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AN EFFICIENT PROTOCOL FOR *IN VITRO* MASS PROPAGATION OF *FUSARIUM WILT* RESISTANT CASTOR (*RICINUS COMMUNIS* L.) PARENTAL LINE SKP-84 THROUGH APICAL MERISTEM

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ABSTRACT
Successful *in vitro* plant regeneration protocol has been described for fusarium wilt resistant castor (*Ricinus communis* L.) parental line SKP-84 through apical meristem. Shoot apex containing apical meristems, were excised from 5-7 days old *in vitro* grown seedlings and cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of cytokinins either alone or in combination. Kinetin had marked effect on shoot initiation and shoot quality. Kinetin (2.325μM) in combination with BAP (1.111μM) produced maximum number of shoot (10.33) and shoot length (5.20 cm). *In vitro* produced shoots were transferred to rooting media containing half strength MS basal media supplemented with NAA at various concentrations. Well developed roots appeared in media supplemented with 0.537μM NAA (69.00 %). Rooted plants were transferred to the pots containing vermicompost with 64.5% survival rate in hardeninng. No genetic variation was detected in plantlets as revealed by RAPD at different stages proved the genetically stability during *in vitro* plant regeneration through apical meristem culture.

INTRODUCTION

Castor (*Ricinus communis* L.) belonging to the family Euphorbiaceae is one of the medicinally important oil seed crop (Ganeshkumari *et al.,* 2008). It has the ability to grow under low rainfall and fertility conditions, and is most suitable for dryland farming. The oil content of the seeds varies from 50 to 55% in different varieties. The importance of oil arises from it richness (85-95%) of ricinoleic acid. Castor oil is being used widely as a lubricant in high speed engines and aeroplanes. Beside its use as lubricant, castor oil is also an important ingredient in manufacture of soaps, printing inks, varnishes, transparent paper, linoleum and plasticizers (Caupin, 1997). After extraction of oil, castor cake is valued as manure. It contains 6.4% N, 2.5% phosphoric acid and 1% K and some micronutrients. Castor is also valued for its anti-termite properties. However, the presence of ricin, an allergen restricts its use as livestock feed. There has been a steady increase in the demand of castor oil and its product in the world market due to their renewable nature, biodegradability and eco-friendliness. In the recent past, use of castor oil as an efficient biodiesel has been reported (Auld *et al.,* 2009). The major castor producing countries are India, China, Brazil, Paraguay, Ethiopia, Philippines, Russia and Thailand. India is the largest producer of castor oil, representing 60% of the global production followed by China and Brazil (Weiss, 2003; FOSTAT, 2009). In India, Gujarat is a leading castor growing state, contributing around 82 per cent of total production in the country and has established a virtual monopolistic grip on the international market.

The chief breeding goals for improvement in castor are insect pest resistance, disease resistance, seed yield, oil content and ricinoleic acid content. The limitation of conventional breeding lies in time, space and maintenance of genetic fidelity. Hence, genetic engineering has become a necessary tool for the improvement of cultivars of this monotypic genus to confer resistance to biotic stresses and to lower the toxicity of seed meal (Sujata and Sailaja, 2005; Lord *et al.*, 1994; Hartley and Lord, 2004).

Genetic improvement through genetic engineering techniques requires an efficient *in vitro* regeneration system, which is rapid and reproducible (Sujata and Sailaja, 2005). Furthermore, *in vitro* regeneration protocol is also required for maintainnace of genetic integrity of higly cross pollinated castor parental lines. However, in the case of castor, research efforts for the last two decades have failed to provide a reliable protocol of *in vitro* plant regeneration. Reports on *in vitro* studies in castor suffer from non-reproducibility, low multiplication rates and involvement of pre-existing meristems (Athma and Reddy 1983; Reddy *et al.,* 1987; Sangduen *et al.*, 1987; Reddy and Bahadur 1989; Sarvesh *et al.,* 1992; Molina and Schobert, 1995; Sujatha and Reddy 1998; Kansara *et al.,* 2010). Therefore, the present investigation was carried out with the objective of developing an efficient regeneration protocol for well established parental lines of castor for large scale multiplication and has further implications in maintaining transgenic lines with desirable characters like insect-pest

resistance, disease resistance or ricin inhibition.

MATERIALS AND METHODS

Source of plant material

Variety SKP-84 is *fusarium wilt* resistant line which has also been used as parent line for some high yielding hybrids like GCH 7. Castor parental line SKP-84 were obtained from the Castor-Mustard Research station, Sardarkrushinagar Dantiwada Agricultural University, Gujarat.

Culture media and growth conditions

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.8% agar was used in all the experiments. Plant growth regulators at different concentrations were incorporated into the basal media as stated below. The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl before autoclaving at 1.06 kg cm^{-2} (121ºC) pressure for 20min. The cultures were incubated at 25 ± 2°C in a culture room with 50 *i* mol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes (40 W; Philips, India) and were exposed to a photoperiod of 16 h and 55 ± 5% of relative humidity (RH).

Shoot bud induction

Excised apical meristems were placed onto MS medium supplemented with different concentrations of BAP (1.111, 2.222, 4.444, 6.666 and 8.888 μM), Kin (1.162, 2.325, 4.651, 6.979 and 9.302 μM) and TDZ (1.136, 2.272, 4.545, 6.818 and 9.090 *i* M). After 3 wk of culture, the frequency of explants producing shoot and the average number of shoots per explants were recorded. All cultures were transferred to fresh medium at every 21 d interval.

Multiplication and elongation of shoots

The established shoots were transferred to MS media added with various concentrations of BAP (1.111, 2.222, 4.444, 6.666 and 8.888 μM), Kin (1.162, 2.325, 4.651, 6.979 and 9.302 μM) and GA_3 (0.289 and 1.445 μM) for multiple shoot induction and elongation.

In vitro root Induction

The elongated individual micro shoots (4-5cm) were excised, and *in vitro* root induction was attempted either on half-strength MS medium without any growth hormones or supplemented with various concentrations of NAA (0.053, 0.268, 0.537, 2.688 and 5.376 μM).

Hardening and acclimatization

In vitro regenerated plantlets with sufficient number and good quality of root were washed carefully in running tap water to remove the traces of agar and transferred to plastic pots containing autoclaved coco pit, vermicompost, vermiculite and garden soil alone and in combinations. Pots were covered with polybags for 4wk to maintain high relative humidity. The plantlets were initially irrigated with quarter-strength inorganic salts of MS medium for 2 wk followed by tap water at 2 days interval for four weeks. Initially the potted plantlets were grown in culture room conditions (25 ± 2ºC, 55 ± 5% RH, under 16h of photoperiod with a light intensity of 40*i* mol m^{-2} s^{-1}. Polybags were removed gradually upon emergence of new leaves and acclimatized plantlets were transferred to the bamboo baskets with sand: soil (1:1).

Genetic stability analysis

Genetic stability analysis of micropropagated plants was examined using RAPD (Random Amplified Polymorphic DNA), a PCR (Polymerase Chain Reaction) based molecular marker system. The genomic DNA was extracted from leaves from every miropropagation stage, using the CTAB (Cetyldimethylethyl Ammonium Bromide) method of Doyle and Doyle, 1990.

Estimation of quantity and quality of DNA

The quantification of DNA was carried out using Nanodrop spectrophotometer by measuring O.D at 260 and 280nm and checked on 0.8 % (w/v) agarose gel prepared in 0.5X TBE (Tris 45mM, Boric Acid 45mM and EDTA 1mM) containing 5 μL of ethidium bromide (EtBr; 50 µg/mL) per 100mL of buffer. Bands were visualized under UV light using a transilluminator. Good quality DNA samples with a ratio of 1.8 – 2.0 at O.D. 260/280 were stored as stock. Stocks were diluted to a final concentration of 50 ng/μl of DNA and used for PCR amplification and was subjected to RAPD analysis.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was performed in a 200μL thin walled PCR tube containing a 25μL volume. The components of PCR consisted of 10X Taq Buffer A (1.5 mM $MgCl_2$), Taq DNA polymerase (3U), dNTP's (0.2 mM each) (Supplied by Banglore Genei), 50 ng DNA, decamer primers 10 pM (Supplied by Biogene, USA) and nano pure water for dilution. Total twenty primers belonging to OPE and OPF series were screened and out of that all four OPE-02 (5'- GGT GCG GGA A -3', GC= 70%), OPE-06 (5'- AAG ACC CCT C -3', GC= 60%), OPF-04 (5'- GGT GAT CAG G -3', GC= 60%) and OPF-10 (5'- GGA AGC TTG G -3', GC= 60%) primers showing proper amplification were used for further stability analysis. The PCR amplification was carried out in thermal cycler following the steps given: **1)** Initial denaturation: 95ºC for 5 minutes, **2)** Denaturation: 95ºC for 1 minute, **3)** Annealing: 38ºC for 1 minute, **4)** Extension: 72ºC for 1 minute, Step 2, 3 and 4 repeated for 35 times, **5)** Final Extension: 72ºC for 7 minutes followed by 4ºC.

PCR products were separated by loading 15μL of each sample and 2μL of loading buffer type II on a 1.2 % agarose gel prepared with 0.5 X TBE buffer. The samples were subject to electrophoresis at 90-100V for 20-25 minutes in 0.5 X TBE buffer. The gel was stained with ethidium bromide and visualized under UV light.

Statistical analysis

All experiments were repeated three times with 30 replicates per treatment. The one-way analysis of variance (ANOVA) was carried out for all experiments and the means were compared using the critical difference (CD) at a 1% level of signiûcance (Panse and Sukhatme 1978).

RESULTS AND DISCUSSION

The present findings demonstrate the possibility for mass propagation of castor through apical meristem. For successful micropropagation apical meristem cultures are preferred as

Table 1: Effect of different concentrations of BAP, Kinetin and TDZ for multiple shoot regeneration through apical meristem (data were recorded after 3 weeks of inoculation).

Sr. No.	Cytokinins (μM)			Multiplication (%)	No. of shoots	Length of shoots (cm)
	BAP	TDZ	Kinetin			
1	1.111	-	-	30.44	7.7	3.2
2	2.222	-	-	27.27	5.7	2.6
3	4.444	-	-	26.80	4.3	1.8
4	6.666	-	-	24.34	5.7	2.4
5	8.888	-	-	24.09	4.0	2.0
6	-	1.136	-	23.05	2.3	1.4
7	-	2.272	-	20.56	2.7	2.1
8	-	4.545	-	21.41	2.7	1.5
9	-	6.818	-	18.42	3.0	1.7
10	-	9.090	-	19.06	3.7	1.1
11	-	-	1.162	26.80	5.7	2.5
12	-	-	2.325	28.88	7.3	3.3
13	-	-	4.651	26.32	4.7	2.5
14	-	-	6.979	24.35	4.3	2.1
15	-	-	9.302	25.84	3.0	2.0
S.Em. ±				0.33	0.49	0.132
C.D. at 5%				0.92	1.42	0.38
C.V. %				2.24	7.05	2.73

Table 2: Effect of combination of cytokinins and GA_3 on shoot multiplication of fusarium wilt resistant *Ricinus communis* parental line SKP-84 (data were recorded after 3 weeks of inoculation).

Sr. No.	Cytokinins (μM)*			Multiplication (%)	No. of shoots	Length of shoots (cm)
	BAP	Kinetin	GA_3			
1	1.111	1.162	-	26.80	8.33	3.83
2	1.111	2.325	-	28.88	10.33	5.20
3	1.111	4.651	-	25.34	8.33	3.07
4	2.222	1.162	-	24.09	6.67	2.70
5	2.222	2.325	-	24.35	7.00	2.40
6	2.222	4.651	-	22.52	7.67	2.83
7	0.444	1.162	-	25.10	7.33	2.40
8	1.111	2.325	1.445	24.09	7.33	2.70
9	1.111	2.325	0.289	26.80	8.33	3.10
10	0.444	1.162	1.445	23.31	6.33	2.83
11	0.444	1.162	0.289	25.10	6.00	2.70
S.Em. ±				0.35	0.31	0.12
C.D.at 5%				1.03	0.91	0.36
C.V. %				2.45	3.32	2.16

Table 3: Effect of subculturing on multiple shoots regeneration and length of shoots (cm) of fusarium wilt resistant *Ricinus communis* parental line SKP-84 (Media: MS + 1.111 μM BAP + 2.325 μM Kin; data were recorded after 3 weeks of inoculation)

No.of Subcultures	No. of shoots	Length of shoots (cm)
1	6.13	3.08
2	6.95	3.25
3	7.75	3.50
4	5.35	3.25
5	5.25	2.98
S.Em. ±	0.471	0.085
C.D.at 5%	1.39	0.25
C.V. %	15.00	5.31

pre-existing meristem easily develop into shoots while maintaining genetic integrity (Sathyanarayana *et al.,* 2008).

Surface sterilization

Seeds from the field are generally laden with large numbers of spores of fungi and bacteria. Decoated castor seeds were surface sterilized with 0.1% $HgCl_2$ for 4 minutes, since it was found to be effective for higher establishment of explants with effective control on contamination. Surface sterilized seeds were further cut from the apices and cultured on basal MS media. Carefully cutting seed can have provided abundant supply of nutrient. About 80% of the incubated seeds were germinated in the MS basal medium (Fig. 1a).

Effect of plant growth regulators on culture establishment

In order to unearth the suitable and optimal cytokinin concentration for *in vitro* regeneration of castor, basal MS media was supplemented with three cytokinins *i.e.* BAP, Kin and TDZ, at various concentrations. The data on the effect of different cytokinins at various concentrations on per cent response to multiplication, number of shoot and length of shoot is presented in Table 1.

It is evident from the Table that out of the three cytokinins used, higher rate of establishment and good quality of shoots could be obtained in media supplemented with either BAP or Kin. Alam *et al.* (2010) also reported superior effect of BA for shoot bud induction. The higher concentration of BAP (more than 4.444μM), was found to produce a negative result as it showed vitrification of shoots in present study which is in acoordance with the finding of Hu and Wang (1983), he reported that the higher concentration of cytokinin reduce the

Table 4: Effect of NAA on *in vitro* root induction in microshoots of fusarium wilt resistant *Ricinus communis* parental line SKP-84 (Media used were ½ MS and data were recorded after 3 weeks of inoculation).

Sr. No.	NAA (μM)	Rooting (%)	Days taken for root initiation	No. Of roots/shoots
1	-	41.00	7.33	2.00
2	0.053	45.33	7.67	3.33
3	0.268	57.33	6.33	4.00
4	0.537	69.00	5.33	5.33
5	2.688	54.33	8.33	3.00
6	5.376	31.00	8.33	2.67
S.Em. ±		0.20	0.26	0.58
C.D.at 5%		0.58	0.76	1.68
C.V. %		1.55	5.84	19.25

Table 5: Effect of different potting mixtures on hardening of fusarium wilt resistant *Ricinus communis* parental line SKP-84 (data were recorded after 6 weeks of inoculation).

Sr. No.	Potting mixtures	Survival (%) of plantlets	Days taken for new sprouting
1	Vermicompost	64.50	11.00
2	Soil	21.75	14.50
3	Coco peat	17.50	18.30
4	Vermicompost : soil (1:1 v/v)	14.50	20.50
5	Vermicompost : soil : Coco peat (1:1:1 v/v)	11.25	21.50
6	Vermiculite	0.0	0.0
S.Em. ±		0.35	0.25
C.D.at 5%		1.04	0.73
C.V. %		2.39	2.05

Figure 1: *In vitro* regeneration and multiplication of fusarium wilt resistant castor parental line (SKP-84) a. Germination of seed, b. Initiation of shoot apices on cytokinins before multiple shoot regeneration, c. Multiplication and Elongation of shoots on combination of cytokinins, d. Subculturing of multiple regenerated plants, e. Rooting of castor plant on established medium containing Auxin, f. and g. Development of plant during hardning

number of micropropagated shoots.

In present study, TDZ had positive effect neither on shoot multiplication nor on shoot quality. The quality shoot as produced by TDZ was fasciculate and with no regeneration and elongation potential even after 3 weeks of culture. Although, Sujatha *et al*. (1998) and Ahn *et al*. (2007) reported better shoot bud multiplication in media supplemented with TDZ, but it has also been reported that TDZ can reduce a number of regenerative processes such as callus formation, somatic embryogenesis and adventitious shoot regeneration (Murthy *et al*., 1998). Surprisingly, our results are in contrast to the findings of Sujatha and reddy (1998) which stated lowest proliferation rate induction by Kin. In our case, best quality of shoot as well as good proliferation rate was achieved in media supplemented with Kin. This difference may be attributed to the difference in genotype.

Effect of cytokinin and GA_3 on shoot Multiplication and Elongation

Since BAP and Kin at lower concentrations showed good results earlier, further investigations were carried out to determine whether the combination of cytokinins and addition of GA_3 in media produce better multiplication and elongation. Effect of combination of cytokinins and addition of GA_3 on shoot multiplication and elongation is shown in Table 2. Addition of GA_3 in growth media showed no marked effect on shoot multiplication or elongation. Sujatha and Reddy (1998) concluded that incorporation of GA_3 significantly enhanced the frequency of elongated shoots but drastically reduced the multiplication ability. Alam *et al*. (2010) used GA_3 in combinations with BAP for elongate proliferated shoots. While in our study combination of 1.111μM BAP + 2.325μM Kin showed good response for elongation and multiplication than addition of GA_3 in media. So, further investigations were carried out with MS media supplemented with 1.111μM BAP and 2.325μM Kin (Fig. 1b).

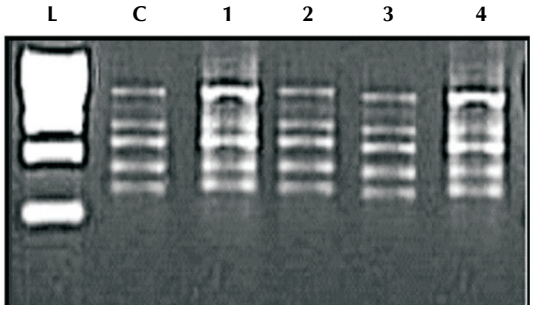
Subculturing on Multiple shoot regeneration through apical meristem

The objective of any micropropagtion protocol is to produce maximum number and good quality of plantlets making the protocol economic. To establish exact number of subcultures to produce maximum number of superior quality plantlets, the best growth regulator combination of previous experiment (1.111 μM BAP + 2.325 μM Kin) were used for sub-culture till four growth cycles (Fig. 1c and d). Multiplication rate gradually increased till third subculture afterwards it showed declining trend and even the length of shoot after third subculture decreased gradually (Table 3).

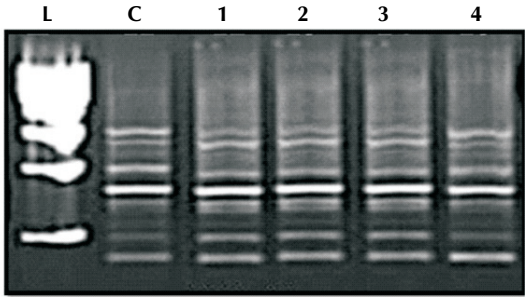
Effect of auxins on *in vitro* root induction

Rooting parameters were significantly influenced by the concentrations of NAA. Half strength MS medium supplemented with 0.537μM NAA took significantly minimum days to initiate the root (5.33 days), maximum rooting per cent (69.00%) and number of root/shoot (Table 4) and (Fig. 1e). Earlier on the experimental basis IBA and IAA were also tried, but since the rooting quality and percent was very less compared to NAA (data not shown) the further rooting experiment conducted using different concentrations of NAA alone. Rooting accomplished on half-strength MS medium supplemented with NAA has already been reported by Sujatha *et al*. (2009) and Alam *et al*. (2010) in *Ricinus communis* L.

Potting mixtures on survival of in vitro raised plantlets and



2a. OPE-6



2b. OPE-10

Figure 2: Molecular stability of Micropropagated plant of castor at each stage of micropropagation. a. OPE-6 random primers, b. OPF-10 random primers (Lane L: 500 bp Molecular marker, Lane C: Control (before *in vitro* regeneration), Lane 1: establishment, Lane 2: multiplication, Lane 3: elongation, Lane 4: rooting)

Acclimatization

After four weeks of incubation on rooting medium the *in vitro* raised plantlets of castor with well developed 4-5 roots were taken out from the culture bottles. The roots were washed thoroughly in remove the adhering agar. The rooted plantlets of castor were then transplanted in plastic cups (200 ml) containing combinations of different potting mixture. The rooted plantlets were initially covered with transparent plastic cups (Fig. 1f). The results on survival per cent and plant growth in hardening are presented in Table 5. The maximum survival (64.50%) with minimum days for new sprouting (11 days) was reported in Vermicompost. Successful hardening of castor has also been reported by Sujatha *et al*. (2009). The reason for better hardening in vermicompost may be due to presence of rich organic matter source providing strength and essential nutrients for survival to the in vitro raised plants. The plantlets were further transferred to bamboo baskets containing sand: soil (1:1) drenched with 0.05 per cent bavistin. Most of the transferred plant showed better growth (Fig. 1g). The hardened plants further transferred to the soil.

Genetic stability during *in vitro* regeneration

Efforts were made to understand whether growth cycles affected the fidelity of genotype. Leaf samples from *in vitro* grow castor shoots were collected after different culture stages (establishment, multiplication, elongation and rooting) and cryopreserved for molecular diversity analysis. The samples were subjected to RAPD using four selected random decamers (OPE-02, OPE-06, OPF-04 and OPF-10) screened from a set of twenty primers. All bands generated from four RAPD decamers were found non-polymorphic (Fig. 2a and 2b) showing gentic integrity during *in vitro* manipulation of explant. Various authors have found RAPD technique useful in examining genetic fidelity of tissue culture-clones. With the use of RAPD markers, clonal fidelity of micropropagated plants has been determined in *Pinus thunbergii* (Goto *et al.,* 1998), *Lilium* (Varshney *et al.,* 2001) and *Tylophora indica* (Jayanthi and Mandal, 2001).

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