ISSN 1996-0700

Asian Journal of Biotechnology

Knowledgia
SCIENTIFIC
A Place to Publish Outstanding Research

http://knowledgiascientific.com

Asian Journal of Biotechnology 3 (2): 165-176, 2011
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2011.165.176
© 2011 Knowledgia Review, Malaysia

Profuse Microtillering of Androgenic Plantlets of Elite Indica Rice Variety IR 72

[1]Bidhan Roy and [2]Asit B. Mandal

[1]Department of Genetics and Plant Breeding, Regional Research Station, Terai Zone, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar 736 165, West Bengal, India
[2]Directorate of Seed Research, Khushmaur, P.O. Kathuli, Maunath Bhanjan 275 101, Uttar Pradesh, India

Corresponding Author: Bidhan Roy, Department of Genetics and Plant Breeding, Regional Research Station, Terai Zone, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar 736 165, West Bengal, India

ABSTRACT

The aim of this experiment was to induce profused microtillering of androgenic plantlets of elite indica rice (*Oryza sativa* L.) variety IR 72. Two microtillers were inoculated in each culture tube containing different medium and growth regulators. Various combinations of growth regulators were also used with each culture medium. Microtillering ability were noted on 45th day after inoculation of microtillers. MS medium with 4-8 mg L^{-1} of kinetin showed very fast microshooting with maximum of 94.7 shootlets per cultured shoot within 45 days of inoculation. Conversely, BAP at high dose (4-8 mg L^{-1}) was found to exert inhibitory effects on shoot development, but induced large number of dormant embryos and pro-embryos. The caulogenic shoots that emerged from the base of the explants were successfully induced root on MS supplemented with 1 mg L^{-1} IAA. Evaluation of clonal fidelity of the microshoots through profiling of isozyme in respect of peroxidase and malate dehydrogenase and RAPD analysis was done to confirm true-to-type nature of the micropropagated plants. Microtillering and subsequent whole plant development may offer ample scope to develop large number of clones from a stock of choice for *in vitro* conservation, characterization and screening for diverse characters of agronomic importance. This approach may have far reaching consequences in transgenic development especially through direct bombardment of miniature shootlets *en route* biolistic and their subsequent multiplication *en masse*.

Key words: *Oryza sativa,* androgenic, microtillering, rooting, isozyme, PCR

INTRODUCTION

Microtillering under *in vitro* culture condition in cereals especially in rice is scanty. It bears tremendous importance in multiplying precious stocks and thus holds a key role in bio-conservation also (Tyagi and Prakash, 2004). On the other hand, microtillering potential can be exploited immensely to supplement transgenic research by multiplying transformed shoots (Peng *et al*., 1992). So, the number of true transgenics may be increased to a larger extent within a short time. Such microtillers are deemed to be true-to-type, if *in vitro* culture system does not create any alteration at the genetic level. In spite of intensive efforts, till date the anther culture response and recovery of androgenic green plantlets remain very less especially in indica rice (Roy and Mandal, 2005; Talebi *et al*., 2007; Niroula and Bimb, 2009). Microtillering and subsequent establishment of whole plants with well developed roots may offer ample scope to develop a large number of sister clones from a important stock, such as IR 72 for subsequent characterization, screening against

diverse biotic and abiotic stresses and for other genetic studies. IR 72 is a high yielding variety developed at International Rice Research Institute, Philippines. It yielded 6.55 t ha^{-1} under standard agronomic management at Central Agricultural Research Institute, Port Blair, India. The grain is medium slender.

Multiplication of doubled haploid lines derived through anther culture may have discernible impact in crop genetic improvement, chromosome stock maintenance and construction of mapping population. Development of androgenic plantlets has been used for crop improvement and development of cultivars (Senadhira *et al.*, 2002). This technique opens new vistas of breeding for resistance and quality of crop plants. It shorten the breeding cycles by immediate fixation of homozygosity and also increase selection efficiency.

To study the clonal fidelity isozyme and RAPD were also studied by many workers. Isozyme analysis is evidenced to be important in classificatory analysis of genotypes and demarcating genomes (Johnson *et al.*, 1967). Isozymes are multiple molecular forms of enzymes with similar substrate specificity and are being used in assessing degree of variations in diverse species. Isozyme pattern frequently varies in intact plants as a function of various physiological and developmental states; are well evident as suitable molecular marker system under physiological stress condition (Maheswaran and Sree-Rangaswamy, 1990). Isozyme studies in rice have been progressed in several lines of research involving gene mapping, gene regulation, developmental genetics and evaluation. In the present study to confirm the clonal fidelity of the micro-propagated androclones, isozyme analysis in respect of peroxidase and malate dehydrogenase were performed.

RAPDs were used extensively across diverse crops to detect changes in tissue culture derived plant progenies to establish plant-progeny relationships. RAPD is primarily based on the use of oligonucleotide primers of arbitrary sequences and were preferred in a variety of applications over RFLPs due to its simplicity (Mullis and Floona,1987). RAPD was used successfully to assess genetic stability among the somatic embryos (Brown *et al.*, 1993; Isabel *et al.*, 1996; Rout *et al.*, 1998). Castillo *et al.* (2010) used SSR and AFLP for genetic stability of cryopreserved shoot tips of *Rubus* germplasm.

In the present study, we tried to standardized a suitable protocol to mass-multiply androgenic microtillers without changing the genetic makeup of the mass-multiplied tillers. Isozymes and RAPD were used to establish clonal fidelity of the micotillers.

MATERIALS AND METHODS

Anther culture: IR 72, an elite indica rice variety was grown in experimental net house during April-July, 1998 at Central Agricultural Research Institute, Port Blair, India. The primary panicles enclosed in leaf sheaths were collected from the main tillers when the distance between the auricle of the flag leaf to that of the first subtending leaf was ~6 cm. The collected panicles along with the boot leaf sheath were covered with moist tissue paper, kept in polyethylene bags and cold treated for 8 days at 8°C prior to anther plating. At the day of inoculation, selected spikelets were surface sterilized with 0.1% freshly prepared $HgCl_2$ solution for 10 min. The $HgCl_2$ was then decanted and the panicles were washed four times in sterile distilled water to remove excess $HgCl_2$. The bottles were kept inverted on sterile tissue paper to soak excess water from the surface of the spikelets. Anthers were plated following method of Chung *et al.* (1991) on 60×15 mm diameter radiation-sterilized petri dishes containing callus induction medium N6 (Chu *et al.*, 1995) supplemented with 2 mg L^{-1} 2, 4-D, 6% sucrose and 0.8% agar. The petri plates were sealed with parafilm and kept

in complete dark at 25±2°C. Embryogenic calli of at least ~2 mm diameter were transferred to 25×150 mm culture tubes containing 10 ml of regeneration medium consisting of MS (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} BAP, 1 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA, 3% (m/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 with 1 N HCl or 1 N NaOH before adding agar and autoclaved at 121°C at 104 kPa for 15 min. The culture tubes were plugged with non-absorbent cotton wrapped in one layer of cheesecloth. Inoculated cultures were kept for four weeks under 16/8 h light (~130 µE/m^2/sec)/dark at 25±2°C. The regenerated plantlets were used as explants for induction of microtillers.

Microtillering: The regenerated shoots of androgenic origin were cultured on MS medium supplemented with 2 mg L^{-1} of BAP. After initial multiplication of microtillers, it was search to find out the best medium and growth regulators combination(s) for rapid microtillering. Thus initially multiplied microtillers of androgenic origin were multiplied recurrently (Roy and Mandal, 2006). These microtillers were again used for mass multiplication during 2004 on MS, N_6 and B5 (Gamborg *et al*., 1968) basal media supplemented with different concentrations of 6-bezylaminopurine (BAP), N-(2-furfurylamino)-1-H-purine-6-amine (kinetin), 1-naphthaleneacetic acid (NAA) singly and in various combinations (Table 2) to induce and optimize microtillering ability. Two androgenic plantlets were inoculated into each culture tubes and for each treatment 20 culture tubes were employed in three replications. Thus, in each treatment 120 androgenic shootlets were inoculated. Culture condition was kept same as used for the regeneration of plantlets from androgenic calli during anther culture. Visual observations like average number of shoots developed per cultured shoot, shoot length and shoot health (Table 2) were taken on the 45th day after culture.

***In vitro* rooting:** Attempt was made to induce root of micropropagated caulogenic androclones. *In vitro* raised shoots measuring 1-4 cm were separated manually under Laminar Airflow cabinet and shoots were cultured individually on MS basal supplemented with different concentrations and combinations of BAP, 1H-indol-3-butyric acid (IBA), 1H-indol -3-acetic acid (IAA) and NAA (Table 4). Two androclones were inoculated in each culture tube and each treatment had 20 culture tubes in three replications. Observations were made on average number of shoot developed per cultured shoot, shoot length, root length, number of roots per cultured shoot, rooting percentage and plant health after 45 days of culture.

Isozyme analysis: Enzymes with some catalytic activity but with different molecular forms are known as isozymes. They are generally made up of a number of subunits and it the varying combination of subunits which give rise to isozymes. Enzymes were extracted following the methods as outlined by Sadasivam and Manickam (1996). As it is well known that isozyme plants may vary with developmental and differentiation stages (Scandalios, 1974), we compared leaf tissues of 45 days old microtillers. Fifty micro-litter of the supernatant was used for isozyme analysis following polyacrylamide gel electrophoresis (PAGE). The gels were run for 30 min at 50 V and then 80 V for 2 h. Staining was also performed as outlined by Sadasivam and Manikam (1996) individually for the two isozymes viz. peroxidase (E.C.1.11.1.7) and malate dehydrogenase (E.C.1.1.1.37). Gels were photographed over an illuminated viewer and Rf value of individual bands were calculated.

PCR-analysis: Twelve arbitrary decamers primers (Operon Technologies USA, Kit D) were employed for PCR amplification using Ready-to-Go-RAPD Analysis Beads (Amersham Pharmacia Biotech Inc., USA). Genomic DNA was extracted from shoots of microtillers of IR 72 following standard CTAB method (Murray and Thomson, 1980). DNA was purified following Sephaglas™ Band Prep Kit (Amersham Pharmacia Biotech Inc., USA). DNA amplification was performed in a Peltier Thermal Cycler (MJ Research, PTC-200). After initial heat denaturation of the DNA at 94°C for 1 min, thermal cycling was performed for 30 times following the temperature regimes -95°C for 1 min, 40°C for 2 min, followed by 72°C for 3 min. The final extension step at 72°C for 5 min was followed by cooling to 8°C to complete the PCR. The amplified products were size fractioned by gel electrophoresis in 0.8% agarose gel with 1×TAE and stained with ethidium bromide. Photographs were taken by using image analysis software, Bio-Profil (Vilber Lourmat, France).

Statistical analysis: The data were subjected to analysis of variance and significant treatment differences selected by Duncan's Multiple Range Test (DMRT).

RESULTS

Callus induction and plantlet regeneration: Cultured anthers of variety IR 72 showed callus induction within 15-30 days on callus induction medium. Most of the responding anthers turned brown and produced 2-4 calli per anther and in a few cases they produced multiple calli. Response of anther to callus formation was 0.60% and regeneration of green plantlets from calli was only 22.2%.

Microtillering: Significant differences were observed in all the treatments for average number of shoots per cultured shoot and shoot length for three different media compositions (Table 1). Rapid and high rate of multiple shooting were observed on MS (Fig. 1c) supplemented with kinetin at 4, 6 and 8 mg L^{-1} (Table 2). They were followed by the combinatorial treatments involving 1 mg L^{-1} BAP with 4 mg L^{-1} kinetin and 1 mg L^{-1} BAP with 3 mg L^{-1} kinetin. In some of the treatments only embryos and pro-embryos were observed without differentiation into plantlets (Fig. 1a, b). Maximum number of dormant embryos and pro-embryos were recorded on MS containing 2 mg L^{-1} BAP+4 mg L^{-1} kinetin followed by 4 mg L^{-1} BAP + 1 mg L^{-1} kinetin (Table 2). BAP singly at a concentration of 4, 6 and 8- mg L^{-1} showed average of 22.6, 26.7 and 14.8 number of dormant embryos, respectively (Table 2).

Table 1: Analysis of variance of micropropagation of androgenic plantlets of an *indica* rice var. IR 72

		Mean sum of square					
		MS		N6		B5	
Source of variation	df	Av. No. of shootlet developed/ cultured shoot	Shoot length (cm)	Av. No. of shootlet developed/ cultured shoot	Shoot length (cm)	Av. No. of shootlet developed/ cultured shoot	Shoot length (cm)
Replication	2	0.55	0.07	0.24	0.01	0.24	0.003
Treatment	25	1479.08**	19.06^{NS}	92.61**	1.36**	20.22**	3.31**
Error	50	5.50	0.16	3.69	0.12	0.63	0.06
Total	77	483.81	6.29	32.47	0.52	6.98	1.11

**Significance at p = 0.01; NS: Non-significant

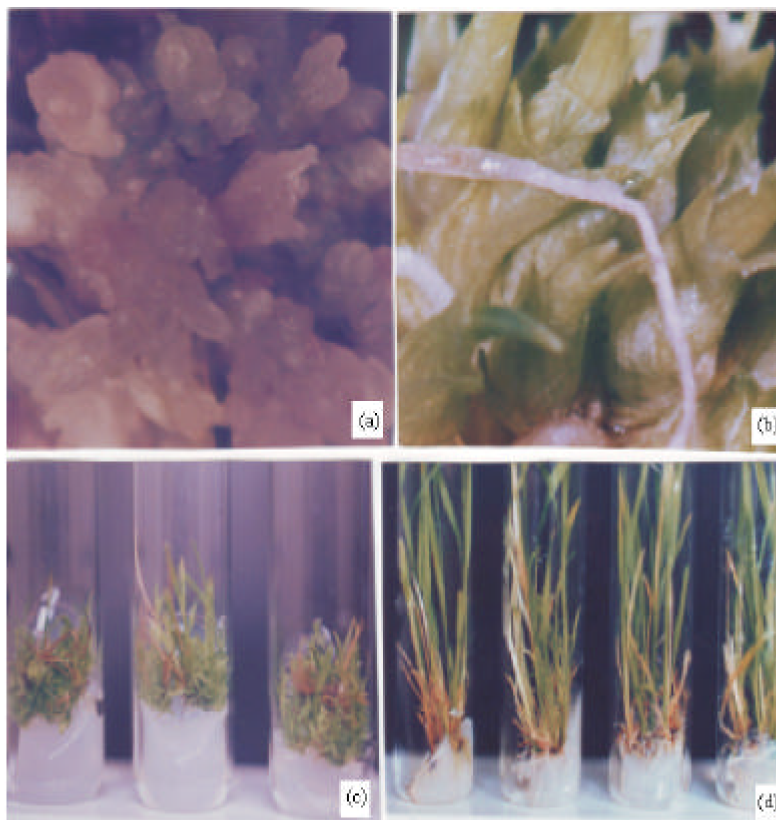

Fig. 1: (a-d) Microtillering in an elite indica rice var. IR 72. (a) Dormant pro-embryos developed at the base of cultured explants on MS with BAP 6 mg L^{-1}. (b) stereo-microscopic view of dormant embryos developed at the base of cultured explants on MS supplemented with BAP 6 mg L^{-1} at 45th day after inoculation of microtiller (10×). (c) profuse microtillering under *in vitro* condition on MS fortified with kinetin 4 mg L^{-1}. (d) Rooted microtillers on MS fortified with 1 mg L^{-1} of IAA

Multiple shoots were also observed on N_6 medium with different growth regulators (Table 2). N_6 medium with supplemented with BAP and kinetin singly (1, 2, 4, 6 and 8 mg L^{-1}) produced multiple shoots that ranged from an average of 9.1 to 25.8 per shoot cultured. The combination of BAP and kinetin in N_6 also produced multiple shoots (8.6 to 23.6). On an average, each treatment in N_6 produced 15 plantlets per shoot cultured except combination of BAP, kinetin and NAA. The latter showed an average of 4.71 shoots in comparison to 2.65 on MS and 5.87 on B_5, respectively. Health of the shoots remained average to weak throughout the treatments involving N_6.

B_5 displayed poor results (Table 2). No treatment showed rapid microtillering. On an average plantlet yield was ~7. Health of plantlets developed on B5 was medium to bad. Microtiller, dormant embryos and pro-embryos were also noticed in case of B_5.

***In vitro* rooting:** Majority of the androclones derived from anther culture was rootless except those developed on MS supplemented with 2 mg L^{-1} BAP. Therefore, an attempt was made to induce root from caulogenic plantlets, which were originated following organogenesis. Significant variation was observed in all the treatments for average number of shoot per culture, shoot length and number

Table 2: Micropropagation of androgenic plantlets of an indica rice var. IR 72

Treatment (mg L^{-1})			Medium								
			MS			N_6			B_5		
BAP	Kinetin	IBA	Av. No. of shootlet or embryo developed/ explant	Shoot length (cm)	Shoot health (1-5 scale)**	Av. No. of shootlet or embryo developed/ cultured shoot	Shoot length (cm)	Shoot health (1-5 scale)**	Av. No. of shootlet or embryo developed/ cultured shoot	Shoot length (cm)	Shoot health (1-5 scale)**
1	-	-	17.6j	*	3	19.2cde	1.1gh	4	4.8efg	0.2g	3
2	-	-	20.7hij	12.0a	2	10.9ij	*	3	13.2a	*	2
4	-	-	22.6ghi	*	2	12.6ghi	1.9cdef	4	5.2ef	2.0c	4
6	-	-	26.7g	*	2	22.7abc	1.3efg	3	4.4fghi	*	4
8	-	-	14.8k	*	3	20.2bcd	0.6h	4	2.5j	1.0def	5
-	1	-	11.9kl	4.3b	3	15.7efg	1.6def	3	7.5cd	*	5
-	2	-	26.2g	4.4b	2	19.1cde	1.2fgh	3	6.1de	0.6efg	4
-	4	-	94.7a	4.1b	2	25.8a	2.8a	3	5.0efg	*	4
-	6	-	71.9b	3.0c	2	12.5ghi	1.4efg	3	4.3fghi	*	5
-	8	-	68.9c	1.5ef	2	12.4ghi	2.4abc	3	3.2hij	0.8def	6
1	2	-	24.6gh	2.1d	2	13.9fghi	2.0bcde	3	2.9ij	4.2a	5
1	3	-	34.8ef	1.6ef	3	11.9ghij	2.0bcde	4	3.7fghi	3.2b	5
1	4	-	38.9e	1.6ef	2	8.6jkl	0.7gh	4	2.3j	*	5
2	3	-	25.2gh	*	3	16.6def	1.1gh	2	4.4fghi	*	4
2	4	-	40.1d	*	2	10.5ij	1.1gh	4	8.9bc	0.5fg	4
3	4	-	19.6ij	*	3	17.1def	2.2abcd	3	8.6bc	*	3
2	1	-	22.2ghi	*	4	11.6hij	1.32efg	3	3.6ghij	*	4
3	1	-	10.2l	*	4	11.6hij	1.4efg	3	9.3b	0.9def	4
4	1	-	26.4g	*	4	23.6ab	2.6abc	2	2.3j	1.1de	5
3	2	-	25.3gh	0.8g	3	10.0ijk	2.7ab	4	4.4fghi	1.2d	5
4	2	-	22.6ghi	0.9fg	3	15.4efgh	1.6def	4	4.2fghi	0.9def	5
4	3	-	9.2l	1.1fg	4	12.2ghij	1.4efg	2	2.8ij	*	5
1	1	0.5	2.9m	2.1d	4	6.3ml	0.8gh	4	6.1de	*	3
2	2	0.5	4.0m	1.3ef	4	6.1ml	1.0gh	5	5.0efg	*	3
1	3	0.5	1.9m	1.6ef	4	6.4klm	1.3efg	4	4.5fgh	1.1de	4
3	1	0.5	1.8m	1.7e	4	5.0m	1.4efg	4	7.9bc	*	3

Values with same letter(s) in column are not significantly different at p 0.05 of DMRT.*Only dormant embryos and pro-embryos produced, further differentiation not observed; **1: Excellent; 2: Good; 3: Medium; 4: Weak and 5: Bad

of roots per culture and root length (Table 3). Excellent rooting with simultaneous shoot elongation was observed on MS containing 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} IAA (Fig. 1d); 1 mg L^{-1} IAA; 1 mg L^{-1} IBA and MS without any growth regulators (Table 4). Maximum number of roots was observed on MS with 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA followed by MS containing 1 mg L^{-1} IAA and 1 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA in combination. Longest root growth was reported on MS with 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA followed by 1 mg L^{-1} IAA. Cent percent rooting was observed in case of MS supplemented with 1 mg L^{-1} IBA; 1 mg L^{-1} IAA. Among the combination treatments 0.5 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA; 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} IAA, 0.5 L^{-1} IBA; 1 L^{-1} IAA and 1 L^{-1} IBA were found to be promising.

Table 3: Analysis of variance of *in vitro* rooting of androgenic shootlets of *indica* rice var. IR 72

		Mean sum of square			
Source of variation	df	Av. no of shootlets/ shoot cultured	Shoot length (cm)	Av. No. of root/ shoot culture	Root length (cm)
Replication	2	0.59	0.002	0.30	0.02
Treatment	11	347.85**	7.12**	170.81**	10.26**
Error	22	2.59	0.24	0.91	0.23
Total	35	139.27	2.39	54.27	3.37

**Significance at p 0.01

Table 4: *In vitro* rooting of androgenic shootlets of *indica* rice var. IR 72

Treatment	Av. no of shootlets/ shoot cultured	Shoot length (cm)	Av. No. of root/ shoot culture	Root length (cm)	Rooting (%)	Plant health (1-5 scale)**
Full MS	10.6cd	4.3a	14.33b	4.26a	100.0	1
½ MS	4.5g	2.1b	8.83d	3.56ab	70.4	2
MS+0.5 mg L^{-1} BAP	43.4a	*	-	-	-	2
MS+0.5 mg L^{-1} BAP+ 0.5 mg L^{-1} NAA	30.8b	*	-	-	-	3
MS+1.0 mg L^{-1} BAP	15.2c	0.6c	-	-	-	4
MS+1.0 mg L^{-1} BAP+ 0.5 mg L^{-1} NAA	21.9b	1.4c	4.83e	3.20b	80.6	2
MS+0.5 mg L^{-1} kinetin+ 0.5 mg L^{-1} NAA	17.6c	2.1b	11.00c	3.51ab	100.0	2
MS+0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} IAA	5.1g	1.9b	-	-	-	2
MS+0.5 mg L^{-1} NAA +0.5 mg L^{-1} IAA+0.5 mg L^{-1} IBA	7.4fg	3.4a	23.00a	3.83ab	100.0	1
MS+1.0 mg L^{-1} NAA	5.5fg	1.6b	-	-	-	2
MS+1.0 mg L^{-1} IAA	6.4fg	4.2a	13.16b	3.58ab	100.0	1
MS+1.0 mg L^{-1} IBA	8.2ef	3.6a	10.33cd	3.28b	100.0	1

Values with same letter(s) in column are not significantly different at p 0.05 of DMRT. *Only dormant embryos and pro-embryos produced, further differentiation not observed. **1: Excellent; 2: Good; 3: Medium; 4: Weak and 5: Bad

Isozyme analysis: To check the clonal fidelity of micro-propagated plantlets derived through anther culture, peroxidase and malate dehydrogenase were analyzed. Peroxidase profile showed no change in the banding pattern in all the samples. All of them showed three bands with Rf 0.12, 0.28 and 0.68, respectively (Fig. 2a). Malate dehydrogenase displayed six bands with Rf values 0.03, 0.15, 0.42, 0.54, 0.64 and 0.69, respectively and all the loci were found to be monomorphic in parent androclones and microtillers of the sister clones (Fig. 2b). Specific variation in metabolic status generally results in discernible differences in isozyme pattern. Similar banding pattern was observed in all the samples in respect of individual isozyme. This confirms the true-to-type nature of the micropropagated plantlets derived from parental androclone.

PCR-analysis: DNA amplification of the *in vitro* developed androgenic microtillers was recorded in the form of score able banding initial and compared with parental androgenic plant. Among the 12-decamer primers used for RAPD analysis only five (OPD 5, 7, 10, 11 and 20) could show amplification in the form of discernible bands on the agarose gel. Five bands were observed in case of OPD-7 and two bands were found in respect of OPD-10 (Table 5).

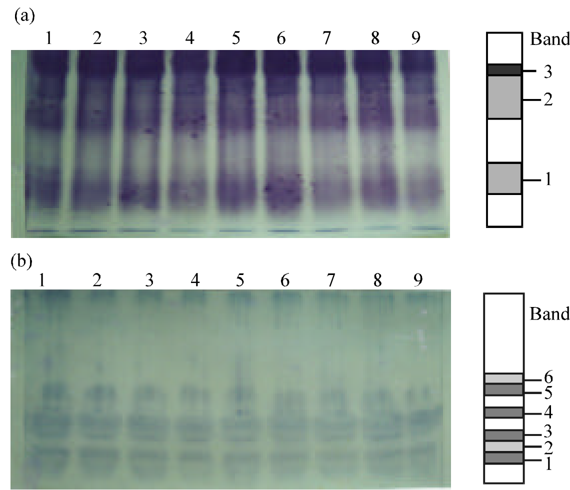

Fig. 2: (a, b) Profiling of microtillers in respect of isozymes. (a) Peroxidase, (b) Malate dehydrogenase. Lane 1: Parent androclone; Lanes 2-9: Randomly selected microtillers

Table 5: Dossier of oligonucleotide decamer primers used

Primer designation	Sequence	No. of amplicons
OPD-5	5'TGAGCGGACA3'	3
OPD-7	5'TTGGCACGGG3'	5
OPD-10	5'GGTCTACACC3'	2
OPD-11	5'AGCGCCATTG3'	4
OPD-20	5'ACCCGGTCAC3'	4

DISCUSSION

Regeneration of green plantlets from anther derived callus is very difficult. Thus, mass-multiplication of androclones using *in vitro* technique has ample importance for further use of those androclones for genetic studies. In this endeavour, we could develop a suitable protocol for rapid and mass-multiplication of anther derived microtillers. BAP was found to have inhibitory effects on shoot growth. The MS with moderate levels of BAP produced high number of dormant embryos and pro-embryos (Table 2). The inoculated shoots were died in such cases and embryos and pro-embryos were produced at the base of the inoculated shoot. Further culture of those embryos and pro-embryos on MS supplemented with 1 mg L^{-1} IAA produced well developed roots and shoots. The regenerated shoot buds rapidly multiplied and elongated on similar induction medium. Bejoy *et al*. (2006) also reported more shoot regeneration of *Cucurma haritha* on MS as compared to White's medium. The rate of multiple shoot formation, however, found to be dependent upon growth regulators as reported earlier in monocots and grasses (Finch *et al*., 1992; Samantaray *et al*., 1995). Rapid and high rate of microtillering was found to be enhanced by kinetin.

Kinetin at 4, 6 and 8 mg L^{-1} was found to be encouraging with optimal performance at 4 mg L^{-1}. This growth regulator produced about 68-95 microtillers from individual androgenic plantlets in many treatments. It could be noted that the moderate concentrations of BAP (2-4 mg L^{-1}) with different doses of kinetin either retarded growth of shoots or produced embryos and pro-embryos with no differentiation. This clearly indicates that higher concentration

of BAP had inhibitory effects on microtillering of androgenic plantlets of rice. This result contradicts with the findings of Medina *et al.* (2004). They reported high multiplication frequency of adventitious shoot of rice when medium supplemented with 5 mg L^{-1} BAP. Combination of BAP and kinetin generated arbitrary results with no clear-cut trend whereas combination of BAP, kinetin and NAA showed very poor response in facilitating microtillering. This result is in resemblance with the contemporary studies in rice by Mahapatra *et al.* (1997). MS was found to be the most effective medium in manipulating microtillering, shoot health (range: 2-4) and shoot length in comparison to the others.

All the microtillers were without root. The rootless microtillers has no use unless it is rooted on appropriate medium composition. The growth regulators- IBA, IAA and NAA showed positive influence on root initiation. Similar kind of effects of growth regulators on rooting was observed by (Das *et al.*, 1997) in *Tryponium trilobatam*, Mandal *et al.* (1998) in *Oryza rufipogon* and Bashir *et al.* (2007) in *Simmondsia chinensis*. The combinations of growth regulators, such as, 0.5 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA; 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} IAA, 0.5 mg L^{-1} IBA; 1 mg L^{-1} IAA and 1 mg L^{-1} IBA were found to be promising. *In vitro* induction of roots from androgenic plantlets bears tremendous importance, since the plantlets regenerated following organogenetic pathway were rootless.

The method based on adventitious shoot culture may results in genetic variation of obtained plantlets (Khanna and Raima, 1998). Variation may results in modification of chromosome number or methylation pattern, chromosome breakage, transposon activation, deletion, genome rearrangement, olyploidy or nucleotide substitution etc. (Bhatia *et al.*, 2005). Isozymes and RAPD profiles of different microtillers of rice, variety IR 72 were assessed for their genetic stability. Both the isozyme system showed monomorphic bands among the microtillers as well as the initial anther derived microtillers. The similar banding pattern among all the samples strongly indicated the clonal fidality of the microtillers. Medina *et al.* (2004) verified genetic fidality of micropropagated plants of rice using isozyme systems. Saker *et al.* (2000) and Zivdar *et al.* (2008) also used isozyme system to find out the genetic stability of tissue culture derived micro-clones of date palm.

The banding pattern for all the five primers in respect of initial microtillers and microtillers had no variation (Fig. 3a-e). By using arbitrary oligonucleotide primers RAPD generally detects variation in DNA sequences by producing amplicons of varying lengths and thus bear key importance in establishing genetic relationships between parental androclone and the androclonal microtillers. Using current strategies for evaluating DNA sequence variation independent of copy number changes in repetitive sequences, the present study showed overall genetic uniformity and true-to-type character of the tillers micropropagated *in vitro* from androgenic plantlets of rice variety IR 72. The monomorphic bands in micropropagated plants by using different primers were reported earlier (Rout *et al.*, 1998). Monomorphism was also observed by Rani and Raina (1998) in enhanced-axillary-branching culture of mature *Eucalyptus tereticornis* Smith. and *E. camaldulensis* Dehn. plants. Genetic uniformity among the microtillers prospects their utility in genetic transformation work where propagation of transgenic plant especially of androgenic origin would be immensely important (Peng *et al.*, 1992). Castillo *et al.* (2010) studied genetic stability of cryopreserved shoot tips of *Rubus* germplasm using SSR and AFLP.

In nutshell, MS supplemented with 4.0 mg L^{-1} kinetin was found to be the most appropriate nutritional and hormonal milieu to achieve very fast microshooting that emerges from the base of the inoculated shoots. Where as, MS with BAP (4.0-8.0 mg L^{-1}) produced a large number of dormant embryos and pro-embryos which can be used for *in vitro* preservation for longer period

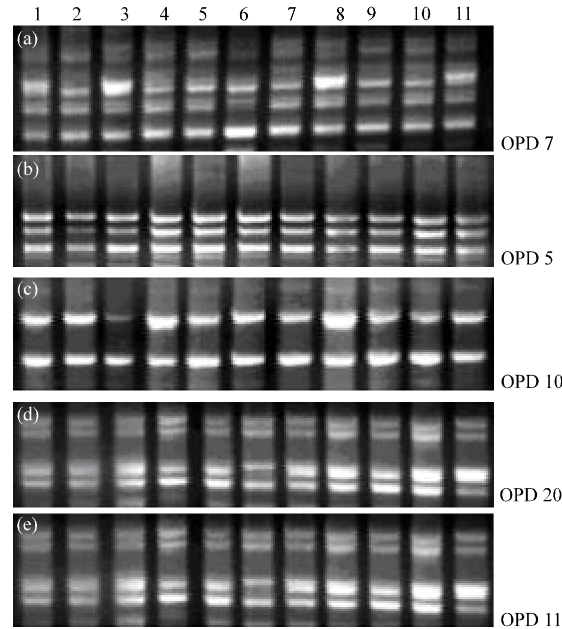

Fig. 3: (a-e) Gel electrophoresis of RAPD fragments obtained from microtillers. (a) OPD-7 (5' TTGGCACGGG 3'). (b) OPD-5 (5' TGAGCGGACA 3'). (c) OPD-10 (5' GGTCTACACC 3'). (d) OPD-20 (5' ACCCGGTCAC 3') and (e) OPD-11 (5' AGCGCCATTG 3'). Lane 1: parental clone and Lanes 2-11: Randomly selected androgenic microtillers of IR 72

(Roy and Mandal, 2006). The MS with IAA (1 mg L^{-1}) NAA (1 mg L^{-1}) and IBA (1 mg L^{-1}) induced multiple rooting and led into vigorous plant growth. In essence isozyme analysis in respect of peroxidase and malate dehydrogenase and RAPD analysis revealed true-to-type nature of the micropropagated plantlets evolved through microtillering reaffirming their suitability in clonal propagation work including use in transgenic development.

REFERENCES

Bashir, M.A., M.A. Anjum and H. Rashid, 2007. *In vitro* root formation in micropropagated shoots of Jojoba (*Simmondsia chinenesis*). Biotechnology, 6: 465-472.

Bejoy, M., M. Dan and N.P. Anish, 2006. Factors affecting the *in vitro* multiplication of the endemic zinziber *Curcuma haritha* Mangaly and Sabu. Asian J. Plant Sci., 5: 847-853.

Bhatia, P., N. Ashwath, T. Senaratna and S. Krauss, 2005. Genetic analysis of cotyledon-derived regenerants of tomato using AFLP markers. Curr. Sci., 88: 85-92.

Brown, P.T.H., F.D. Lange, E. Kranz and H. Lorz, 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. General Genet., 237: 311-317.

Castillo, N.R.F., N.V. Bassil, S. Wada and B.M. Reed, 2010. Genetic stability of cryopreserved shoot tips of *Rubus germplasm. In vitro* Cell. Dev. Biol. Plant, 46: 246-256.

Chu, C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu and F.Y. Bi, 1995. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Scientia Sinica, 5: 659-668.

Chung, G.S., S.J. Yang and B.G. Oh, 1991. Reliable, simple methods for producing rice plants through anther culture. Proceedings of the 2nd International Rice Genetics Symposium, May 14-18, IRRI, Philippines, pp: 791-793.

Das, P., A. Patra, A.B. Das, S.K. Palai and T.K. Bose, 1997. *In vitro* regeneration of *Tryponium trilobatam* Schott. a medicinal plant, through cell culture. Orissa J. Hort., 25: 57-61.

Finch, R.P., A. Baset, I.H. Slamet and E.C. Cocking, 1992. *In vitro* shoot culture of wild oryzae and other grass species. Plant Cell Tissue Organ Culture, 30: 31-39.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Isabel, N., L. Tremblay, M. Michaud, F.M. Tremblay and J. Bousquet, 1996. Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea gluca*, Pinaceae). Am. J. Bot., 83: 1121-1131.

Johnson, B.L., D. Barnhart and O. Hall, 1967. Analysis of genome and relationships in the polyploid wheat by protein electrophoesis. Am. J. Bot., 54: 1089-1098.

Khanna, H.K. and S.K. Raina, 1998. Genotype culture medium interaction effect on regeneration response of three indica rice cultivars. Plant Cell Tiss. Org. Cult., 52: 145-153.

Mahapatra, K., L.K. Bose, S.C. Sahu, S. Rajamani and K.C. Mathur, 1997. *In vitro* multiplication of the rice gall midge. Rice Biotech. Q., 29: 8-8.

Maheswaran, M. and S.R. Sree-Rangaswamy, 1990. Peroxidase isozyme system: A potential marker in *Oryza* culture *in vitro*. Oryza, 27: 129-132.

Mandal, N., S.K. Sinha and S. Gupta, 1998. Anther culture of *Oryza rufipogon* Griff. Oryza, 35: 232-236.

Medina, R., M. Faloci, M.A. Marassi and L.A. Mroginski, 2004. Genetic stability in rice micropropagation. Biocell, 28: 13-20.

Mullis, K.B. and F.A. Faloona, 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Method Enzy., 155: 335-350.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Murray, M.G. and W.F. Thompson, 1980. Rapid isolation of high molecular weight DNA. Nucleic Acids Res., 8: 4321-4325.

Niroula, R.K. and H.P. Bimb, 2009. Effect of genotype and callus induction medium on green plant regeneration from anther of Nepalese rice cultivars. Asian J. Plant Sci., 8: 368-374.

Peng, J., H. Kononowicz and T.K. Hodges, 1992. Transgenic indica rice plants. Theor. Applied Genet., 83: 855-863.

Rani, V. and S.N. Raina, 1998. Genetic analysis of enhanced-auxiliary-branching-derived *Eucalyptus tereticornis* Smith and *E. camaldulensis* Dehn Plants. Plant Cell Rep., 17: 236-242.

Rout, G.R., P. Das, S. Goel and S.S. Raina, 1998. Determination of genetic stability of micropropagated plants of ginger showing random amplified polymorphic DNA (RAPD) markers. Bot. Bull. Acad. Sinica, 39: 23-27.

Roy, B. and A.B. Mandal, 2005. Anther culture in *indica* rice and variation in major agronomic characters in androclones of Karnal local. Afr. J. Biotechnol., 4: 235-240.

Roy B. and A.B. Mandal, 2006. Rapid and recurrent mass-multiplication of androgenic embryos in indica rice. Indian J. Biotechnol., 5: 239-242.

Sadasivam, S. and A. Manickam, 1996. Biochemical Methods. New Age International Publishers, New Delhi, pp: 1-255.

Saker, M.M., S.A. Bekheet, H.S. Taha, A.S. Fahmy and H.A. Moursy, 2000. Detection of somaclonal variations in tissue culture-derived date palm plants using isozyme analysis and RAPD fingerprints. Biol. Plant., 43: 347-351.

Samantaray, S.S., G.R. Rout and P. Das, 1995. *In vitro* plant regeneration from leaf base and mesocotyl culture of Echinocloa colona. Plant Cell Tiss. Org. Cult., 40: 37-41.

Scandalios, J.G., 1974. Isozyme in development and differentiation. Ann. Rep. Plant Physiol., 25: 225-258.

Senadhira, D., F.J. Zapata-Arias, G.B. Gregorio, M.S. Alejar, H.C. De la Cruz, T.F. Padolina and A.M. Galvez, 2002. Development of the first salt-tolerant rice cultivar through *indica/indica* anther culture. Field Crop Res., 76: 103-110.

Talebi, R., M.R. Rahemi, H. Arefi, M. Nourozi and N.l. Bagheri, 2007. *In vitro* Plant Regeneration Through Anther Culture of Some Iranian Local Rice (*Oryza sativa* L.) Cultivars Pak. J. Biol. Sci., 10: 2056-2060.

Tyagi, R.K. and S. Prakash, 2004. Genotype and sex specific protocol for *in vitro* micropropagation and medium term conservation of jojoba. Biol. Plantarum, 48: 19-23.

Zivdar, S., M. Mousawi and N. Alemzadeh Ansari, 2008. Genetic stability in date palm micropropagation. Asian J. Plant Sci., 7: 775-778.