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In Vitro Direct Organogenesis Using Mature Embryo With Cotyledons In Chickpea

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A B S T R A C T

The present study was conducted to find out an in vitro efficient method for multiple shoot regeneration of two local chickpea varieties. The mature embryos were excised of two chickpea varieties i.e. Bittle-98 and Dasht-2000 (with cotyledon and without cotyledon) used as explants. The explants were cultured on Murashige and Skoog (MS) medium supplemented with three concentrations of (2, 3, 4 mg/l) 6-benzylaminopurine (BAP) using explant with and without cotyledon. Further, 0.5 mg/l α-naphthaleneacetic acid (NAA) along with varied concentrations of BAP (2, 3, 4 mg/ l) was also tested using explant with cotyledon. 3 mg/l BAP alone and 3 mg/l BAP with 0.5 mg/l NAA were found the most effective cytokinin in multiple shoot induction in both tested varieties. Bittle-98 and Dasht-2000 showed 82% and 76% elongation in shoots induction with 0.2 mg/l Indole-3-acetic acid (IAA). Root formation was recorded 80% and 60% in Bittle-98 and Dasht-2000 with 1.0 mg/l Indole-3-butyric acid (IBA). Whereas, recorded root formation was 40 and 20% in Bittle-98 and Dasht-2000 with 1.0 mg/l NAA. The best response for rooting observed in Bittle-98 as its roots were thick, long and strong. Plantlets of Bittle-98 were acclimatized in solid medium for 7-14 days. The successful *invitro* regeneration of Bittle-98 was observed, when excised embryo with cotyledon were used as explant, made it valuable for genetic transformation.

Introduction

Chickpea (*Cicer arietinum*) is an important legume with two distinct types; small seeded Desi (with brown seed coat) and large seeded Kabuli (with white seed coat) (Gaur et al., 2010). *C. arietinum* is main dietary constituent of food in under developing countries. The crude protein ranges from 12.4 to 31.5 percent in this crop. (Ignacimuthu and Prakash, 2006). *C. arietinum* plays an important role in the sustainability of rain fed agriculture system due to having ability to tolerate drought (Daryanto et al., 2015). It is a low input crop that often completes its lifecycle in drought and heat stress. It is also valued for its ability to improve the soil fertility through fixing atmospheric nitrogen (Fatima et al., 2008).

Conventional breeding methods for incorporation of resistance are often costly and time consuming and limited to lack of proper gene in gene pool (Chahal and Gosal, 2002). Recent advances in biotechnology such as plant tissue culture and genetic transformation can significantly contribute to better sustainability of this important food crop through incorporation of alien genes to develop required resistance (Somer et al., 2003). The ability to regenerate plants from cultured cells, tissues or organs constitute the basis of producing transgenic crops (Geetha et al., 1998). Tissue-culture techniques provide rapid means for vegetative plant propagation (Reinert, 1977) and also provide a method for experimentation and manipulation of plant material within the laboratory.

There are several reports available on *C. arietinum* shoot regeneration from immature or mature cotyledons (Aasim et al., 2013; Ghanti et al., 2010; Pawar et al., 2012; Tripathi et al., 2013). Embryonic axes with half portion of cotyledons was more responsive explant (Singh et al., 2002; Chakraborty et al., 2006; Paul et al., 2008; Yousefiara et al., 2008). However, cotyledonarynodes from mature seeds have been noted as most responsive for the induction of multiple shoots via organogenesis in chickpea (Sarker et al., 2005; Sujatha et al., 2007). The present research was designed to optimize and increase regeneration under *in vitro* conditions using dissected half embryos with and without cotyledon explants to study the

response of two chickpea genotypes. Multiple shooting, elongation, rooting and acclimatization of plantlets were also investigated.

Materials and Methods

Seeds of *C. arietinum* were obtained from Pulses Program, Crop Science Institute NARC, Islamabad, Pakistan. The seeds of two chickpea varieties (Bittle-98 and Dasht, 2000) were used as explants source. Two types of explants *i.e.* embryonic axes with endosperm and without endosperm were tested for their *in vitro* regeneration response. These seeds were surface-sterilized with 70% ethanol for one minute and with 40% chlorex for 20 minutes followed by 4-5 washing with sterilized dH_2O. Surface sterilized seeds were soaked in sterile double distilled water for 16–18 hours. After discarding water, seeds were allowed to germinate for one day. The seed coats were removed, the radicals were discarded and a longitudinal slit was made. The dissected half embryos with and without cotyledon were chosen as explants.

Multiple Shoot Induction

Explants were cultured on MS media (Murashige and Skoog, 1962) supplemented with BAP (2, 3 and 4 mg/l) and NAA (0.5 mg/l), 3% sucrose and 0.8% agar. The explants were kept under a photoperiod for 16/8 hours light/dark cycle at 22±2 °C. Regular sub culturing was done at 7 days intervals. Twenty four explants from each genotype in triplicate sets were cultured. Number of shoots produced from each explant was counted after 14 days.

Shoot Elongation

Explants with emerging shoots from the best regeneration medium were further cultured in medium containing IAA (0.2, 0.25 and 0.5 mg/l) for another 20-25 days. Three replicas of 46 regenerating explants were used for each treatment.

Rooting

Five elongated shoots were transferred in semisolid medium supplemented with plant growth regulator i.e. IBA (1.0 and 0.5) and NAA (1.0, 0.5 mg/l), 3% sucrose and 0.5% agar for rooting. Root regeneration responses were recorded after 10 and 14 days of the culture.

Acclimatization and Establishment of Plantlets

Plantlets that developed long and strong roots were used for acclimatization. The regenerated plantlets were shifted in pots containing sand-soil-manure mixture (1:1:1). Pots were covered with polythene bags for one week and put in to a growth chamber, in order to protect humidity and better establishment. After one week, holes were made in polythene bags and three weeks later pots were finally shifted in green house.

Data Analysis

Each of the experiment conducted thrice and single experiment was treated as one replication. Complete Randomize Design (CRD) was used as experimental design. The mean values along with standard error (SE) were calculated for all experimental treatments. Statistical differences among means were estimated at 5% level of probability using Duncan's Multiple Range Test (DMRT) with the help of Statistical Software version 5.0 (Stat Soft, 1995).

Results and Discussion

Concentrations Impact of PGRs on Multiple Shoot Induction

*Effect of BAP on explant embryos having cotyledon***:** The three concentrations of BAP on embryos with cotyledon showed variable results regarding multiple shoot formation between both varieties (Tables 1). The BAP treated embryo having cotyledon promoted multiple shoot formation on the both tested cultivars (Fig. 1). In cultivar *Bittle-98* had maximum shoot induction 72.2 percent on MS medium containing 3 mg/l BAP, followed by 62.5 percent with 2 mg/l BAP and 59.7 percent with 4 mg/l BAP respectively. Whereas, cultivar *Dasht-2000* showed maximum shoot induction 73.6 percent with 3 mg/l BAP, 69.2 percent with 2 mg/l BAP and 61.1 percent with 4 mg/l BAP respectively (Table 1).

Effect of BAP on explant embryos without cotyledon: The impact of tested concentrations of BAP on embryos without cotyledon for multiple shoot formation is shown (Table 2). The embryo without cotyledon promoted multiple shoot formation by the use mentioned PGRs in both tested varieties (Fig. 2). In cultivar *Bittle-98* maximum shoot induction was recorded 58.3 percent on MS with 3 mg/l BAP followed by 55.5 percent with 2 mg/l BAP and 34.7 percent with 4 mg/l BAP respectively. Whereas, cultivar *Dasht-2000* showed maximum shoot induction 20.8.6 percent with 3 mg/l BAP, 16.6 percent with 2 mg/l BAP and 12.5 percent with 4 mg/l BAP respectively (Table 2).

Table1 Effect of different concentrations of BAP on multiple shoot induction from half embryo along with cotyledon explants of chickpea.

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE	Percentage	Mean ± SE	Percentage
BAP (2 mg/l)	15.00 ± 0.57	62.5	16.66 ±1.33	69.2
BAP (3 mg/l)	17.33 ± 0.88	72.2	17.66 ± 1.45	73.6
BAP (4 mg/l)	14.33 ± 0.88	59.7	14.66 ± 1.20	61.1

*Means followed by the different letters indicates statistically significant differences at 5% probability level.

Table 2 Effect of different concentrations of BAP on multiple shoot induction from half embryo without cotyledon explants of chickpea.

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE*	Percentage	Mean ± SE*	Percentage
BAP (2 mg/l)	13.33 ± 0.33a	55.5	4 ± 0.57b	16.6
BAP (3 mg/l)	14.00 ± 0.57a	58.3	5 ± 0.57a	20.8
BAP (4 mg/l)	8.33 ± 0.66b	34.7	3 ± 0.57b	12.5

*Means followed by the different letters indicates statistically significant differences at 5% probability level.

Table 3 Effect of different concentrations of BAP and NAA on multiple shoot induction from half embryo along with cotyledon explants of chickpea.

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE*	Percentage	Mean ± SE*	Percentage
BAP+NAA (2 + 0.5 mg/l)	11.00 ± 0.66b	45.8	10.00 ± 0.88b	41.6
BAP+NAA (3 + 0.5 mg/l)	14.00 ± 0.57a	58.3	12.00 ± 1.00a	50
BAP+NAA (4 + 0.5 mg/l)	10.00 ± 0.88b	41.6	9.00 ± 0.66b	37.5

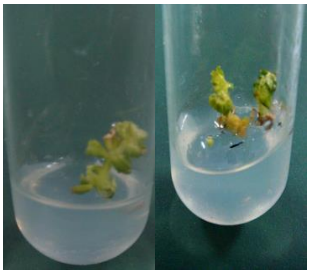
*Means followed by the different letters indicates statistically significant differences at 5% probability level

A. with cotyledon

B. without cotyledon

Fig. 1 Regeneration of whole plant from half embryo explants.

A. with cotyledon



B. without cotyledon

Fig. 2 Regeneration of multiple shoots from an explants after 14 days of incubation

Effect of BAP and NAA on explant embryos without cotyledon: The impact of NAA along with varied concentrations of BAP on embryos with cotyledon for multiple shoot formation is shown (Table 3). In cultivar *Bittle-98* maximum shoot induction was recorded 58.3% on MS with 3 mg/l BAP+ 0.5 mg/l NAA followed by 45.8 percent with 2 mg/l BAP + 0.5 mg/l NAA and 41.6 percent with 4 mg/l BAP+ 0.5 mg/l NAA respectively. Whereas, cultivar *Dasht-2000* showed maximum shoot induction 50 percent with 3 mg/l BAP + 0.5 mg/l NAA, 41.6 percent with 2mg/l BAP + 0.5 mg/l NAA and 37.5 percent with 4 mg/l BAP+ 0.5 mg/l NAA respectively (Table 3).

Effect of all concentrations of BAP was non-significant in Bittle-98. Further, explants of Bittle-98 showed differences in terms of multiple shoot production. Significant differences in multiple shoot formation was recorded in Dasht-2000 to various hormonal concentrations, which indicated that there might be some indigenous plant factors residing in cotyledon necessary for promotion of multiple shoot formation. Singh et al. (2002) also reported that the presence of cotyledon attached to embryo was essential for shoot production. It can be estimated that selection of embryo with cotyledon combined with additional PGR might be served as useful explants for genetic transformation point of view. Similarly, Chakraborti et al. (2006) reported that combination of cytokinins with a relatively lower concentration of auxin was useful to have efficient frequency of multiple shoot production in Indian origin chickpea cultivars. Such kind of combination was also tested in the current investigation. Various concentrations of BAP (2, 3 and 4 mg/l) were tested in combination with NAA (0.5 mg/l). Different concentrations of BAP along with NAA did not improve the frequency of shoot formation as compared to alone BAP concentration (Table 3). Yousefiara et al. (2008) also reported that use of BAP alone showed best response for multiple shoot induction.

Effect of IAA on elongation of shoots with cotyledon: The effect of three concentrations of IAA on elongation of shoot induction from half embryo along with cotyledon explants of chickpea (Fig. 3). It was observed that further growth of regenerated shoots was inhibited by either alone with BAP or BAP in combination with NAA. So, it was attempted to elongate shoots with selected (0.2, 0.25 and 0.5 mg/l) IAA concentrations. It was observed that IAA 0.2 mg/l was the most effective for elongation of regenerated shoots with 82 percent in Bittle-98 and 76 % in Dasht-2000 cultivars respectively (Table 4).

The average elongated shoot induction in Bittle-98 and Dasht-2000 treated with IAA 0.2 mg/l were (38 ± 1.15) and (35 ± 1.15) respectively. Whereas, elongated shoot induction in Bittle-98 and Dasht-2000 treated with IAA 0.25 mg/l were (30 ± 1.15) and (28 ± 1.15) respectively. The gradual increase of IAA had negative impact and reduced the percentage of shoot elongation, as shoot induction became nil when concentration of IAA was raised to 0.49 mg/l (Table 4).

Table 4 Effect of different concentrations of IAA on elongation of shoot induction from half embryo along with cotyledon explants of chickpea after 20 days.

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE*	Percentages	Mean ± SE*	Percentages
IAA 0.2 mg/l	38 ± 1.15a	82	35 ± 1.15a	76
IAA 0.25 mg/l	30 ± 1.15b	65	28 ± 1.15b	60
IAA 0.5 mg/l	0	0	0	0

*Means followed by the different letters indicates statistically significant differences at 5% probability level.

Table 5 Effect of different concentrations of IBA on root formation from elongated shoots

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE*	Percentage	Mean ± SE*	Percentage
IBA 0.5 mg/l	2 ± 0.57a	40	1 ± 0.00a	20
IBA 1.0 mg/l	4 ± 0.57b	80	3 ± 0.57b	60

*Means followed by the different letters indicates statistically significant differences at 5% probability level.

Table 6 Effect of different concentrations of NAA on root formation from elongated shoots

Plant Growth Regulator Concentrations	Bittle-98		Dasht-2000	
	Mean ± SE*	Percentage	Mean ± SE*	Percentage
NAA 0.5 mg/l	1± 0.00a	20	1 ± 0.00a	20
NAA 1.0 mg/l	2 ± 0.57a	40	1 ± 0.00a	20

*Means followed by the different letters indicates statistically significant differences at 5% probability level.

Fig. 3 Elongated shoots induction treated with IAA from half embryo with cotyledon explant after 7 days of incubation.

Fig. 4 Roots formation (3-5cm) from elongated shoots after 10-14 days of incubation.

Concentrations impact of PGRs on Root Formation in Regenerated Shoots

Effect of IBA: The effect of tested concentrations of IBA on root formation from elongated shoots is shown (Fig. 4).In order to get full plant recovery, shoot (3-5 cm) were obtained after 20 days of incubation in elongated media and shifted in rooting media containing full strength of MS, 0.5 % agar and IBA. Two concentrations of IBA (0.5 and 1.0 mg/l) were used. The cultivars Bittle-98 and Dasht-2000 showed best response with 80% and 60% root formation when treated with 1 mg/l IBA respectively. The present results are agreement with Aasim et al., (2013b) and Barpete et al., (2014). They observed that IBA is the most responsive growth regulator during the rooting of chickpea and Grasspea. The average numbers of rooting for Bittle-98 and Dasht-2000 were (4 ± 0.577) and (3 ± 0.577) respectively. Whereas, less promising results in favoring roots formation were recorded when 0.5 mg/l IBA was used resulting 40 and 20 percent root formation in Bittle-98 and Dasht-2000 respectively (Table 5). Similar results were showed by Sujatha et al. (2007) and Paul et al. (2008).

Effect of NAA: The effect of NAA concentrations on the root formation from elongated shoots shown in Table 6. Two concentrations of NAA (0.5 and 1.0 mg/l) showed that NAA have not enhanced the frequency of root formation. The mean number of root formation in *Bittle-98* were (2 ± 0.57) and (1 ± 0.00) in NAA 1.0 mg/l and NAA 0.5 mg/l respectively. Whereas, root elongation in *Dasht-2000* was recorded (1 ±0.00) when NAA was used 1.0 mg/l and 0.5 mg/l respectively.

Acclimatization

In order to study acclimatization parameters, plantlets of cultivar Bittle-98 with developed strong and long roots were used in experiments.

Conclusion

The results of present study will contribute to future studies in improvement of local chickpea cultivars. The successful culture of Bittle-98 will have a number of benefits in future. Explants excised embryo with

cotyledon makes it a valuable for *Agrobacterium tumefaction* transformation point of view. It is strongly hoped that traits like insect resistance, herbicide resistance, drought tolerance which are lacking in prevailing germplasm, would be transformed into Bittle-98 using our optimized protocol.

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