BIHAREAN BIOLOGIST 10 (1): 28-32
Article No.: e151404

http://biozoojournals.ro/bihbiol/index.html

Callus induction is affected by explant type and plant growth regulators in chickpea (*Cicer arietinum* L.)

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Received: 30. October 2014 / Accepted: 04. February 2015 / Available online: 01. June 2016 / Printed: June 2016

Abstract. Chickpea (*Cicer arietinum* L.) is one of the most important plant protein resources. Plant breeding is important for ensuring food security by developing new varieties that are higher-yielding. One of the methods for increasing of plant variation is somaclonal variation. The most common factors affecting somaclonal variation are explant type and plant growth regulators. The present study was carried out for comparison of different explant types and plant growth regulators in chickpea (Bivanij cultivar) in order to callus induction. In this experiment four explants (embryo, cotyledon, node and hypocotyl) were cultured on the best growth regulators compositions that have been previously reported. The evaluated parameters include percentages of callus induction, number of days for callus induction and callus growth. Results showed that the embryos showed 100% callus induction in almost in all mediums. Cotyledons, nodes and hypocotyls medium supplemented by 0.5 mg/l BAP+ 0.5 mg/l NAA showed 95, 70 and 100 percentage callus induction respectively. The highest callus growth was belong to embryo in medium supplemented by 1.5 mg/l BAP+ 2.5mg/l NAA (0.165 mm d/d) and the lowest was belong to cotyledon in medium supplemented by 0.1mg/l NAA+ 0.5 mg/l 2,4-D (0.004 mm d/d).

Key words: Plant growth regulators, explant type, chickpea, callus.

Introduction

Chickpea (*Cicer arietinum* L.), commonly known as gram, as a member of the Legumine family is one of the most important plant protein resources and a vital grain legume of large areas of Asia. Based on the genotype and environment, the protein content in chickpea seed ranged between 20-23% and average seed yield is about 1.0 tons/ha (Pradhan et al. 2014). This crop stands second as for occupied area (about 10 million ha) in the area under cultivation and third in production (about 7 million tons) among the cultivated pulses in the world (Soorni et al. 2012). It is a significant source of protein, phosphorus, iron and certain water soluble vitamins and low content of fat. Protein content of chickpea can be improved via tissue culture and gene transformation (Soorni et al. 2012).

Chickpea made it the most cultivated pulse crop and the most appreciated protein source among vegetarians all over the world. It is able to drive more than 70% of nitrogen symbiotic nitrogen fixation; which makes it a proficient crop for "alternative agriculture" that is now attracting substantial interest in the industrialized world (Sujatha et al. 2007, Zarei et al. 2011).

Different methods of breeding are employed in plant breeding since the last century. Nevertheless recent advances made in the field of plant tissue culture have brought about new emerging technologies for plant breeding. The potential value of cell, tissue, organ, anther, microspore and embryo culture as tool for use in the plant breeding has been reported (Green 1977, Vasil 1987, Kahrizi et al. 2011).

Application of tissue culture techniques for genetic upgradation of economically important plants has been reported. Several researchers (Kartha et al. 1981, Islam et al. 1995, Barna & Wakhlu 1994, Barna & Wakhlu 1995, Anju & Chawla 2005, Kiran et al. 2010) have described on the *in vitro* of *C. arietinum*. The callus induction may be affected by chemical, physical and biological factors (Soorni et al. 2012, Kahrizi et al. 2013, Kahrizi & Soorni 2013).

The somaclonal variation depends upon reliable callus culture and plant regeneration procedures. The frequencies of callus induction and plant regeneration in chickpea are influenced by many factors such as culture medium composition, explant type, genotype and environment etc (Khanna & Raina 1998, Khatun et al. 2003). The aim of current research was investigate the effect of different explant type and concentrations of plant growth regulators on callus induction in chickpea (Bivanij cultivar).

Materials and methods

The plant material in this experiment was chickpea (Bivanij cultivar) that is known as a high yield. However this cultivar is sensitive to *Ascochyta* blight disease caused by *Ascochyta rabiei* (Pass.).

For providing of sterilized stock plants, the mature chickpea seeds were surface-sterilized with 70% ethanol for 1 min and cultured on basal MS medium. Then the culture was incubated in phytotron at 25 °C under a 16/8 hour low light/dark cycle. Explants such as cotyledon, leaf and hypocotyl were removed from two weeks sterile plants. The embryo explants were isolated directly from sterilized seeds. This experiment was carried out in order to comparison of various explants and different growth regulators in chickpea (Bivanij cultivar).

The experiment was conducted in MS medium supplemented by different concentrations of plant growth regulators in four explants (embryo, cotyledon, node and hypocotyl). The experiment was conducted based on completely randomized design (CRD).

For this purpose the explants were cultured on the best plant growth regulators compositions that have been previously reported (Table 1).

The factors include callus induction percentage (CIP), days to callus induction (DCI) and callus diameter. The callus diameters were recorded after 14, 21 and 28 days. Then Callus Growth (CG) was evaluated based on diameter changes.

Percentage of callus induction: Percentage of callus induction was calculated according to the following formula: Percentage of callus induction= (Number of explants formed calli/number of cultured explants) × 100 (Arzani & Mirodjagh 1999).

Table 1. Different plant growth regulators compositions that have been used in callus induction experiment in current research.

Plant growth regulators compositions	Explant Type
(1mg/l BAP+1.5 mg /l NAA) – (1mg/l BAP+2mg /l NAA) – (1mg/l BAP+1mg /l NAA) – (2mg/l BAP+3mg/l 2,4-D) – (1.5mg/l BAP+2.5mg/l 2,4-D) – (0.5mg/l Kin+2.5mg/l 2,4-D) – (0.5mg/l Kin+1mg/l 2,4-D) –(0.5mg/l Kin+0.5mg/l 2,4-D) –(0.5mg/l 2,4-D+0.1mg /l NAA) – (0.5mg/l 2,4-D+0.5mg /l NAA) – (0.5mg/l BAP+0.1mg /l NAA) –(0.5mg/l BAP+0.5mg /l NAA)	Embryo
(1 mg/l BAP+0.5 mg /l NAA) – (1.5 mg/l BAP+1 mg/l NAA) – (3 mg/l BAP+3 mg/l NAA) – (1 mg/l BAP+3 mg/l 2,4-D) – (3 mg/l BAP+2 mg/l 2,4-D) – (3 mg/l BAP+3 mg/l 2,4-D) – (0.5mg/l 2,4-D+0.1mg /l NAA) – (0.5mg/l 2,4-D+0.5mg /l NAA) – (0.5mg/l BAP+0.1mg /l NAA) – (0.5mg/l BAP+0.5mg /l NAA)	Cotyledon
(0.5mg/l 2,4-D+0.1mg /l NAA)-(0.5mg/l 2,4-D+0.5mg /l NAA)–(0.5mg/l BAP+0.1mg /l NAA)–(0.5mg/l BAP+0.5mg /l NAA)	node
(0.5mg/l 2,4-D+0.1mg /l NAA) – (0.5mg/l 2,4-D+0.5mg /l NAA) –(0.5mg/l BAP+0.1mg /l NAA) – (0.5mg/l BAP+0.5mg /l NAA)	Hypocotyl

Callus diameter: To calculate calli diameters, length and width of each were measured and the following formula was served: Callus diameter= √Length × √Width (Compton 1994).

Callus growth (*CG*): To calculate callus growth (mm diameter per day), calli diameters after 28 days culture were firstly measured and then the following formula was served: CG= (d2-d1)/(t2-t1) (Compton 1994).

Specific analyses and the results are noted in the appropriate figure and table footnotes and text. Mean comparison was performed using Duncan's Multiple Range test at 0.05 probability level after ANOVA. Statistical analyses were done using SPSS (vers. 19) and SAS (ver. 9.1).

Results

Four explants were used such as embryo, cotyledon, node and hypocotyl. They show variation in terms amount of callus induction, days taken for callus initiation and percentage of callus induction. The 3-5 cm sterile stock plants achieved after seed incubation for three weeks (Fig. 1).

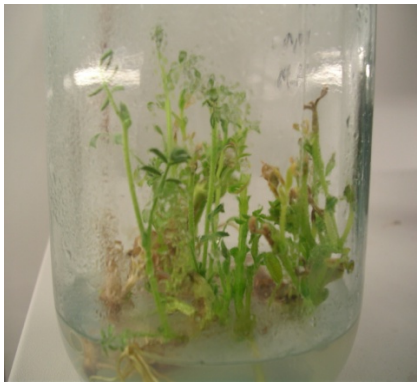

Figure 1. The serialized stock plant for explant preparation in order to callus induction experiment.

Effect of plant growth regulators on the callus induction in embryo explants

In the embryo experiment, showed that there are significant differences among plant growth regulators (PGRs) levels for callus induction percentage (CIP), days to callus induction (DCI) and callus growth (CG) (Table 2).

Obtained results showed that the most PGRs had the highest callus induction percentage (about 100%) in embryo explant and the 0.1 NAA + 0.5 BAP composition produced the lowest callus percentage (23.66%) (Fig. 2A).

Table 2. Mean squares for effect of growth regulators (PGRs) for callus induction percentage (CIP), days to callus induction (DCI) and callus growth (CG) in embryo explant. Where SOV: source of variations, DF: degree of freedom, CV: Coefficient of variations, * and **: significant at 0.05 and 0.01 levels respectively.

SOV	DF	Mean Squares		
		CIP	DCI	CG
PGRs	11	0.142**	7.89**	0.015*
Error	24	0.000015	1.08	0.097
CV		1.37	13.77	13.60

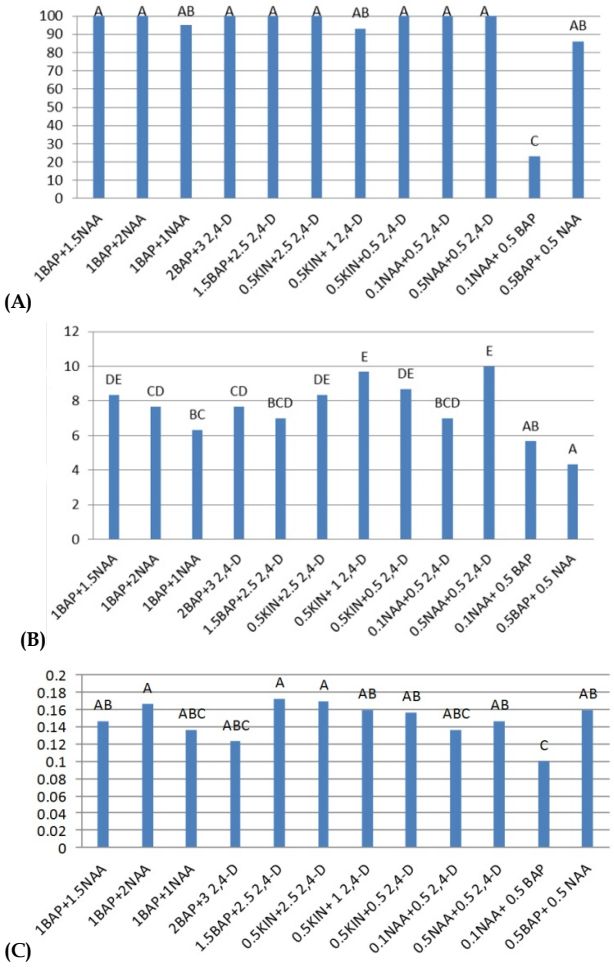

Figure 2. Mean comparison for plant growth regulators effects on the callus induction embryo derived in chickpea. A: Callus induction percentage (%); B: Days to callus induction (days); C: Callus growth (mm/day).

The result showed that the shortest period for embryo callus induction (4.33 days) is related to 0.5 mg/l BAP + 0.5 mg/l NAA composition. The 0.5 mg/l Kin + 1 mg/l 2,4-D and 0.5 mg/l NAA + 0.5 mg/l 2,4-D compositions showed the highest amounts for days to callus induction (9.66 and 10.00 days respectively) (Fig. 2B).

Results showed that (1mg/l BAP+2mg/l NAA), (1.5mg/l BAP+2.5mg/l 2,4-D), and (1.5mg/l BAP+2.5mg/l 2,4-D) compositions had the highest callus growth (CG) in embryo explant (166, 173 and 170 µm diameter/day respectively). The 0.1 mg/l NAA + 0.5mg/l BAP treatment showed the lowest CG (100 µm diameter/day) (Fig. 2C).

Effect of plant growth regulators on the callus induction in cotyledon explants

In the cotyledon experiment, showed that there are significant differences among plant growth regulators (PGRs) levels for callus induction percentage (CIP) and days to callus induction (DCI) (Table 3). The effects of PGRs on CG weren't significant (Table 3).

The 0.5 mg/l BAP + 0.5 mg/l NAA had the highest percentage of callus induction (about 95.33%) in cotyledon explant and the lowest callus produced in a concentration of (3 mg/l BAP+3mg/l 2,4-D) (45.33, 42.33 and 35.00 % respectively) (Fig. 3A).

Results showed that the shortest period for embryo callus induction (7.66 days) is belong to (3 mg/l BAP + 3 mg/l 2,4-D) and (1.5 mg/l BAP + 1mg /l NAA) compositions. The 0.5 mg/l BAP + 0.5mg/l NAA composition showed the highest amounts for days to callus induction (14.66 days) (Fig. 3B). Mean comparison that (1mg/l BAP+0.5 mg/l NAA), (3 mg/l BAP+3 mg/l NAA), (3 mg/l BAP+2 mg/l 2,4-D) and (3 mg/l BAP + 3 mg/l 2,4-D) compositions had the highest callus growth (CG) in cotyledon explant. However there is not significant different among these four composition for this callus trait (Fig. 3C). In the node experiment, showed that there are significant differences among plant growth regulators (PGRs) levels for callus induction percentage (CIP). The effects of PGRs on CG and days to callus induction (DCI) weren't significant (Table 4).

The 0.5 mg/l BAP + 0.5 mg/l NAA had the highest callus induction percentage (about 70.00%) in node explant and the (0.1 mg/l NAA + 0.5 mg/l BAP) composition produced the lowest callus percentage (43.66%) (Fig. 4A). The shortest period for node callus induction is belong to (0.1 mg/l NAA + 0.5 mg/l 2,4-D) and (0.5 mg/l BAP + 0.5 mg/l NAA) compositions. However there is not significant different among these four composition for this callus trait (Fig. 4B). The 0.5 mg/l BAP+0.5 mg/l NAA composition had the highest callus growth (CG) in node explant. The lowst CG is belong to 0.5 mg/l BAP+0.5 mg/l 2,4-D composition (Fig. 4C).

Comparative study of plant growth regulator on hypocotyl explant

In the hypocotyl experiment, showed that there are significant differences among plant growth regulators (PGRs) levels for callus induction (CIP). The effects of PGRs on CG and days to callus induction (DCI) weren't significant (Table 5).

Results showed that the (0.5 mg/l NAA + 0.5 mg/l 2,4-D) and (0.5 mg/l BAP + 0.5 mg/l NAA) had the highest callus induction (100%) in hypocotyl explant and the (0.5mg/l BAP+0.1 mg/l NAA) and (0.1mg/l NAA+0.5mg/l 2,4-D) medium-produced lowest callus percentage (83 and 64% respectively) (Fig. 5A).

The shortest period for hypocotyl callus induction is belonging to (0.1 mg/l NAA + 0.5 mg/l BAP) composition (Fig. 5B).

Results showed that 0.5 mg/l BAP+0.5 mg/l NAA composition had the lowest callus growth (CG) in hypocotyl explant. There were not significant difrences amon other PGRs compositions for CG in checkpea hypocotyl (Fig. 5C). A sample of callus induction is shown in Figure 6.

Table 3. Mean squares for effect of growth regulators (PGRs) for callus induction percentage (CIP), days to callus induction (DCI) and callus growth (CG) in cotyledon explant. Where SOV: source of variations, DF: degree of freedom, CV: Coefficient of variations, **: significant at 0.01 levels, ns: non-significant.

SOV	DF	Mean Squares		
		CIP	DCI	CG
PGRs	9	0.12**	20.35**	0.00041ns
Error	20	0.0073	0.83	0.000165
CV		13.91	8.89	18.19

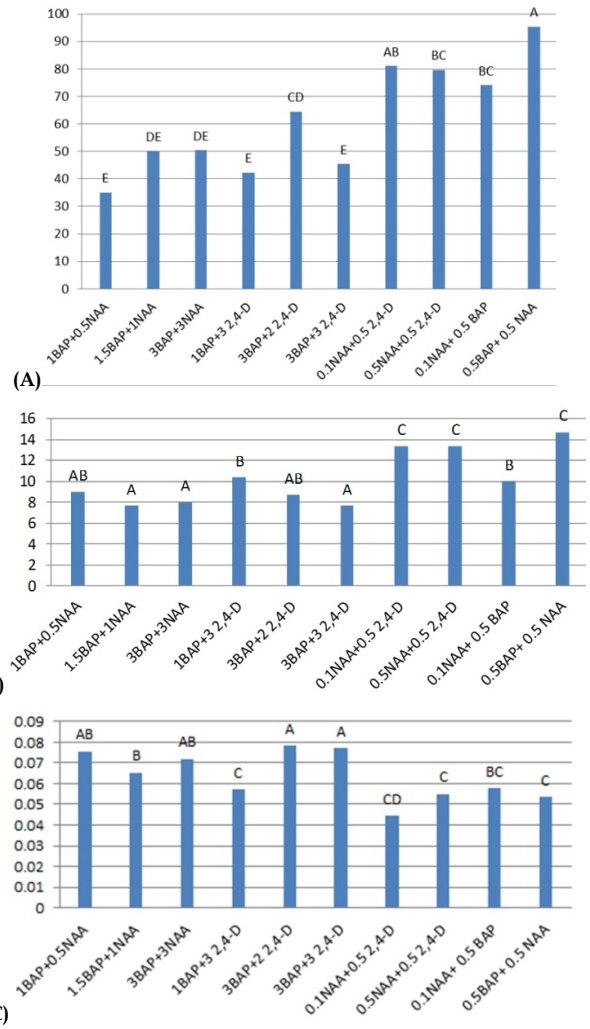

Figure 3. Mean comparison for plant growth regulators effects on the callus induction cotyledon derived in chickpea. A: Callus induction percentage (%); B: Days to callus induction (days); C: Callus growth (mm/day)

Table 4. Mean squares for effect of growth regulators (PGRs) for callus induction percentage (CIP), days to callus induction (DCI) and callus growth (CG) in node explant. Where SOV: source of variations, DF: degree of freedom, CV: Coefficient of variations, **: significant at 0.01 levels, ns: non-significant.

SOV	DF	Mean Squares		
		CIP	DCI	CG
PGRs	3	0.0465**	1.19ns	0.0003ns
Error	8	0.00059	1.33	0.00023
CV		4.13	20.01	11.01

Table 5. Mean squares for effect of growth regulators (PGRs) for callus induction percentage (CIP), days to callus induction (DCI) and callus growth (CG) in hypocotyl explant. Where SOV: source of variations, DF: degree of freedom, CV: Coefficient of variations, **: significant at 0.01 levels, ns: non-significant.

SOV	DF	Mean Squares		
		CIP	DCI	CG
PGRs	3	0.087**	0.66ns	0.0029ns
Error	8	0.00068	1.33	0.0074
CV		3.007	21.65	20.82

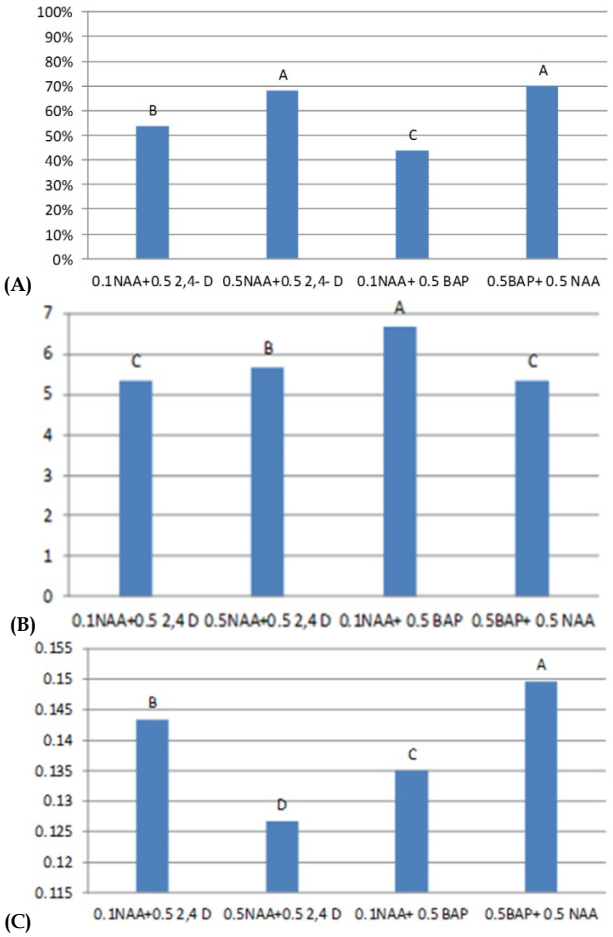

Figure 4. Mean comparison for plant growth regulators effects on the callus induction node derived in chickpea. A: Callus induction percentage (%); B: Days to callus induction (days); C: Callus growth (mm/day).

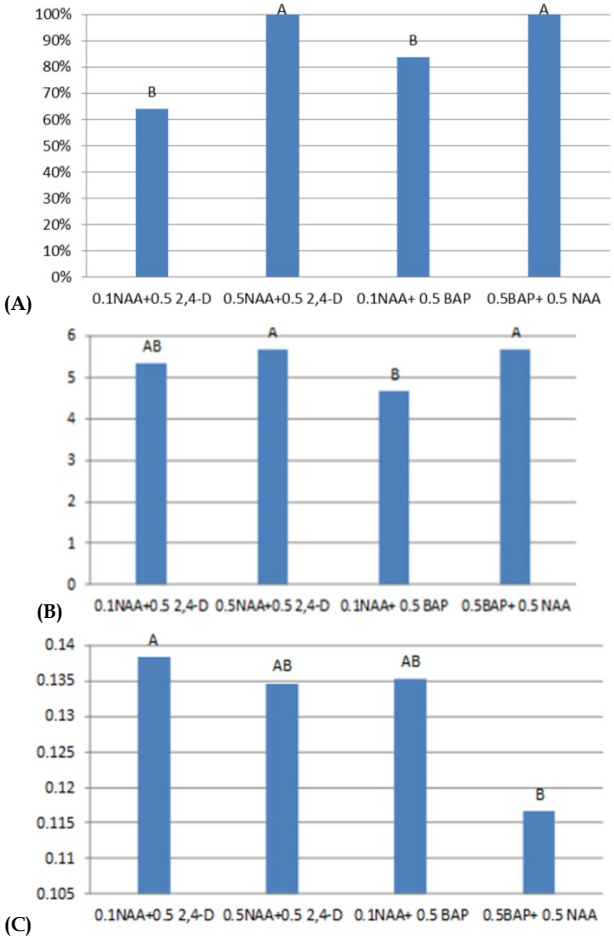

Figure 5. Mean comparison for plant growth regulators effects on the callus induction hypocotyl derived in chickpea. A: Callus induction percentage (%); B: Days to callus induction (days); C: Callus growth (mm/day).

Discussion

In the embryo callus induction experiment, there are significant differences among PGRs for CIP, DCI and CG. It seems that not only 2,4-D effects on callus induction percentage, but also increasing in NAA concentration leads to increasing in callus induction percentage. Anju & Chawla (2005) reported that the highest callus induction percentage in chickpea embryos was achieved in 1mg/l BAP+1.5 mg/l NAA and 1mg/l BAP+ 0.5 mg/l NAA. According to Zaman et al. (2010), 100% callus induction was achieved on 1/2MS that supplemented by 0.5 and 2.0 mg/l 2,4-D that is in agreements with our results. Our results showed that lower concentrations for 2,4-D and NAA leads to callus induction in shorter periods. There are significant differences among PGRs for CIP and DCI in the cotyledon experiment. The effects of PGRs on CG weren't significant.

Huda et al. (2003) reported that the highest percentage for cotyledon callus induction was 95%. This result achieved in (3 mg/l 2,4-D + 3 mg/l BAP) that is agreement with our results. Difference in amount is related to difference in chickpea genotype. Huda et al. (2003) showed the minimum days to cotyledon callus induction is belong to (3 mg/l BAP + 3 mg/l 2,4-D) and (3 mg/l BAP + 3 mg/l NAA) (about 8 days) that is near to our results. Results showed that there are significant differences among PGRs for CIP in the node

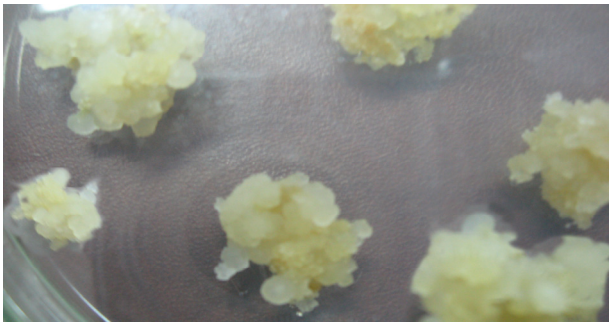

Figure 6. Callus induction hypocotyl derived in chickpea.

experiment. The effects of PGRs on CG and DCI weren't significant. There is not any report for callus induction in chickpea node. This explant has been applied for direct regeneration (Kiran et al. 2010). In the hypocotyl experiment, showed that there are significant differences among PGRs for CIP. The effects of PGRs on CGR and DCI weren't significant. It seems that hypocotyl callus induction occurs in equal amounts of auxins and cytokinin. It may equal amounts of auxins and cytokinin keeps the cells in undifferentiated stage.

Neelam et al. (1986) cultured the chickpea hypocotyls on B5 medium that has been supplemented by PGRs. They achieved the highest callus induction percentage in 1mg/l BAP + 1mg/l Kin. The highest callus induction from hypocotyl was resulted in Kaberi et al. (2013) expriment on MS medium supplimented by 2 mg/l 2,4-D + 0.5 mg/l NAA. This result is similar to our findings.

Darvishi et al. (2014) reported that among interaction effects the leaf explant in 1 mg/l 2,4-D and BAP-free found as the highest effect for callus induction (12.35 mm in callus diameter at 28 days after culture) in Pennyroyal. Results of current experiment showed that the embryos and hypocotyls explant of chickpea showed complete callus induction. The highest callus growth induced from embryo explant in medium supplemented by 1.5 mg/l BAP+ 2.5mg/l NAA.

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