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BRIEF COMMUNICATION

The effect of phenyl acetic acid on shoot bud induction, elongation and rooting of chickpea

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Abstract

A highly efficient protocol for plant regeneration from cotyledonary node of two chickpea (*Cicer arietinum* L.) cultivars ICCV-10 and Annigeri used phenylacetic acid (PAA). The Murashige and Skoog (MS) medium supplemented with 2.0 mg dm^{-3} 6-benzylaminopurine (BAP) and 1.0 mg dm^{-3} PAA was used for induction of bud formation. Buds were elongated on MS medium supplemented either with only 0.75 mg dm^{-3} gibberellic acid (GA_3) or 0.2 mg dm^{-3} GA_3 + 0.6 mg dm^{-3} PAA. The elongated shoots were then transferred onto rooting medium containing 1 mg dm^{-3} PAA. The frequency of multiple shoot induction and rooting was higher in Annigeri as compared to ICCV-10. The complete plantlets with well-developed roots were transferred to pots containing sterilized soil and sand in the ratio 3:1 where they survived (74 %) and set normal seeds.

Additional key words: auxins, cytokinins, elongation, gibberellic acid, multiple shoots.

Chickpea (*Cicer arietinum* L.) is an important grain legume. Conventional breeding approaches seem to be inadequate due to restricted direct gene pool and sexual incompatibility amongst the wild relatives (Sing and Sing 1989). In order to exploit direct gene transformation, an efficient regeneration system is pre-requisite. Shoot regeneration from different explants (Surya Prakash *et al*. 1992, Shri and Davis 1992, Murthy *et al*. 1996, Subhadra *et al*. 1998, Sharma and Amla 1998, Chakrabarty *et al*. 2000, Singh *et al*. 2002, Jayanand *et al*. 2003) as well as somatic embryogenesis (Rao and Chopra 1991, Barna and Wakhulu 1993, Sagare *et al*. 1993, Kumar *et al*. 1994, 1995, Kiran *et al*. 2005) have been reported in chickpea. Direct organogenesis from pre-existing meristem may provide a reproducible high frequency regeneration system. Though several protocols were developed, rooting of regenerated shoot was limited. These protocols were specific for some genotypes and often not reproducible. So there is a need for further improvement in shoot induction and specifically *in vitro* rooting. We tried phenylacetic acid (PAA), naturally occurring auxin present in many lower and higher plant species (Abe *et al*. 1974, Schneider and Wightman 1986), for shoot regeneration, elongation and root formation in chickpea cultivars ICCV-10 and Annigeri. To the best of our knowledge there are no reports on the direct regeneration of chickpea using PAA without the intervention of callus.

Seeds of chickpea cultivars ICCV-10 and Annigeri were obtained from the Agricultural Research Station, Gulbarga, Kranataka, India. Seeds were surface sterilized with 0.1 % (m/v) mercuric chloride for 4 - 5 min. Seeds were rinsed with sterile distilled water about 4 - 5 times and germinated on filter paper bridges containing sterile distilled water. Cotyledonary node were excised aseptically from 6 to 7-d-old germinating seeds and inoculated on to Murashige and Skoog's (1962; MS) medium supplemented with different concentrations of N^6-benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (KIN) alone or in combination with PAA or indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). Observations were recorded on a weekly basis. After a 1 month, shoots

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Abbreviation: BAP - N^6-benzylaminopurine; GA_3 - gibberellic acid; KIN - kinetin; MS - Murashige and Skoog; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; PAA - phenylacetic acid ; TDZ - thidiazuron.
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more than 3 cm in length were transferred onto rooting medium. Those less than 3 cm long were cultured on shoot elongation medium containing MS salts and supplemented with various concentrations and combinations of GA_3, BAP and PAA. After 30 d, shoots (4 - 5 cm long) with at least 3 - 4 internodes were excised from shoot clumps and transferred to half-strength MS medium containing different concentration of PAA, IBA and NAA. All the media contained 3 % (m/v) sucrose, pH adjusted to 5.8 and solidified with 0.8 % agar before autoclaving at 121 °C for 20 min. All the cultures were maintained at a temperature of 25 ± 2 °C, 16-h photo-period with irradiance of 50 - 100 μmol m^{-2} s^{-1} provided by cool white, fluorescent lamps. Rooted plantlets were transferred to medium without hormones for two weeks for pre hardening. Finally plantlets were transferred to pots containing sterilized soil and sand in the ratio 3:1. For histological studies, tissues were fixed in formalin, acetic acid and alcohol, dehydrated in a tertiary butyl alcohol series, embedded in paraffin wax (MP 58 °C), sectioned at 10 μm, stained with saffranin and mounted on DPX.

The experimental design was random and factorial with auxins and cytokinins as independent variable. The data pertaining to number of shoot and root formation were subjected to analysis of variance (*ANOVA*). Mean separation was done using Duncan's new multiple range test. Thirty cultures were raised for each treatment and all experiments were repeated thrice.

The age of the explants has been reported to be an important factor in obtaining regeneration in chickpea (Polisetty *et al*. 1997). Fruitful results have been obtained using cotyledonary node taken from 6 to 7-d-old seedlings (Chakraborti *et al*. 2006). In our preliminary experiments shoot-bud were elicited from cotyledonary node explants, cultured on MS medium supplemented with different concentration of BAP (0.5 - 3.0 mg dm^{-3}), KIN (0.5 - 3.0 mg dm^{-3}) and TDZ (0.25 - 1.0 mg dm^{-3}) (data not shown). Cotyledonary node explants as the best source of multiple shoot induction have been suggested in chickpea (Sanyal *et al.* 2005) and bean (Gang and Wei 2009). In earlier reports, IBA was the auxin most frequently used for achieving regeneration in chickpea (Sharma and Amla 1998). In the present study, however, the response to IBA was poorer as it induced callusing along with shoot buds, which hindered further development. Consequently, the shoot buds remained as a crumpled mass or distorted leafy structures, which is a well-known problem encountered during regeneration in chickpea (Batra *et al.* 2002). Conversely, when PAA was added along with BAP into the primary induction medium much better results were obtained. There was no callusing, and a large number of shoot buds were induced (Table 1). PAA at all concentrations induced multiple shoots with varied frequency, but at 1.0 mg dm^{-3} along with BAP (2.0 mg dm^{-3}) yielded the best results (100 % frequency and 44 shoots per explant; Fig. 1*a*,*b*). Multiple shoots were induced better in the presence of PAA followed by IBA; such a response of PAA was also observed by Husain *et al*. 1999 in chilli pepper and Dhaka and Kothari 2002 in sunflower. The direct formation of buds became visible on the proximal end of cotyledonary node after 6 - 8 d of culture, and after 25 - 30 d these buds were proliferates. Between the two

Table 1. Bud organogenesis in cultured cotyledonary node of. *C. arietinum* on MS medium supplemented with BAP along with PAA, IBA or NAA. The experiment was repeated thrice. Means ± SE, $n = 25$. Means followed by same superscript in a column are not significantly different at $P = 0.05$.

PGR	Concentration [mg dm^{-3}]	ICCV- 10 frequency [%]	shoot number [$explant^{-1}$]	Annigeri frequency [%]	shoot number [$explant^{-1}$]
BAP+PAA	2.0 + 0.25	78	35.0 ± 0.57^{d}	80	36.0 ± 0.57^{c}
	2.0 + 0.50	90	41.3 ± 0.88^{b}	95	43.6 ± 1.20^{b}
	2.0 + 1.00	100	44.5 ± 0.57^{a}	100	48.0 ± 0.33^{a}
	2.0 + 1.50	80	38.0 ± 1.20^{c}	78	41.5 ± 0.88^{b}
	2.0 + 2.00	62	21.5 ± 0.68^{g}	53	15.2 ± 0.44^{f}
	2.2 + 2.50	30	5.0 ± 0.86^{j}	38	8.0 ± 0.54^{h}
BAP+IBA	2.0 + 0.25	70	15.9 ± 0.96^{h}	76	19.5 ± 0.45^{e}
	2.0 + 0.50	64	31.0 ± 0.57^{e}	68	36.0 ± 0.78^{c}
	2.0 + 1.00	45	27.0 ± 0.33^{f}	69	30.5 ± 0.48^{d}
	2.0 + 1.50	28	14.6 ± 0.65^{h}	21	11.0 ± 0.59^{g}
	2.0 + 2.00	15	4.4 ± 0.56^{j}	20	6.0 ± 0.78^{i}
BAP+NAA	2.0 + 0.25	30	13.4 ± 1.14^{h}	26	11.0 ± 0.65^{g}
	2.0 + 0.50	50	22.6 ± 0.88^{g}	62	21.6 ± 0.69^{e}
	2.0 + 1.00	19	15.6 ± 1.20^{h}	28	14.0 ± 0.98^{f}
	2.0 + 1.50	15	8.6 ± 0.95^{i}	12	4.0 ± 0.59^{j}
	2.0 + 2.00	11	5.2 ± 1.25^{j}	8	3.5 ± 0.62^{j}

Table 2. Elongation of shoot buds (formed in primary cultures) upon sub-culturing on MS medium supplemented with different plant growth regulators. The experiment was repeated thrice. Means ± SE, $n = 25$. Means followed by same superscript in a column are not significantly different at $P = 0.05$.

Longer than 1 cm			Shorter than 1 cm					
GA_3 [mg dm^{-3}]	frequency [%]	shoot number [$explant^{-1}$]	GA_3+PAA [mg dm^{-3}]	frequency [%]	shoot number [$explant^{-1}$]	GA_3+IBA [mg dm^{-3}]	frequency [%]	shoot number [$explant^{-1}$]
0.25	80	6.0 ± 0.59^{d}	0.2 + 0.1	50	6.0 ± 0.45^{d}	0.2 + 0.1	10	4.0 ± 0.69^{b}
0.50	90	9.5 ± 0.65^{c}	0.2 + 0.2	70	12.5 ± 0.96^{c}	0.2 + 0.2	15	3.5 ± 0.54^{c}
0.75	100	18.6 ± 0.85^{a}	0.2 + 0.4	86	15.0 ± 0.89^{b}	0.2 + 0.4	12	5.0 ± 0.63^{a}
1.00	80	12.5 ± 0.68^{b}	0.2 + 0.6	100	18.5 ± 0.65^{a}	0.2 + 0.6	0	0
1.25	70	5.0 ± 0.23^{d}	0.2 + 0.8	75	11.0 ± 0.76^{c}	0.2 + 0.8	0	0
1.50	50	1.0 ± 0.45^{e}	0.2 + 1.0	50	5.5 ± 0.63^{d}	0.2 + 1.0	0	0

Table 3. *In vitro* rooting of elongated shoots supplemented with different concentration of IBA, NAA and PAA The experiment was repeated thrice. Means ± SE, $n = 25$. Means followed by same superscript in a column are not significantly different at $P = 0.05$.

PGR	Concentration [mg dm^{-3}]	ICCV- 10 frequency [%]	root number [$explant^{-1}$]	Annigeri frequency [%]	root number [$explant^{-1}$]
PAA	0.25	80	4.5 ± 0.58^{d}	90	5.3 ± 0.68^{c}
	0.50	100	10.0 ± 0.68^{a}	100	15.5 ± 0.46^{a}
	1.00	80	7.5 ± 0.74^{b}	100	13.0 ± 0.66^{b}
	2.00	70	5.0 ± 0.76^{d}	75	4.5 ± 0.98^{d}
IBA	0.50	60	6.5 ± 0.66^{c}	50	4.3 ± 0.86^{d}
	1.00	30	3.0 ± 0.89^{e}	40	2.0 ± 0.64^{e}
NAA	0.50	20	2.0 ± 1.00^{f}	30	1.5 ± 0.58^{f}
	1.00	30	3.5 ± 1.20^{e}	25	2.5 ± 1.20^{e}

cultivars, ICCV-10 produced more shoots per culture compared to Annigeri (Table 1) only in some hormone combinations, suggesting genotypic dependency on shoot regeneration as earlier reported in other legumes (Ma and Wu 2008).

Our studies revealed that the morphogenic response of cotyledonary node was asynchronous. A large number of shoot buds differentiated within 3 - 4 weeks in the primary shoot-bud induction medium (PAA + BAP). Three types of shoot formed in the primary culture. Those that were 3 cm or longer and could be directly transferred on the rooting medium. The shoot buds that were 1 cm or longer elongated best on medium supplemented with 0.75 mg dm^{-3} GA_3 in the first subculture (Table 2). The presence of GA_3 in the medium also prevented hyper-hydricity in elongated shoots (Fiore *et al*. 1997). The third category of buds (smaller than 1 cm), were elongated on PAA plus GA_3 and IBA plus GA_3 (Table 2). On medium supplemented with 0.6 mg dm^{-3} PAA + 0.2 mg dm^{-3} GA_3, larger numbers of shoots elongated as compared to medium containing 0.4 mg dm^{-3} IBA + 0.2 mg dm^{-3} GA_3 (Table 2, Fig. 1*c*). This is a pioneering report on the use of PAA for the direct regeneration of chickpea from cotyledonary node cultures. The above results demonstrate that PAA is a very effective plant growth regulator for producing normal, elongated shoots in chickpea cultures, whereas NAA and IBA showed a marked proclivity towards abnormal morphogenesis as earlier reported by Shri and Davis (1992). Small and Morris (1990) and Husain *et al*. (1999) reported that PAA had a positive effect in producing normal, elongated shoots in *Phaseolus vulgaris* and *Capsicum annuum*.

To understand the origin of these shoots, longitudinal sections were taken at 3 - 9 d old regenerated shoots. Microphotographs (Fig. 1*f-i*) indicated that adventitious shoots arose sequentially from the basal peripheral region of an already emerged shoot which was adjacent to a cotyledonary node. Initially, small protuberances emerged on the epidermal cell layer at the basal portion of the already developed shoot (Fig. 1*f*), which later developed into a bulge (Fig. 1*g*) and subsequently differentiated into a shoot (Fig. 1*h*,*i*).

In chickpea, shoot regeneration by organogenesis or somatic embryogenesis has been reported earlier by several authors with limited success in whole plant regeneration. In most of the studies (Kar *et al*. 1996, Polisetty *et al*. 1996, 1997, Subhadra *et al*. 1998, Singh *et al*. 2002) roots were only sporadically observed in regenerated shoots even after prolonged incubation periods. Polisetty *et al*. (1997) suggested a possible inhibitory effect of BAP on *in vitro* root formation in chickpea. In present experiments, elongated shoots were

transferred to ½ strength MS medium supplemented with PAA or IBA or NAA at different concentration (0.25 - 2.0 mg dm^{-3}). The highest rooting frequency and number of roots were observed on medium containing 0.5 mg dm^{-3} PAA followed by 1.0 mg dm^{-3} PAA (Table 3; Fig. 1*d*.). From the results it is evident that PAA is a very effective plant growth regulator for production of normal, elongated whitish roots in chickpea, whereas NAA and IBA showed a marked tendency towards poor rhizogenesis (Table 3). These results are in agreement with Dhaka and Kothari (2002) in sunflower. To our knowledge, such a predictable rooting using PAA has not been reported earlier in chickpea. Half strength MS medium containing PAA elicited more number of roots than full strength MS medium (data not shown).

Regenerants were successfully transferred to polycups containing a mixture of soil and sand in a ratio of 3:1 (Fig. 1*e*) and later to the field with 74 % survival rate. Tissue culture regenerated plants produced normal flowers and set the seeds and no morphological variations were observed.

This protocol has several distinct advantages over the earlier published protocols: *1*) the initial explants, cotyledonary node, is available irrespective of season; *2*) in direct organogenesis time period is reduced by avoiding callus phase; and *3*) PAA is a less expensive than IBA, which offers great economy for routine regeneration. This protocol can be also used in transformation experiments.

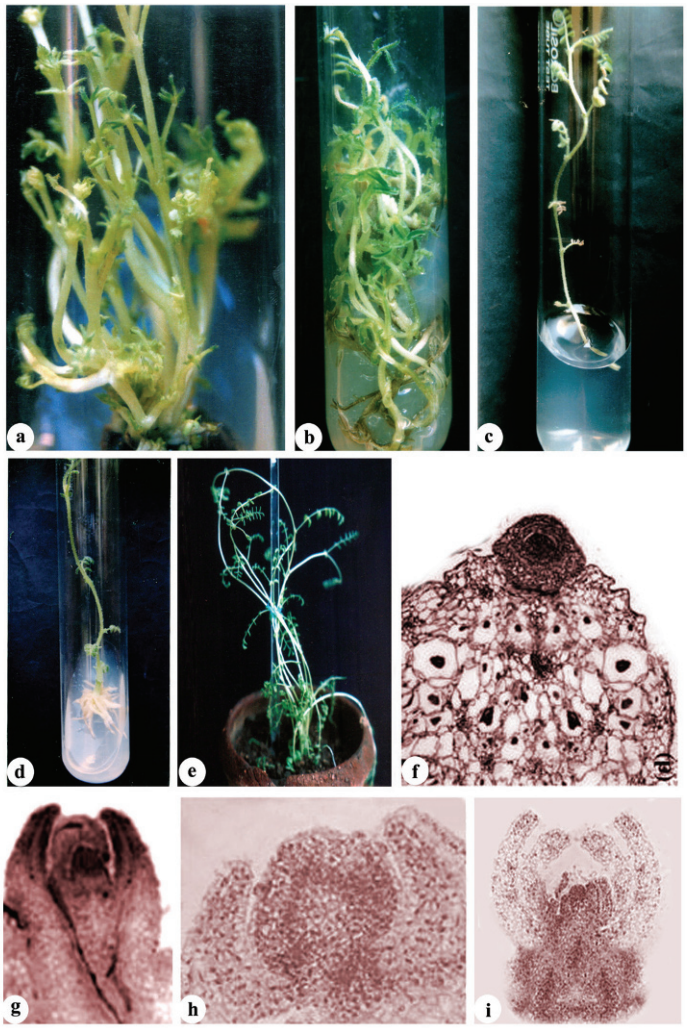

Fig. 1. Regeneration of *Cicer arietinum* from cotyledonary node cultured in the presence of PAA: *a* - multiple shoot buds formed at the proximal portion of cotyledonary node after 2 weeks on medium supplemented with 2 mg dm^{-3} BAP + 1.0 mg dm^{-3} PAA; *b* - proliferation of multiple shoot in primary culture after 4 weeks on medium supplemented with 2 mg dm^{-3} BAP + 1.0 mg dm^{-3} PAA; *c* - elongated shoots in first subculture on MS medium + 0.4 mg dm^{-3} PAA + 0.2 mg dm^{-3} GA_3; *d* - rooting on ½ strength MS medium with 1 mg dm^{-3} PAA; *e* - regenerated plants established in polycups under field conditions; *f* - protuberance appearing on the basal portion of the shoot near the cotyledonary node axis; *g* - *de novo* formation of shoot buds at initial stage of development; *h* - bulge transformed to shoot bud at early stage after 4 - 5 d (bar = 100 μm); *i* - shoot buds surrounded by leaf primordia in longitudinal section.

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