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In vitro micropropagation through cotyledonary node culture of castor bean (*Ricinus communis* L.)

Iftekhar Alam[1]*, Shamima Akhtar Sharmin[1], Sanjoy Chandra Mondal[2], Md. Jahangir Alam[2], Muhammad Khalekuzzaman[3], M. Anisuzzaman[2] and Mohammad Firoz Alam[2]

[1] Division of Applied Life Science (BK21), Gyeongsang National University, Jinju 660-701, Republic of Korea
[2]Department of Botany, Rajshahi University, Rajshahi-6205, Bangladesh
[3]Department of Genetic Engineering and Biotechnology, Rajshahi University, Rajshahi-6205, Bangladesh

***Corresponding author: alami@gnu.ac.kr**

Abstract

An efficient plant regeneration protocol was described for castor (*Ricinus communis* L.) using whole cotyledonary nodes as explant. Seeds were surface sterilized with 0.1% (w/v) mercuric chloride and germinated in growth regulator-free MS medium. Cotyledonary nodes were excised from 5-7 days old seedlings and were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, Kin singly or in combination with NAA. Use of BAP at 3.0 mg.l^{-1} induced the highest frequency (85%) of shoot induction as well as maximum number of shoots per explant (12.56). Proliferated shoot clusters were elongated in 1.0 mg.l^{-1} BAP in combination with 0.25 mg.l^{-1} GA_3 . For root induction, *in vitro* shoots were transferred to rooting media containing NAA or IBA. The highest rooting frequency (87.5%) as well as highest number of roots (10.5) was observed in MS medium supplemented with 1.0 mg.l^{-1} NAA. Regenerated plantlets were acclimatized successfully in the growth room for further development.

Keywords: *In vitro*, micropropagation, medicinal plant, *Ricinus communis*

Abbreviations: BAP_6-Benzylaminopurine; NAA_α-naphthalene acetic acid; PGR_plant growth regulator; Kin_kinetin

Introduction

Castor (*Ricinus communis* L.) belongs to the *Euphorbiaceae* family and is one of the medicinally important oil seed crop (Kumari et al., 2008). It is a semi tropical perennial non-edible oilseed crop, widely cultivated throughout tropical regions. The seeds contain approximately 60% oil and are the only commercial source of ricinoleic acid that is used as industrial lubricants, paints, coatings, and plastics (Caupin, 1997). India is the largest producer of castor oil, representing 60% of the global production followed by China and Brazil (FAO, 2006). Castor contains some toxic compounds like ricin (Hartley and Lord, 2004) and hyperallergenic 2S albumins (Shewry et al., 2002) in its seeds, which restrict its commercial cultivation in USA (Ahn et al., 2007). 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 lower the toxicity of seed meal and to confer resistance to biotic stresses. The success of using such approach largely depends on an efficient *in vitro* regeneration system, which is rapid, reproducible and applicable to a broad range of genotypes. 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 tissues as explants was either inefficient or difficult to reproduce (reviewed by Ahn et al., 2007). While shoot induction from embryonic tips and shoot apex involved preexisting meristem and inefficient in adventitious shoot formation (Molina and Schobert, 1995; Sujatha and Reddy, 1998). The transformation efficiency of meristem-based protocols was found very low (0.04%) (Sujatha and Sailaja, 2005). Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques which would widen the possibilities of developing transgenic lines and/or

Table 1. Effect of different concentrations and combinations of BAP and NAA in MS medium for shoot bud induction from cotyledonary explants. Data were recorded after 4 weeks of inoculation.

PGR mg.l^{-1}	Frequency (%)	No. of shoot Mean ±SE	Mean length (cm) Mean ±SE
BAP			
1.0	55.8	4.56±0.27	2.15±0.70
2.0	75.6	8.80±0.66	2.40±0.42
3.0	85.1	12.56±0.46	3.10±0.51
4.0	65.4	7.19±0.32	2.65±0.29
Kin			
1.0	59.4	3.00±0.08	3.90±0.73
2.0	70.7	6.20±0.16	5.28±0.40
3.0	49.8	4.50±0.12	4.95±0.38
5.0	42.7	3.50±0.12	3.91±0.34
BAP+NAA			
2.0+0.1	56.7	4.38±0.49	3.05±0.21
2.0+0.5	50.9	5.60±0.84	3.25±0.48
3.0+0.1	67.3	9.86±0.62	4.20±0.46
3.0+0.5	48.9	5.58±0.37	3.92±0.57
4.0+0.5	36.0	4.91±0.39	3.75±0.29

somaclonal variants. So, the objective of this work was to establish a high frequency plant regeneration system from whole cotyledonary explant.

Materials and methods

Plant materials and culture conditions

Seeds collected from the Botanic Garden, Rajshahi University were partially decoated and surface-sterilized in 0.1% (w/v) mercuric chloride for 4 min followed by 4-5 times rinses in sterile deionized water. Five to seven seeds were placed on petri dish containing MS medium (Murashige and Skoog, 1962) devoid of PGR and incubated in the dark condition for germination. After 5-7 days, primary leaves and the epicotyls were detached from the seedling using sterile surgical blades. Then the seedling radicle was excised, leaving approximately 3–5 cm long hypocotyls intact. Explants consisting of cotyledon and axillary meristem regions with hypocotyls (3–5 cm long) were inoculated vertically on different shoot induction medium to induce shoots (Table 1). Cultures were maintained by periodic subculturing on fresh medium once every 3 weeks. After 3-6 weeks of culture the multiple shoot clusters were elongated in a medium containing 1.0 mg.l^{-1} BAP plus 0.25 mg.l^{-1} GA_3. When the regenerated shoots were attained a height of 2-3 cm at approximately 3-4 weeks, adventitious shoots were excised and transferred to rooting medium for root induction (Table 2). In all cases, 3% sucrose (w/v) was used as a carbon source. After adjusting the pH to 5.7±0.01 prior to gelling with 0.8% agar (w/v) (BHD, England), the media were sterilized by autoclaving at 121^0 C for 20 min (1.06 kg cm^{-2}). Cultures were maintained in a growth chamber at 25±1°C under a 16/8-h (light/dark) photoperiod with a light intensity of 58-60 µmol m^{-2} s^{-1} (supplied by cool-white fluorescent lamps).

Acclimatization and Transfer to Soil

Plantlets with a well-developed root system were washed carefully to remove agar and then transferred to the pots containing sterile vermiculite (Pal et al., 2007). After watering, plantlets were maintained in a growth chamber at 27±1°C under 16 h illuminations (145-150 µmol m^{-2} s^{-1}) with fluorescent lamps. After 3 weeks of acclimatization, plantlets were transferred to larger pots containing vermiculite for further growth.

Data recording and statistical analysis

To test the efficiency of shoot multiplication media, frequency (percentage) of shoot induction, number of shoot per culture and mean of length were calculated following Misra et al., (1996). Frequency of root induction and number of root per shoots were recorded for determining rooting efficiency. Mean values and standard error were calculated for the total number of shoots or roots in a given treatment. Each treatment consists of at least 12 replications and the entire experiment was repeated twice.

Results and Discussion

Shoot multiplication

About 80% of the incubated seeds were germinated in the MS basal medium. Excised cotyledonary nodes (3-5 cm) of 5-7 day old seedlings were cultured on MS medium supplemented with various concentrations of BAP or Kin individually or BAP in combination with NAA for shoot regeneration (Table 1). Among the different growth regulators tested, BAP at the concentration of 3.0 mg.l^{-1} indicated the most promising results. Particularly, about 85% of the explants produced shoots within 3 weeks with an average of 12.56 shoots per explant (Fig A) in this treatment. Superiority of BAP in inducing shoots has been described previously in castor (Sujatha and Reddy, 1998). In the present study, higher concentrations of

Table 2. Effect of different concentrations of IBA and NAA in MS medium for root induction in microshoots. Data were recorded after 4 weeks of inoculation.

PGR mg.l^{-1}	**Rooting Frequency (%)**	**No. of Root** Mean ±SE
NAA		
0.1	33.3	3.25±0.33
0.5	67.5	7.14±0.76
1.0	87.5	10.5±0.81
2.0	60.0	4.05±0.33
3.0	42.5	3.75±0.33
IBA		
0.1	25.0	2.90±0.43
0.5	65.0	5.14±0.66
1.0	80.0	8.55±0.41
2.0	57.5	3.25±1.03
3.0	37.5	3.20±0.14

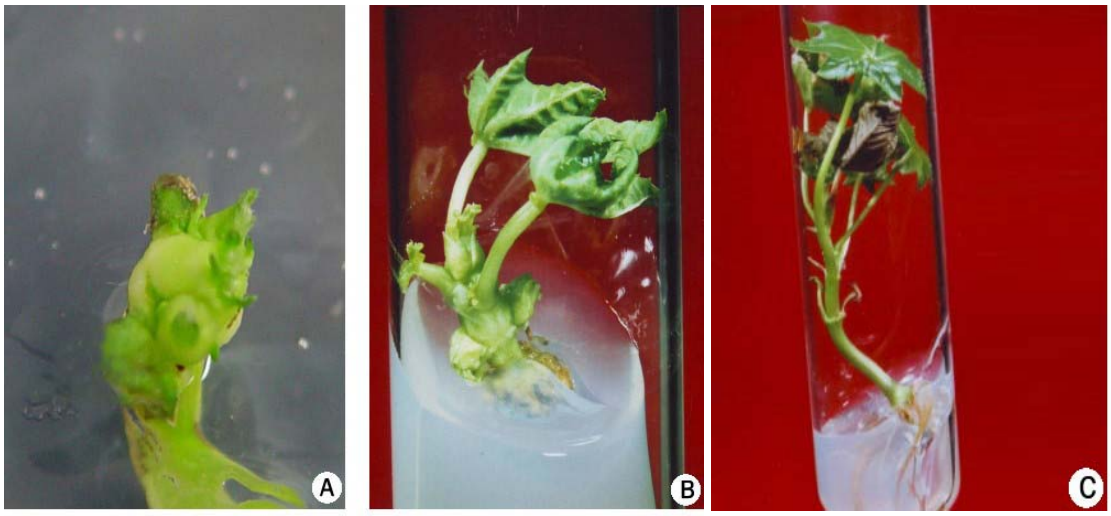

Fig 1. *In vitro* shoot regeneration in castor from cotyledonary explant.
A: Bud initiation from cotyledonary explant after two weeks of culture on shoot induction medium.
B: Shoot multiplication.
C: Development of roots in *in vitro* developed shoots.

BAP reduced the shoot number as well as shoot length. Hu and Wang (1983) reported that higher concentrations of cytokinin reduced the number of micropropagated shoots. Different concentrations of Kin were also induced shoots, but the number of shoots was lower than that in BAP and was not encouraging. However Kin has a profound effect on shoot length compared with BAP. Similar results were obtained earlier by Sujatha and Reddy (1998). Green compact calli were often produced along the cotyledon vein but did not resulted in shoot regeneration. Similar type of result was also observed in castor by Ahn and Chen (2008). However callusing is less in the medium containing Kin. When NAA was added with BAP, it did not enhance shoot number, rather it encourage basal callusing.

Shoot elongation

It was observed that, 1 or 2 main shoots were elongated faster that the other buds of the cluster. The elongated shoots (2-3cm) were excised from the cluster and placed on rooting medium. On the other hand, the remaining clusters, containing a bud of few millimeters, elongated slowly. Those shoot-clusters were subcultured in a reduced BAP level (1.0 $mg.l^{-1}$ BAP) in addition of 0.25 $mg.l^{-1}$ GA_3 for shoot elongation (Fig B). However, this combination was not found effective during shoot induction phase from the initial explant (data not shown). Addition of GA_3 was found to favor shoot elongation (Deore and Johnson, 2008; Najaf-Abadi and Hamidoghli, 2009). It promotes cell division and elongation in the sub apical zone of the shoots (George 1993). Furthermore application of GA_3 in elongating regenerated shoots has been described in many species including *Euphorbiaceae* members (Baburaj et al., 1987; Sujatha and Reddy 1998). However, in our experiment combination of GA_3 with BAP was not found effective during shoot initiation from the initial explant. Root development was not observed while plantlets were maintained on the multiplication or elongation med- ium.

Rooting of the in vitro shoots and acclimatization of young plantlets

Induction of rooting is an important step for *in vitro* plant propagation. Excised shoots were inoculated on MS medium with two different types of auxin for proper root development. The rooting responses were summarized in Table 2. Maximum rooting (87.5%) was observed on the medium supplemented with 1.0 $mg.l^{-1}$ NAA (Fig C) followed by that observed on the medium supplemented with 1.0 $mg.l^{-1}$ IBA (80%). Root induction was strengthened within 3 weeks of culture. Least callus formation occurred in all rooting medium. Sujatha and Reddy (1998) obtained highest rooting with least callusing using 1.0 $mg.l^{-1}$ IBA. While Ahn et al., (2007) observed two different rooting pattern using IBA and NAA and percentage of rooting was inversely correlated to shoot development. Plantlets with a well-developed root system were transferred to soil pot. After 3 weeks of acclimatization, more than 60% of the regenerated plants were survived in soil under growth chamber condition. In conclusion, adventitious shoot regeneration was achieved through the induction of shoot bud from cotyledonary node explant of castor seed. Shoot buds were induced by using 3.0 $mg.l^{-1}$ BAP. Low concentration of GA_3 was found useful during shoot elongation. This system shortened the process of shoot regeneration and more adventitious shoots were harvested. This protocol has potential for large-scale micropropagation and application in molecular plant breeding research programs.

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