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Short communication. Micropropagation of two Pakistani soybean (*Glycine max* L.) cultivars from cotyledonary nodes

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Abstract

Soybean is an important oilseed crop throughout the world and there are continuous efforts to improve it through various techniques from field to laboratory. Although soybean has been grown in Pakistan since a long period, there are no or limited factors involving its improvement through biotechnological techniques in this country. This study aimed to optimize a regeneration protocol for two soybean cultivars, NARC-4 and NARC-7, using cotyledonary nodes as explant. Cultivar NARC-4 showed higher percentage of regeneration (88%) and mean number of shoots per explant (7.3 shoot per explant) compared to cv. NARC-7 with maximum frequency of 82% shoot regeneration and maximum mean number of 6.4 shoots per explant. However, variants of cyotkinins in the media had variable effects on regeneration and shoot length. Generally 6-benzylamino purine was better compared to zeatin riboside and kinetin. The results showed that half cotyledon could be effectively used as explant for direct micropropagation in soybean. The results could also be exploited positively for *Agrobacterium*-mediated genetic transformation.

Additional key words: 6-benzylamino purine, kinetin, organogenesis, regeneration, shooting response, zeatin roboside.

Resumen

Micropropagación de dos cultivares paquistaníes de soja (*Glycine max* L.) a partir de nodos de cotiledones

La soja es un cultivo oleaginoso de gran importancia en todo el mundo y hay un esfuerzo continuo para mejorarla a través de diversas técnicas desde el campo hasta el laboratorio. Aunque la soja se ha cultivado en Pakistán desde hace mucho tiempo, en este país hay pocos factores implicados en su mejora a través de técnicas biotecnológicas. Este estudio tuvo como objetivo la optimización de un protocolo de regeneración de dos cultivares de soja, NARC-4 y NARC-7, utilizando nodos cotiledonares como explantes. El cultivar NARC-4 mostró un mayor porcentaje de regeneración (88%) y número de brotes por explante (7,3) en comparación con NARC-7, con un máximo de 82% de regeneración de brotes y 6,4 brotes por explante. Sin embargo, diferentes citoquininas en los medios tuvieron efectos variables sobre la regeneración y la longitud de brotes. En general, la 6-benzilamino purina dio mejores resultados que el ribósido de zeatina y la kinetina. Los resultados mostraron que la mitad de los cotiledones podrían ser utilizados eficazmente como explante para la micropropagación directa en la soja. Estos resultados también podrían utilizarse en la transformación genética mediada por *Agrobacterium*.

Palabras clave adicionales: 6-benzilamino purina, kinetina, organogénesis, regeneración, respuesta de los brotes, ribósido de zeatina.

Soybean (*Glycine max L.*) is a legume that grows in tropical, subtropical, and temperate climates. It was originally domesticated in China around 1700-1100 B.C., soybean is now cultivated throughout East and Southeast Asia. It is also cultivated in Brazil and to a very limited extent in sub-Saharan Africa and West Asia, South Asia. Demand for soybean remains strong and continues to grow because it is used as an ingredient in the formulation of a multitude of food, feed and industrial products. In addition, soybean is a primary

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Abbreviations used: 2,4-D (2,4 dichloro phenoxy acetic acid), B5 (Gamborg B5), BAP (6-benzylamino purine), IBA (indole 3-butyric acid), Kin (kinetin), MES (4-morpholine etanesulfonic acid), MS (Murashige and Skoog), NARC (National Agriculture Research Center), TDZ (thidiazuron), ZTR (zeatin roboside).

source of high-value secondary co-products such as lecithin, vitamins, nutraceuticals and anti-oxidants. Its seeds are the dominant oil-seeds in world trade, accounting for about 56% of global oilseed production. The U.S., Brazil and Argentina are the predominant soybean producing countries (Wilson, 2008).

Soybean breeders are using different approaches including conventional techniques to achieve desired improvements throughout the world; however, most of these approaches have their own limitations. Therefore, it is very important to supplement conventional breeding techniques with plant biotechnology. Several tissue culture studies have been done for effective improvement of soybean. Reviews of previous studies show genotype, explant, age of explant, combination and concentration of plant growth regulators affect regeneration from *in vitro* cultures of soybean. Tissue culture has been reported using immature cotyledons (Lippmann and Lippmann, 1984; Li *et al*., 1985; Parrott *et al*., 1988; Santarem *et al*., 1997; Bonacin *et al*., 2000; Ko and Korban, 2004), hypocotyls and ovaries (Beversdorf and Bingham, 1977), primary leaf tissues (Wright *et al*., 1987; Droste *et al*., 1993), hypocotyls and epicotyl (Rajasekaran and Pellow, 1997; Reichert *et al*., 2003) and cotyledonary nodes of few days germinated seedlings (Christianson *et al*., 1983; Wright *et al*., 1986). Similarly, Cheng *et al*. (1980) were able to get multiple shoot bud formation from cotyledonary nodes on medium containing high concentrations of 6-benzylamino purine (BAP) but bud growth improved when the cultures were transferred to low concentrations of BAP. Multiple shoots were also observed from mature embryos when cultured in the presence of BAP (Buising *et al*., 1994). They reported that by the treatment of BAP, the cells in embryonic axes do not remain quiescent and are reprogrammed to produce multiple somatic foci. However, addition of thidiazuron (TDZ) (Franklin *et al*., 2004; Shan *et al*., 2005) or kinetin (Ma and Wu, 2008) with BAP has been reported as better combination for embryoid formation from cotyledonary node explants. Radhakrishnan and Ranjithakumari (2007) produced callus from mature half seed's nodal segment using different combinations of BAP and 2,4 dichloro phenoxy acetic acid (2,4-D). The callus produced a number of plants when cultured on Gamborg B5 medium supplemented with BAP while addition of 2,4-D enhanced somatic embryo production.

The study aimed to develop an efficient regeneration system for two Pakistani soybean cultivars NARC-4 and NARC-7. The seeds of NARC-4 and NARC-7 were collected from National Agriculture Research Center (NARC) Islamabad, Pakistan. Micropropagation of the two cultivars was achieved using mature soybean cotyledonary node (half seed) following Paz *et al*. (2006) with some modifications.

The healthy seeds were separated and washed under running tap water. These seeds were surface sterilized with 0.1% (w/v) mercuric chloride for 5 min under aseptic conditions followed by three rinses with autoclaved distilled water. The seeds were imbibed with sterilized water for 16 hours in Petri plates in dark at room temperature (25°C). Thereafter, the seed coat was removed and they were vertically bisected along the hilum to separate the cotyledons. This made the mature seed cotyledonary node exposed and each seed produced two explants.

For direct organogenesis, whole procedure was divided into four steps:

i) Resting stage (Stage I)

The explants (bisected soybean seeds) were inoculated on the medium containing Gamborg B5 medium (Gamborg *et al*., 1968) supplemented with 3% sucrose, 3.9 g L^{-1} 4-morpholine ethanesulfonic acid (MES). The media was solidified with 0.7% noble agar (Sigma) after adjusting pH to 5.7. Filter sterilized B5 vitamins and 1.0 or 2.0 mg L^{-1} 6-benzylamino purine (BAP); kinetin or zeatin roboside (ZTR) were added after autoclaving at 121°C, under pressure of 115 psi for 20 min. In each Petri plate (100 × 15 mm), 10-15 mL media was poured and six explants were cultured with abaxial side touching the media in Petri plates. The plates were incubated in the growth room for 5 days.

ii) Shoot induction stage (Stage II, SI I-II)

Thereafter, the explants were transferred to shoot induction medium containing Gamborg B5 medium and 3% sucrose, 0.59 g L^{-1} MES and 0.7% agar. pH was adjusted at 5.7 before autoclaving. Gamborg B5 vitamins and 1.0 or 2.0 mg L^{-1} BAP; kinetin or ZTR were added after autoclaving. Each Petri plate contained 5-6 explants cultured with adaxial side touching the media. The media was refreshed after 14 days and a fresh cut was made at the base of each explant at each transfer.

iii) Shoot elongation stage (Stage III, SE I-IV)

Thereafter, the explants were transferred to MS medium (Murashige and Skoog, 1962) for shoot elongation containing 3% sucrose, 0.59 g L^{-1} MES and 0.7% agar (pH 5.7). Filter sterilized 1.0 or 2.0 mg L^{-1} BAP; Kinetin or ZTR, B5 vitamins, asparagine (50 mg L^{-1}), L-pyroglutamic acid (100 mg L^{-1}) were added after autoclaving. The media was dispensed in Magenta vessels. The explants were subcultured on fresh medium for 4×14 days by making a cut at the base of each explant at each subculture.

All *in vitro* cultures were incubated in growth chamber at 23 ± 1°C, in 16/8 h light/dark photoperiod under 10,000 lux illuminated with white florescent light (Philips).

iv) Rooting (Stage IV)

When the regenerated shoots attained height 3-4 cm, they were excised and rooted on half strength MS macro, micro elements and vitamins, 0.59 g L^{-1} MES, 2% sucrose supplemented with 0, 1 and 5 mg L^{-1} of indole 3-butyric acid (IBA) solidified with 0.7% agar at pH 5.7.

The rooted plants (2-3 roots or 3-4 cm long root) were transferred to plastic pots containing sterilized clay and sand (1:1). The pots were completely covered with transparent polythene bags to retain high humidity and were kept in growth room for 10-14 days. The plants were watered with Hoagland solution when required. Thereafter, the polythene bags were gradually removed and the plants were transferred to soil containing clay and manure (2:1) in clay pots and shifted to greenhouse.

For each experiment, 100 explants were regenerated per treatment. The percentage response, number of shoots per explant and shoot length was recorded after 60-80 days of first culture and analyzed by ANOVA using Statistical Analysis System package (SAS Institute v. 9.1). The data about rooting was recorded after 30 days of culture. The means were further analyzed using LSD or t test at probability level $P < 0.05$.

It was observed that half cotyledonary explants of cv. NARC-4 induced more number of shoots per explant with high frequency of regeneration on medium containing BAP and ZTR compared to cv. NARC-7.

Both cultivars showed improved frequency of shoot regeneration, number of shoots per explant and mean shoot length with 1 mg L^{-1} BAP, kinetin and ZTR. However, the best frequency of shoot regeneration and number of shoots per explant was obtained on 1 mg L^{-1} BAP. Contrarily this concentration of BAP had inhibitory effect on shoot length. The maximum shoot length was obtained on 1 mg L^{-1} ZTR.

Maximum frequency of 88 and 84% shoot regeneration and the highest number of 7.3 and 7.1 shoots per explant on cv. NARC-4 was recorded on culture medium containing 1.0 mg L^{-1} either BAP or ZTR, respectively. Maximum shoot regeneration frequency of 82 and 76% and the highest number of 6.4 and 5.8 shoots per explant on cv. NARC-7 was recorded on culture medium containing 1.0 mg L^{-1} BAP or 1.0 mg L^{-1} ZTR, respectively (Fig. 1). However, both BAP and kinetin had inhibitory effect on shoot length compared to ZTR. Maximum shoot length of 3.08 and 2.95 cm was recorded in cvs. NARC-4 and NARC-7, respectively on regeneration medium containing 1 mg L^{-1} ZTR. Irrespective of the cultivar, increasing the concentration from 1.0 mg L^{-1} to 2.0 mg L^{-1} of BAP, kinetin or ZTR was inhibitory

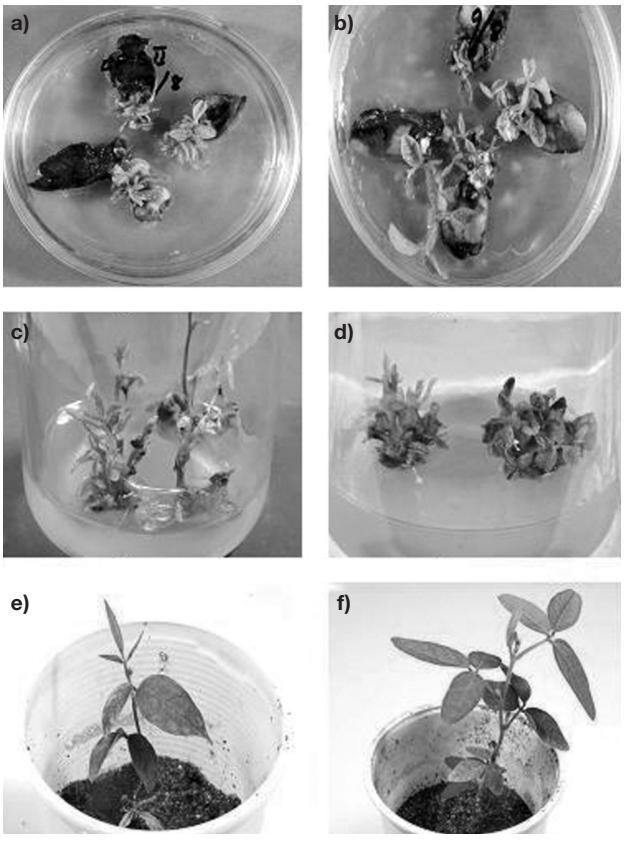

Figure 1. *In vitro* shoot regeneration (a) from nodal portions of cv. NARC-7, (b) from NARC-4 on MS medium containing 1.0 mg L^{-1} BAP at stage II. Shoot elongation in (c) cv. NARC-7 on MS medium containing 1.0 mg L^{-1} kinetin at stage II, and (d) cv. NARC-4 on MS medium containing 1.0 mg L^{-1} ZTR at stage II. Acclimatization of (e) cv. NARC-7 and (f) cv. NARC-4.

Table 1. Effects of different cytokinins on shoot regeneration from cotyledonary nodal explants of soybean cv. NARC-4 and NARC-7

Conc. (mg L^{-1})	Frequency (%) of shoot regeneration			No. of shoots per explant			Mean shoot length (cm)		
	BAP[1]	Kin[2]	ZTR[3]	BAP	Kin	ZTR	BAP	Kin	ZTR
NARC-4									
1.0	88^{aA}	71^{aB}	84^{aA}	7.3^{aA}	3.4^{aB}	7.1^{Aa}	2.81^{aB}	1.89^{aC}	3.08^{Aa}
2.0	68^{bB}	60^{bB}	80^{aA}	2.9^{bB}	1.2^{bC}	3.4^{bA}	2.17^{bB}	1.43^{bC}	2.87^{Aa}
NARC-7									
1.0	82^{aA}	68^{aB}	76^{aB}	6.4^{aA}	4.0^{aB}	5.8^{aA}	2.74^{aB}	5.8^{aA}	2.95^{aB}
2.0	54^{bB}	56^{bB}	77^{aA}	1.7^{bB}	1.0^{bB}	3.3^{bA}	2.11^{bB}	3.3^{bA}	2.36^{bB}

[1] BAP (6-benzylamino purine). [2] Kin (Kinetin). [3] ZTR (zeatin roboside). Mean values within a column for each block of cv. NARC-4 and NARC-7 followed by different small letters are significantly different at the 0.05 probability level using t test. Mean values within a row followed by different capital letters are significantly different at the 0.05 probability level using LSD test.

and decreased regeneration percentage, number of shoots per explant and shoot length in both cultivars (Table 1).

The results showed reduced rooting in cvs. NARC-4 and NARC-7 on half strength MS medium without vitamins (Control). However, 1 mg L^{-1} IBA was more favorable compared to 5 mg L^{-1} IBA for induction of roots. The results showed 76.3 and 55.3% rooting and a number of 4.7 and 2.2 roots per explant in cvs. NARC-4 and NARC-7, respectively (Table 2).

The results showed that regeneration was genotype dependent and affected by the concentration of cytokinins in the regeneration medium. It was further observed that, reduced concentration of cytokinin had promotory and increased concentration had inhibitory effect on shoot regeneration. ZTR was better compared to other two cytokinins in general. Previous reports suggest use of ZTR for shoot elongation during *Agrobacterium* mediated transformation (Zhang *et al*., 1999; Liu *et al*., 2004; Paz *et al*., 2006; Olhoft *et al*., 2007). It was observed that exogenous cytokinin application alters axillary meristem development, promotes shoot proliferation and regeneration of the cell in meristemic tissues. This phenomenon has been reported previously in cytokinin pretreated seedlings (Shan *et al*., 2005). The results reported in this study are also in agreement with previous studies suggesting soybean organogenesis by continuous culture on BAP containing medium (Wright *et al*., 1987) or by culturing in alternative cycle of BAP and TDZ (Barwale and Widholm, 1990) or combination of BAP with auxin mainly 2,4-D or NAA (Tripathi and Tiwari, 2003; Radhakrishnan and Ranjithakumari, 2007) or ZTR for plant regeneration (Kamenicka *et al*., 1998; Steinitz *et al*., 2006; Barik *et al*., 2007) either through organogenesis, callogenesis or protoplast culture.

Present findings conclude that cotyledonary node explants of soybean can be efficiently used for plant regeneration under *in vitro* conditions. Moreover, the plant regeneration protocol could be efficiently used for *Agrobacterium* mediated genetic transformation of these and other soybean cultivars.

Table 2. Effect of indole 3-butyric acid (IBA) on root induction of soybean cultivars NARC-4 and NARC-7

IBA (mg L^{-1})	NARC-4		NARC-7	
	Percentage roting	No. of roots/shoot	Percentage rooting	No. of roots/shoot
0.0	28.8	2.3^{b}	34.2	1.8^{b}
1.0	76.3	4.7^{a}	55.3	2.2^{a}
5.0	47.0	4.4^{a}	32.5	1.4^{c}

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using LSD test.

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