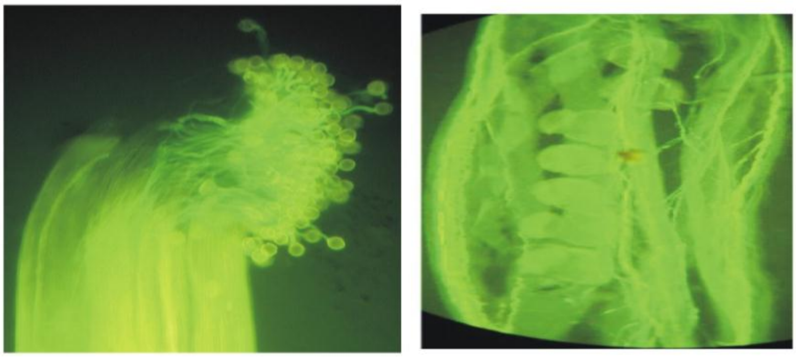

Fig.3: L-R *In vivo* germination of *C. serrata* pollen on the pistil stub of *C. tetragonoloba* smeared with pollen germination medium.