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Studies on *in vitro* Micro Propagation for Regeneration of Chickpea Plant

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A B S T R A C T

Chickpea is best nutritional crop in leguminous species, and biotic and abiotic stress affects the yield loss around 50 to 60 % .For preventing yield loss from this stress, it is necessary to developed high yield, qualitative variety of chickpea. I*n vitro* methods are best technique for regeneration and development of transgenic variety of chickpea. In this investigation research to determine chickpea in vitro micropropagation was studied in genotypes; of chickpea JG62, JG63 JG315, VIJAY and Bhupdachana with explants immature cotyledonary and embryonic axis. Explant were cultivated on Murashige and Sckoog (1962) medium with different concentration of plant growth regulators NAA (Naphtyl acetic acid) /BAP (Benzyl amino purine) and 2,4-D.Explant morphogenetic response was recorded after one month incubation. Results were expressed as embryogenesis and organogenesis regeneration frequency (shoot and root development from explants where JG62, JG63 genotype was more callogenic than VIJAY, JG315 and Bhupdachana. Under this investigation we found that callusing take place when basal medium is 2,4-D, whereas direct shoot differentiation occurs on basal medium supplemented with different concentration and combination of BAP with NAA, and both explants of chickpea immature cotyledonary node and embryonic axis have good response *in vitro* condition and can be used for development of transgenic variety in chickpea.

Introduction

Chickpea pulse crop is the most balanced nutritional compositions, and its protein digestibility is the best among the dry season food legumes. Chickpea is an important food legume and a protein rich cash crop has been classified into two main types; dark-seeded desi type of I and large light-seeded Kabuli. Chickpea seeds contain 20-30% protein, 40% carbohydrates and many other useful nutrients present. Chickpea best for crop rotation programs because its have to ability of fixing nitrogen from atmosphere. It leaves have large amount of residual nitrogen behind for subsequent crops and adds much needed organic matter to maintain and improve soil health, long term fertility and sustainability. Chickpea is primarily a crop of developing countries contributing to a large part of human food and animal feed in these areas. Current global yield average of chickpea is

0.9 t/ha, is much lower than its estimated potential of 6 t/ha under optimum growing conditions. India is the largest producer of chickpea, but still is the largest importer. Chickpea yield in India has remained at 0.89 t/ha, which is much lower than the maximum yield reported in china i.e. 3.2t/ha (FAO 2018). Chickpea yield and productivity are adversely affected by various biotic and abiotic stresses like *Ascochyta* blight, *Fusarium* wilt, *Helicoverpa* pod borer, *Botrytis* grey mold. Abiotic stresses drought and cold stand to be the number one problem in chickpea growing regions causing a 50 -60 % reduction in yield globally.

There are many scientist has reports on plant regeneration and also a some on genetic transformation of chickpea, to obtain efficient in vitro culture system with little success. It is also desirable that biotechnology work should be conducted with locally adopted cultivars, to avoid of evolved cultivar, high frequency regeneration is needed for introduction of gene of interest in high yielding released genotypes therefore, an, attempt is being made through the present research to culture explants and develop an efficient protocol for regeneration identify suitable culture media and condition for efficient plantlet regeneration from cotylendory node and embryonic axis of chickpea.

However, the chickpea crop is relatively recalcitrant to regeneration by applied tissue culture techniques. Further, Regeneration via direct shoot induction has been reported in chickpea from various explants, viz. mature embryo axes, immature embryo' seed and cotyledonary node, hypocotyl and shoot apex. Thus, plant regeneration from callus cultures has been obtained with a very low frequency.

In view of the above, present investigation attempts to obtain morphogenesis response of chickpea, immature cotylendonary and embryogenic axis mature explants and identify the role of commercially available high yielding genotypes and growth regulator in callusing medium on the callus induction and plantlet regeneration ability.

Materials and Methods

The plant material of chickpea variety JG62, JG315, and VIJAY were obtain from Department of Biotechnology, college of Agriculture, RVSKVV Gwalior. M.P. and all experiments were done in Tissue culture laboratory.

The explants (immature embryonic axis and immature cotyledonary nodes) were excised from immature seeds were collected from immature pods of chickpea plants, grown in a greenhouse or field. immature pods were surface sterilized by immersing in 70% ethyl alcohol for 1 min followed by 5 min in 0.1% mercuric chloride ($HgCL_2$) solution followed by 3 subsequent rinsing with sterilized double distilled water.

Cotyledonary node and embryonic axis were excised from 45 days old plants and were cut into pieces separately. Explants were inoculated in Petri dishes containing MS medium with different composition of hormones (Table 1). Cultured petridish were incubated at 25+-2^{o}C and subjected to a photoperiod of 12 hours at 1200lux.

Observation recorded

Number of callus forming explants – cultured explants on different media were recorded for callus formation after 25-30 days

Number of Embryogenic calli- During 2nd stage observation a count was made for embryogenic calli identified by their phenotypic appearance such calli were compact

Number of organogenic calli- identified by their phenotypic such cali were yellow green in colour with dense and glossy appearance and regenerating.

Results and Discussion

Morphogenesis in cultured explants

Two explants immature embryonic axis and immature cotyledonry nodes were cultured on ten different MS with different concentration of plant Hormones. The first response of cultured explants was similar after 7 days and mostly independent of cultured media combination and accession, during the first week, explants became swollen and no callus, proliferation was evident. After 7 days of cotyledons in culture, callus proliferation started from full length of embryonic axis. Later, callus proliferation was observed from most of the explants, first set of observation were recorded after 4 weeks of cultured callus initiating explants were counted. culture media played an imperative role in the formation of morphogenic calli.

Immature cotyledonary node culture

Immature cotyledonary nodes of chickpea were cultured on different combination of MS media the effect of culture media and accession on immature cotylendory nodes are presented.

Callus induction – The callus induction from IC(immature cotylendonary) culture varied from 75% to 18% (Table 2). Maximum callus induction was evident in JG 62, and minimum callus found JG 315. In terms of cultured media performance of cultured media MS-4, 75 % followed by MS-5 74% was found the best callus initiation and the minimum response was observed by inoculation media MS-10, (10%).

Embryonic callus initiation

The formation of embryoniccalli from IC cultured varied from 72.4% to 40 % (Table 3) and calli formation JG16 is maximum number of callus reported and minimum JG315 and other genotype of chickpea was response was good in different cultured media, in terms of cultured media response to in vitro culture the performance of cultured media MS-6, followed byMS-4 responding for embryonic callus initiation, the minimum response was exposed by inoculation media MS-1 was around average 40 % along with media MS-3, 41%, MS-2, 42% and MS10D 43%.

Organogenic callus formation

The mean organogenic callus formation from immature cotylendonary node culture varied from 72 to 38% (Table 4). Maximum Organogenic calli formation was observed from. JG62 around average was 53% and VIJAY, JG315 was along as performance was good and minimum by BHUPDA CHANA and JG63, among the 10 media tested six media viz.MS-5,MS-4, MS-6, MS10 was good response for organogenic callus formation followed by two nutrient media MS-1, MS-7 responded very poorly as per average percentage of performance.

Immature embryonic axis

Callus induction

The mean callus induction frequencies from immature embryonic axis cultures varied from 80.4% to 39,8% (Table 5) Maximum callus induction was evident in JG63,vijay, JG315 was around 62% and minimum both by JG62,Bhupda chana around 60 % and respectively. Among different culture media MS-1, MS-2, MS-5.MS-6, ms-4, MS-3 (80%-68%) was found the highest. The minimum response was exposed by inoculation media

MS10, MS-9 around 39 %).

Embryonic callus initiation

The overall formation of embryogenic callus varied from 71.2% to 38%. (Table 6) Most embryogenic callus was generated from JG62 and least by JG315. The performance of culture mediaMS-7, MS-6 and MS-5 was highest around 71%. The low responsive inoculation media were MS-3, MS-4 (39.0%) respectively.

Organogenic callus formation

The mean organogenic callus formation from immature embryonic axis cultures varied from 71% to 16% (Table 7). Higher organogenic callus formation was observed from JG62. JG63 and J315 and low by Vijay, Bhupdachana among cultured media response to in vitro culture the cultured performance of cultured media, MS-1, MS-2, MS-3, MS-4,MS-5,MS-6 was found to be higher. The low response was exposed by inoculation media MS-10, MS-8 was very poorly.

Table.1 Different plant growth regulators used for experiment

	Growth regulators mg/l			
S.N	Composition	BAP	NAA	2,4-D
1.	MS1	0.1	1.0	-
2.	MS2	0.2	1.0	-
3.	MS3	0.5	1	-
4.	MS4	2.0	2.0	-
5.	MS5	4.0	2.0	-
6.	MS6	3.0	6.0	-
7.	MS7	0.1	8.0	-
8.	MS8	0.1	10	-
9.	MS9	-		5.0
10.	MS10	-		10.0

Table.2 Callus induction

Sr. No	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	Mean
1.	MS1	75	65	70	60	65	67
2.	MS2	75	60	65	80	75	71
3.	MS3	70	60	75	65	80	70
4.	MS4	80	85	75	70	65	75
5.	MS5	85	80	70	75	60	74
6.	MS6	25	15	20	10	20	18
7.	MS7	15	20	25	20	10	18
8.	MS8	35	30	20	25	15	25
9.	MS9	10	15	25	20	30	20
10.	MS10	25	20	15	10	20	18
Mean		49.5	45	46	43.5	44	

Table.3 Embryonic callus initiation

Sr, No	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	MEAN
1.	MS1	45	40	40	35	40	**40**
2.	MS2	46	42	44	37	41	42
3.	MS3	42	41	39	45	38	41
4.	MS4	76	77	69	58	74	70.8
5.	MS5	80	70	70	65	75	72
6.	**MS6**	70	69	74	72	77	**72.4**
7.	MS7	50	54	52	56	51	52.6
8.	MS8	51	48	47	44	41	46.2
9.	MS9	53	51	55	57	59	55
10.	MS10	49	47	44	40	39	43.8
Mean		56.2	53.9	53.4	50.9	53.5	

Table.4 Organogenic callus formation

Sr. No	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	MEAN
1.	MS1	40	35	40	30	45	**38**
2.	MS2	80	60	65	50	65	**64**
3.	MS3	20	10	20	25	25	**20**
4.	**MS4**	70	70	70	78	72	**72**
5.	MS5	69	69	69	71	64	**68.4**
6.	MS6	51	51	51	60	62	**55**
7.	MS7	44	44	44	43	1.5	**35.3**
8.	MS8	42	42	42	41	39	**41.6**
9.	MS9	57	57	57	50	63	**56.8**
10.	MS10	62	62	62	65	64	**63**
Mean		53.5	50	52	51.3	50.05	

Table.5 Callus induction

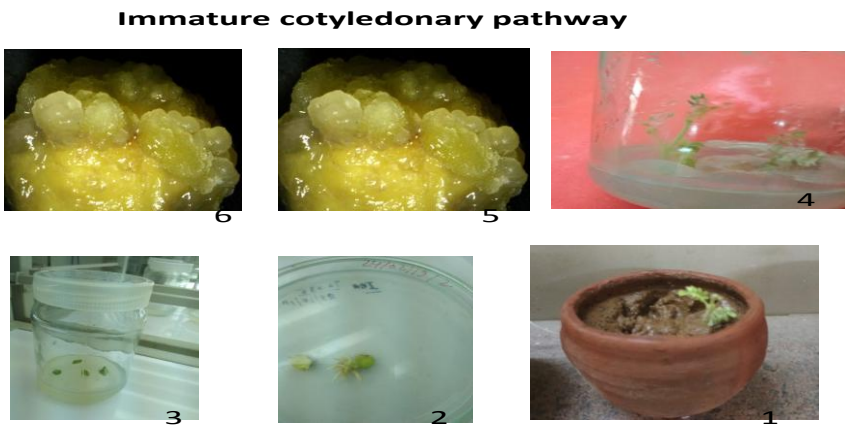
Sr .No	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	MEAN
1.	MS1	77	79	80	81	85	80.4
2.	MS2	70	74	69	67	65	80.4
3.	MS3	65	69	67	71	70	69
4.	MS4	79	78	80	77	76	68.4
5.	MS5	80	81	79	78	77	78
6.	MS6	60	59	58	57	54	79
7.	MS7	55	53	52	49	45	57.6
8.	MS8	40	42	45	40	41	50.8
9.	MS9	41	40	42	44	47	41.6
10.	MS10	40	39	41	39	40	39.8
Mean		60.7	**61.4**	61.3	60.3	60	

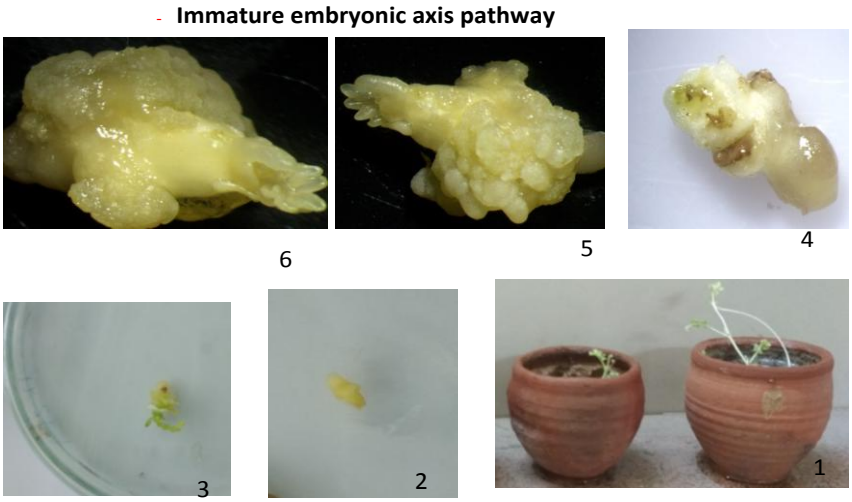
Table.6 Embryonic callus initiation

Sr.	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	MEAN
1.	MS1	43	48	46	42	38	43.4
2.	MS2	43	39	38	34	38	38.4
3.	MS3	40	39	38	44	36	39.4
4.	MS4	43	39	42	35	39	39.6
5.	MS5	76	76	68	61	72	**70.6**
6.	MS6	78	69	69	64	74	**70.8**
7.	MS7	69	68	72	71	76	71.2
8.	MS8	49	52	51	55	49	51.2
9.	MS9	48	47	46	43	40	44.8
10.	MS10	52	50	54	56	57	53.8
Mean		54.1	52.7	52.4	50.5	51.9	

Table.7 Organogenic callus formation

Sr.N0	Composition	JG62	JG63	VIJAY	JG315	Bhupdachana	MEAN
1.	MS1	70	25	20	10	15	70
2.	MS2	70	60	65	55	50	60
3.	MS3	70	55	60	75	65	65
4.	MS4	65	55	60	75	65	64
5.	**MS5**	75	75	70	70	65	**71**
6.	MS6	65	75	6.5	60	65	54.3
7.	MS7	15	20	25	40	15	23
8.	MS8	20	25	15	10	15	17
9.	MS9	25	20	15	10	20	18
10.	MS10	20	25	10	15	10	16
Mean		**49.5**	**45.55**	36.27	**45.55**	41.1	





During recent years, many attempts for generating plant via in vitro protocol for chickpea have not yielded considerably, demanding further studies with such system. In pulses plant regeneration is possible via either embryogenesis or organogenesis with the most efficient system using embryonic axis and cotyledonary node as the initial culture explants. The organogenic culture system is more productive for development of transgenic variety in chickpea in a shorter duration as compared to the embryogenesis culture system as per reports. Plant regeneration appeared to be a stepwise process starting from callus induction, callus proliferation, morphogenesis followed by plant regeneration. Direct somatic embryogenesis or formation of embryoids in callus cultures was obtained without complete plantlet regeneration. similar observation were recorded by Cauhan and singh 2002, H.S Chawla and Arora 2005, Naz *et al.*, 2008, and Ahmed amer *et al.*, 2018 in chickpea. During present investigation three types of media were used for culture establishment a basal MS medium supplemented with plant Hormones, where the result exhibit that the somatic embryo induction largely depends upon the nature of initial culture medium ie. the first medium, whereas other 2 media supported the germination of somatic embryos. For immature cotyledonary node culture, during investigation 2 auxin (NAA and 2,4-D) and cytokinin (BAP) were used for morphogenesis. Result clearly indicated the PGR some combination led to organogenesis some followed the process of embryogenesis from explants, while other produced only callus with high or low growth rate. We evaluated callus initiation the highest 95 % callus obtainMS+3.0 mg/l 2, 4-D + 3 mg/l BAP cotyledon explants of chickpea according to Hudda *et al.*, 2003. In the present investigation MS-4, MS-5 initiation highest number of callus, embryonic callus induction MS-6 and organogenic callus was found in MS-4 from explants of chickpea and lowest percentage of callus initiation in MS-10. From immature embryonic axis the maximum number of calli was found in MS.-1,MS-2, embryonic callus MS-5, MS-6 and organogenic MS-5 however, the lowest percentage of calli found in MS-10 %. Among the JG62, JG 63 and proved the best responsive for callus induction from immature cotyledonary node where as JG63 and JG62 proved the best responsive embryonic calli as well as organogenesis in case of both immature cotyledonary node and immature embryonic axis, and other genotypes was like JG315,Vijay, was good but in Bhupdachana poorly response found.

In conclusion the under this investigation we found that callusing take place when basal medium is 2,4-D, whereas direct shoot differentiation occurs on basal medium supplemented with different concentration and combination of BAP with NAA, and both explants of chickpea immature cotyledonary node and embryonic axis have good response *in vitro* condition and can be used for development of transgenic variety in chickpea.

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