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Guar Species Regenerated through Plant Tissue Culture Technique

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Abstract: ***With the objective to optimize medium receipe and cultural conditions for plant regeneration in Cyamopsis species viz. C. tetragonoloba cv. HG 563, C. serrata and C. Senegalensis. Different explants viz. cotyledon, hypocotyls, cotyledonary node measuring 4-5mm obtained from asceptically grown seedlings were inoculated on the surface of culture medium. Embryo explants were excised from surface sterilized 10 day old green pods taken from net house and three explants per flask were cultured. Direct shoot regeneration from hypocotyls was observed in medium adjuncted with 1mg/l BAP in C. serrata. Cotyledonary node produced multiple shoot on MS medium supplemented with 2mg/l BAP. C. senegalensis showed direct multiple shoot formation only from hypocotyl explants in medium supplemented with 1mg/l BAP. Maximum callus induction from cotyledon explant was evident in C. serrata and C. senegalensis on MS medium with B5 vitamins and supplemented 2, 4-D (2mg/l). Hypocotyl explants of all the tested species of Cyamopsis showed very good callus induction response in media supplemented with 2mg/l 2, 4-D. Half strength MS + NAA(2mg/l) was the best for rooting of shoots in C. tetragonoloba while ½ MS with 0.5 mg/l and 1 mg/l IBA supported best rooting in C. senegalensis and C. serrata. C. tetragonoloba showed flowering in MS medium adjuncted with NAA (2mg/l) with BAP (2mg/l) from shoots obtained from cotyledonary node explant after 80-90 days of inoculation. In vitro regenerated plantlets were successfully transferred to paper cups. Micropropagation of C. serrata and C. senegalensis on the above media will help to produce true to type plants and reduce dependence on seeds for plant production.***

Abbreviations: BAP- 6-benzylaminopurine; CH- casein hydrolysate; 2,4-D- dichlorophenoxyacitic acid; DAA- days after anthesis; DOI- days of inoculation; IAA- indole-3-acetic acid; IBA- indole-3-butyric acid; Kn- kinetin; NAA- 1-naphthaleneacetic acid.

Keywords: Cluster bean (*Cyamopsis tetragonoloba*), *C. serrata*, *C. senegalensis*, Guar, Hypocotyl

1. Introduction

Guar (*Cyamopsis tetragonoloba* (L.) Taub.) syn. *C. psoraliodes* (lamk; D.C.2n=2x= 14) (family leguminaceae), is one of the most important *kharif* legume crop and is well adapted to arid and semi-arid regions of the world. In India, 3.34 million hectares of land was under guar cultivation during the year 2006-2007 (Ministry of Agri. & Coop, GOI, 2009). Guar does not exist in a wild state and is believed to have originated from an African species imported to India as horse fodder by Arabian traders (Ecoport, 2010). India where demand for guar for fracking produced an agricultural boom as of 2012 (Gardiner 2012). 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).

The crop is mainly grown in the dry habitat of Rajasthan, Haryana, Gujarat and Punjab and to limited extent in U.P. and M.P. Guar is important source of guar gum (guar galactomanans) which is used as viscosity enhancer for both food and non-food purposes. Galactomanans are major storage food reserve of endosperm of guar seeds and endosperm constitutes about 30-35 per cent of the whole seed. The primary use is for galactomannan gum (also known as guar gum) which is extracted from guar seed and used as a stabilizer in ice cream and other frozen deserts (Morris et al. *2004*). Currently guar gum is used in numerous nutraceutical and pharmaceutical additives (Morris *2004*). It is used in textile, paper, mining, pharmaceuticals, food processing, cosmetics, petroleum, explosives, oil industries, well drilling, photography, refining and also recently its uses in tissue culture media as a gelling agent has been reported (Babbar *et al.*, 2005; Jain and Babbar , 2005 and Jain *et al.*,2005). 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). Thus significant genotypic variability for both total dietary fiber and soluble dietary fiber was found in seed from several guar accessions (Kays et al. *2006*). Several guar accessions also showed significant variability for seed derived daidzein, genistein, quercetin, and kaempferol (Wang and Morris *2007*). 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). 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).*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 dyspepsia and anorexia Anti-ulcer, anti-secretory, cytoprotective, hypoglycemic, hypolipidemic and anti-hyperglycemic effects (Mukhtar et al., 2006). 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).

The application of tissue culture technology as a central tool or an adjunct to other methods is at the vanguard in plant modification and improvement of agriculture (Brown *et al.*, 1995). Thus, plant biotechnology has opened new opportunities to improve classical plant breeding programs to improve various agronomic traits of crop species. Efficient regeneration protocol for tissue culture are lacking in legumes. Legumes, in general, are considered as recalcitrant (Kaviraj *et al.*, 2006; Chakarbarti *et al.*, 2006).

Very few studies have been conducted on tissue culture in guar till date.

2. Materials and Methods

Seed sterilization: The seeds of the all the three tested species *viz. C. tetragonoloba* cv. HG 563, *C. serrata* and *C. Senegalensis* seeds after washing in tap water were kept in dilute teepol solution for 10-15 minutes followed by thorough rinsing in distilled water and then surface sterilized with 70% alcohol for 1 minute and then with 0.1% mercuric chloride solution under laminar hood for 4-5 minutes. Mercuric chloride was then removed by 8-10 washing with plenty of sterile distilled water under the hood of laminar flow to remove all traces of mercury.

Culture conditions and inoculation of explants: The sterilized seeds were germinated on a germination medium containing 3% sucrose, 8% agar under asceptic conditions initially under dark condition until germination and then shifted to light conditions. Cotyledons, hypocotyls, and cotyledon node explants were obtained from 7-10 day old seedlings grown in aseptic conditions. Then these explants were germinated on different regeneration medium and shoots were obtained from them and these shoots were taken as explant for further study. Embryo explants were excised from surface sterilized 10 day old green pods taken from net house and three explants per flask were cultured. Culture was kept in culture room at 25±1ºC temperature, under photo period of 16h light and 8h darkness.

MS salts supplemented with B_5 vitamins was used as basal medium. Various concentrations of growth regulators alone and in combination were tried for regeneration, root induction and flowering of shoots. (Table 1).

3. Results and Discussion

Different concentration of hormones were tested for morphogenic response of different explants of the three species of *Cyamopsis*. According to our study, 2, 4-D and BAP induced callusing from cotyledons in all three species of *Cyamopsis.* The maximum callus induction was evident in *C. serrata* and *C. senegalensis* on a medium supplemented with 2, 4-D (2mg/l) and the callus was yellowish white in color. On the other hand, in *C. tetragonoloba* poor callus formation was observed on the same medium and the callus was brown in color . Addition of 0.5mg/l BAP to 2,4-D supplemented medium yielded green fragile callus (Table 1). This adduces support to the finding of Prem (2005). Haque *et al.* (2007) recently reported shoot morphogenesis in *Vigna radiata* from cotyledonary explants on medium supplemented with 0.5 mg/l BAP. However, higher concentrations of kinetin/ cytokinin resulted in condensed and vitrified shoots.

MS medium supplemented with 2mg/l 2, 4-D induced callusing in all the three tested species from hypocotyl explants. Interestingly BAP (0.5 and 2 mg/l) induced indirect multiple shoot formation while 1 mg/l BAP induced direct multiple shoot formation without intervening callusing phase in *C. senegalensis* . However, differentiation of multiple shooting was evident at all the three concentrations of BAP tried in *C. serrata* and number of multiple shoots increased with the increase in BAP concentration (Table 2) Fig 16. Chema and Bawa (1991) reported *de novo* formation of shoot buds from hypocotyl explants of pigeonpea on the medium containing BAP and IAA.

Among the various explants tried, cotyledonary node was the most responsive explant for plant regeneration in all the three tested species of *Cyamopsis*. 2, 4-D (1mg/l) induced callusing and indirect shoot regeneration in *C. serrata* and *C. senegalensis* while its higher dose (2mg/l) induced callusing from explant in all three species tested. The callus was yellowish green in *C. serrata* and *C. senegalensis* whereas it was brownish in *C. tetragonoloba*. Supplementation of MS medium with BAP alone lead to indirect shoot regeneration via callusing and its frequency decreased with increasing its concentration for 0.5 mg/l to 2.0 mg/l (Fig 1 A, B). Interestingly 2mg/l BAP support multiple shoot formation from cotyledonary explants in *C. serrata*(Table 3) . Virender (2008) observed direct shoot morphogenesis by 1.5mg/l BAP in *Cyamopsis tetragonoloba* and *C. Serrata.* Shoot regeneration from cotyledonary node has also been reported in other legumes on BAP supplemented medium like *Cajanus cajan* (Prakash *et al.,* 1994), *Phaseolus vulgaris (*McClean and Graftan, 1989 and Mohamed *et al.,* 1992), *V. radiata* (Mathew, 1987 and Gulati and Jaiwal, 1990, 1992, 1994) and chickpea (Subhadra *et al.,* 1998). Surekha and Arundhati (2007) also observed regeneration from cotyledonary node of peanut cultured on BAP and in combination with kinetin.

Culturing of immature embryos (10-12 DAA) of cultivated species resulted in indirect shoot regeneration at 0.5 mg/l 2, 4-D in *C. tetragonoloba* and *C. serrata* whereas high concentration of 2, 4-D induced callusing only in both these species. Callus growth was maximum at 1 mg/l BAP in *C. serrata* while *C. tetragonoloba* showed indirect shoot regeneration on the same medium and similar response was evident in *C. tetragonoloba* on 2mg/l BAP however, *C. serrata* showed indirect shoot regeneration on the same media (Table 4).

Direct shoot regeneration in *C. tetragonoloba* was evident on MS medium supplemented with 0.5 mg/l BAP after 60-70 DOI. Prem *et al.* (2005) reported shoot regeneration in guar using embryo as explants via somatic embryogenesis on BAP and NAA supplemented medium.

Rooting and Flowering

For rooting of *in vitro* generated shoots, different rooting media shown in table 5 were tried. Shoots rooted on a ½ strength MS medium supplemented with NAA (2mg/l) in the *C.tetragonoloba* and pod formation (Fig 2 C) was also evident in this species in ½ strength MS+IBA (0.5 mg/l) medium (Fig 3A,B,C) while 0.5 mg/l and 1.0 mg/l IBA showed rooting in wild species. *C. serrata* also shows good root formation in A6 medium (MS + 0.2 mg/l NAA + 0.001 mg/l BAP + 500 mg/l CH).

Flowering was observed from shoots of nodal segments in ¼ strength MS medium alone in *C. serrata* while *C. senegalensis* it was evident in both ½ strength and ¼ strength MS medium supplemented with 0.1 mg/l IBA,

1mg/l IBA (Fig 2A, B). *C. tetragonoloba* showed flowering in MS medium adjuncted with NAA (2mg/l) with BAP (2mg/l) from shoots obtained from cotyledonary node explant after 80-90 days of inoculation whereas in *C. senegalensis* flowering was also observed from shoots of hypocotyl explants in MS medium supplemented with 2,4-D (2mg/l) and BAP (0.5 mg/l) after longer period of innoculation (table 5).

Virender (2008) found best rooting response of excised shoots on ½ strength MS medium supplemented with 1 mg/l IBA and 0.1 mg/l GA_3. Similar results were obtained in *Phaseolus vulgaris* (Zambre *et al.* 1998) and cluster bean (Prem *et al.*2005). Prem *et al.* (2003) observed rooting response of regenerated shoot of cotyledonary node explants in guar on MS medium supplemented with 4.9 □ M IBA. Mathiyazhagan (2009) found ½ MS + IBA (0.5 mg/l) with 3 g/l charcoal to be the best rooting medium.

The plantlets with sufficient rooting were taken out of the medium and washed properly with tap water. These were then transferred to small cups or pots containing sterilized dune sand and farm yard manure in 3:1 ratio. These were irrigated with ¼ strength MS nutrient solution, covered with polythene bags to maintain high humidity and maintained in culture room at 26±2ºC. Potted cups were irrigated with nutrient solution from time to time. (Fig 3D, E, F).

4. Conclusions

In the present study the cotyledon produced callusing only on the entire medium tried. BAP at concentrations of 0.5 and 2.0 mg/l supported indirect multiple shoot regeneration via callusing in *C. senegalensis* whereas 1 mg/l BAP supported direct multiple shoot regenerationfrom hypocotyl . On the other hand, BAP induced differentiation of multiple shoots in *C. serrata* and the number of shoots per hypocotyl increased with the increasing concentration of BAP.

Cotyledonary node explant was the most responsive explant for plant regeneration in all the three species of *Cyamopsis* under investigation.

BAP induced indirect shoot regeneration via callusing and its frequency decreased with increasing its concentration for 0.5 mg/l to 2.0 mg/l. Interestingly, 2mg/l BAP supported multiple shoot formation from cotyledonary nodes in *C. serrata.* Direct shoot regeneration without intervening callus stage was evident in *C. tetragonoloba* from immature embryos cultured on medium adjuncted with 0.5 mg/l BAP after 60–70 days after inoculation. Half strength medium with + NAA(2mg/l) was the best for rooting of shoots in *C. tetragonoloba* while ½ MS with 0.1 mg/l and 0.5 mg/l IBA supported best rooting in *C. senegalensis* and *C. serrata*, respectively. Plantlets were successfully transferred to paper cups.

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Table 1: Morphogenic responses of cotyledons taken from 7-10 day old aseptically grown seedlings of three species of *Cyamopsis* to plant growth regulators

Sr. No.	Adjuvants to MS Medium	Morphogenic response		
		C.tetragonoloba	*C.serrata*	*C.senegalensis*
1	MS basal medium	No response	No response	No response
2	2,4D (1 mg/l)	Adventious root formation from explant	Callusing (+)	Swelling of explant
3	2,4D (2 mg/l)	Callusing (+)	Callusing (+++)	Callusing (+++)
4	BAP (0.5 mg/l)	Swelling of explant	Callusing (+)	Callusing (+)
5	BAP (1 mg/l)	Callusing (+)	Swelling of explant	Callusing (+)
6	BAP (2 mg/l)	Callusing (+)	Callusing (+)	Callusing (++)
7	2,4D(2 mg/l) + BAP (0.5 mg/l)	Callusing(+)	Callusing (+)	Callusing (++)
8	2,4D (2 mg/l) + BAP (1 mg/l)	Callusing(+)	Callusing (+)	Callusing (+)

+ = Low, ++ = Medium +++ = Good

Table 2: Morphogenic responses of hypocotyls taken from 7-10 day old aseptically grown seedlings of three species of *Cyamopsis* to plant growth regulators

Sr. No.	Adjuvants to MS Medium	Morphogenic response		
		C.tetragonoloba	*C.serrata*	*C.senegalenisis*
1	MS basal medium	No response	No response	No response
2	2,4D (1 mg/l)	Callusing (+)	Callusing	Callusing (+)
3	2,4D (2 mg/l)	Callusing (+)	Callusing (+++)	Callusing (+++)
4	BAP (0.5 mg/l)	Callusing (+)	Callusing + multiple shoot formation + differentation	Callusing + multiple shoot formation
5	BAP (1 mg/l)	Callusing (+)	Multiple shoot formation + differentation	Multiple shoot formation
6	BAP (2 mg/l)	Callusing (+)	Callusing + multiple	Callusing + multiple shoot formation

			shoot formation + differentation	
7	2,4D (2 mg/l) + BAP (0.5 mg/l)	No response	Callusing (+)	Callusing (++)
8	2,4D(2 mg/l) + BAP (1 mg/l)	Callusing (+)	Callusing (+)	Callusing (++)

+ = Low, ++ = Medium +++ = Good

Table 3: Morphogenic responses of cotyledonary nodes taken from 7-10 day old aseptically grown seedlings of three species of *Cyamopsis* to plant growth regulators

Sr. No.	Adjuvants to MS Medium	Morphogenic response		
		C.tetragonoloba	*C.serrata*	*C.senegalensis*
1	MS basal medium	No response	No response	No response
2	2,4D (1 mg/l)	Browning of explant	Callusing + shoot regeneration	Callusing + shoot regeneration
3	2,4D (2 mg/l)	Callusing (+)	Callusing (++)	Callusing (++)
4	BAP (0.5 mg/l)	Callusing + shoot regeneration	Callusing + multiple shoot formation	Callusing + shoot regeneration
5	BAP (1 mg/l)	Callusing + shoot regeneration	Callusing + multiple shoot formation	Callusing + shoot regeneration
6	BAP (2 mg/l)	Callusing + shoot regeneration	Multiple shoot formation	Callusing + shoot regeneration
7	2,4D(2 mg/l) + BAP (0.5mg/l)	Callusing + shoot regeneration	Callusing (+)	Callusing + shoot regeneration
8	2,4D (2 mg/l) + BAP (1 mg/l)	Callusing (+)	Callusing (+)	Callusing (+)

+ = Low, ++ = Medium +++ = Good

Table 4: Morphogenic responses of immature embryos excised 10-12 days after anthesis (DAA) in three species of *Cyamopsis* to plant growth regulators and other adjuvants

Sr. No.	Adjuvants to MS Medium	Morphogenic response		
		C.tetragonoloba	*C.serrata*	*C.senegalensis*
1	MS basal medium	No response	No response	No response
2	2,4D (0.5 mg/l)	Callusing + shoot regeneration + adventious root formation from explant	Callusing + shoot regeneration	No response
3	2,4D (1 mg/l)	Callusing (+)	Callusing (+)	No response
4	2,4D (2 mg/l)	Callusing (+)	Callusing (+)	No response
5	BAP (0.5 mg/l)	No response	Root formation	No response
6	BAP (1 mg/l)	Callusing + shoot regeneration	Callusing (+++)	Callusing
7	BAP (2 mg/l)	Callusing + shoot regeneration	Callusing + shoot regeneration	No response

+ = Low, ++ = Medium +++ = Good

Table 5: Different combination of growth regulators used for rooting and flowering in different species of *Cyamopsis*

S.No	Medium	*C.tetragololoba*	*C.serrata*	*C.senegalensis*
1.	½ MS	-	-	-
2.	¼ MS	-	-, Flowering	-
3.	½ MS + IBA 0.1 mg/l	-, Flowering	-	-, Flowering
4.	½ MS + IBA 0.5 mg/l	-, Flowering	+	+
5.	½ MS + IBA 1 mg/l	-	+	+, Flowering
6.	½ MS + IBA 1.5 mg/l	-	-	-
7.	½ MS + IAA 1 mg /l	-	-	-
8.	½ MS + NAA 2mg/l + IAA 1mg/l	-	-	-
9.	½ MS + NAA 2 mg/l	+	-	-
10.	¼ MS + IBA 0.1 mg/l	-	-	-, Flowering
11.	¼ MS + IBA 0.5 mg/l	-	-	-
12.	¼ MS + IBA 1 mg/l	-	-	-, Flowering
13.	¼ MS + IBA 1.5 mg/l	-	-	-
14.	¼ MS + IAA 1 mg /l	-	-	-
15.	¼ MS + NAA 2mg/l + IAA 1mg/l	-	-	-
16.	¼ MS + NAA 2 mg/l	-	-	-
17.	MS + NAA 0.2 mg/l + BAP 0.001 mg/l + 500 mg/l CH.	-	+	-
18.	MS + 2,4-D 2mg/l	-	-	Flowering
19.	MS + BAP 0.5 mg/l	-	-	Flowering
20.	MS + NAA 2 mg/l + BAP 2mg/l	Flowering	-	-

– = Nil + = Rooting

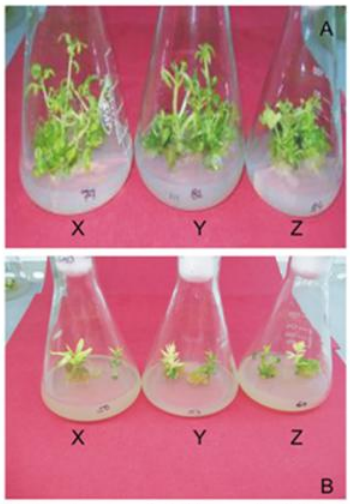

Fig 1 : Morphogenic response of cotyledonary nodes of C. serrata (A) and C. senegalensis (B) to different concentration of BAP (X=0.5mg/l, Y=1.0mg/l and Z=2mg/l

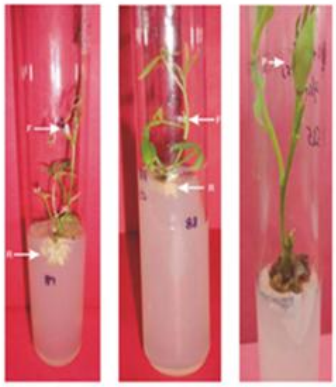

Fig 2 : Rooting and flowering of shoots on 1/2 Strength MS medium supplemented with 0.1 mg/l IBA in C. senegalensis (A) and 0.5 mg/l IBA in C. serrata (B). In vitro fruiting on 0.5 mg/l IBA in Cyamopsis tetragonoloba ©

F = Flower
R = Root
P = Pod

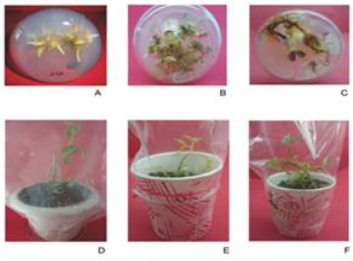

Fig 3: A-C Rooting of shoots on 1/2 strength MS supplemented with 2.0 mg/l NAA (C.tetragonoloba, A), 0.5 mg/l IBA (C.senegalensis, B) and MS + 0.2 mg/l NAA + 0.001 mg/l + 500mg/l CH (C.serrata, C)
D-F Acclimation of rooted plants of Cymopsis tetragonoloba (D), C. senegalensis(E) and C. serrata(F).