Somatic organogenesis and plant regeneration in *Ricinus communis*

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Abstract

An *in vitro* propagation system was developed for castor-bean (*Ricinus communis* L. cv. TMV 6) through cotyledon derived callus cultures. The impact of different concentrations of auxins, cytokinins, additives, amino acids and sugars were evaluated for callus induction and shoot proliferation. Green compact nodular organogenic callus was obtained on the medium fortified with Murashige and Skoog (MS) salts, B5 vitamins, 2.0 mg dm^{-3} 6-benzyladenine and 0.8 mg dm^{-3} α-naphthalene acetic acid (NAA). Multiple shoot proliferation from the callus cultures was achieved on the medium with MS salts, B5 vitamins, 2.5 mg dm^{-3} thidiazuron (TDZ), 0.4 mg dm^{-3} NAA and 15 mg dm^{-3} glutamine. During multiple shoot induction the phenolic secretion was controlled by the addition of 15 mg dm^{-3} polyvinylpyrolidone. The proliferated shoots were elongated on the medium comprising MS salts, B5 vitamins, 1.5 mg dm^{-3} TDZ and 0.3 mg dm^{-3} gibberellic acid. The elongated shoots were rooted on the medium containing MS salts, B5 vitamins, 0.3 mg dm^{-3} indole-3-butyric acid and 0.6 mg dm^{-3} silver nitrate. After root induction, the plants were hardened in earthen pots containing sand, soil and vermiculite.

Additional key words: additives, amino acids, auxins, callus cultures, cotyledon explants, cytokinins, root induction.

Introduction

The castor-bean (*Ricinus communis* L.) is one of the medicinally important crops, largely cultivated for its essential oil and ricin. Due to some limitations in the plant breeding techniques, the yield and development of the new cultivars was significantly affected by biotic and abiotic stresses. Now-a-days, the transgenic plants produced through tissue culture methods showed superior abiotic and biotic stress tolerance (Ganesan and Jayabalan 2006). Hence, standardization of regeneration protocol for castor-bean regeneration becomes essential and important. Regarding castor-bean regeneration, few reports were reported for whole plant regeneration through shoot tip and embryo axes culture (Sujatha and Reddy 1998) and genetic transformation through direct regeneration methods (Sujatha and Sailaja 2005). Unfortunately, no reports were published for callus induction and callus-mediated plant regeneration. Hence, we planned to regenerate the plantlets through callus cultures. We also analyzed different factors like type and age of explants, supplementation of different types and concentrations of plant growth regulators, carbon sources, additives and amino acids for enhanced production of organogenic callus and plant regeneration.

Materials and methods

***In vitro* seed germination:** The castor-bean (*Ricinus communis* L. cv. TMV 6) seeds procured from Tapioca and Castor Research Station, Tamil Nadu Agricultural University, Yethapur, India, and were surface sterilized by the method of Ganesan and Jayabalan (2004). The sterilized seeds were inoculated on germination medium containing of Murashige and Skoog (1962; MS) salts, B_5 vitamins (Gamborg *et al.* 1968), 6-benzyladenine (BA; 0.2 - 2.0 mg dm^{-3}), gibberellic acid (GA_3; 0.1 - 1.0 mg dm^{-3}), 30 g dm^{-3} sucrose and 0.8 g dm^{-3} agar. The pH of the

Received 23 March 2006, *accepted* 17 December 2006.

Abbreviations: BA - 6-benzyladenine; B5 medium - Gamborg medium; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; 2iP - 6-(γ,γ,-dimethylallylamino)-purine; Kin - kinetin; MS medium - Murashige and Skoog medium; NAA - α-naphthalene acetic acid; PGR - plant growth regulator; PVP - polyvinylpyrolidone; TDZ - thidiazuron.

Acknowledgement: We thank the Director, Tapioca and Castor-bean Research Station, Tamil Nadu Agricultural University, Yethapur, Tamil Nadu, India. for providing the seeds.

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medium was adjusted to 5.7 - 5.8, using 0.1 M NaOH before autoclaving at 121 °C for 15 min. Initially the cultures were maintained in the dark for 48 h at 25 ± 2 °C and then under 16-h photoperiod with an irradiance of approximately 15 μmol m^{-2} s^{-1}.

Initiation, proliferation and selection of organogenic callus: Cotyledon, hypocotyls, epicotyls, and immature leaves were used as explants and placed horizontally in callus initiation medium which contained MS salts, B5 vitamins and varying concentrations of BA (0.5 to 4.0 mg dm^{-3}), kinetin (Kin; 1.0 to 3.0 mg dm^{-3}) and 6-(γ,γ,-dimethylallylamino)-purine (2iP; 1.0 to 3.0 mg dm^{-3}) were tested. After the identification of suitable concentration of cytokinin for callus induction, combinations of different concentrations of α-naphthalene acetic acid (NAA; 0.2 to 2.0 mg dm^{-3}) were tested for enhanced callus production. After 6 weeks of culture, callus formation was observed from the cut end of the explants. From the obtained mass, the organogenic nature of the callus was identified by the presence of green colour with compact texture (GCN). The organogenic portions were isolated and subcultured in the same medium. Greenish friable (GF), brown compact (BC), brown friable (BF) and yellowish green (YG) colored non-organogenic callus were also observed and they were not selected and discarded for further studies due to zero response. The selected organogenic callus was weekly subcultured for another two weeks for the induction of matured green compact organogenic callus. For callus induction, maximum of 75 explants were tested and these experiments were repeated for three times with six replicates.

Adventitious shoot proliferation: 8-week-old organogenic callus (250 mg) was transferred to 200 cm^3 narrow bottles containing 25 cm^3 of shoot initiation medium. Then the cultures were subcultured weekly for 2 months for the initiation of shoots. During each subculture removal of dead, dark brown cells was necessary. Otherwise, the whole callus tissues become necrotic and become dead. The plant growth regulators like, BA (0.5 - 2.5 mg dm^{-3}), thidiazuron (TDZ; 0.5 - 2.5 mg dm^{-3}) and 2iP (0.5 - 2.5 mg dm^{-3}) with different concentrations and combination of indole-3-butyric acid (IBA; 0.05 - 0.8 mg dm^{-3}) and indole-3-acetic acid (IAA; 0.05 - 0.8 mg dm^{-3}) were tested for proliferation of shoots along with the medium consisting of MS salts, B5 vitamins, sucrose 30 g dm^{-3}, and 0.8 % agar (pH 5.75). During multiple shoot proliferation, maximum of 65 callus cultures were tested and these experiments were repeated for three times with six replicates.

Effect of carbon source, additives and amino acids on multiple shoot proliferation: The effects of sucrose, glucose, fructose and maltose (10 - 40 mg dm^{-3}) were tested for organogenic callus induction and multiple shoot proliferation. In that same way, the influences of different amino acids like alanine, glutamine, proline and serine (5 - 25 mg dm^{-3}) were also tested. During plant regeneration of castor-bean, browning of explants as well as medium was noticed. To control phenolic exudation, different concentrations of activated charcoal (50 - 250 mg dm^{-3}), citric acid (5 - 25 mg dm^{-3}), ascorbic acid (5 - 25 mg dm^{-3}) and polyvinylpyrolidone (PVP: 5 - 25 mg dm^{-3}) were tested.

Root induction and hardening: Root induction from the elongated shoots were obtained in the medium fortified with MS salts, B5 vitamins, IAA (0.1 - 0.5 mg dm^{-3}), IBA (0.1 - 0.5 mg dm^{-3}), $AgNO_3$ (0.1 - 0.5 mg dm^{-3}), glucose (30 g dm^{-3}) and agar (0.8 %). After complete regeneration of shoots with tertiary roots (35 - 40 d after root induction) the regenerated plants were transferred to plastic pots containing sand, soil and *Vermiculite* in 1:1:1 ratio for hardening in the greenhouse. The plants were completely covered with plastic bags for 2 weeks to maintain high humidity and it was progressively removed to adapt to normal environmental condition. The survival percentage was recorded regularly. The selected regenerated plants, adapted to normal environmental condition, were transferred to earthen pots for further growth and development. For root induction studies, 90 elongated shoots were tested for each concentration. The experiments were repeated for three times with six replicates.

Statistical analysis: Means and standard errors were used throughout the study and the values were assessed using a parametric Moods median test (Snedecor and Cochran 1989). The data were further analyzed for variance by Duncan's multiple range test (DMRT) using the *SAS* programme (SAS Institute, Cary, NC, USA).

Results and discussion

Seed germination and organogenic callus induction: The surface sterilized seeds exhibited 95 % germination after 10 d of inoculation on the medium supplemented with MS basal salts, B5 vitamins, 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} GA_3 The cotyledons, hypocotyls, epicotyls and immature leaves were collected from 5- to 12-d-old seedlings. Five days after inoculation, callusing was observed from all the explants. Hypocotyls, epicotyls and immature leaves showed very poor callus induction and from these explants, yellowish compact and brownish nodular calli were induced after 15 d of culture. The cotyledon explants showed best callus induction. Individual treatment of Kin, BA and 2iP showed low response for callus induction. Among the three cytokinins tested

media comprising BA (2.0 mg dm^{-3}) showed superior response (25.3 %). Hence, along with BA (2.0 mg dm^{-3}) different concentration of NAA was tested for enhanced callus production. High proficiency callus induction of cotyledon explants was noticed on the medium comprising of MS salts, B5 vitamins, BA (2.0 mg dm^{-3}) and NAA (0.8 mg dm^{-3}) callus was observed when compared with other concentrations on the medium

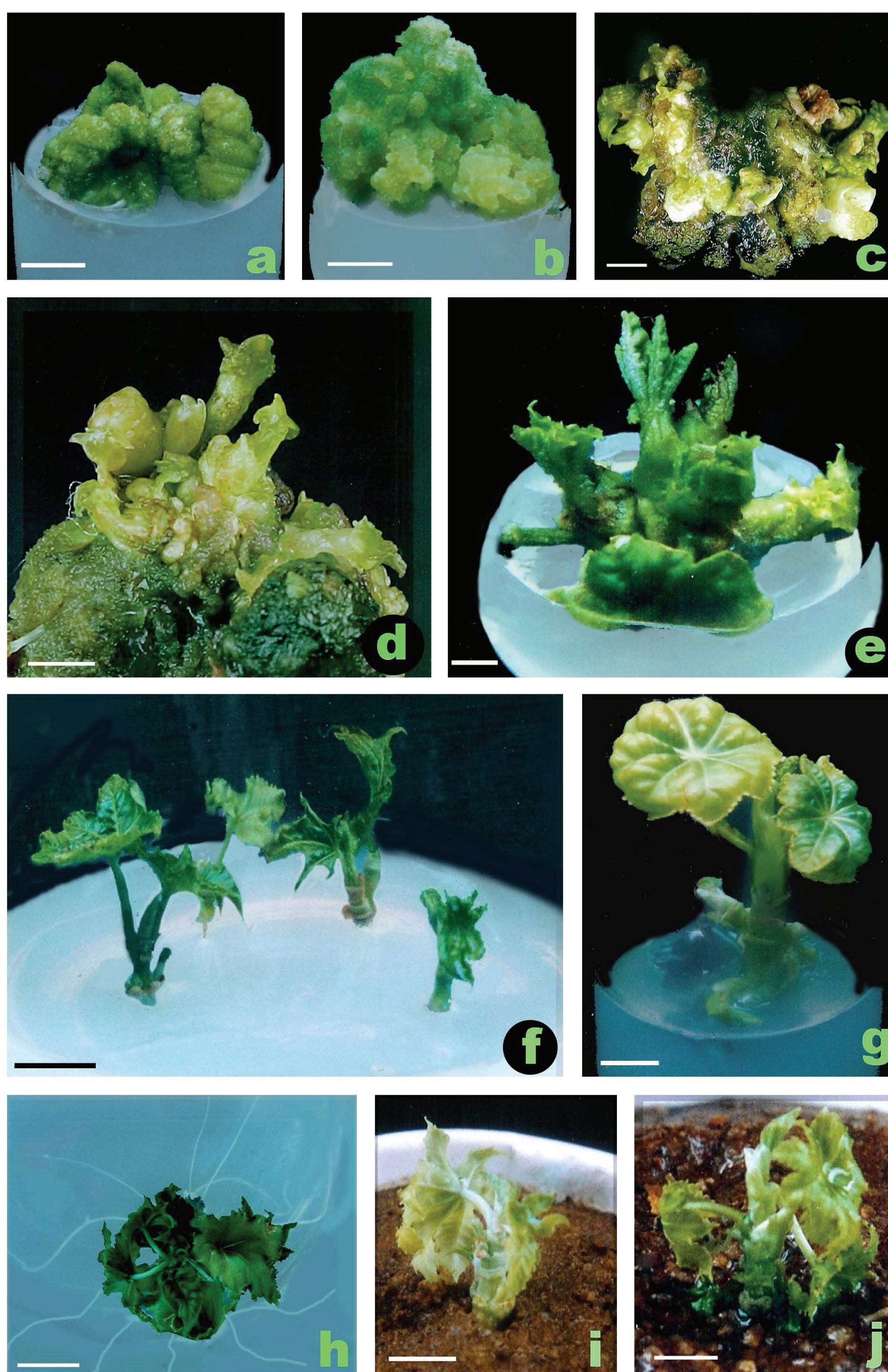

Fig. 1. *a* - Two weeks old green compact nodular organogenic callus obtained from cotyledon explants (*bar* = 0.5 cm); *b* - 4-week-old green compact nodular organogenic callus (*bar* = 0.65 cm); *c*, *d* - multiple shoot proliferation from the callus culture after 2 weeks of culture (*bar* = 0.5 cm); *e* - 6-week-old proliferated multiple shoots (*bar* = 1.0 cm); *f* - isolated single shoots on elongation medium after 2 weeks (*bar* = 1.0 cm); *g* - 4-week-old elongated shoot (*bar* = 0.75 cm); *h* - 2-week-old rooted plantlet on rooting medium (*bar* = 1.0 cm); *i*, *j* - 4- and 6-week-old hardened plants in plastic pots (*bar* = 2.0 cm).

fortified with MS salts, B5 vitamins, 30 g dm^{-3} glucose and 8 g dm^{-3} agar. The induced calli was green, compact and nodular in nature (Fig. 1*b*).

Similar to our results, in *Melia azedarach,* organogenic callus cultures were initiated by using combination of BA (4.4 µM) and NAA (0.46 µM) and successful regeneration of plantlets was obtained by using the callus cultures (Vila *et al.* 2003). Like this result, in most of the dicot plants, a combination of high amount of auxins (2,4-D or NAA) with low amount of cytokinins (BA or Kin) was widely used for the initiation of organogenic callus (Caboni *et al.* 2000, Rugini and Muganu 1998, Haliloglu 2006, Rani *et al*. 2006) and sometimes cytokinins alone (BA or Kin) was also used for the induction of organogenic callus (Yam *et al.* 1990). Our experiments proved that combined effect of BA and NAA showed best response for organogenic callus induction.

Table 1. Effect of plant growth regulators (PGRs) on organogenic callus induction from cotyledon explants of *Ricinus communis*. GF - green friable, GC - green compact, YC - yellow compact, BC - brown compact, BF - brown friable, GCN - green compact nodular. Means ± SE of four repeated experiments. Each treatment consisted of six replicates. Means within a row followed by the same letters are not significant at P = 0.05 according to DMRT.

PGRs	[mg dm^{-3}]	Response [%]	Callus mass [g]	
Kin	1.0	12.3 ± 0.3 f	0.4 ± 0.1 no	GF
	1.5	14.1 ± 1.1 e	0.7 ± 0.2 m	GF
	2.0	17.6 ± 1.4 cd	1.9 ± 0.1hi	GC
	2.5	15.2 ± 1.0 d	1.7 ± 0.3 ijk	GC
	3.0	10.9 ± 0.7 g	1.5 ± 0.1 jk	GC
BA	0.5	8.5 ± 0.6 i	1.2 ± 0.1 kl	YC
	1.0	21.2 ± 1.7 b	2.0 ± 0.2 ghi	GC
	1.5	25.3 ± 0.3 a	2.5 ± 0.5 e	GC
	2.0	24.7 ± 1.0 ab	2.4 ± 0.7 ef	GC
	2.5	20.5 ± 0.9 bc	2.1 ± 0.2 g	GC
	3.0	18.1 ± 1.5 c	2.2 ± 0.1 fg	GC
	3.5	13.2 ± 0.7 ef	1.8 ± 0.3 i	GC
	4.0	7.6 ± 0.6 jk	1.1 ± 0.1 l	YC
PiP	1.0	5.2 ± 0.9 m	0.3 ± 0.8 o	GF
	1.5	6.3 ± 1.0 l	0.5 ± 0.2 n	GF
	2.0	9.6 ± 0.6 hi	1.3 ± 0.6 k	GC
	2.5	7.7 ± 0.5 ijk	1.0 ± 0.7 j	BC
	3.0	6.9 ± 1.3 k	0.7 ± 0.4 m	BF
BA+NAA	2.0+0.2	46.5 ± 1.85 e	2.9 ± 0.11 d	GC
	2.0+0.4	52.6 ± 2.12 de	3.1 ± 0.11 c	GC
	2.0+0.6	55.5 ± 2.45 cd	3.3 ± 0.34 b	GCN
	2.0+0.8	69.5 ± 1.45 a	3.5 ± 0.22 a	GCN
	2.0+1.0	61.5 ± 1.24 b	3.4 ± 0.14 ab	GCN
	2.0+1.2	59.3 ± 1.12 c	3.3 ± 0.11 b	GC
	2.0+1.4	53.5 ± 1.87 d	3.0 ± 0.10 cd	GC
	2.0+1.6	44.8 ± 2.64 f	2.5 ± 0.21 e	GC
	2.0+1.8	41.5 ± 2.39 fg	2.3 ± 0.11 f	GC
	2.0+2.0	38.5 ± 1.92 g	2.0 ± 0.14 gh	GC

During organogenic callus formation, calli with various texture was noticed and only the hard and compact green calli responded well for the induction of shoots. Hence, shoot induction and multiplication was achieved by using the above said calli. Individual effect of different concentrations of BA, Kin and combined effect of Kin + NAA were also tested for the induction of organogenic callus, unfortunately, in these concentrations, abnormalities like, induction of roots, bulging of explants and necrosis of tissues were clearly observed. It is also observed that BA or Kin when applied alone induced only waste brownish friable callus from cotyledon explants (Sudha *et al.* 1998, Tang and Guo 2001, Martin 2002). In our studies combination of cytokinins with auxins showed best response because, the nature of the callus varied according to the application of the cytokinin and also on the auxin:cytokinin ratio (Marks and Simpson 1994, Soniya and Sujitha 2006). In *Tylophora indica,* organogenic callus induction was achieved by the supplementation of cytokinin alone (Manjula *et al.* 2000, Handro and Floh 2001). In these cases, endognous IAA content played a vital role in callus induction (Fracaro and Echeverrigary 2001).

Like our results, organogenic callus induction and plantlet multiplication was obtained in several plants

Table 2. Effect of different concentrations of additives on multiple shoot proliferation from callus cultures of *Ricinus communis*. Means ± SE of four repeated experiments. Each treatment consisted of six replicates. Means within a row followed by the same letters are not significant at P = 0.05 according to DMRT.

Additives	[mg dm^{-3}]	Response [%]	Shoot number [callus^{-1}]
Charcoal	50	58.4 ± 0.2 mn	16.8 ± 0.4 i
	100	62.1 ± 0.3 k	17.2 ± 0.8 fgh
	150	65.6 ± 0.5 hi	17.6 ± 0.5 ef
	200	63.2 ± 0.2 j	17.0 ± 0.2 h
	250	60.4 ± 1.0 l	16.5 ± 0.6 ijk
PVP	5	69.3 ± 0.6 cd	18.4 ± 0.2 de
	10	71.8 ± 0.5 ab	19.2 ± 1.0 b
	15	72.2 ± 0.2 a	20.0 ± 0.3 a
	20	70.2 ± 0.1 b	19.0 ± 0.1 bcd
	25	67.3 ± 0.3 de	18.6 ± 0.9 d
Ascorbic acid	5	67.5 ± 1.0 f	17.1 ± 0.5 gh
	10	68.7 ± 0.6 e	17.9 ± 0.1 e
	15	69.1 ± 0.3 d	19.5 ± 0.2 ab
	20	66.2 ± 0.7 g	18.9 ± 0.1 cd
	25	65.0 ± 0.1 i	17.6 ± 0.2 ef
Citric acid	5	56.5 ± 1.0 p	15.4 ± 0.5 l
	10	59.6 ± 0.5 m	16.0 ± 0.2 jk
	15	60.1 ± 0.2 lm	16.5 ± 0.1 ijk
	20	58.2 ± 0.1 n	15.0 ± 0.4 lm
	25	57.4 ± 0.3 op	14.4 ± 0.3 m

species. In *Iris ensata, I. setosa* and *I. sanguinea* supplementation of BA with NAA showed best callus induction but NAA or BA alone showed poor response (Boltenkov and Zarembo 2003). Callus induction from the different explants *Malus domestica* for organogenesis was also obtained by the combined treatment of BA and NAA (Caboni *et al.* 2000, Martin 2002). In *Dianthus caryophyllus* also organogenic callus induction was successfully induced by the supplementation of auxin and cytokinin synergistically (Kallak *et al.* 1997).

Previous experiments in our laboratory proved that phenolic excretion and oxidation is a severe problem during callus induction and callus-mediated regeneration and this problem was solved by the addition of some additives along with plant growth regulators (Ganesan and Jayabalan 2005). Unexpectedly, in this present investigation, excretion of phenolic compounds from explants to the medium was strictly avoided by regular sub-culturing of callus and without addition of additives.

Shoot proliferation from the callus: During shoot proliferation, all the treatments of BA + NAA (Fig. 2*A*), TDZ + NAA (Fig. 2*B*) and 2iP + NAA (Fig. 2*C*) showed shoots proliferation from the obtained callus cultures. After one month, maximum of 17.8 shoots were regenerated from the callus in the media fortified with MS salts, B5 vitamins, TDZ (2.5 mg dm^{-3}) and NAA (0.4 mg dm^{-3}). In the case of BA and NAA combination, BA (2.0 mg dm^{-3}) and NAA (0.4 mg dm^{-3}) produced 16 shoots from the callus cultures (Fig. 1*c*,*d*). At the same time, 2iP + NAA combination showed poor response for multiple shoot induction. Only 9.0 shoots were induced after 45 d of culture. In the present investigation, we confirmed that along with any type of cytokinin, addition of NAA favors shoot organogenesis from the callus cultures of castor-bean.

Similar to this result, TDZ-mediated shoot proliferation was obtained in several plants (Fiola *et al.* 1990, Malik and Saxena 1992). Usually TDZ was used

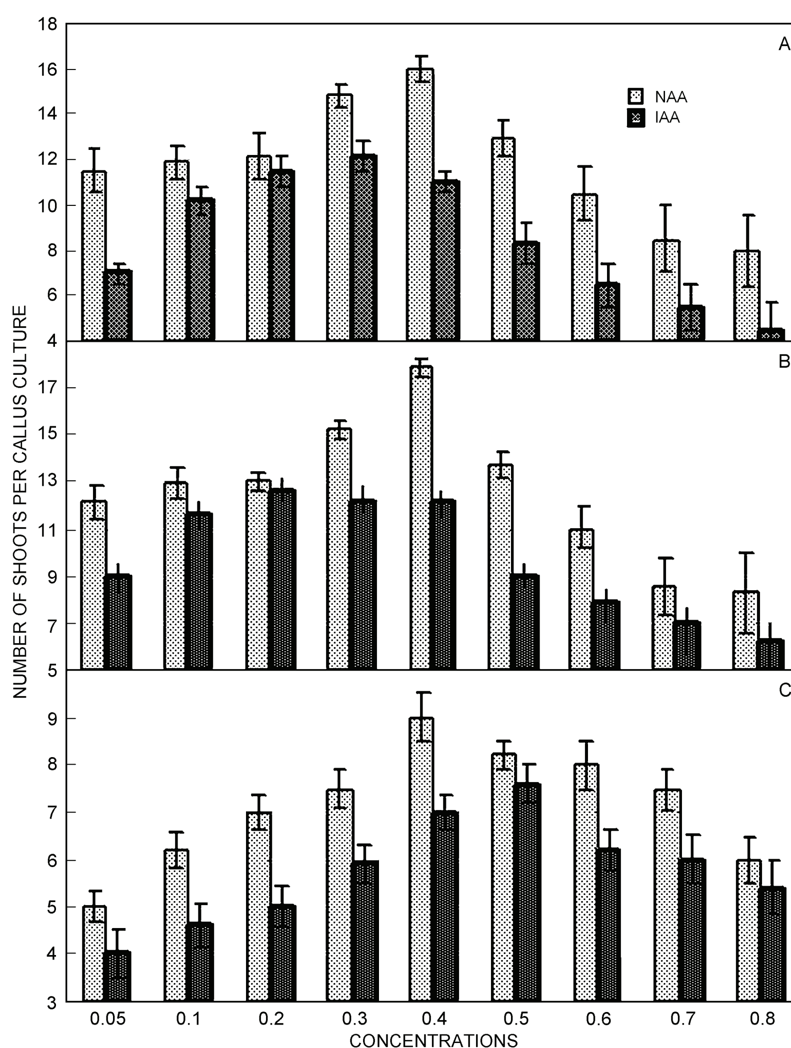

Fig. 2. Effect of 2.0 mg dm^{-3} BAP (*A*), 2.5 mg dm^{-3} TDZ (*B*) and 2.0 mg dm^{-3} 2iP (*C*) combined with different concentrations of NAA and IAA on multiple shoot induction from cotyledon derived callus cultures of castor-bean. Number of explants tested 65. Each treatment consisted of six replicates.

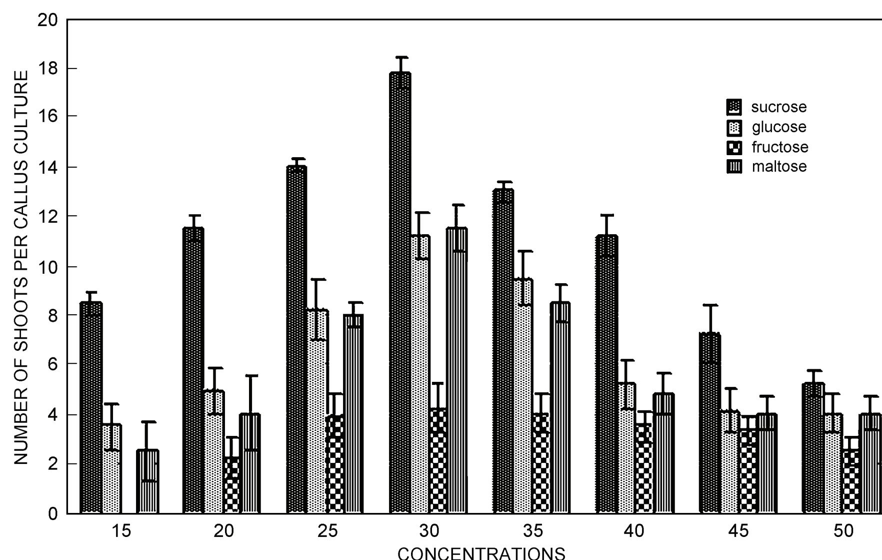

Fig. 3. Effect of different concentrations of sugars on multiple shoot proliferation of callus cultured on the medium fortified with MS salts and B5 vitamins. Number of explants tested 65. Each treatment consisted of six replicates.

Table 3. Effect of different concentrations of amino acids on the multiple shoot proliferation from the callus. Means ± SE of four repeated experiments. Each treatment consisted of six replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

Amino acids	[mg dm^{-3}]	Response [%]	Shoot number [callus^{-1}]
Alanine	5	56.3 ± 0.2 m	18.7 ± 0.3 n
	10	57.2 ± 0.1 l	19.0 ± 0.1 lm
	15	57.6 ± 0.5 k	19.3 ± 0.2 kl
	20	52.2 ± 0.1 n	18.3 ± 0.5 o
	25	49.3 ± 0.6 o	17.5 ± 0.1 p
Proline	5	64.6 ± 0.7 i	19.2 ± 0.1 l
	10	65.7 ± 0.4 gh	19.9 ± 0.4 ij
	15	66.5 ± 0.2 g	20.5 ± 0.2 gh
	20	65.4 ± 0.1 h	19.6 ± 0.3 jkl
	25	63.2 ± 0.2 j	19.0 ± 0.7 m
Serine	5	73.5 ± 0.1 e	20.3 ± 0.4 h
	10	74.0 ± 0.5 de	21.5 ± 0.3 bcd
	15	74.3 ± 0.3 d	21.7 ± 0.1 b
	20	73.5 ± 0.6 e	20.6 ± 0.2 fgh
	25	72.7 ± 0.8 f	19.7 ± 0.1 j
Glutamine	5	84.3 ± 0.2 b	21.1 ± 0.3 d
	10	84.9 ± 0.5 ab	21.4 ± 0.1 cde
	15	85.2 ± 0.1 a	22.1 ± 0.2 a
	20	83.6 ± 0.3 c	21.0 ± 0.4 de
	25	83.1 ± 0.4 cd	20.7 ± 0.2 e

for somatic embryogenesis in many plant species, either alone or in combination with other growth regulators (Murthy *et al.* 1998, Faisal and Anis 2006). In our experiments, TDZ along with NAA were proved as best cytokinin for organogenesis from cotyledon callus cultures. In some cases, shoot multiplication and regeneration was efficiently achieved by 2iP (0.17 μM) from the callus derived rhizomes of *Cymbidium ensifolium* (Chang and Chang 2000). In *Fraxinus angustifolia* and *Carica papaya* also 2iP showed a vital role in the shoot organogenesis from different explants derived callus (Tonon *et al.* 2001, Khatoon and Sultana 1994). Usually, BA or Kin was widely used for multiple shoot initiation from the callus cultures (Martin 2002).

Effect of carbon sources, additives and amino acids on shoot proliferation studies from callus cultures: During multiple shoot proliferation, effects of different carbon sources were tested (Ganesan and Jayabalan 2005). Among them, sucrose 30 g dm^{-3} showed best response. With the other sugars and concentrations reduced percentage of multiple shoot induction was observed (Fig. 3). At the same time, severe browning of callus was noticed in medium with glucose, fructose or maltose. Similarly, sucrose-mediated shoot multiplication in *Corylus avellana* was effectively achieved and maximum of 3 - 4 shoots were regenerated (Yu *et al.* 1993). In nature, sugars are transported within the plant as sucrose and the tissue may have the inherent capacity for uptake, transport and utilization of sucrose (Sul and Korban 2004).

During multiple shoot proliferation from callus cultures of cotyledon explants, severe browning of callus was noticed. Previous results proved that addition of some additives controlled the browning of callus and tissues and simultaneously increased the percentage of multiple shoot induction (Amin and Jaiswal 1988, Quraishi and Mishra 1998). We have evaluated four additives to control the phenolic oxidation and all suppressed browning of callus tissues. Among them, supplementation of PVP (15 mg dm^{-3}) was superior. At

the same time, addition of PVP along with multiple shoot induction medium enhanced the multiple shoot induction percentage. Maximum of 20 shoots per callus clump were initiated by the addition of PVP and in the case of controls only 17.8 shoots were obtained (Table 2).

The influence of various amino acids like alanine, glutamine, proline and serine were also evaluated for the multiple shoot initiation from the obtained callus. All the four amino acids enhanced multiple shoot induction. From the four, 15 mg dm^{-3} glutamine showed the best response for multiple shoot proliferation and maximum of 22.1 shoots per callus clump was regenerated (Table 3; Fig. 1*e*). Similar effect of glutamine-mediated plant regeneration was obtained in barley by using microspore explants (Ritala *et al.* 2001). Generally, glutamine has been used for the induction of embryogenic callus and direct and indirect induction of somatic embryos (Ipekci and Gozukirmizi 2002, Kim *et al.* 1997).

Table 4. Effect of GA_3 in combination with BA, TDZ and 2iP on shoot elongation of *Ricinus communis*. Means ± SE of four repeated experiments. Each treatment consisted of six replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

PGRs	[mg dm^{-3}]	Response [%]	Shoot length [cm]
BA+GA_3	2.5+0.1	41.2 ± 0.1 g	5.2 ± 0.3 f
	2.5+0.2	42.3 ± 0.3 ef	5.3 ± 0.1 ef
	2.5+0.3	47.1 ± 0.2 d	6.2 ± 0.2 c
	2.5+0.4	46.5 ± 0.6 de	6.0 ± 0.6 cd
	2.5+0.5	42.7 ± 0.4 e	5.6 ± 0.4 de
TDZ+GA_3	1.5+0.1	49.0 ± 0.3 cd	5.3 ± 0.2 ef
	1.5+0.2	49.1 ± 0.3 c	5.6 ± 0.1 de
	1.5+0.3	55.1 ± 0.2 a	7.4 ± 0.5 a
	1.5+0.4	54.3 ± 0.1 ab	6.8 ± 0.4 b
	1.5+0.5	50.2 ± 0.4 b	6.0 ± 0.2 cd
2iP+GA_3	2.5+0.1	30.1 ± 0.1 I	5.0 ± 0.2 g
	2.5+0.2	33.4 ± 0.5 hi	5.1 ± 0.4 fg
	2.5+0.3	41.9 ± 0.3 f	5.8 ± 0.3 d
	2.5+0.4	41.7 ± 0.2 fg	5.6 ± 0.5 de
	2.5+0.5	39.2 ± 0.4 h	5.4 ± 0.6 e

There are several standardized protocols for callus-mediated somatic organogenesis and embryogenesis of economically important crops (Wilkins *et al.* 2000, 2004). Unfortunately, organogenic callus induction and plant regeneration from the callus cultures of castor-bean was not reported. Our results proved that organogenic callus induction and direct shoot regeneration was possible in castor-bean. Usually, addition of GA_3 with shoot proliferation medium was used for the elongation of shoots (Caboni *et al.* 2000). In our studies also shoot elongation was achieved by combined treatments of TDZ (1.5 mg dm^{-3}) with GA_3 (0.3 mg dm^{-3}) (Table 4). Regular weekly sub-culturing of induced shoots was done on the same medium for complete elongation and maturation and complete maturation of shoots required total of 2 - 4 weeks (Fig. 1*f*,*g*). Wilting of leaves was completely avoided by this weekly interval subculture (Pretto and Santarem 1997, Reddy *et al.* 2002).

Table 5. Effect of IBA, IAA and $AgNO_3$ on root induction from elongated shoots obtained from cotyledon derived callus cultures of *Ricinus communis*. Means ± SE of four repeated experiments. Each treatment consisted of six replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT (n.d. - no response)

PGRs [mg dm^{-3}]		Response [%]	Root number [explant^{-1}]	Root length [cm]
IBA	0.1	35.2 ± 1.0 k	1.9 ± 0.2 l	1.1 ± 0.5 i
	0.2	38.6 ± 1.1 j	2.3 ± 0.7 j	1.7 ± 0.5 h
	0.3	46.3 ± 0.4 f	3.8 ± 0.5 g	3.4 ± 0.3 de
	0.4	43.4 ± 0.3 fgh	3.5 ± 0.6 h	2.9 ± 0.4 f
	0.5	41.0 ± 1.0 i	2.7 ± 0.4 i	2.2 ± 0.1 g
IAA	0.1	n.d.	n.d.	n.d.
	0.2	n.d.	n.d.	n.d.
	0.3	n.d.	n.d.	n.d.
	0.4	20.2 ± 0.2 l	2.0 ± 0.1 k	1.2 ± 0.2
	0.5	18.3 ± 0.3 m	1.3 ± 0.4 m	0.5 ± 0.3
$AgNO_3$	0.2	41.4 ± 0.4 h	3.3 ± 0.3 hi	1.9 ± 0.2 gh
	0.4	43.1 ± 0.6 gh	3.7 ± 0.2 gh	3.7 ± 0.5 d
	0.6	55.4 ± 0.2 d	4.8 ± 0.1 e	5.2 ± 0.1 bc
	0.8	51.2 ± 0.4 de	4.6 ± 0.3 ef	4.7 ± 0.6 cd
	1.0	47.5 ± 0.3 e	4.3 ± 0.3 f	3.2 ± 0.4 e
IBA+	0.3+0.2	65.0 ± 1.2 d	5.2 ± 0.25 c	5.2 ± 0.5 bc
$AgNO_3$	0.3+0.4	69.5 ± 1.9 c	5.4 ± 0.32 bc	5.5 ± 0.9 ab
	0.3+0.6	72.5 ± 2.2 a	5.9 ± 0.51 a	5.6 ± 0.8 a
	0.3+0.8	70.5 ± 3.2 b	5.5 ± 0.16 b	5.3 ± 0.4 b
	0.3+1.0	68.5 ± 2.5 cd	5.1 ± 0.25 d	5.0 ± 0.5 c

Root induction and hardening: Induction of rooting is an important step in plant propagation *in vitro*. The classical root induction method uses high auxin concentration, however, the roots are often stunted and malformed (Moncousin 1991, Rao and Purohit 2006). In the present investigation, root induction was achieved after 7 d of culture. Our results proved that root induction in castor-bean was difficult compared to other crop plants. Hence, along with different auxins we tested $AgNO_3$ for root induction. In the present study, compared with individual treatment of auxins or $AgNO_3$, combination of IBA (0.3 mg dm^{-3}) with $AgNO_3$ (0.6 mg dm^{-3}) showed best response for root induction from elongated shoots and maximum of 4.8 roots was induced (Table 5; Fig. 1*h*). These results demonstrated that $AgNO_3$ can influence root emergence and growth and can improve rooting efficiency (Bais *et al.* 2000). Similar to our results, it has been accepted that interaction of thiol compounds stimulate rooting *in vitro* (Biddington 1992). He demonstrated that the use of ethylene inhibitors such as $AgNO_3$ might promote root formation in shoot cultures of apple.

In the case of IBA (0.3 mg dm^{-3}) also root induction was noticed but in all the concentrations of IBA tested root induction was low when compared with combined treatment of IBA and $AgNO_3$. Supplementation of IAA showed zero response for root induction, instead basal callus formation was noticed. Usually, IBA mediated root induction has been reported for several plants including *Hemidesmus indicus* (Sreekumar *et al.* 2000) and *Cunila galiodes* (Fracaro and Echeverrigary 2001).

The increased root length leads to increase in the survival percentage of hardened and field grown plants. For hardening process sand, soil and *Vermiculite* were used in 1:1:1 ratio (Fig. 1*i,j*). After proper acclimatization the hardened plants were transferred to field.

In conclusion, an efficient and simple protocol for *in vitro* adventitious shoot multiplication from callus cultures, and whole plant regeneration has been described. The protocol was optimized by manipulations of different PGRs, amino acids, sugars and additives for enhanced multiplication. This protocol could be used for the somaclonal variation induction, and producing transgenic plants in castor-bean through *Agrobacterium* and biolistic methods.

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