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HORMONAL EFFECTS ON *IN VITRO* REGENERATION OF *RICINUS COMMUNIS* L. CULTIVAR SHABJE

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Abstract

Context: Castor is the best candidate plant to the venture of green energy sector. In Bangladesh castor is widely grown and we can reap the benefit from castor by the application of different biotechnological approaches. Genetic engineering appears as one of the necessary tools for the improvement of cultivars of castor. Therefore, an efficient regeneration protocol for castor needs to be established, which is rapid, reproducible and applicable to a broad range of genotypes.

Objectives: To establish a high frequency plant regeneration system from the seedling explants of *Ricinus communis* L. cultivar shabje using tissue culture technique.

Materials and Methods: The proliferating axillary shoots were established on modified MS medium supplemented with different concentrations of BA, Kn and 2ip using explants from the *in vitro* grown seedlings of *R. communis*.

Results: Explants from the seedlings raised on cytokinin enriched medium showed early sprouting and development of axillary shoots than those taken from the seedlings grown on cytokinin omitted medium. Shoot proliferation efficiency of cotyledonary node explant was noted two times greater than that of shoot tip explant. MMS_1 medium fortified with 1.5-2.5 mg/l BA showed the best performance and yield 5-10 axillary shoots within 6 weeks of culture. Microcuttings prepared from *in vitro* proliferated shoots were rooted on MMS_2 medium. The best rooting (92.7%) obtained on ½MS fortified with 0.2 mg/l of IBA accompanied by 0.6 mg/l of $AgNO_3$.

Conclusion: Best media formulation for shoot proliferation in *Ricinus* cultivar was MS medium fortified with 2.0 mg/l BA and its recalcitrant property could be minimized in some extent by using of $AgNO_3$ and charcoal in case of root induction.

Keywords: *In vitro* regeneration, seedling explants, *Ricinus communis*.

Introduction

Castor (*Ricinus communis* L.) is a member of the family Euphorbiaceae or spurge family, mostly native of the tropical and subtropical regions (Weiss 2000). It is medicinally important oil seed crop containing approximately 60% oil (Kumari *et al*. 2008) and is the only commercial source of ricinolic acid that is used as industrial lubricants, paints, coatings, and plastics (Moshkin 1986, Caupin 1997). In world trade, India is the largest castor oil producer, representing 60% of production, followed by China and Brazil (Weiss 2000, FAO 2006).

Its cultivation poses serious health concerns due to the presence of the ricin toxin (Lord *et al*. 1994, Hartley and Lord 2004) and hyperallergenic 2S albumins in its seeds (Pastorella *et al*. 2001, Shewry *et al*. 2002) which restrict its commercial cultivation in USA (Ahn *et al*. 2007). Ricin toxin inactivates ribosomes by cleaving the *N*-glycosidic bond between an adenine and ribose in 28S rRNA, thus inhibiting protein synthesis (Endo and Tsurugi 1987). 2S albumin was identified as the major allergen of castor seed a total of 96% castor sensitive patients had the lgE antibody that was specific to the 2S storage albumin (Thorpe *et al*.1988).

Most of the world's castor cultivars are vulnerable to insect attack. Reliable sources of resistance to the major insect pests are rather limited in the available germplasm of this monotypic genus (Sujatha and Sailaja 2005). Genetic engineering appears as one of the necessary tools for the improvement of cultivars of this species to reduce the levels of these hazardous proteins. Therefore, an efficient regeneration protocol for castor needs to be established, which is rapid, reproducible and applicable to a broad range of genotypes.

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However, castor is extremely recalcitrant to *in vitro* regeneration (Ahn *et al*. 2007). The previous reports on *in vitro* shoot multiplication of castor (Athma and Reddy 1983, Reddy *et al*. 1987, Sangduen *et al*. 1987, Reddy and Bahadur 1989, Sarvesh *et al*. 1992) using vegetative tissue as explants was either inefficient or difficult to reproduce (Ahn *et al*. 2007). Then researchers started to focus on meristematic tissues to improve regeneration efficiency (Molina and Schbert 1995, Lakshmi and Bhadur 1997, Sujatha and Reddy 1998). Sujatha and Reddy (1998) reported the first reliable protocol using embryonic tips and shoot apex. However, all the shoots were derived from the preexisting meristem, and adventitious shoot formation was not observed. When applied in *Agrobacterium* mediated transformation, this protocol resulted in a low rate of putative transformant recovery (0.08%, one putative transformant in, 1,200 embryonic tips used (Sujatha and Sailaja 2005). The transformation efficiency of meristem based protocol was also developed (McKeon and Chen 2003), but the efficiency was also very low. Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques which would widen the possibilites of developing transgenic lines and/or somaclonal variants. The present investigation was, therefore, undertaken to establish a high frequency plant regeneration system from the seedling explants of a local *R. communis* cultivar shabje using tissue culture technique.

Materials and Methods

The explants used in this investigation were collected from 4 week old seedlings raised *in vitro* on MS medium. Seeds were collected from the research field of Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh. Seeds were washed throughly under running tap water and then treated with 1% savlon supplied by ACI and 2-3 drop of Tween-80 for about 10 min. This was followed by successive three washing with distilled water to make free the seeds from savlon and Tween-80, surface sterilization was carried out with 0.1% $HgCl_2$ for 6-7 min followed by gentle shaking. After this treatment, the seeds were rinsed 4-5 times in sterile distilled water to make free the seeds from $HgCl_2$. Sterilized seeds were partially decoated and aseptically germinated in glass bottle containing 50 ml semisolid MS (Murashige and Skoog 1962) medium either with or without 1 mg/l BA. The aseptic seedlings attained a height of 8-10 cm within 3-4 weeks under incubation. Two types of explants (1 cm shoot segments) consisting of cotyledonary node and shoot tips were prepared from *in vitro* raised seedlings under completely sterile condition and cultured on different nutrient media containing cytokinin for proliferating axillary shoots. Following sequential subculturing and multiplication of primary culture, a stock of axillary shoot cultures was raised. For *in vitro* rooting of shoots, 1-3 cm long microcuttings were prepared from *in vitro* proliferated shoots. Rooting was induced by culturing microcuttings on media containing different concentrations of auxins.

For axillary shoot proliferation MMS_1 (MS with half strength of macronutrients and full strength of micronutrients and vitamins) media supplemented with three types of cytokinins were used. For root formation in the *in vitro* regenerated shoots, only MMS_2 (MS with half strengths of both macro and micronutrients and full strength of vitamins) with different concentrations and combinations of auxins and additives were used. The media were supplemented with 20 g/l of sucrose and gelled with 8 g/l of agar (BDH). The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at 121ºC under 1.1 kg/cm^2 pressure for 20 minutes. Unless otherwise mentioned, all cultures were maintained in growth room under 16 h photoperiod with a light intensity of 2000-3000 Lux (50-70 mE. m^{-2}. S^{-1}).

Results

Proliferation of shoots: In vitro grown 12-15 mm long axillary shoot segments consisting of either cotyledonary node or shoot tips were cultured on proliferation MS medium fortified with only one concentration (1mg/l) of BA. Seedlings grown on BA-containing medium were morphologically characterized

by thick and bushy shoots. Both types of explants from such seedlings produced axillary shoots but the growth of axillary shoot was about double in medium containing 1 mg/l BA (Table 1). The overall proliferation efficiency of cotyledonary node explants (Fig. A) was significantly higher than that of shoot tip explants when evaluated after 6 weeks of culture. Therefore, only cotyledonary node segments from seedlings grown on BA containing medium were used in subsequent proliferation experiments for assessing optimum cytokinin concentration. However, for the maintenance and further multiplication of proliferating cultures, nodal segments from *in vitro* proliferated shoots were cultured in this optimum concentration of cytokinin.

Table 1. Axillary shoot proliferation from two types of explants from seedlings grown on medium with or without 1 mg/l of BA. Data (mean ± SE) were recorded after 6 weeks of culture of the explants on MMS_1 medium containing 1 mg/l of BA. Each treatment consisted of 15-20 explants.

Explant type	% of explant showing proliferation	Total shoot per culture	*No. of usable shoot per culture	Days to sprout the axillary buds
Seedlings raised on medium with BA				
Cotyledonary node	100.0	9.5±1.23	5.5±0.92	5-7
Shoot tip	81.7	4.6±0.75	2.7±0.65	7-10
Seedlings raised on medium without BA				
Cotyledonary node	85.8	6.5±0.85	3.3±0.31	8-12
Shoot tip	61.0	3.3±0.47	2.1±0.32	12-14

*Shoots length ≥ 10 mm were considered only.

Table 2: Proliferation of axillary shoots from cotyledonary node segments of seedlings grown on medium with BA. Data (mean ± SE) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 culture.

Concentrations of growth regulators (mg/l)		% of explants showing proliferation	total shoot / culture	* No. of usable shoot / culture	Average length of shoot (mm)	Days to sprout the axillary buds
BA	0.5	75.0	4.7±0.91	2.2±0.68	19.0±3.0	8-12
	1.0	82.0	6.0±0.95	2.7±0.91	20.1±3.0	8-12
	1.5	100.0	7.4±1.16	3.9±0.95	22.0±3.2	5-10
	2.0	100.0	9.4±1.23	5.3±1.10	26.2±4.3	5-7
	2.5	100.0	8.5±1.22	4.8±1.00	24.1±3.5	5-7
	5.0	-	-	-	-	-
Kn	0.5	70.2	3.0±0.65	1.5±0.27	16.1±1.8	10-12
	1.0	75.1	3.9±0.87	2.4±0.63	17.5±2.1	10-12
	1.5	90.0	5.1±0.92	3.5±0.71	20.1±2.6	8-12
	2.0	100.0	7.5±1.23	4.1±0.91	22.3±3.5	5-10
	2.5	92.3	6.3±1.18	3.6±0.88	20.1±3.0	5-10
	5.0	-	-	-	-	-
2ip	0.5	62.0	2.8±0.37	1.4±0.18	14.2±1.3	12-14
	1.0	68.3	3.2±0.34	2.1±0.23	15.4±1.6	10-12
	1.5	72.6	4.0±0.91	2.3±0.27	18.2±1.5	10-12
	2.0	78.9	5.8±0.91	3.4±0.68	21.0±1.9	8-12
	2.5	75.5	5.7±0.92	3.3±0.69	20.6±1.8	8-12
	5.0	-	-	-	-	-

*Shoots length ≥ 10 mm were considered only

Observations after 6 weeks following one subculture on MMS_1 supplemented with different concentrations (0.5-5.0 mg/l) of BA, Kn and 2ip indicated that fifteen concentrations successfully produced axillary shoots and three concentrations failed to produce any axillary shoot (Table 2).Three of the treatments showed enhanced proliferation of axillary shoots (Fig. B&C). Satisfactory performance was observed in the media supplemented with 1.5, 2.0 and 2.5 mg/l BA and the media containing 2.0 mg/l Kn. In all the above cases, 100% of the cultured explants produced shoots. Among them, the total number of shoot per culture, number of usable (length 1 cm) shoot per culture and the average length of shoot per culture were the highest on the medium supplemented with 2.0 mg/l BA. But in case of Kn, the total number of shoot per culture, number of usable shoot per culture and average length of shoots per culture were also the highest on the medium

containing 0.2 mg/l of Kn. Among the different concentrations of 2ip, the percentage of explants showing proliferation was the highest 78.9% in 2.0 mg/l. The total number of shoot per culture, number of usable shoot per culture and average length of shoots per culture were also the highest on the same hormone concentration. From overall observations, it was clear that cotyledonary node explants were more responsive and BA found more effective on axillary shoot proliferation in *R. communis* cultivar shabje.

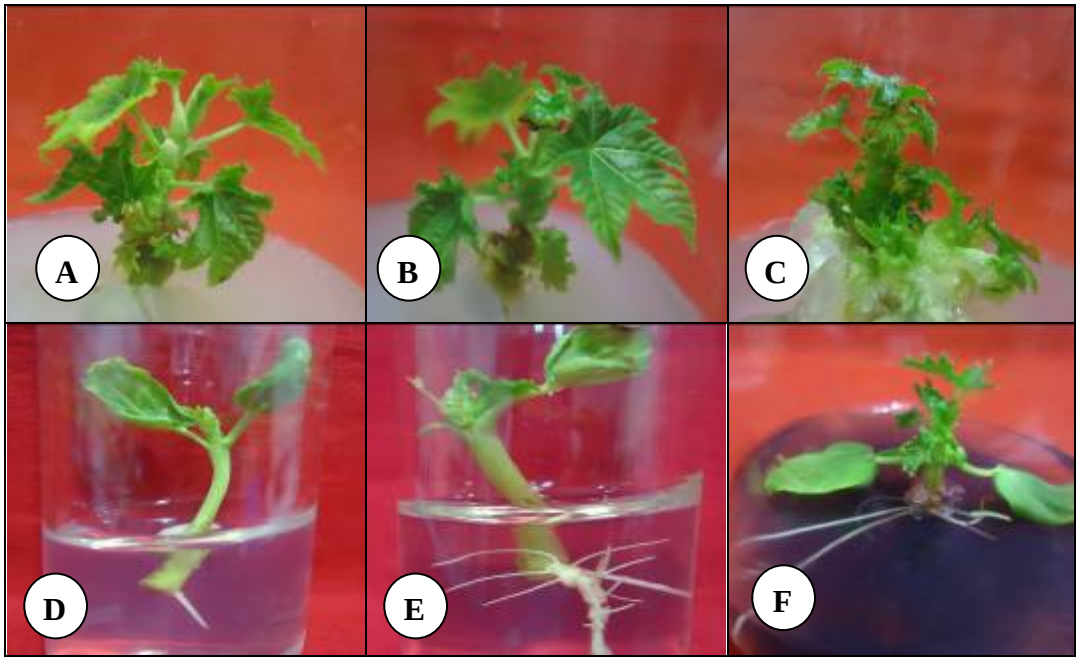

Fig. 1. *In vitro* plantlet regeneration from the seedling explants of *Ricinus communis* L. cultivar shabje; A. Proliferation of axillary shoots from a cotyledonary node explant cultured for 4 weeks on 2.0 mg/l BA containing medium; B. Proliferating axillary shoots formed on 2.0 mg/l BA supplemented media after 6 weeks of culture; C. A clump of proliferating axillary shoots mass formed after 8 weeks of culture on 2.0 mg/l of BA. D. & E. Root formation from microcuttings cultured on medium containing 0.2 mg/l IBA with 0.6 mg/l $AgNO_3$ after 3 and 5 weeks of culture respectively. F. Rooting of a microcutting cultured on a medium containing 0.2 mg/l of IBA with 150 mg/l of activated charcoal.

Rooting of shoots: Root induction performance was tested in microcuttings (1-3 cm) prepared from *in vitro* grown shoots. They were cultured in 25x150 mm tubes containing MMS_2 medium supplemented with different concentrations (0.1-1.0 mg/l) of IAA, IBA and NAA, with various concentrations of $AgNO_3$ and activated charcoal for inducing adventitious roots. Of the three auxins tested singly, observations after 5 weeks of incubation revealed that percentage of cutting rooted, the number of roots per microcutting, the average length of root and callus formation at the microcutting bases increased gradually with increasing levels of auxins in the media ranging 0.1-0.4 mg/l. Further increase in the level of auxin did not produce any improvement of rooting instead, profuse basal callusing was observed (Table 3). NAA tended to produce callus at the microcutting bases; the effect was maximum on 0.8-1.0 mg/l NAA supplemented media accompanied by malformation of roots. Contrary to that, no callus formation and no malformed roots were found in microcuttings rooted on media supplemented with 0.1-0.4 mg/l IBA (Fig. D) and in the same concentrations of IAA. However, slight callusing was found in microcuttings rooted on the media supplemented with 0.6-0.8 mg/l IBA and 0.6 mg/l IAA and considerable callusing when the medium contained 1.0 mg/l IBA and 0.8 mg/l IAA. But when 0.2 mg/l IBA was used with $AgNO_3$ and activated

charcoal in various combination, the percentage of cuttings rooted was the highest 92.3% (Table 4) and average length of roots was also found highest in 0.6 mg/l $AgNO_3$ containing medium (Fig. E). The number of roots per cuttings was the highest in 200 mg/l activated charcoal containing medium with 0.2 mg/l IBA (Fig. F). No malforming root was observed in 0.1-0.6 mg/l $AgNO_3$ and activated charcoal containing media rather, slight callusing was found in microcuttings rooted on the media supplemented with 0.8-1.0 mg/l $AgNO_3$ (Table 4). Above all, adventitious root formation performance of IBA proved best than that of NAA and IAA. Among the concentrations of auxins tested, 0.2 mg/l of IBA with 0.6 mg/l $AgNO_3$ was found to be the best for inducing adventitious roots on *R. communis* cultivar shabje.

Table 3. Effect of different concentrations of auxins on adventitious root formation of microcuttings. Data (mean ± SE) were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/l)	% of cutting rooted	No. of roots /rooted cutting	Average length of roots (mm)	Days to root emergence	* Callus formation
IAA	0.1	17.5	1.4±0.17	19±1.7	10-12	-
	0.2	34.7	2.5±0.34	22±2.1	10-12	-
	0.4	26.2	2.3±0.21	20±2.0	10-12	-
	0.6	15.3	2.2±0.16	1.7±1.5	12-15	+
	0.8	9.4	2.1±0.18	12±1.2	15-20	++
	1.0	-	-	-	-	++
IBA	0.1	44.7	2.7±0.39	23±2.0	7-10	-
	0.2	85.9	4.7±0.52	31±3.2	7-10	-
	0.4	78.5	4.0±0.41	26±2.8	7-10	-
	0.6	72.1	3.5±0.32	25±2.2	10-12	+
	0.8	43.0	2.3±0.19	17±1.5	10-12	+
	1.0	21.2	1.5±0.17	12±0.9	12-15	++
NAA	0.1	28.4	2.3±0.26	19±1.3	10-12	+
	0.2	52.7	3.5±0.25	24±1.9	10-12	+
	0.4	44.3	3.2±0.30	23±1.9	12-15	++
	0.6	35.7	2.6±0.35	20±1.1	12-15	++
	0.8	21.0	2.0±0.21	16±1.0	15-20	+++
	1.0	10.6	1.3±0.19	11±0.9	15-20	+++

* Intensity of callusing: - indicates no response, + slight callusing, ++ considerable callusing, +++ profuse callusing

Table 4 Effect of activated charcoal and $AgNO_3$ with IBA on adventitious root formation of microcuttings. Data (mean ± SE) were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Conc. (mg/l)	% of cuttings rooted	No. of roots /rooted cuttings	Average length of roots (mm)	Days to root emergence	* Callus formation
IBA + $AgNO_3$	0.2+0.1	85.0	4.9±0.63	32±3.0	7-10	-
	0.2+0.2	87.2	5.1±0.75	35±3.7	7-10	-
	0.2+0.4	88.6	5.4±0.81	39±3.9	5-8	-
	0.2+0.6	92.7	5.8±0.95	42±4.2	5-8	-
	0.2+0.8	90.3	5.5±0.77	40±4.1	5-8	+
	0.2+1.0	86.1	5.2±0.69	35±3.6	7-10	+
IBA + AC	0.2+ 25	75.3	6.1±0.52	26±2.1	7-10	-
	0.2+ 50	79.2	6.5±0.49	28±2.3	7-10	-
	0.2+100	83.6	7.0±0.53	31±2.8	6-8	-
	0.2+150	85.2	7.9±0.72	38±3.9	6-8	-
	0.2+200	80.1	7.3±0.61	35±3.7	6-8	-
	0.2+250	79.4	7.0±0.69	36±3.6	6-8	-

* Intensity of callusing: - indicates no response, + slight callusing

Discussion

The comparative *in vitro* response of the two explants of *R. communis* was evaluated, the cotyledonary node and shoot tip were used as explants and grown in the same MS medium supplemented with cytokinin. In

comparison to shoot tip, better performance was noted for cotyledonary node in all parameters like percentage of proliferated explants, number of total shoot per culture, number of usable shoot per culture and days to sprout the axillary buds. Better performance for cotyledonary node was also reported by Dong and Jia (1991) in the culture of watermelon. Differential response of various explants from the same plant is considered to be specific (Debergh and Read 1990) rather than due to variation in the indigenous hormonal level of buds present in different regions of the stem as suggested by Lane (1978). Shoot tip is well known juvenile tissue holding the highest potential of *in vitro* regeneration for some other plants but in *R. communis* cotyledonary explant proved to be best efficient in regeneration performance in respect to shoot tip.

Explants taken from the seedling grown in cytokinin enriched medium showed early sprouting of axillary buds and better shoot development. Better performance of cytokinin in developing axillary bud and shoot development was also reported in monkey jack (Rahman and Amin 1995). Seedlings growing in the medium supplemented with cytokinin had the better efficiency in producing shoot development. Cytokinin stimulates cell division, shoot initiation and bud formation in tissue culture of plant. In *R. communis* performance of cytokinin in shoot proliferation also evaluated. Three cytokinins were tested like BA, Kin. and 2ip on cotyledonary node explant for its proliferation. Among the three cytokinins tested BA was found superior to Kn. and 2ip in shoot proliferation in *R. communis.* Similar effect for better performance of BA in shoot proliferation was also reported in some other cultivar of castor (Alam *et al*. 2010). It has been mentioned that BA is the cytokinin of choice at a concentrations around 1 mg/l for producing axillary shoots from explants of most of the plants (Hutchinson 1981, Litz *et al*. 1985, Litz and Jaiswal 1990). The present experiment similarly established that BA is most efficient cytokinin in shoot proliferation in local cultivar of castor.

Root induction in artificial media is very difficult in castor as the plant was denoted as recalcitrant by several authors (Ahn *et al*. 2007). Under the present investigation tremendous efforts has been made to produce root induction under different hormone treatments. IBA, IAA and NAA were tested in different concentrations but IBA was found to be more effective than NAA and IAA. Similar superior performance of IBA was observed during root induction in castor (Kumari *et al.* 2008) and watermelon (Ahad *et al*. 1994). Hutchinson (1981) and Litz and Jaiswal (1990) emphasized that the preferred auxin for adventitious rooting is IBA. But when $AgNO_3$ and activated charcoal were added with IBA, the root formation was comparatively better than IBA used singly. Similar effect was also reported in castor (Kumari *et al*. 2008) when they used $AgNO_3$.

Conclusions

Best media formulation for shoot proliferation in *R. communis* cultivar shabje was MS medium fortified with 2.0 mg/l BA and its recalcitrant property could be minimized in some extent by using of $AgNO_3$ and charcoal in case of root induction.

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