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To develop an efficient and reproducible protocol for plant regeneration in cultivated and wild species of cluster bean

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Abstract
In the present research work plant regeneration studies were done in cultivated and wild species of cluster bean (*Cyamopsis* spp.).Three genotypes *viz*., HG2-20, HG563 and *C. serrata* were used for present investigation. For direct shoot regeneration *C. tetragonoloba* cultivars showed best response on MS medium supplemented with TDZ (5µM) as compared to wild species *C. serrata*. Maximum shoot induction response in immature cotyledon explants was observed (73%) on MS medium supplemented with TDZ (5µM) in cv. Minimum days to shoot induction (25 days) from immature cotyledons was observed on MS medium supplemented with TDZ (5µM) in *C. tetragonoloba* cv. HG 563. Maximum regeneration response from immature embryo axes explant was observed (97.7%) on MS medium supplemented with TDZ (5µM) in C. *tetragonoloba* cv. The best shoot regeneration response from cotyledonary node explants was observed (99.66%) on MS medium containing TDZ (5µM) in *C. tetragonoloba* cv. Out of three genotypes, highest callus induction response (82.80%) was observed on MS medium supplemented with TDZ (5 µM) in cultivar HG 563 and minimum days to callus induction (Sixteen days) was also observed on the same medium in all genotypes.MS basal supplemented with TDZ (5 µM) & NAA (2 mg/l) was observed as best medium for shoot induction and multiple shoot formation in all explants of three genotypes. Among all genotypes of *C. serrata* Shinz. was observed as best genotype for multiple shoot formation. Among all explants, Cotyledonary node was best explant for plant regeneration.

Keywords: *Cyamopsis tetragonoloba*, cytokinin, plant tissue culture, regeneration, TDZ

Introduction
Cyamopsis tetragonoloba (L.) Taub. Commonly known as cluster bean or guar (2n = 14) belongs to family *Leguminosae* [1]. The term guar has arisen from its most common use in India as cattle feed "*Gowahaar (Gow means cow and Ahaar means feed)*". It is an extremely drought resistant, deep-rooted, summer-growing, annual legume that is divided into three species, *C. tetragonoloba* (L.) Taub., *C. serrata Schinz., C. senegalensis. C. serrata* is early maturing (40-50 days) and low growing annual herb with trifoliate leaves. *C. senegalensis* is also low growing, annual herb having narrow pentafoliate leaves and small pods. It matures in 120-130 days [2]. Both these wild relatives have some desirable attributes like drought tolerance photo and thermo-insensitivity and disease resistance [3]. The Indian states such as Rajasthan, Haryana, Gujarat and Punjab contribute more than 99 per cent of the total guar seed production in India (NHBC). Rajasthan is the major producer of guar seed in the country (contributing about 81% of Indian guar seed production) followed by Haryana (Nearly 13%) and Gujarat (about 1%) (NHBC).Major portion (75%) of the guar gum produced in India is exported mainly to USA and European countries enabling its export to 65 countries. Currently, guar gum is used in numerous nutraceutical and pharmaceutical additives as well as laxatives, oil well drilling, food and the mining industry. On Account of its hydrophilic property and the ability to form bulky, jelly like masses, it is used in appetite depressants as a bulking agent in laxatives, and in gastric ulcer treatment. Young pods are good source of protein, carbohydrate, vitamins ('A' and 'C'), and important minerals like calcium and iron [4].
Traditional plant breeding has led to the release of a number of commercial varieties but their development is constrained by the cleistogamous floral morphology of guar [5]. Supplementing conventional plant breeding with plant biotechnological techniques is anticipatedthrough to go head way in supporting guar improvement. Interspecific hybridization among the *C. tetragonoloba* and its wild relatives is anticipated to produce hybrid with trait of early maturity [6]. Plant tissue culture offers new strategies for improvement of agricultural

crops and economically important crops.The present study was planned with the objective to develop an efficient and reproducible protocol for plant regeneration in cultivated and wild species of cluster bean.

Methodology

The study was carried out in the Plant tissue culture laboratory, Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University, Hisar. Theexplant (immature pods) and the plant materials, *Cyamopsis tetragonoloba* (L.) Taub. (var. HG 563, HG2-20) and Wild species *Cyamopsis serrata* Schinz. and *Cyamopsis senegalensis* were collected from the research farm area of Forage Section, Department of Genetics and Plant Breeding, CCS HAU, Hisar. The chemicals used for this investigation were of high purity with AR grade and were obtained from HiMedia Laboratories (India).

Preparation, sterilization and storage of culture media

Murashige and Skoog's (1962) medium along with B5 vitamins were used as basal medium with 30 g/l sucrose, 0.1 g/l inositol, 8 g/l agar during the course of present study. Various growth regulators like α-naphthalene acetic acid (NAA), Indole 3-butyric acid (IBA), Indole acetic acid (IAA) kinetin, thiadizuron (TDZ), 6- benzyl amino purine (BAP) and gibberellic acid (GA3) were used alone or in various combinations with other growth regulators.

Surface sterilization of explants: *Seed sterilization:* The seeds and pods of *C. tetragonoloba* cvs. HG2-20 and HG 563, *C. serrata* and *C. seneglensis* were soaked in tap water for 20 minutes then washed with dilute teepol solution for 5 min [7]. The cotyledon explants were excised after removing the seed coat. The embryonic axis was cultured on MS medium supplemented with different concentrations and combinations of growth regulators (Table 1).

Inoculation of explants

Ten seeds were placed in jam bottle with medium without growth regulators. Cotyledonary node and hypocotyl explants were excised from 15 day old seedling grown in aseptic conditions. Immature cotyledon and immature embryo axis were excised from pods (8). The explants were inoculated in petriplates containing different MS modified media for each experiment (Table1).Each experiment was repeated thrice.

Statistical data analysis

The data of all the experiments recorded during the present investigation were subjected to statistical analysis. All the values have been presented as the mean of three replicates and standard error using OPSTAT statistical analysis software program available on CCSHAU, Hisar [8].

Regeneration

Data on number of shoots per explants, days to shoot formation and number of explants responding to shoot induction were recorded.

$$\text{Per cent shoot induction} = \frac{\text{No. of explants responding to shoot induction}}{\text{Total no. of explants}}$$

$$\text{Shoot induction efficiency} = \frac{\text{Per cent shoot induction} \times \text{Mean no. of shoots/ explants}}{\text{Callus formation}}$$

$$\text{Per cent callus induction} = \frac{\text{No. of explants responding to callus induction}}{\text{Total no. of explants}}$$

Rooting

The regenerated shoots obtained were surgically separated after three to four weeks and plated on following media listed in Table 1. Cultures were incubated at 25±1 °C in culture room under photoperiod of 16 hour light and 8 hour dark.

Table 1: MS modified media used for root induction studies in cluster bean

S. No	Medium used
1.	Half MS + NAA (1 mg/l)
2.	Half MS+ IBA (2 mg/l)
3.	Half MS+ GA3(0.1 mg/l)+IBA(1 mg/l)
4.	Half MS + NAA(1 mg/l)+IBA(1 mg/l)

Photomicrography

Photomicrography of the cultures was done using Carl Zeiss Stereo microscope mounted with a Cannon IXUS 170 (20 MegaPixel with 12x IS zoom) camera.

Result

Immature cotyledons

The results showed that immature cotyledon explants of *C. tetragonoloba* cultivars HG2-20, HG 563 and wild species *C. serrata* on MS medium supplemented with different concentrations and combinations gave variable responses (Fig. 1). Cotyledon expanded after four to five days of inoculation. Shoot initiation was observed after 4-5 weeks of inoculation of the explants. Off white colored callus initiation was observed after 15-20 days at cut end only (Fig. 2: A, B & C). Callus of *C. serrata* was compact and green in colour. In wild species *C. serrata* (Fig. 3: A & B) and cultivar HG 563 multiple shoot regenerated by embryogenic callus (Fig. 3: C). Direct shoot regeneration was observed without callus intervention phase in cultivar HG2-20 (Fig. 3: D).

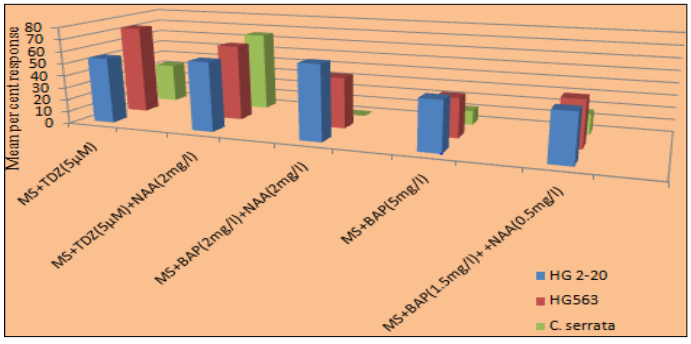

Fig 1: Per cent shoot induction response of cultured immature cotyledons of Cluster bean on different media

In cultivar HG 2-20 the maximum shoot induction response (60.95%) was on MS medium supplemented with BAP (2 mg/l) and NAA (2 mg/l) and least response (12.03%) was observed on MS medium supplemented with kinetin (0.5 mg/l) with NAA (0.5 mg/l) (Table 2). The best shoot induction response (73%) was observed on MS medium supplemented with TDZ (5 µM) in HG 563 and the least shoot induction response (31.86%) was observed on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) (Table 3). In *C. serrata* the maximum shoot induction response (65.28%) was noticed on MS medium supplemented with TDZ (5 µM) and NAA (2 mg/l) and no shoot induction response was observed on MS medium supplemented with different concentrations of BAP with auxins (Table 4).

Fig 2: *In vitro* response of cultured immature cotyledon explants of cluster bean A: HG2-20; B: *C. serrata* and C: HG 563

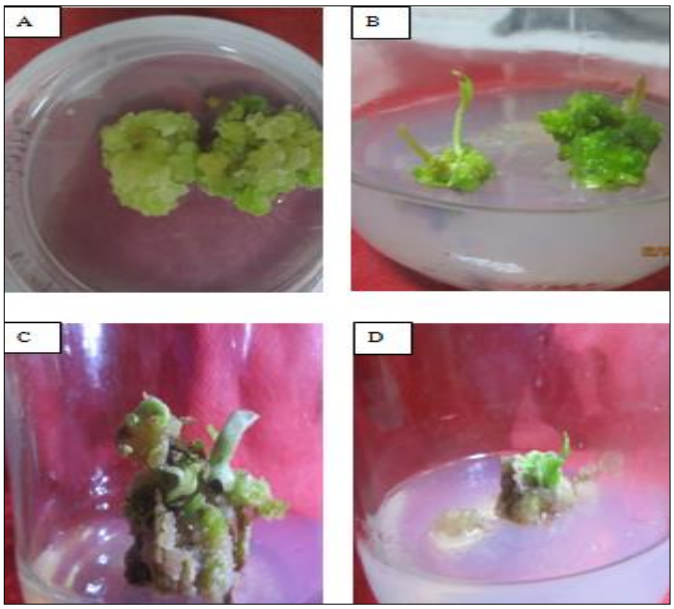

Fig 3: *In vitro* regeneration response of cultured immature cotyledon explants of cluster bean A: callus induction in *C. serrata;* B: multiple shoot induction in *C. serrata*; C: shoot induction in cv. HG563; D: shoot induction in HG2-20

Table 2: Response of immature cotyledon explants cultured on different media in cultivar HG 2-20 of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Soot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ(2.5µM) +NAA (0.5mg/l)	54.76 (47.85±5.72)	1-2	1.20±0.02	65.71	35-44	40.16±0.55
2.	MS+TDZ (2.5µM) +NAA (2mg/l)	49.41 (44.63±4.87)	1	1.00±0.00	49.41	29-31	30.16±0.11
3.	MS+ TDZ(5µM)	54.00 (47.29±1.96)	1-2	1.50±0.03	81.00	28-30	29.33±0.17
4.	MS+ TDZ (5µM) +NAA (2mg/l)	56.71 (48.93±4.48)	1-2	1.30±0.03	73.72	26-35	29.83±0.62
5.	MS+BAP (0.5mg/l) +NAA (0.5mg/l)	18.16 (25.09±2.01)	1-2	1.10±0.02	19.97	34-50	43.00±1.22
6.	MS+BAP (1.5 mg/l) +NAA (0.5 mg/l)	39.67 (39.00±1.70)	1	1.00±0.00	39.67	40-50	47.50±0.41
7.	MS+BAP (2 mg/l) +NAA (2 mg/l)	60.95 (51.46±4.44)	1-2	1.40±0.02	85.33	30-42	38.00±0.78
8.	MS+BAP(5 mg/l)	40.73 (39.44±7.05)	1-2	1.06±0.01	43.17	34-44	39.50±0.75
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	41.66 (40.04±5.10)	1-2	1.30±0.04	54.15	31-34	32.33±0.22
10.	MS+Kinetin(0.5mg/l) +NAA (0.5 mg/l)	12.03 (20.28±0.41)	1	1.00±0.00	12.03	28-36	32.76±0.59
11.	MS+Kinetin (2 mg/l) +NAA(2 mg/l)	39.86 (39.02±4.42)	1-2	1.06±0.01	42.25	32-34	33.66±0.15
12.	MS+Kinetin(5 mg/l)	30.76 (33.01±6.22)	1-2	1.13±0.03	34.75	42-52	42.83±1.30
13.	MS+Kinetin(5 mg/l) +NAA(2 mg/l)	29.44 (32.81±1.53)	1-2	1.06±0.01	31.20	26-34	31.00±0.59

(Values in parenthesis are angular transformed data)

Table 3: Response of immature cotyledon explants cultured on different media in cultivar HG563 of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots / explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (2.5 µM) +NAA (0.5 mg/l)	50.93 (45.52±3.51)	1-2	1.50±0.07	76.39	38-42	40.16±0.26
2.	MS+TDZ (2.5 µM) +NAA(2 mg/l)	54.61 (47.63±1.68)	1-2	1.33±0.08	72.63	26-34	29.50±0.59
3.	MS+ TDZ (5 µM)	73.00 (59.63±5.78)*	1-3	2.26±0.76	164.98	25-30	27.66±0.37
4.	MS+ TDZ (5 µM) +NAA (2 mg/l)	62.24 (52.07±0.81)*	1-2	1.63±0.02	101.45	21-32	27.16±0.79
5.	MS+BAP (0.5 mg/l) +NAA (0.5 mg/l)	31.86 (34.32±1.28)	1	1.00±0.00	31.00	45-52	47.33±0.60
6.	MS+BAP (1.5 mg/l) +NAA (0.5 mg/l)	38.48 (39.29±2.12)	1-2	1.10±0.04	42.32	32-42	38.00±0.78
7.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	41.85 (40.07±9.39)	1-2	1.63±0.02	68.21	29-40	34.66±0.82
8.	MS+BAP (5 mg/l)	32.52 (34.70±1.96)	1-2	1.30±0.04	42.27	30-40	35.00±0.74
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	50.00 (44.98±3.32)	1	1.00±0.00	50.00	26-37	32.33±0.77
10.	MS+Kinetin (0.5 mg/l) + NAA (0.5 mg/l)	39.52 (38.74±4.92)	1	1.0±0.00	39.00	30-40	32.40±0.34
11.	MS+Kinetin (2 mg/l) +NAA (2 mg/l)	36.66 (37.10±4.12)	1-2	1.2±0.05	43.99	35-45	39.36±0.53
12.	MS+Kinetin (5 mg/l)	35.74 (36.64±2.49)	1-2	1.33±0.08	47.53	36-48	42.83±0.87
13.	MS+Kinetin (5 mg/l) + NAA (2 mg/l)	42.77 (40.78±2.88)	1-2	1.1±0.02	47.04	38-48	41.90±0.57

(Values in parenthesis are angular transformed data) (* shows shoot formation by embryogenic callus)

Table 4: Response of immature cotyledon explants cultured on different media in *C. serrata*, wild species of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (2.5 µM) +NAA (0.5 mg/l)	39.28 (38.72±2.78)*	1-2	1.40±0.07	54.99	30-38	34.53±0.34
2.	MS+TDZ (2.5µM) + NAA (2 mg/l)	38.11 (38.04±2.66)*	1-2	1.06±0.02	40.39	30-40	34.83±0.63
3.	MS+TDZ (5 µM)	32.52 (34.00±8.44)*	1-7	4.3±0.45	139.83	30-35	31.66±0.43
4.	MS+TDZ (5 µM) +NAA (2 mg/l)	65.28 (54.41±7.24)*	2-4	2.6±0.09	169.72	30-32	30.66±0.17
5.	MS+ BAP (0.5 mg/l) +NAA (0.5 mg/l)	0.00 (0.00±0.00)	0	0.00±0.00	0	0	0.00±0.00
6.	MS+ BAP (1.5 mg/l) +NAA (0.5 mg/l)	0.00 (0.00±0.00)	0	0.0±0.00	0	0	0.00 ±0.00
7.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	0.00 (0.00±0.00)	0	0.0±0.00	0	0	0.00±0.00
8.	MS+ BAP (5 mg/l)	11.63 (19.96±0.86)	1-2	1.4±0.07	16.28	30-42	35.66±0.90
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	0.00 (0.00±0.00)	0	0.00±0.00	0	0	0.00±0.00
10.	MS+Kinetin (0.5 mg/l) +NAA(0.5 mg/l)	17.79 (24.86±1.62)	1	1.0±0.00	17.79	45-50	46.95±0.60
11.	MS+Kinetin (2 mg/l) +NAA(2 mg/l)	20.47 (26.86±1.07)	1-2	1.2±0.03	24.56	30-40	41.16±0.50
12.	MS+Kinetin (5 mg/l)	24.49 (29.01±5.56)	1	1.00±0.00	24.99	28-45	37.50±0.97
13.	MS+Kinetin (5mg/l) +NAA (2 mg/l)	23.36 (28.61±3.70)	1-2	1.07±0.02	25.26	40-55	46.13±0.55

(Values in parenthesis are angular transformed data) (* shows shoot formation by embryogenic callus)

Moderate shoot induction response (56.71% and 54.76%) in cultivar HG2-20 was observed on MS medium fortified with TDZ (5 µM) & NAA (2 mg/l) and TDZ (2.5 µM) with NAA (0.5 mg/l). In cultivar HG 563 moderate response (62.24%) was observed on MS medium supplemented with TDZ (5 µM) and NAA (2 mg/l) followed by *C. serrata* (39.28%) on MS medium supplemented with TDZ (2.5 µM) and NAA (0.5 mg/l). Maximum number of shoots per explant (Seven) were observed in wild species *C. serrata* on MS medium supplemented with TDZ (5 µM) (Fig. 4: B & C) and no shoots were observed on MS medium supplemented with different concentrations and combinations of BAP with NAA. In cultivar HG 563 the maximum number of shoots per explant (Three) were observed on MS medium supplemented with TDZ (5 µM). Multiple shoots induction was not observed in cultivar HG2-20 (Fig. 3: D). Shortest duration (21 days) to shoot induction was observed in cv. HG 563 on MS medium containing TDZ (5 µM) and NAA (2 mg/l)and longest duration (52 days) to shoot induction was observed on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l). Shortest duration (28 days) was observed in HG 2-20 on MS medium supplemented with TDZ (5 µM) and longest duration (52 days) was observed on MS medium supplemented with kinetin (5 mg/l). In wild species *C. serrata* shortest duration to shoot induction (30 days) on the MS medium supplemented with TDZ (5µM) and longest duration to shoot induction was observed (55 days) on MS medium supplemented with kinetin (5 mg/l) with NAA (2 mg/l).

Among the three genotypes, *C. serrata* was the most responsive genotype with the highest shoot induction efficiency (169.72) on MS medium containing TDZ (5 µM) and NAA (2 mg/l), followed by HG 563 (164.98) on MS medium containing TDZ (5 µM) and HG2-20 (85.33) on MS medium supplemented with BAP (2 mg/l) and NAA (2 mg/l). In *C. serrata* the least shoot induction efficiency (0) was observed on MS medium supplemented with BAP alone or in combination with NAA and in cultivar HG 563 least shoot induction efficiency (31.00) was observed on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l). In cultivar HG2-20 least shoot induction efficiency (12.0 3) was observed on MS medium supplemented with kinetin (0.5 mg/l) with NAA (0.5 mg/l). TDZ (5 µM) and NAA (2 mg/l) was observed as best medium among all type of media combinations tried (Fig. 1).

Immature embryo axes

In present investigation shoot regeneration was visible within 2-3 weeks (Fig: 4). The wild species *C. serrata* (Fig. 5: A, B, C) and cultivar HG2-20 (Fig. 5: D) showed shoot induction by embryogenic callus. Direct shoot regeneration was observed in cultivar HG 563 from immature embryo axes. Explants were sub cultured to the same medium which enhanced the shoot regeneration and shoot length of both explants (Fig. 6: A, B & C).

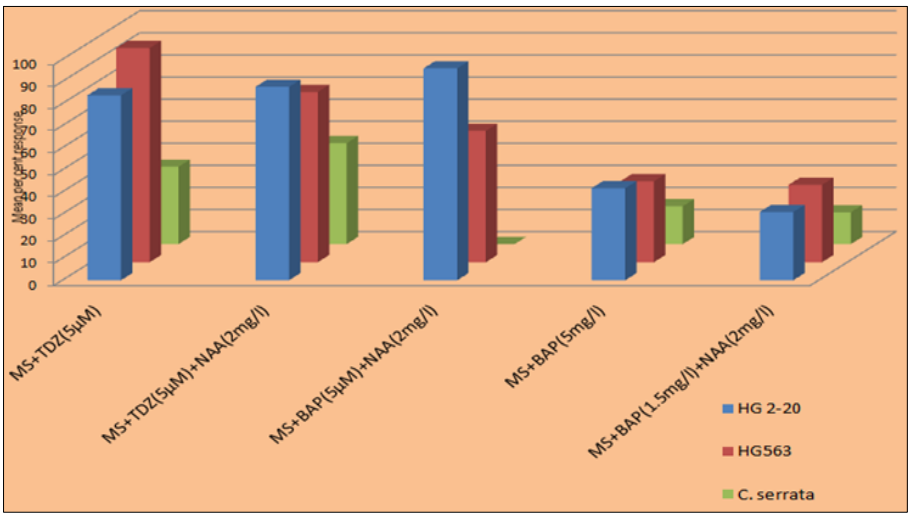

Fig 4: Per cent shoot induction response of cultured immature embryo axes of cluster bean on different media

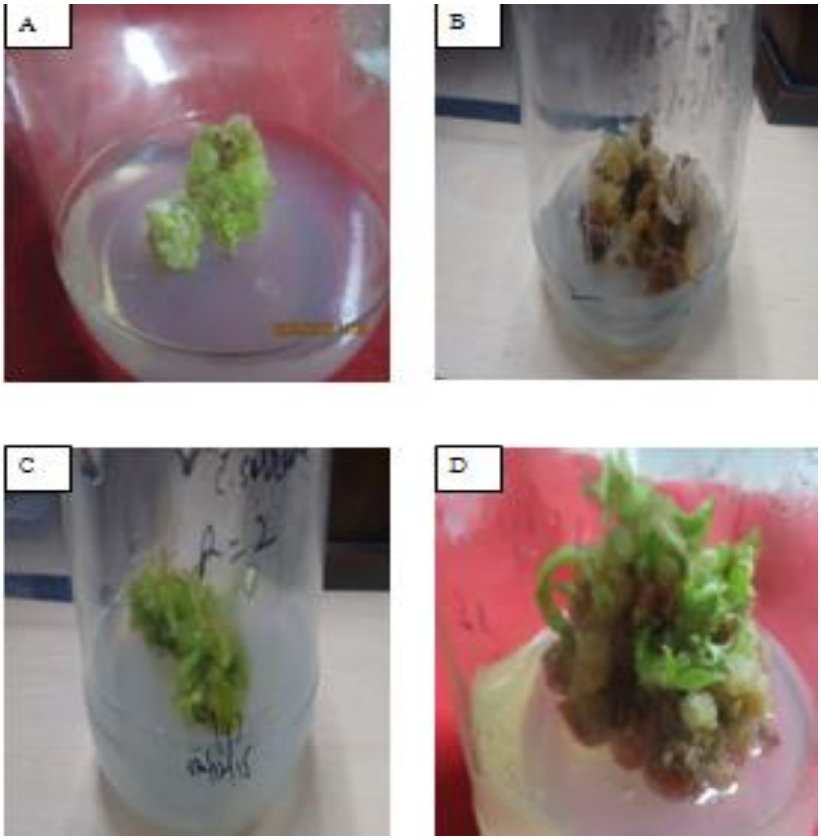

Fig 5: *In vitro* multiple shoot induction response in cultured immature embryo axes of cluster bean A, B and C: *C. serrata*; D: *C. tetragonoloba* cv. HG2-20

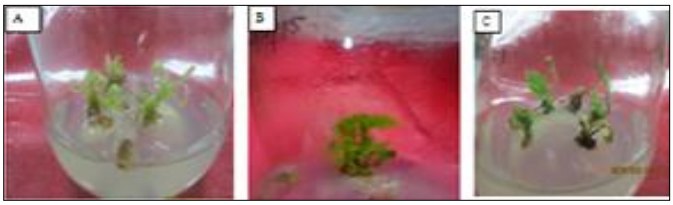

Fig 6: *In vitro* shoot proliferation from immature embryo axes of cluster bean A: *C. serrata;* B: HG 563; C: HG 2-20

In HG 2-20, shoot induction response was maximum (95.98%) on MS medium supplemented with BAP (2 mg/l) and NAA (2 mg/l) and least shoot regeneration response (23.27%) was observed on MS medium supplemented with kinetin (0.5 mg/l) with NAA (0.5 mg/l) (Table 5). The frequency of shoot induction was highest (97.7%) observed in HG 563 on MS medium supplemented with TDZ (5 µM) and lowest response of shoot induction (15.97%) was observed on MS medium supplemented with kinetin (5 mg/l) (Table 6). In *C. serrata* maximum regeneration response (45.74%) observed on MS medium supplemented with TDZ (5 µM) with NAA (2 mg/l) and no shoot induction response was observed on MS medium supplemented with BAP (concentration less than 5 mg/l along with NAA) (Table 7).

Table 5: Response of immature embryo axis explants cultured on different media in cultivar HG 2-20 of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (2.5 µM) +NAA (0.5 mg/l)	60.30 (51.02±4.07)	1-2	1.16±0.02	69.94	19-25	20.20±0.54
2.	MS+TDZ (2.5 µM) +NAA (2 mg/l)	60.39 (51.20±4.85)	1-2	1.06±0.02	64.01	15-22	16.73±0.19
3.	MS+TDZ (5 µM)	83.76 (66.76±4.16)*	1-7	1.3±0.04	108.88	9-15	11.60±1.40
4.	MS+TDZ (5 µM) +NAA (2 mg/l)	87.60 (73.23±8.97)*	1-3	1.2±0.03	105.12	12-15	10.33±0.23
5.	MS+BAP (0.5 mg/l) +NAA (0.5 mg/l)	29.03 (32.32±4.17)	1	1.00±0.00	29.03	23-30	26.36±0.39
6.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	95.98 (80.68±4.93)*	1	1.0±0.00	95.98	20-30	24.33±0.60
7.	MS+BAP (1.5 mg/l) +NAA (0.5 mg/l)	30.91 (33.76±0.75)	1	1.0±0.00	30.91	21-30	23.00±0.39
8.	MS+BAP (5 mg/l)	41.70 (40.20±1.20)	1-2	1.0±0.02	41.7	19-21	20.33±0.15
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	55.24 (48.02±3.84)*	1-2	1.13±0.34	62.42	25-32	27.23±0.43
10.	MS+ Kinetin (0.5 mg/l) +NAA (0.5 mg/l)	23.27 (28.60±3.16)	1-2	1.00±0.00	23.27	20-25	22.56±0.18
11.	MS+Kinetin (2 mg/l) + NAA (2 mg/l)	27.27 (31.30±3.01)	1-2	1.06±0.02	28.90	18-28	22.03±0.46
12.	MS+Kinetin (5 mg/l)	33.76 (35.37±3.18)	1	1.00±0.00	33.76	15-20	16.46±0.30
13.	MS+Kinetin (5 mg/l) +NAA (2 mg/l)	41.92 (40.25±3.39)	1-2	1.20±0.03	50.30	18-24	21.66±0.28

(Values in parenthesis are angular transformed data) (* shows shoot formation by embryogenic callus)

Table 6: Response of embryo axis explants cultured on different media in cultivar HG 563 of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (2.5 µM) + NAA (0.5 mg/l)	48.49 (44.11±1.39)	1-2	1.3±0.09	63.03	12-18	14.31±0.34
2.	MS+TDZ (2.5 µM) +NAA (2 mg/l)	62.69 (52.69±6.24)	1-2	1.2±0.03	75.22	10-20	15.80±0.45
3.	MS+ TDZ (5 µM)	97.7 (84.91±5.08)	1-2	1.4±0.06	136.78	5-6	5.60±0.04
4.	MS+ TDZ (5 µM) +NAA (2 mg/l)	77.12 (62.03±5.04)	1-2	1.6±0.04	123.39	5-7	6.00±0.15
5.	MS+BAP (0.5 mg/l) +NAA (0.5 mg/l)	17.89 (24.96±1.28)	1-2	1.0±0.00	17.89	20-25	22.33±0.37
6.	MS+BAP (1.5 mg/l) +NAA (0.5 mg/l)	35.04 (36.20±2.57)	1-2	1.3±0.09	45.55	12-17	14.00±0.39
7.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	59.49 (50.70±8.36)	1-2	1.3±0.06	77.33	10-13	11.50±0.20
8.	MS+ BAP (5 mg/l)	36.68 (37.20±2.27)	1-2	1.0±0.02	36.68	15-20	17.33±0.37
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	27.71 (31.52±3.52)	1-2	1.2±0.03	33.25	10-15	21.00±0.34
10.	MS+Kinetin (0.5 mg/l) +NAA (0.5 mg/l)	19.46 (26.13±1.08)	1	1.0±0.00	19.46	22-30	25.5±0.47
11.	MS+Kinetin (2 mg/l) +NAA (2 mg/l)	23.63 (28.94±2.47)	1	1.0±0.00	23.63	12-18	15.4±0.14
12.	MS+Kinetin (5 mg/l)	15.97 (23.46±1.55)	1-2	1.1±0.02	17.56	16-18	16.83±0.15
13.	MS+Kinetin (5 mg/l) +NAA (2 mg/l)	38.16 (38.12±1.45)	1-2	1.2±0.00	45.79	7-9	8.00±0.15

(Values in parenthesis are angular transformed data)

Table 7: Response of embryo axis explants cultured on different media in *C. serrata*, wild species of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (2.5 µM) +NAA (0.5 mg/l)	31.48 (33.92±3.93)*	1-2	1.1±0.03	34.62	12-17	18.30±0.22
2.	MS+TDZ (2.5 µM) +NAA (2 mg/l)	28.15 (32.21±1.77)*	1-2	1.60±0.33	45.04	11-16	13.36±0.15
3.	MS+TDZ (5 µM)	35.05 (35.30±11.62)*	2-8	6.16±1.83	215.90	6-8	7.00±0.15
4.	MS+TDZ (5 µM) +NAA (2 mg/l)	45.74 (42.53±1.25)*	2-3	2.70±0.27	123.49	20-30	26.00±0.79
5.	MS+BAP (0.5 mg/l) +NAA (0.5 mg/l)	0.00 (0.00±0.00)	0	0.00±0.00	0	0	0.00±0.00
6.	MS+ BAP (1.5 mg/l) +NAA (0.5 mg/l)	0.00 (0.00±0.00)	0	0.00±0.00	0	0	0±0.00
7.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	0.00 (0.00±0.00)	0	0.00±0.00	0	0	0±0.00
8.	MS+BAP (5 mg/l)	17.17 (24.39±1.54)	1-2	1.08±0.07	18.54	21-25	22.00±0.39
9.	MS+BAP (5 mg/l) +NAA (2 mg/l)	16.97 (24.21±1.85)	1-2	1.33±0.09	22.57	15-20	17.66±0.37
10.	MS+Kinetin (0.5mg/l) +NAA (0.5 mg/l)	11.13 (19.36±1.67)	1-2	1.06±0.11	11.79	15-22	19.53±0.54
11.	MS+Kinetin (2 mg/l) +NAA (2 mg/l)	19.90 (26.46 ±0.83)	1	1.00±0.00	19.90	15-18	15.63±0.15
12.	MS+Kinetin (5 mg/l)	24.52 (31.82±0.95)	1-2	1.13±0.03	27.70	14-18	15.00±0.15
13.	MS+Kinetin (5 mg/l) +NAA (2 mg/l)	18.38 (25.08±3.26)	1-2	1.23±0.04	22.60	15-18	15.63±0.11

(Values in parenthesis are angular transformed data) (* shows shoot formation by embryogenic callus)

Moderate shoot induction (77.12% and 62.69%) in cultivar HG 563 was observed on MS medium fortified with TDZ (5 µM) with NAA (2 mg/l) and TDZ (2.5 µM) with NAA (2 mg/l) respectively. In cultivar HG2-20 moderate response (87.60% and 83.76%) was observed on MS medium supplemented with TDZ (5 µM) with NAA (2 mg/l) and TDZ (5 µM) followed by *C. serrata* (35.05%) on MS medium supplemented with TDZ (5 µM).Out of three genotypes maximum number of shoots per explant (Eight) were observed in wild species *C. serrata* on MS medium supplemented with TDZ (5 µM) followed by (Seven) in cultivar HG 2-20 on the same MS medium supplemented with TDZ (5 µM). Multiple shoots induction was not observed in cultivar HG 563 (Fig. 7: B). The least number of shoot (one) per explant was observed in *C. serrata* on MS medium supplemented with kinetin (2 mg/l) with NAA (2 mg/l) and observed one in cultivar HG2-20 on medium supplemented with BAP (concentration less than 5 mg/l in combination with NAA). The medium containing TDZ (5 µM) gave best shoot induction response in shortest duration in all the three genotypes, HG 563 (Five days), followed by (Seven days) in *C. serreta* and (Nine days) in HG2-20, while the MS medium supplemented with other combinations gave delayed shoot induction.

Among the three genotypes, highest shoot induction efficiency (215.90) was observed in *C. serrata* followed by HG 563 (136.38) and HG2-20 (108.88) on the same MS medium supplemented with TDZ (5 µM). The lowest shoot induction efficiency (0) was observed in *C. serrata* on MS medium supplemented with BAP (concentration less than 5mg/l) and in cultivar HG 563 least shoot induction efficiency (17.56) was observed on MS medium supplemented with kinetin (5 mg/l), in cultivar HG2-20 least regeneration response (23.27) was observed on MS medium supplemented with kinetin (0.5 mg/l) with NAA (0.5 mg/l). TDZ (5 µM) and NAA (2 mg/l) was observed as best medium among all type of media combinations tried (Fig. 5).

Cotyledonary node

The cotyledonary node explants of three genotypes underwent expansion during 4-5 initial days (Fig: 7) of culture. Shoot regeneration occurred after 2-3 weeks (Fig. 8 A, B, C and D).

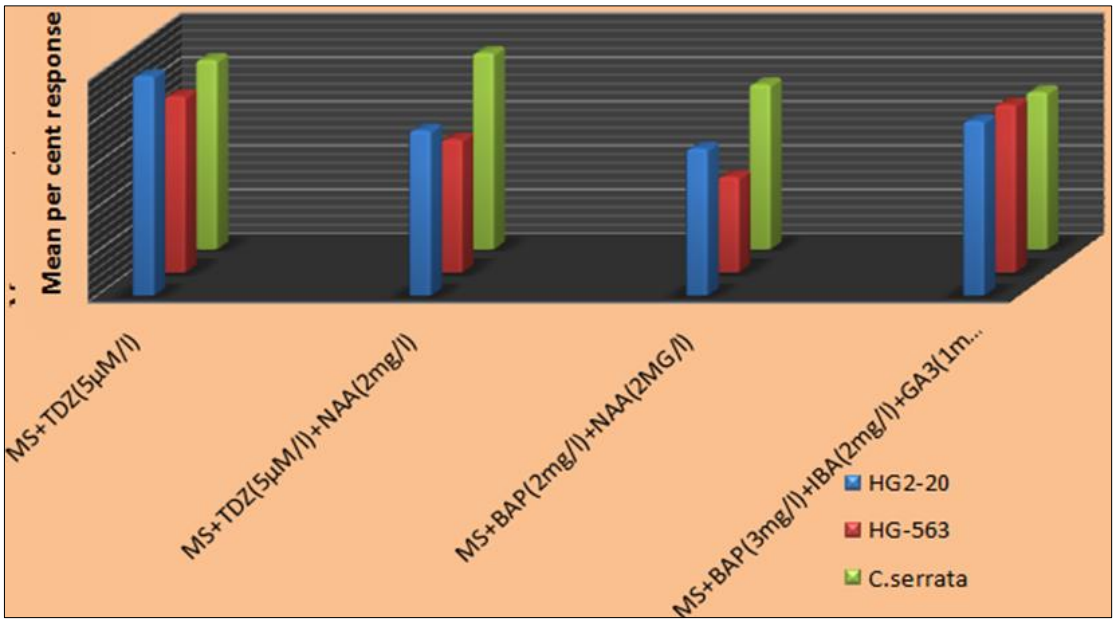

Fig 7: Per cent shoot induction response of cultured cotyledonary node explants of cluster bean on different media

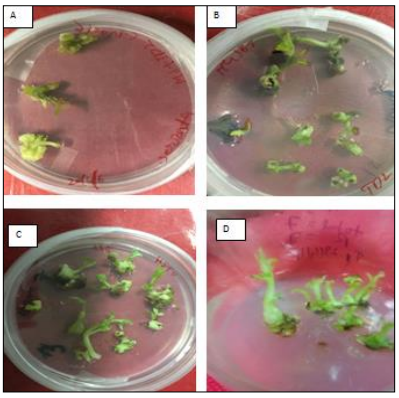

Fig 8: *In vitro* shoot induction response in cultured cotyledonary node explants of cluster bean on different media A: *C. serrata*; B: HG 563; C and D: Multiple shoots induction in cv. HG2-20

The results of the Cotyledonary node explants cultured on different combinations and concentration of growth regulators are shown in (Table 8, 9, 10). A total six media combinations used for this investigation. The ratio of hormones with respect to each other as well their concentrations affected the frequency of shoot induction. Both the cultivars HG2-20 and HG 563 showed maximum frequency of shoot induction on same medium i.e. MS medium supplemented with TDZ (5 µM); highest (99.66%) in HG 2-20, followed by (80%) in HG 563 (Table 8 & 9). In *C. serrata* maximum shoot induction response (89.16%) was observed on MS medium supplemented with TDZ (5 µM) & NAA (2 mg/l) (Table 10). All three genotypes showed lowest response of shoot induction on MS medium supplemented with kinetin (5 mg/l) & NAA (2 mg/l); (32.68%) in HG2-20, (32.68%) in HG 563 and (42.12%) in *C. serrata*.

Table 8: Response of cotyledonary node explants cultured on different media in cultivar HG 2-20 of cluster bean.

S. No.	Medium	Per cent shoot regeneration Response	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (5 µM)	99.66 (79.99±10.0)	1-3	2.3±0.07	229.21	7-8	7.3±0.09
2.	MS+TDZ (5 µM) +NAA (2 mg/l)	75.00 (64.98±13.2)	2-4	2.6±0.17	195	6-8	6.3±0.08
3.	MS+ BAP (2 mg/l) +NAA (2 mg/l)	66.69 (54.76±5.76)	1-2	1.3±0.05	86.69	8-15	12.0±0.44
4.	MS+BAP (3 mg/l) +IBA (2 mg/l) +GA3 (1 mg/l)	79.16 (67.39±11.52)	1-2	1.4±0.03	110.82	6	6.0±0.00
5.	MS+Kinetin (5 mg/l)	31.96 (34.22±2.79)	1-2	1.0±0.01	31.96	10-18	13.6±0.43
6.	MS+Kinetin (5 mg/l) +NAA(2 mg/l)	28.33 (32.07±2.09)	1-2	1.4±0.08	39.66	10-17	13.5±0.45

(Values in parenthesis are angular transformed data)

Table 9: Response of cotyledonary node explants cultured on different mediain cultivar HG 563 of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+ TDZ (5 µM)	80.00 (73.07±16.92)	1-3	3.0±0.00	240.00	8-10	8.6±0.09
2.	MS+ TDZ (5 µM) + NAA (2 mg/l)	60.33 (51.49±7.57)	1-2	2.0±0.15	120.66	8-12	9.0±0.15
3.	MS+BAP (2 mg/l) + NAA (2 mg/l)	43.45 (41.02±6.41)	1-2	1.5±0.07	65.17	10-15	12.3±0.37
4.	MS+ BAP (3mg/l) +IBA (2 mg/l)+GA_3 (1 mg/l)	76.16 (64.40±13.33)	1-3	2.3±0.09	175.16	9-12	7.6±0.56
5.	MS+ Kinetin (5 mg/l)	45.52 (42.34±5.86)	1-2	1.0±0.02	45.52	10-16	14.16±0.30
6.	MS+ Kinetin (5 mg/l) +NAA (2 mg/l)	32.68 (34.77±2.49)	1-2	1.2±0.03	39.21	14-19	17.03±0.20

(Values in parenthesis are angular transformed data**)**

Table 10: Response of Cotyledonary node explants cultured on different media in *C. serrata*, wild species of cluster bean

S. No.	Medium	Per cent shoot regeneration	No. of shoots/explant		Shoot induction Efficiency	Days to shoot induction	
			Range	Mean		Range	Mean
1.	MS+TDZ (5 µM)	86.02 (72.07±9.50)	3-4	3.1±0.02	266.66	7-10	9.00±0.15
2.	MS+TDZ (5 µM+ NAA (2 mg/l)	89.16 (74.22±8.06)	2-3	2.6±0.04	231.81	12-15	12.00±0.30
3.	MS+BAP (2 mg/l) + NAA (2 mg/l)	75.00 (60.05±1.91)	2-3	2.1±0.04	157.5	10-20	16.66±0.62
4.	MS+BAP (3mg/l) +IBA (2 mg/l) +GA_3 (1 mg/l)	71.46 (57.72±1.56)	2-3	2.8±0.04	200.08	13-20	18.33±0.70
5.	MS+Kinetin(5 mg/l)	49.64 (44.76±4.75)	1	1.0±0.00	49.64	14-18	15.7±0.12
6.	MS+Kinetin (5 mg/l) +NAA (2 mg/l)	42.12 (40.38±3.95)	1-2	1.1±0.02	46.33	14-20	16.5±0.20

(Values in parenthesis are angular transformed data)

Moderate shoot induction response was observed (79.16% and 75%) in cultivar HG2-20 on MS medium supplemented with BAP (3 mg/l) & IBA (2 mg/l) + GA_3 (1 mg/l) and TDZ (5 µM) & NAA (2 mg/l) respectively. In cultivar HG 563 moderate response (76.16%) was observed on MS medium supplemented with BAP (3 mg/l) & IBA (2 mg/l) + GA3 (1 mg/l) followed by *C. serrata* (86.02%) on MS medium supplemented with TDZ (5 µM).Maximum number of shoots per explant (Four) were observed in wild species *C. serrata* on MS medium supplemented with TDZ (5 µM) followed by (Four) cultivar HG 2-20 on MS medium supplemented with TDZ (5 µM) with NAA (2mg/l) (Fig. 9: C & D) and (Three) in cultivar HG 563 on MS medium supplemented with TDZ (5 µM) (Fig. 9: B). Similar results (Three shoots per explants) in cultivar HG 563 was also observed on MS medium supplemented with BAP (3 mg/l) & IBA (2 mg/l) & GA3 (1 mg/l). All the three genotypes showed lowest number of shoots per explant (one shoot per explant) on rest of the media combinations. The MS medium containing TDZ (5 µM) & NAA (2 mg/l) and BAP (3 mg/l) & IBA (2 mg/l) & GA3 (1 mg/l) gave best shoot induction response in shortest duration in both cultivars, HG 2-20 (Six days), followed by (Eight days) in HG 563. In *C. serrata* minimum days (Seven days) to shoot induction was observed on MS medium supplemented with TDZ (5 µM) while the MS medium supplemented with other combinations gave delayed shoot induction.

Among the three genotypes, highest shoot induction efficiency (266.66) was observed in *C. serrata* followed by HG 563 (240) and HG2-20 (229.21) on the same medium, MS medium supplemented with TDZ (5 µM). The lowest shoot induction efficiency (46.33) and (39.21) was observed in *C. serrata* and cultivar HG 563 on MS medium supplemented with kinetin (5 mg/l) with NAA (2 mg/l). In cultivar HG2-20 lowest shoot induction efficiency (31.96) was observed on MS medium supplemented with kinetin (5 mg/l). TDZ (5 µM) and BAP (3 mg/l) with IBA (2 mg/l) and GA3 (1 mg/l) was observed as best medium among all the media combinations tried (Fig. 8).

Hypocotyl

Hypocotyl expands after 6-7 days of inoculation (Fig 9). The visible callus on the explants started appearing after 12 days (Fig. 10 A, B, C).

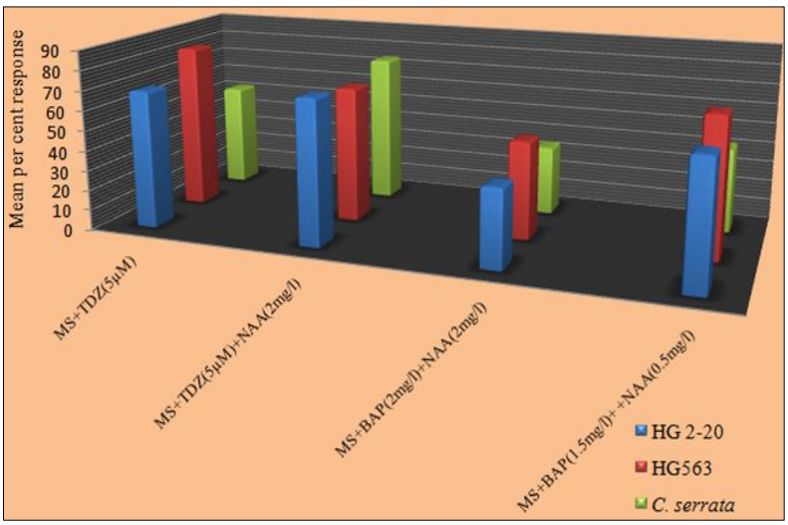

Fig 9: Per cent callus induction response of cultured hypocotyl explants of Cluster bean on different media

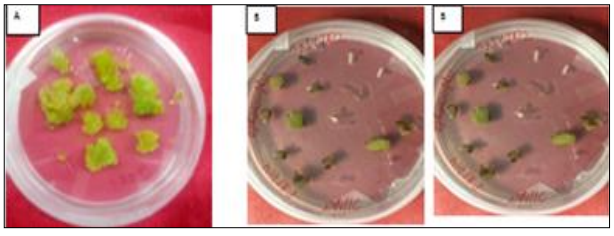

Fig 10: *In vitro* callus induction response of cultured hypocotyl explants of cluster bean on different media A: *C. serrata*; B: HG 563; C and D: HG2-20

C. serrata callus was observed bright green in colour and friable (Fig. 10: A). Shoot morphogenesis was not observed in cultured hypocotyl explants. Out of three genotypes, highest callus induction response (82.80%) was observed in cultivar HG 563 on MS medium supplemented with TDZ (5 µM) (Table 11) followed by (74.52%) in *C. serrata* on MS medium supplemented with TDZ (5 µM) & NAA (2 mg/l) (Table 12) and (72.65%) was observed in cultivar HG2-20 on MS medium supplemented with TDZ (5 µM) with NAA (2 mg/l) (Table 13). The least callus induction response; (48.76% in HG 563, 34.62% in *C. serrata* and 38.98% in HG 2-20) was observed

on MS medium supplemented with BAP (2 mg/l) and NAA (2 mg/l).The minimum days to callus induction (Sixteen days) was observed in all the three genotypes on MS medium supplemented with TDZ (5 µM). The delayed (29 days) callus induction was noticed in HG 563 on MS medium supplemented with BAP (2 mg/l) and NAA (2 mg/l) and (26 days) in cultivar HG2-20 and *C. serrata* on the same medium, MS medium supplemented with TDZ (5 µM).MS medium supplemented with TDZ (5 µM) & NAA (2 mg/l) was observed as best medium among all type of media combinations tried (Fig. 10).

Table 11: Callus induction in hypocotyl explants cultured on different media in cultivar HG2-20 of cluster bean

S. No.	Medium	Per cent callus induction	Days to callus formation	
			Range	Mean
1.	MS+TDZ (5 µM)	69.22 (56.74 ±5.97)	16-25	20.66±0.62
2.	MS+TDZ(5 µM) +NAA (2 mg/l)	72.65 (58.75±3.95)	16-22	20.00±0.30
3.	MS+BAP (2 mg/l) +NAA (2 mg/l)	38.98 (38.46±5.09)	20-26	23.66±0.37
4.	MS+ BAP(3mg/l)+IBA(2 mg/l) +GA_3 (1 mg/l)	62.12 (52.02±2.11)	14-24	19.00±0.65

(Values in parenthesis are angular transformed data)

Table 12: Callus induction in hypocotyl explants cultured on different media in HG 563 of cluster bean

S. No.	Medium	Per cent callus induction	Days to callus formation	
			Range	Mean
1.	MS+TDZ (5 µM)	82.80 (66.39±5.36)	16-27	20.66±0.85
2.	MS+TDZ (5 µM) +NAA (2 mg/l)	67.99 (56.12±6.34)	17-21	18.00±0.39
3.	MS+BAP (2 mg/l) +NAA (2 mg/l)	48.76 (44.23±3.91)	22-29	25.66±0.52
4.	MS+BAP (3 mg/l)+IBA (2 mg/l)+GA_3 (1mg/l)	69.41 (57.61±8.09)	20-24	22.00±0.29

(Values in parenthesis are angular transformed data)

Table 13: Callus induction in hypocotyl explants cultured on different media in *C. serrata*, wild species of cluster bean

S. No.	Medium	Per cent callus induction	Days to callus formation	
			Range	Mean
1.	MS+TDZ (5 µM)	52.61 (46.68±6.66)	16-24	19.66±0.60
2.	MS+TDZ (5 µM)+NAA (2 mg/l)	74.52 (59.79±2.61)	21-24	21.00±0.44
3.	MS+ BAP(2 mg/l)+NAA(2 mg/l)	34.62 (35.33±7.61)	17-26	20.66±0.70
4.	MS+ BAP(3 mg/l)+IBA(2 mg/l) +GA_3 (1 mg/l)	42.24 (43.39±3.73)	18-25	21.66±0.52

(Values in parenthesis are angular transformed data)

Rooting

Four rooting media, half strength MS basal medium supplemented with NAA (1 mg/l), IBA (2 mg/l), GA_3 (0.1 mg/l) with IBA (1 mg/l) and NAA (1 mg/l) with IBA (1 mg/l) were used for rooting in all genotypes of cluster bean. No rooting response was observed on all the media tried. On rooting medium, callusing was observed in cultivars i.e. HG2-20 and HG 563 on half strength medium supplemented with NAA (1mg/l). In cultivar HG563 callus induction response observed was (20%) and in cultivar HG2-20, (28%) callus induction response was observed (Table 14).

Table 14: Rooting response in regenerated shoots of cluster bean on different media.

Medium	Per cent Root induction	Per cent Callus formation
HG 563		
Half MS+NAA (1 mg/l)	0	20
Half MS+IBA (2 mg/l)	0	0
Half MS+GA_3 (0.1 mg/l)+IBA (1 mg/l)	0	0
Half MS+NAA (1 mg/l)+IBA (1 mg/l)	0	0
HG2-20		
Half MS+NAA (1 mg/l)	0	28
Half MS+IBA (2 mg/l)	0	0
Half MS+GA3 (0.1 mg/l)+IBA (1 mg/l)	0	0
Half MS+NAA (1 mg/l)+IBA (1 mg/l)	0	0
C. serrata		
Half MS+NAA (1 mg/l)	0	0
Half MS+IBA (2 mg/l)	0	0
Half MS+GA3 (0.1 mg/l)+IBA (1 mg/l)	0	0
Half MS+NAA (1 mg/l)+IBA (1 mg/l)	0	0

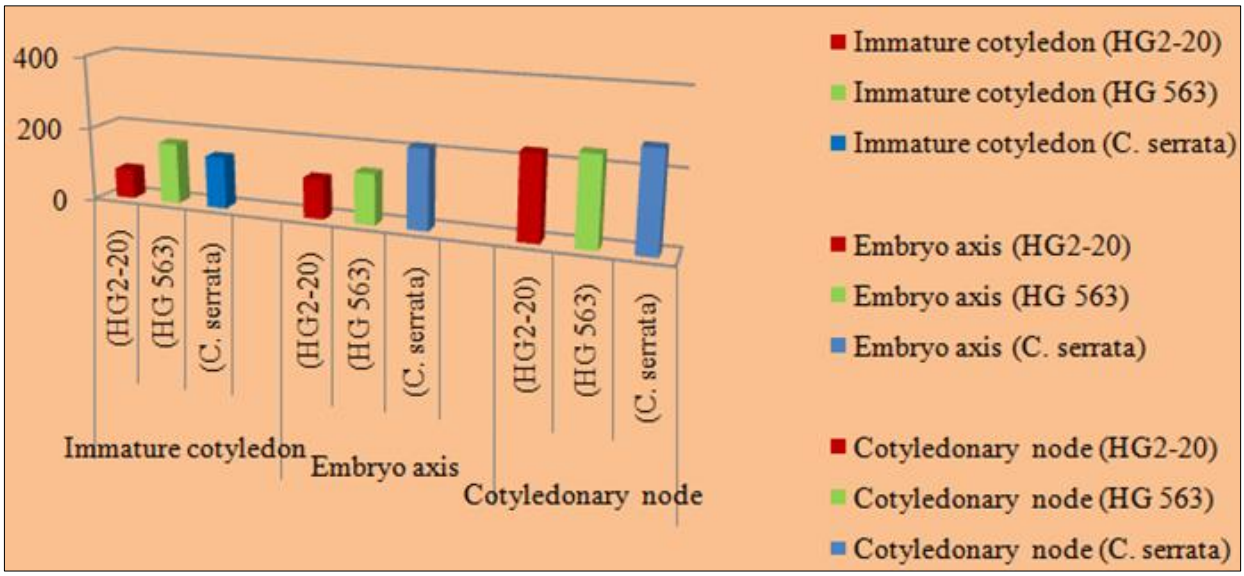

Fig 11: Shoot induction efficiency of different cultured explants in cluster bean on MS medium supplemented with TDZ (5 µM)

Discussion

Cyamopsis tetragonoloba L (Taub.), a drought tolerant multipurpose grain legume, mainly grown in the semi-arid regions of north-west India and to a limited extent in Pakistan, Brazil, Australia, South Africa and the USA [10]. Plant tissue culture can be used for the development of hybrids, embryo rescue, transformation and analysis of gene expression [11]. The present study was undertaken to develop an efficient and reproducible protocol for plant regeneration in cultivated and wild species of cluster bean. Cytokinins in different concentrations and combination were used for multiple shoot formation using immature cotyledon, immature embryo axes, cotyledonary node and hypocotyl explants. Two genotypes of cultivated cluster bean (HG563 and HG 2-20) and *C. serrata* Shinz, a wild species of cluster bean were used. A total of fourteen MS modified media were used in the present study.

Immature cotyledons

In the present investigation, it was found that in immature cotyledon explants regenerated shoots directly or through embryogenic callus. The best shoot induction response (73%) was observed on MS medium supplemented with TDZ (5 µM) in cv. HG 563 in shortest duration of 21 days. In *C. serrata*, shoots regenerated through embryogenic callus on MS medium supplemented with TDZ (5 µM) and NAA (2 mg/l) developing upto seven shoots per explant in 30 days. BAP and Kinetin alone or in combination with auxin showed comapratively less response as compared to TDZ supplemented medium. Direct shoot induction was observed in HG 2-20 cultivar on MS medium supplemented with different concentrations and combinations of TDZ (5 µM). In the present study, the explants were placed abaxially on MS medium supplemented with growth regulators. Tripathi also reported that the initiation of meristematic activity was achieved in immature cotyledon of chickpea when explants were cultured adaxially on MS medium supplemented with 13.68 µM zeatin, 24.6 µM 2-iP, 0.29 µM IAA and 0.27 µM α-NOA [12]. The shoot regeneration response was highly genotype dependent as the two cultivated genotypes and wild species responded differently. This observation is supported by other workers [13]. On the other hand, scientist reported genotype-independent *in vitro* regeneration from immature cotyledons of chickpea on MS medium supplemented with zeatin and 2-iP [14]. Present investigation showed that TDZ is a potent hormone for multiple shoot induction from immature cotyledon explants as compared to other cytokinins in cluster bean. Similarly, Murthy reported regulatory role of thidiazuron (TDZ) and explant factors in imparting somatic embryogenic potential in relation to endogenous growth regulator levels in peanut (*Arachis hypogaea* L. cv. Tango) [15]. It appeared that TDZ induced somatic embryogenesis in peanut by influencing endogenous levels of both auxin and cytokinins. TDZ triggers meristematic activity in cotyledon cells [16]. The highest frequency (90.5%) of somatic embryos was obtained on MS medium supplemented with 2, 4-D (10.0 µM) and BAP (1.0 µM) with the production of a maximum of 22.8 embryos per explant. In present study, BAP showed reduced response as compared to TDZ. Tivarekar investigated that immature cotyledons of mungbean produced the best response of shoot regeneration (100%) on MS medium supplemented with BAP (2 mg/l) in combination with IAA (0.5 mg/l) [17]. We also used BAP with auxin but TDZ showed more synergistic effect with auxin as compared to BAP. Ouyang reported regeneration pathway via direct shoot organogenesis and somatic embryogenesis for an endangered species, *Metabriggsia ovalifolia* [18]. They found that TDZ and BAP induced a high frequency of shoot organogenesis. TDZ induced adventitious shoots at a low concentration but BAP induced both shoot organogenesis and somatic embryogenesis at higher concentration.

Immature embryo axes

Immature embryonic axes were cultured on MS medium supplemented with different concentrations and combinations of growth regulators i.e. α-naphthalene acetic acid (NAA), cytokinins, *viz*., 6-benzyl aminopurine (BAP) and kinetin and TDZ alone and in combination with each other. In the present study, it was observed that immature embryonic axes produced highest shoot induction frequency (97.7%) in cultivar HG 563 on MS medium supplemented with TDZ (5 µM) in minimum 5 days. It was also observed that immature embryo explants were better in comparison with immature cotyledon (shoot induction efficiency). TDZ was found superior hormone for *in vitro* regeneration and multiple shoot induction as compared to other hormones. Eapen reported that immature embryonic axes produced a higher number of somatic embryos in comparison

with immature cotyledons in peanut [19]. The present study showed that out of three genotypes, maximum number of shoots per explants (Eight) was observed in wild species *C. serreta* on MS medium supplemented with TDZ (5 µM). Prem reported shoot regeneration in guar using embryo as explant via somatic embryogenesis on BAP and NAA supplemented medium [20]. But the present study showed that TDZ in combination with NAA was superior than BAP with NAA. Many factors affect the response such as genotypes, culture medium and hormones concentrations. Present study showed that all three genotype showed variable response on different media combinations.

Cotyledonary nodes

In the present investigation, highest shoot induction response (99.66%) in cotyledonary node explants was observed in cultivar HG 2-20 on MS medium supplemented with TDZ (5 µM). Significantly, all genotypes showed more shoot induction and multiple shoot formation response on MS medium containing TDZ alone or in combination with auxin. Verma reported highest percentages of shoot and root induction from cotyledonary node explants of cluster bean on the medium containing IBA (2 mg/l), BAP (3 mg/l) and GA_3 (1 mg/l) [21]. But the present results indicate that TDZ supplemented medium showed best response as compared to media used in earlier studies. Shoot regeneration from cotyledonary node explants has also been reported in other legumes, Maximum frequency of multiple shoot induction was observed on MS medium supplemented with BAP (3 mg/l) in combination with IAA (2 mg/l). In the present investigation, callus induction was not reported from cotyledonary node explants. The present study showed that TDZ is superior hormone than all cytokinin used for cotyledonary node explants in the three genotypes.

Hypocotyls

Shoot morphogenesis was not observed in cultured hypocotyl explants. The visible callus started appearing after 14 to 42 days in culture. The highest callus induction response (82.80%) was observed in cultivar HG 563 on MS medium supplemented with TDZ (5 µM) in minimum 16 days. Among the different media combinations TDZ (5 µM) and NAA (2 mg/l) recorded best medium for hypocotyl callus culture in all three genotypes of cluster bean. There are reports that suggest that hypocotyl explant is highly genotype dependent than the cotyledonary node and embryonic axes explants. This genotypic variation in hypocotyl may be due to absence of meristamatic tissues. Premfound that 2, 4-D (1.2 mg/l) in combination with BAP (0.6 mg/l) was the best medium for hypocotyl callus culture (20). Vermafound that hypocotyl explants did not produced shoots and gave callus induction on MS medium supplemented with IBA (2 mg/l), BAP (3 mg/l) and GA_3 (1 mg/l) (21). But present study showed that TDZ (5 µM) gave good response of callus induction as compared to IBA (2 mg/l), BAP (3 mg/l) and GA3 (1 mg/l). Thus, our study showed that shoot formation using the three different cytokinins gave response in descending order TDZ > BAP > Kinetin.

In a nutshell, our findings strongly support the use of TDZ in cluster bean for shoot regeneration. A short exposure to TDZ effectively induces a range of different morphogenetic responses. Combination of TDZ with other phyto hormones (including other cytokinins or auxins) can be more effective than when used alone. TDZ promotes organogenesis as well as other metabolic processes. Its use as growth regulators in woody species is considered most positive innovation in tissue culture. Role of TDZ in shoot formation and multiple shoot formation is well documented. Dewir emphasized that TDZ inhibits shoot formation and shoot inhibition is due to increased cytokinin levels which are produced in response to TDZ used. Further, they found that shoot regeneration is greatly emphasized by explant orientation [22]. We tried to induce rooting in the developed shoot using six rooting media but none of these initiated roots earlier workers reported root formation in cluster bean but they used different genotypes (Prem *et al.*, 2003, 2005). None the less, the study provides a protocol for shoot regeneration in three genotypes of cluster bean. It also demonstrates the use of immature cotyledons for shoot induction for the first time. Our study strongly suggests the use of TDZ in lower concentration (5 µM) with or without other cytokinins. The genotypes dependence, orientation of explants and media composition is some of the factors which decides the individual genotype response for shoot regeneration. Further efforts are needed to explore other combinations and concentration of growth regulators for complete plant regeneration with high efficiency.

Conclusion

The present study was undertaken to develop plant regeneration system. Three genotypes *viz*., HG2-20, HG563 and *C. serrata* were used for present investigation. For direct shoot regeneration *C. tetragonoloba* cultivars showed best response on MS medium supplemented with TDZ (5µM) as compared to wild species *C. serrata*. Somatic embryogenesis was observed in cultivars HG2-20 and HG 563 on MS medium supplemented with TDZ (5µM) & NAA (2mg/l) and MS medium supplemented with BAP (2mg/l) & NAA (2mg/l).Maximum shoot induction response in immature cotyledon explants was observed (73%) on MS medium supplemented with TDZ (5µM) in cv. HG 563 and in wild species maximum shoot induction response was (65.28%) on same medium. Maximum shoots per explant (Seven) in immature cotyledon explants were observed in wild species *C. serrata* on MS medium supplemented with TDZ (5µM) as compared to cvs. HG 563 (Three) and HG2-20 (Two) on the same medium. Minimum days to shoot induction (25 days) from immature cotyledons was observed on MS medium supplemented with TDZ (5µM) in *C. tetragonoloba* cv. HG 563. In *C. serrata* shoot regeneration was observed by embryogenic callus cultured on MS medium supplemented with TDZ alone or in combination with NAA. Maximum regeneration response from immature embryo axes explant was observed (97.7%) on MS medium supplemented with TDZ (5µM) in *C. tetragonoloba* cv. HG 563 and maximum shoots per explant (Eight) was observed from embryo axes explant in *C. serrata* on the same medium. The best shoot regeneration response from cotyledonary node explants was observed (99.66%) on MS medium containing TDZ (5µM) in *C. tetragonoloba* cv. HG 2-20 and maximum shoots per explants (Four) was observed in wild species *C. serrata* on MS medium containing TDZ (5µM).Out of three genotypes, heighest callus induction response (82.80%) was observed on MS medium supplemented with TDZ (5 µM) in cultivar HG 563 and minimum days to callus induction (Sixteen days) was also observed on the same medium in all genotypes. Hypocotyl explants did not show shoot morphogenesis on all media tried. Over all, maximum shoot induction efficiency (266.66) was observed from cotyledonary node explants of *C. serrata* on MS

medium supplemented with TDZ (5µM).Out of four media tried for root induction, none of medium produced roots in all the genotypes.MS basal supplemented with TDZ (5 µM) & NAA (2 mg/l) was observed as best medium for shoot induction and multiple shoot formation in all explants of three genotypes. Among all genotypes *C. serrata* Shinz. was observed as best genotype for multiple shoot formation. Among all explants, Cotyledonary node was best explant for plant regeneration.

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