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Article

DETECTION OF SOMACLONAL VARIATION IN MICROPROPAGATED AND ACCLIMATIZED PLANTLETS OF *Oryza sativa* MRQ 74 FROM STEM EXPLANTS

*Detecção de Variação Somaclonal em Plantas Micropropagadas e Aclimatizadas de **Oryza sativa** MRQ 74 a Partir de Explantes do Caule*

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ABSTRACT - In plant tissue culture system, the excessive supply of plant growth hormones may inhibit further growth and development of the regenerants and sometimes it may induce somaclonal variation. In this study, the optimum concentration of plant growth hormones for *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74 was identified and subsequently the occurrence of somaclonal variation of regenerated plantlets was determined through cytological analysis of *in vivo* and *in vitro* grown plantlets. MS media supplemented with 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA was found to be the optimum concentration for shoots and roots induction of *Oryza sativa* L. cv. MRQ 74. Plantlets derived from MS media supplemented with 0.5 mg L^{-1} 2,4-D showed higher survival rate than that of MS media supplemented with 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA. The plant height, number of leaf, leaf length and number of seeds per stalk of acclimatized plants were significantly lower ($p<0.05$) as compared with plants grown *in vivo*. Cytological analysis of *in vivo* and *in vitro* grown *Oryza sativa* L. cv. MRQ 74 revealed that the presence of 2,4-D in the culture media had significantly decreased the mitotic index (MI) and increased the ploidy level of the cell nuclei. The findings of the present study would be useful for plant breeders and biotechnologist since somaclonal variation provides a useful source of genetic variation for crop productivity and quality improvement.

Keywords: plant growth hormones, *Oryza sativa* L. cv. MRQ 74, survival rate, agronomic parameters, mitotic index, ploidy level.

*RESUMO - No sistema de cultura de tecidos de plantas, o fornecimento excessivo de hormônios de crescimento vegetal pode inibir o crescimento e desenvolvimento dos regenerantes e, às vezes, induzir a variação somaclonal. Neste estudo, a concentração ideal de hormônios de crescimento para regeneração in vitro de **Oryza sativa** L. cv. MRQ 74 foi identificada, e, posteriormente, a ocorrência de variação somaclonal de plântulas regeneradas foi determinada através da análise citológica de plântulas cultivadas in vivo e in vitro. O meio de cultura MS suplementado com 0,1 mg L^{-1} de BAP em combinação com 0,1 mg L^{-1} de ANA foi a concentração ideal para a indução da parte aérea e das raízes de **Oryza sativa** L. cv. MRQ 74. As plântulas derivadas de meio MS suplementado com 0,5 mg L^{-1} de 2,4-D apresentaram maior taxa de sobrevivência do que as do meio MS suplementado com 0,1 mg L^{-1} de BAP em combinação com 0,1 mg L^{-1} de ANA. A altura de plantas, o número de folhas, o comprimento das folhas e o número de sementes por caule de planta aclimatizada foram significativamente menores*

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(p<0,05) do que nas plantas cultivadas in vivo. A análise citológica de ***Oryza sativa*** *L. cv. MRQ 74 cultivada in vivo e in vitro revelou que a presença de 2,4-D no meio de cultura diminuiu significativamente o índice mitótico (IM) e aumentou o nível de ploidia dos núcleos celulares. Os resultados do presente estudo podem ser úteis para criadores de plantas e biotecnologistas, uma vez que a variação somaclonal fornece uma fonte útil de variação genética para a produtividade e melhoria da qualidade das culturas.*

Palavras-chave: hormônios de crescimento vegetal, *Oryza sativa* L. cv. MRQ 74, taxa de sobrevivência, parâmetros agronômicos, índice mitótico, nível de ploidia.

INTRODUCTION

Oryza sativa L. cv. MRQ 74 is an aromatic rice cultivar known as 'Maswangi'. It was introduced to Malaysian farmers in 2005. The cultivar was developed from crosses between aromatic rice from India, Q 34 and Khaw Dok Mali from Thailand in 1991. It was then crossed again with Kasturi from India in 1994 to produce high quality aromatic rice. This cultivar is currently planted both as an inorganic and an organic practice (Rosniyana et al., 2010). It was also proposed to be grown as aerobic rice because of its high potential for commercialization. Consumption of aromatic rice has been gaining popularity in Malaysia and around the world as a result of low starch content and low glycemic index (GI), which are good for health and suitable for diabetic people (Golam et al., 2012). For example, rice imported into the United States is mostly aromatic Thai jasmine, Indian and Pakistan basmati (Napasintuwong, 2012).

Since the demand of aromatic rice is increasing in many countries in the world, mass propagation through the tissue culture technique and an efficient protocol for acclimatization of the propagated plantlets must be developed. Despite the fact that *in vitro* propagation is the most promising method for rapid and mass propagation of many plant species, in Malaysia, this technique is not widely used especially in monocot plants such as rice because of its difficulty to propagate *in vitro*. Various types of explants such as mature seeds (Saharan et al., 2004: Bano et al., 2005), root segments (Hoque and Mansfield, 2004) coleoptiles and mature embryos (Khanna and Raina, 1998) have been used for *in vitro* regeneration of rice. However, the use of stem explants of rice is still scanty. In addition to type of explants, the success of *in vitro* regeneration of a species is determined by the presence of plant growth hormones in the culture medium. Kinetin, 6-Benzylaminopurine (BAP) and α-Naphthalene acetic acid (NAA) are some of the widely used plant growth hormones for induction of shoots and roots. Thidiazuron (TDZ) is also extensively applied in culture media which influences shoot production. TDZ is a powerful plant growth hormone for *in vitro* regeneration and subsequent growth in many plant species (Çöçü et al., 2004; Faisal et al., 2005). In fact, TDZ induced better response than BAP in shoot regeneration of peanut (Gairi and Rashid, 2004). Unfortunately, the presence of certain plant growth hormones such as 2,4-D and kinetin may lead to somaclonal variation among the regenerants. Hence, the aims of this study were to determine the optimum concentration of plant growth hormones for *in vitro* regeneration of *Oryza sativa* L. cv. MRQ 74 and to identify the ability of the plantlets derived from *in vitro* regeneration to survive under ex vivo conditions. Moreover, the effects of 2,4-D on somaclonal variation of the regenerated plantlets were also investigated.

MATERIALS AND METHODS

Explants Source

Mature seeds of rice were obtained from Malaysian Agricultural Research and Development (MARDI) Seberang Prai, Penang, Malaysia. Dehusked mature seeds were surface sterilized in 70% (v/v) Clorox with two drops of 1 mL L^{-1} Tween 20 followed by 50%, 30%, 20% and 10% Clorox. The dehusked seeds were then rinsed once in sterilised distilled water. Finally, the seeds were rinsed in 70% (v/v) ethanol for one minute and in sterilized distilled water three times for complete removal of Clorox and ethanol in a laminar air flow cabinet. The sterilized seeds were then cultured in MS medium (Murashige and Skoog, 1962) containing sucrose (30 g L^{-1}) and

agar (8 g L^{-1}). The cultures were incubated in the culture room at 25 ± 1 °C under 16 hours light and 8 hours dark with 1000 lux of light intensity. The seedlings were maintained in the culture room for 4 weeks before they were used as explant sources.

In vitro regeneration

Stem explants (basal segments) of four-week-old aseptic seedlings were approximately excised into 10.0 mm segments and were cultured in MS media supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} technical agar fortified with BAP (0.1, 0.5, 1.0 and 1.5 mg L^{-1}), kinetin (0.1 and 0.5 mg L^{-1}), NAA (0.1, 0.5 and 1.0 mg L^{-1}), TDZ (0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1}) and 2,4-D (0.5 mg L^{-1}) either applied singly or in combinations. The stem explants were also cultured in MS basal media as a control. The pH of the media was adjusted to 5.8 prior to autoclaving process at 121 °C, 105 kPa for 21 minutes. The cultures were maintained in the culture room at 25 ± 1 °C under 16 hours light and 8 hours dark with 1000 lux of light intensity for two months.

Hardening

Two-month-old well rooted plantlets were carefully taken out from the MS media containing 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA, and MS media supplemented with 0.5 mg L^{-1} 2,4-D. The roots were washed thoroughly under running tap water to remove all traces of medium attached to the roots. The plantlets were planted in plastic containers containing black soil, red soil and a mixture of black and red soil in the ratio of 1:1. The plastic containers were covered with plastic bags and were maintained in the culture room at 25 ± 1 °C, under 16 hours light and 8 hours dark for two months. The observation was made on the survival rate of plantlets after 4 and 8 weeks of hardening. The survived plantlets with new leaves were transplanted to plastic containers containing garden soil before they were transferred to a greenhouse. The plantlets were irrigated every alternate day throughout the experiment. Fertilizers in the form of liquid urea (100 mg L^{-1}) were applied every three weeks after acclimatization. Several parameters such as survival rate, plant height, number of leaves and number of seeds per stalk were measured after 8 weeks in the green house. Rice mature seeds were also germinated on garden soil as *in vivo* grown plants for comparison.

Histological studies

Leaf and root specimens were fixed in glutaraldehyde-paraformaldehyde-caffeine fixative solution for 24 to 48 hours at room temperature. After fixation, the specimens were then dehydrated in a series of ethanol, 30%, 50%, 70%, 80%, 90%, 95%, and 100% for 30, 45, 45, 60, 60, 60 and 60 minutes, respectively. After the dehydration process, the specimens were treated with xylene for clearing. Subsequently, the specimens were embedded in paraffin wax and sectioned at 35 µm. The specimens were stained with Schiff's reagent and naphthol blue black. The specimens were then mounted with Surgipath mounting medium and were dried for one day prior to observation.

Cytological studies

The standard growth of primary roots of *Oryza sativa* MRQ 74 was determined by germinating the rice seeds on moist cotton wool in Petri dishes and MS basal medium (for comparison). The growth of primary roots was measured daily (at the same time) until the secondary roots appeared. The mean primary root length of the samples was determined and the roots were used in preparing the permanent slides for Mitotic Index (MI), cell and nuclear areas, nuclear to cell areas ratio, nuclear DNA content and chromosome number determination.

The roots with standard length of 8.09 ± 0.79 mm were excised from the plantlets cultured in MS media supplemented with 0.5 mg L^{-1} 2,4-D and MS media supplemented with 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA after 8 weeks of culture. The roots were fixed in 3:1 (v/v) absolute alcohol:glacial acetic acid for 24 hours. The roots were hydrolyzed in 1N HCl at 63 °C for 13 minutes and were stained in Feulgen reagent for 2 hours. The stained roots were

then treated with pectinase for 40 minutes. The apical region of a root tip (1-2 mm) was placed on a clean slide in a small drop of 45% acetic acid, sprayed with freeze spray and rinsed. Cover slides were then mounted on the slides by DPX (Di-N-Butyle Phthalate in Xylene). The prepared slides were then viewed using Axioskop Zeiss (Germany microscope attached to AxioCam MRc video camera) and were analyzed using the AxioVision 4.7 software to measure the MI, cell and nuclear areas, nuclear to cell areas ratio, nuclear DNA content and chromosome number.

Soil analysis

The growing substrates were analyzed using X-Ray Fluorescence (XRF) method. The red and black soils were dried in an oven at 40 °C for 24 hours. The dried soils were screened with a 2 mm mesh to remove large objects. The samples were then ground into powder form. Approximately 3 to 5 g of the dried samples were placed in an XRF sample cup for analysis.

Statistical Analysis

All data were analyzed using the IBM SPSS Statistics 20 software (International Business Machines Corp., Armonk, NY). Statistical variance analysis was conducted using ANOVA (Duncan's Multiple Range Test) and t-test at 5% significance level. Values are presented as mean ± SE (standard error of mean).

RESULTS AND DISCUSSION

***In vitro* regeneration**

Vegetative propagation through tissue culture system has become possible for many agricultural and horticultural crops. However, there are still many species, varieties and cultivars that remained unresponsive even though major efforts had been made. For example, monocot plants such as rice are difficult to propagate because of lack of meristematic tissues (Habibi et al., 2009). Therefore, mature seeds are the most preferred explants in tissue culture of rice as compared to stem, leaf and root (Niroula et al., 2005). However, in this study, *in vitro* regeneration of *Oryza sativa* MRQ 74 was successfully achieved from stem explants of four-week-old aseptic seedlings. BAP was found to be more effective than kinetin for shoot proliferation (Table 1). Similar results were reported in *Salix* (Khan et al., 2011), *Bacopa chamaedryoides* (Sk Moquammel and Biswajit, 2013) and *Passiflora foetida* (Shekhawat et al., 2015). The presence of BAP alone at a concentration of 1.5 mg L^{-1} in the MS media gave the highest mean number of shoots per explant (4.03 ± 0.3). The combinations of BAP and NAA at all concentrations applied - except for the concentrations of 1.0 mg L^{-1} BAP in combination with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP in combination with 0.5 mg L^{-1} NAA - produced a significantly lower mean number of shoots per explant as compared to application of BAP alone. These findings were supported by Saini and Jawal (2005) who reported that the efficiency of BAP for shoot regeneration in *V. mungo* was significantly decreased when it was combined with IAA, NAA or IBA. On the other hand, there were different effects for root formation when both BAP and NAA were supplemented in the culture media. The highest mean number of roots per explant (25.53 ± 1.89) was found in MS media supplemented with 0.1 mg L^{-1} in combination with 0.1 mg L^{-1} NAA with the longest root length ranging from 0.5 cm to 7.0 cm. As expected, the addition of BAP alone in MS media produced a lower mean number of roots per explant.

The inefficiency of kinetin for shoot proliferation was observed in this study. In general, the mean number of shoots produced in MS media supplemented with kinetin in combinations with NAA was not significantly different in most treatments as compared to control (Table 2). The plantlets produced in MS media supplemented with 0.5 mg L^{-1} kinetin had the highest root length ranging from 0.7 cm to 9.0 cm. However, the mean number of roots produced was among the lowest ones (6.00 ± 0.58). As compared with BAP, TDZ was highly effective in promoting shoot formation (8.23 ± 1.09) at a low concentration (0.1 mg L^{1}) and drastically inhibited (4.20 ± 0.33) at high concentration (0.5 mg L^{-1}) (Table 3). However, the plantlets produced at the concentrations of 0.3 to 0.5 mg L^{-1} TDZ showed abnormal growth. These results were in agreement with the

Table 1 - The effects of BAP and NAA on induction of shoots and roots of *Oryza sativa* MRQ74

MS + Hormone (mg L^{-1})		No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)	Root length (cm)
BAP	NAA			
0.0	0.0	2.30 ± 0.16 abc	5.27 ± 0.76 d	0.2 - 4.0
0.1	0.0	3.00 ± 0.27 cde	5.83 ± 1.18 d	0.3 - 4.0
	0.1	2.80 ± 0.23 bcde	25.33 ± 1.89 f	0.5 - 7.0
	0.5	1.97 ± 0.16 a	5.37 ± 0.74 d	0.2 - 4.5
	1.0	2.13 ± 0.21 ab	6.80 ± 0.70 d	0.4 - 4.5
0.5	0.0	3.13 ± 0.23 de	1.23 ± 0.25 a	0.4 - 2.7
	0.1	2.37 ± 0.15 abcd	5.53 ± 0.86 d	0.3 - 4.2
	0.5	3.03 ± 0.24 cde	2.57 ± 0.27 abc	0.2 - 0.7
	1.0	2.30 ± 0.18 abc	4.80 ± 0.58 cd	0.3 - 1.7
1.0	0.0	3.13 ± 0.26 de	1.27 ± 0.25 a	0.3 - 2.5
	0.1	2.70 ± 0.25 abcd	5.13 ± 0.66 d	0.3 - 4.3
	0.5	2.57 ± 0.22 abcd	9.80 ± 1.35 e	0.4 - 5.0
	1.0	3.53 ± 0.43 ef	4.40 ± 0.69 bcd	0.2 - 2.5
1.5	0.0	4.03 ± 0.31 f	1.37 ± 0.24 a	0.2 - 2.5
	0.1	2.30 ± 0.28 abc	2.67 ± 0.56 abc	0.3 - 2.0
	0.5	2.67 ± 0.24 abcd	2.13 ± 0.26 ab	0.2 - 2.3
	1.0	2.87 ± 0.18 bcde	1.87 ± 0.28 a	0.2 - 1.1

Mean values with the same letters in a column are not significantly different at p>0.05.

Table 2 - The effects of NAA and kinetin on induction of shoots and roots of *Oryza sativa* MRQ74

MS + Hormone (mg L^{-1})		No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)	Root length (cm)
NAA	kinetin			
0.0	0.0	2.30 ± 0.16 bc	5.27 ± 0.76 a	0.2 - 4.0
	0.5	2.57 ± 0.20 c	6.00 ± 0.58 ab	0.7 - 9.0
0.1	0.0	1.63 ± 0.21 a	24.37 ± 1.91 e	0.3 - 3.0
	0.1	2.40 ± 0.20 bc	8.63 ± 0.83 bc	0.2 - 3.2
	0.5	2.03 ± 0.18 abc	5.27 ± 0.69 a	0.2 - 2.0
0.5	0.0	2.10 ± 0.19 abc	9.90 ± 1.03 cd	0.3 - 5.5
	0.1	2.00 ± 0.19 abc	9.73 ±0.79 cd	0.4 - 5.0
	0.5	2.03 ± 0.19 abc	5.10 ± 0.62 a	0.2 - 2.5
1.0	0.0	1.80 ± 0.16 ab	12.17 ± 0.87 d	0.2 - 2.8
	0.1	2.07 ± 0.19 abc	10.30 ± 1.51 cd	0.3 - 5.7
	0.5	2.60 ± 0.18 c	8.77 ± 1.00 bc	0.4 - 3.0

Mean values with the same letters in a column are not significantly different at p>0.05.

findings of Mithila et al., (2003) in African violet. In order to stimulate more shoot formation, TDZ was added into MS media containing BAP and NAA. However, TDZ in combination with BAP and NAA at the concentrations of 0.1 mg L^{-1} each yielded the least mean number of shoots per explant (2.57 ± 0.22). In terms of mean number of roots produced, the lowest concentration of TDZ showed the highest mean number of roots per explant.

Meanwhile, the presence of 2,4-D at the concentration of 0.5 mg L^{-1} in MS medium resulted in callus formation and, subsequently, in production of shoots and roots (Table 4). The MS media supplemented with 0.5 mg L^{-1} 2,4-D produced plantlets with shorter and bigger size of roots as compared with MS media supplemented with 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA,

Table 3 - The effects of TDZ, NAA and BAP on induction of shoots and roots of *Oryza sativa* MRQ74

MS + Hormone (mg L^{-1})			No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)	Root length (cm)
TDZ	NAA	BAP			
0.0	0.0	0.0	2.30 ± 0.16 a	5.27 ± 0.76 b	0.2 - 4.0
0.1			8.23 ± 1.09 e	0.30 ± 0.16 a	0.2 - 0.7
0.2			6.47 ± 0.86 d	NR	NR
0.3			5.40 ± 0.73 cd	NR	NR
0.4			5.40 ± 0.60 cd	NR	NR
0.5			4.20 ± 0.33 abc	0.03 ± 0.03 a	0.2
0.1	0.1		3.17 ± 0.41 ab	0.03 ± 0.03 a	0.4
0.2	0.1		4.67 ±0.66 bcd	NR	NR
0.1	0.1	0.1	2.57 ± 0.22 a	0.07 ± 0.05 a	0.2 - 0.5

Mean values with the same letters in a column are not significantly different at p>0.05. (NR: no response).

Table 4 - The effects of 2,4-D on shoots and roots induction *Oryza sativa* MRQ74

MS + Hormone (mg L^{-1})		No. of shoots per explant (Mean ± SE)	No. of roots per explant (Mean ± SE)	Root length (cm)
2,4-D	Observation			
0.0	No callus formation	2.30 ± 0.16 a	5.27 ± 0.76 a	0.2 - 4.0
0.5	Callus formation	4.51 ± 0.45 b	20.05 ± 2.34 b	0.3 - 5.0

Mean values with the same letter in a column are not significantly different at p>0.05.

as well as a lesser number and smaller size of shoots. Based on the mean number of shoots and roots and root length, the plantlets regenerated in MS media supplemented with 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA and MS media fortified with 0.5 mg L^{-1} 2,4-D were chosen for acclimatization purposes.

Effect of different growing substrates on acclimatization

The plantlets derived from MS media supplemented with 0.5 mg L^{-1} 2,4-D were well adapted in two types of growing substrates after being acclimatized for four weeks (Figure 1A, Table 5). The highest survival rate was achieved by the mixture of black and red soil (90.00 ± 1.53%), followed by black soil (80.00 ± 2.52%) and red soil (3.33 ± 0.33%). However, the percentages of survival rates were significantly lower in all treatments on week eight: they were 83.33 ± 1.20%, 40.00 ± 1.15% and 0% in the mixture of black and red soil, black soil and red soil, respectively.

A similar pattern of the survival rate was found for the plantlets derived from MS media containing 0.1 mg L^{-1} BAP in combination with 0.1 mg L^{-1} NAA. Nevertheless, the percentage of the survived plantlets was significantly lower as compared with the plantlets derived from MS media containing 0.5 mg L^{-1} 2,4-D. The highest percentage was 10.00 ± 0.04%, followed by 3.33 ± 0.03% and 0% which were recorded after four weeks on the mixture of black and red soil, black soil and red soil, respectively. None of the plantlets was able to survive until week eight of acclimatization. It was found that the initial morphological features of *Oryza sativa* MRQ 74 plantlets prior to acclimatization greatly influenced the success of acclimatization of this species. In addition, the different survival rates might also be due to the different types and amount of nutrient content in the soil. This was confirmed through soil nutrient content analysis using the *XRF* (X-Ray Fluorescence) method as shown in Table 6. Higher values of compounds containing important nutrients for plant growth and development such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) were found in black soil as compared to red soil.

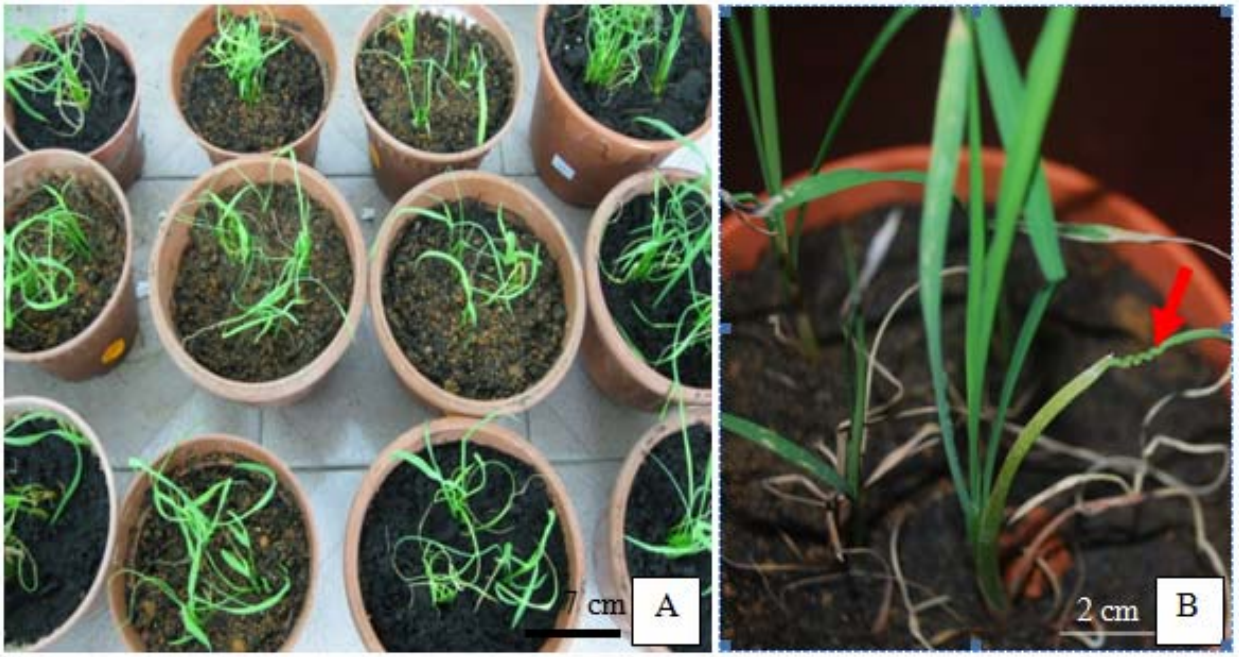


Figure 1 - One-month-old acclimatized plantlets on black soil and mixture of black and red soil (A), abnormal leaf morphology shown by red arrow (B).

Table 5 - The survival rate of plantlets derived from MS media supplemented with 2,4-D and MS media supplemented with BAP in combination with NAA

Growing substrate	Survival rates of the plantlets (%)	
	MS + 0.5 mg L^{-1} 2,4-D	MS + 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA
(4 weeks)		
Black Soil	80.00 ± 2.52 b	3.33 ± 1.93 a
Red Soil	3.33 ± 0.33 a	0
Black + red soil	90.00 ± 1.53 c	10.00 ± 1.91 b
(8 weeks)		
Black Soil	40.00 ± 1.15 a	0
Red Soil	0	0
Black + red soil	83.33 ± 1.20 b	0

Mean values with the same letter within a column are not significantly different at p>0.05.

After 8 weeks in the culture room, the survived plantlets from the mixture of black and red soil were then transferred to plastic containers containing garden soil and were maintained in a green house for further growth and development. The acclimatized plants experienced new environmental conditions such as high light intensity, low air humidity and high temperature. As a result, only 80% of the plants survived and 60% reached maturity period and started fruiting after 8 weeks. Mohammed and Vidaver (1990) reported that only 33% of *in vitro* plantlets of Fouglas fir (*Pseudotsuga menziesii*) survived when they were maintained under relative humidity level of 40-70% and at temperatures ranging from 22 - 28 °C, but a significantly higher survival rate (89%) was shown under relative humidity level of 80-90%, at 15-25 °C. Variations in number of tillers per plant, plant height, panicle length, fertility and number of seeds produced are commonly found in tissue culture derived plants (Schaeffer et al., 1984; Murai and Kinoshita, 1986). These variations were also observed in the present study. The analysis of variance (t-test, $p<0.05$) showed that all the measured parameters were significantly higher for *in vivo* grown plants than that for acclimatized plants (Table 7). The mean number of seeds per stalk of acclimatized plants was more than 50% lower than that of *in vivo* plants. These results indicate the low

growth rate of acclimatized plants as they need to adapt to low humidity, high light intensity and temperature.

Histological studies on leaf and root of *in vivo*, *in vitro* and acclimatized plants

The addition of plant growth hormones in the culture media not only stimulated better cell growth, but sometimes also caused morphological changes, especially in leaf morphology and structure. In the present study, abnormal morphology of leaf was found among the regenerants whereby the leaf blade became wavy (Figure 1B). The cross section of *in vitro* grown leaves showed a single layer of upper and lower epidermis with almost no cuticle in both sides, adaxial and abaxial leaf surfaces (Figure 2A). The similar epidermal structural was observed for *in vivo* and acclimatized leaves

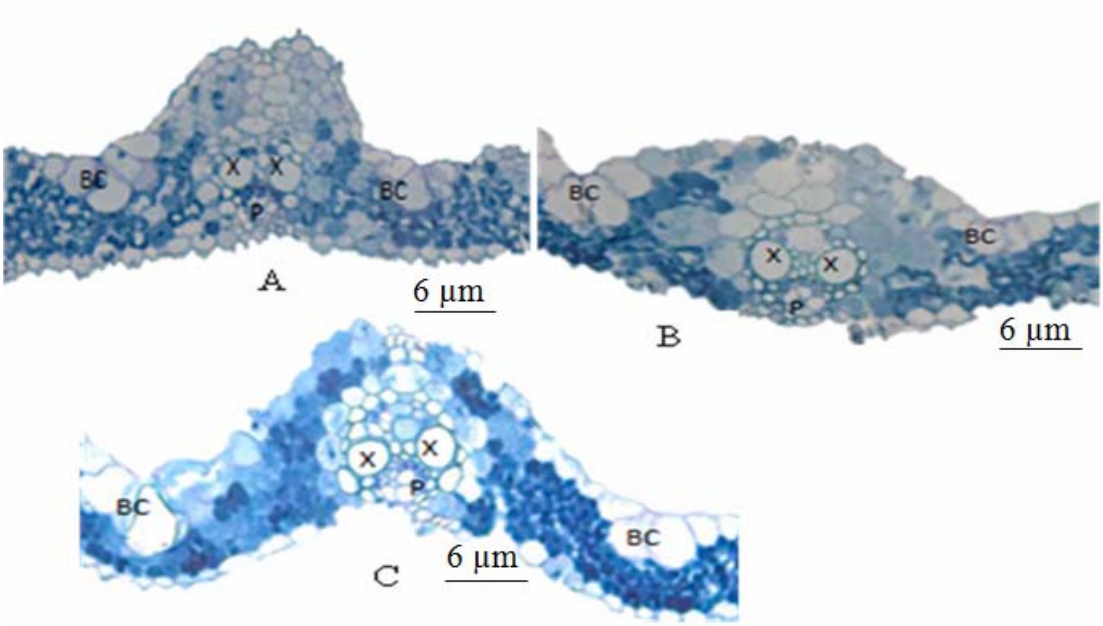
Table 6 - Soil nutrient content in black and red soil

Compound	Value (%)	
	Black soil	Red soil
Aluminium oxide (Al_2O_3)	9.158	43.165
Calcium oxide (CaO)	37.584	0.420
Cloride (Cl)	0.795	0.024
Iron(III) oxide	5.234	6.830
Lead(II) oxide (PbO)	0.120	0.010
Magnesium oxide (MgO)	1.598	0.000
Manganese(II) oxide (MnO)	0.151	0.021
Phosphorus pentoxide (P_2O_5)	1.758	0.108
Potassium oxide (K_2O)	2.380	0.447
Silicon dioxide (SiO_2)	35.445	l47.823
Sodium oxide (Na_2O)	0.286	0.053
Sulfur trioxide (SO_3)	5.162	0.183
Titanium dioxide (TiO_2)	1.094	0.773
Zinc oxide (ZnO)	0.080	0.015
Zirconium oxide (ZrO_2)	0.070	0.052

Table 7 - Performance of *in vivo* and acclimatized plantlets

Parameter	*In vivo*	Acclimatized plantlet
Plant height (cm)	49.70 ± 1.08*	37.80 ± 0.91*
Number of leaves	8.07 ± 0.78*	5.37 ± 0.67*
Leaf length (cm)	40.83 ± 0.58*	26.80 ± 0.47*
Number of seeds per stalk	38.60 ± 2.07*	16.40 ± 0.81*

* Significant difference (t-test, $p<0.05$).

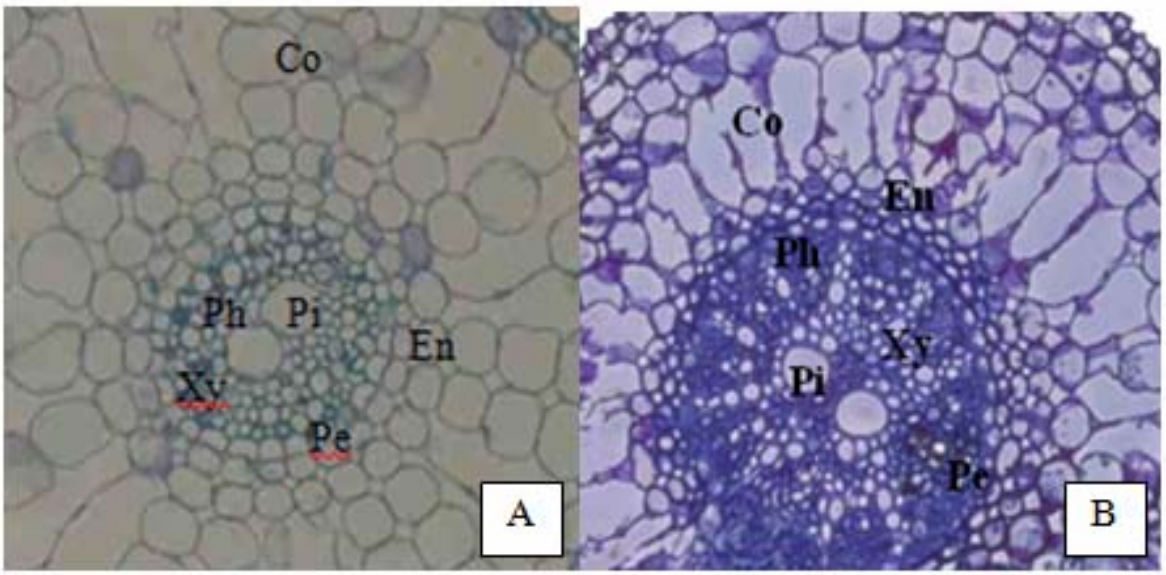


BC: Bulliform cell, X: Xylem, P: Phloem. Magnification 200x.

Figure 2 - Cross-section of *in vitro* leaf of the plantlets regenerated in MS medium supplemented with 0.5 mg L-1 2,4-D (A), *in vivo* leaf (B) and leaf of acclimatized plants (C).

(Figures 2B, 2C). The bulliform cells were well developed on adaxial surfaces of all leaf samples (*in vitro*, *in vivo* and acclimatized). However, the bundle sheath cells of *in vitro* grown leaf could not be observed clearly. In fact, abnormal differentiation of cells in the bundle sheath had occured. This caused the number of the cells to increase as compared with *in vivo* and acclimatized leaves. According to Abe et al. (2010), the growth of the central region of the leaf would fail to synchronize with the lateral region. Generally, *in vitro* conditions cause rapid growth and shoot proliferation, resulted in abnormal histological features of regenerated plantlets such as absence of thick cuticle, altered leaf morphology and poor water transport system (Pati et al., 2013). The vascular tissues, xylem and phloem can be seen apparently in all samples of leaves. Unlike vascular tissues, stoma and guard cells were almost absent. Compact mesophyll cells were clearly present between the epidermal layers and the vascular bundles.

A histological study of root showed that the cross section of *in vivo* root consists of epidermis, cortex, endodermis, pericycle, xylem, phloem and pith (Figure 3A). All the sections were also present in the acclimatized root (Figure 3B). However, the cortex of the root was poorly developed. Moreover, starch granules were also found in all root samples.



Co: cortex, En: Endodermis, Pe: Pericycle, Xy: Xylem, Ph: Phloem, Pi: Pith. Magnification 100x.

Figure 3 **-** Cross section of root of *in vivo* grown (A) and acclimatized (B) *Oryza sativa* L. cv. MRQ 74.

Cytological studies on root tip meristem cells of *in vivo* and *in vitro* grown *Oryza sativa* MRQ 74

The mitotic index (MI) of *in vivo* grown *Oryza sativa* MRQ 74 was not significantly different than that of *in vitro* (MS basal medium), as shown in Table 8. However, the presence of 2,4-D in MS medium has significantly decreased the MI of the root tip meristem cells (3.78 ± 0.22%). The effects of plant growth hormones on cellular behaviour of plant species have been reported by many researchers (Selma and Signem, 2012; Taha and Wafa, 2012; Sk Moquammel and Biswajit, 2013). The results found from the current work were in line with the findings of Ud-Deen and Kabir (2009), who reported that the MI and cell size of onion decreased with increasing concentration of plant growth hormones such as GA_3.The chromosome number was also slightly different (22.00 ± 2.00) as compared to *in vivo* (24.00 ± 1.15) and MS basal medium (24.00 ± 2.65). It has been reported that the diploid rice has 24 chromosome number (Cheng et al., 2001). These findings suggested that *in vitro* conditions contributed to the occurrence of polyploid cells and the addition of plant growth hormones increased the ploidy level of the cells. The presence of 2,4-D in the tissue culture system is often associated with genetic abnormalities such as polyploidy and simulation of DNA synthesis (Mohanty et al., 2008).

Table 8 - The mitotic index and number of chromosomes of root tip meristem cells

Treatment		Mitotic index, MI (%)	Number of chromosomes (mean)
In vivo		10.45 ± 0.31 b	24.00 ± 1.15 ab
In vitro	MS basal medium	10.22 ± 0.59 b	24.00 ± 2.65 ab
	MS medium supplemented with 0.5 mg L^{-1} 2,4-D	3.78 ± 0.22 a	22.00 ± 2.00 a

Mean values with the same letters within a column are not significantly different at p>0.05.

The mean nuclear area, cell area and the ratio of nuclear to cell areas of *in vivo* and *in vitro* grown *Oryza sativa* MRQ 74 are shown in Table 9. The *in vitro* (MS basal medium) grown *Oryza sativa* MRQ 74 had the lowest nuclear and cell areas, 48.92 ± 2.03 µm² and 145.40 ± 6.37 µm², respectively. On the other hand, the highest mean cell area (237.93 ± 19.71 µm²) was recorded from cells grown in MS media fortified with 0.5 mg L^{-1} 2,4-D with the lowest nuclear to cell areas ratio (0.29 ± 0.01 µm²). Most of the root meristem cell nuclei of *Oryza sativa* MRQ 74 grown *in vivo* were at the G1 phase (62.75%). The results also showed that a small portion (1.34%) of the cell nuclei was polyploid (c value>4.8) as shown in Table 10. The ploidy level of the meristematic cells of *in vitro* grown *Oryza sativa* MRQ 74 increased in MS basal medium (5.34%) and became 13.37% in MS medium supplemented with 0.5 mg L^{-1} 2,4-D.

Table 9 - The mean nuclear and cell areas and their ratios in root meristem cells

Treatment		Mean (µm²)		
		Nucleus (N)	Cell (C)	Ratio (N/C)
In vivo		62.20 ± 1.96 b	191.00 ± 4.95 b	0.33 ± 0.03 b
In vitro	MS basal medium	48.92 ± 2.03 a	145.40 ± 6.37 a	0.34 ± 0.03 b
	MS + 0.5 mg L-1 2,4-D	64.67 ± 2.51 b	237.93 ± 19.71 b	0.29 ± 0.01 a

Mean values with the same letters within a column are not significantly different at p>0.05.

Table 10 - Frequency of nuclei in root tip meristem cells at different cell cycle phases

MS + hormones (mg L^{-1})		Phase (%)			
		G1	S	G2	> 4.8
In vivo		62.75 c	24.16 a	12.75 a	1.34 a
In vitro	MS basal medium	35.33 b	33.33 b	26.00 b	5.34 b
	MS + 0.5 mg L^{-1} 2,4-D	11.33 a	51.33 c	24.00 b	13.37 c

Mean values with the same letters within a column are not significantly different at p>0.05.

Based on the cytological observation, it was shown that after 8 weeks of culture, the root tip meristem cells of *in vivo* and *in vitro* grown (MS basal medium) *Oryza sativa* MRQ 74 remained diploid, whereby only a small percentage of polyploid cells had been observed (Figures 4A and 4B). However, supplementing the culture media with plant growth hormone such as 2,4-D increased the percentage of polyploid cells (14.0%) as compared with *in vivo* and *in vitro* values of 2.0% and 4.0%, respectively (Figure 4C). The cells are considered polyploid when the DNA C value is more than 4.8.

In summary, the most effective plant growth hormones for *in vitro* regeneration of *Oryza sativa* MRQ 74 from stem explants were BAP in combination with NAA with regard to the mean number of shoots, roots and root length, without any intermediate callus phase. However, the *in vitro* raised plantlets showed poor survival rate during acclimatization as compared with plantlets derived from MS media fortified with 2,4-D whereby the intermediate callus phase was observed. The occurrence of somaclonal variation was observed among the regenerants in terms of leaf morphology, plant height and number of seeds per stalk. The presence of 2,4-D in the culture media had a significant effect on the mitotic index and ploidy level of the cell nuclei.

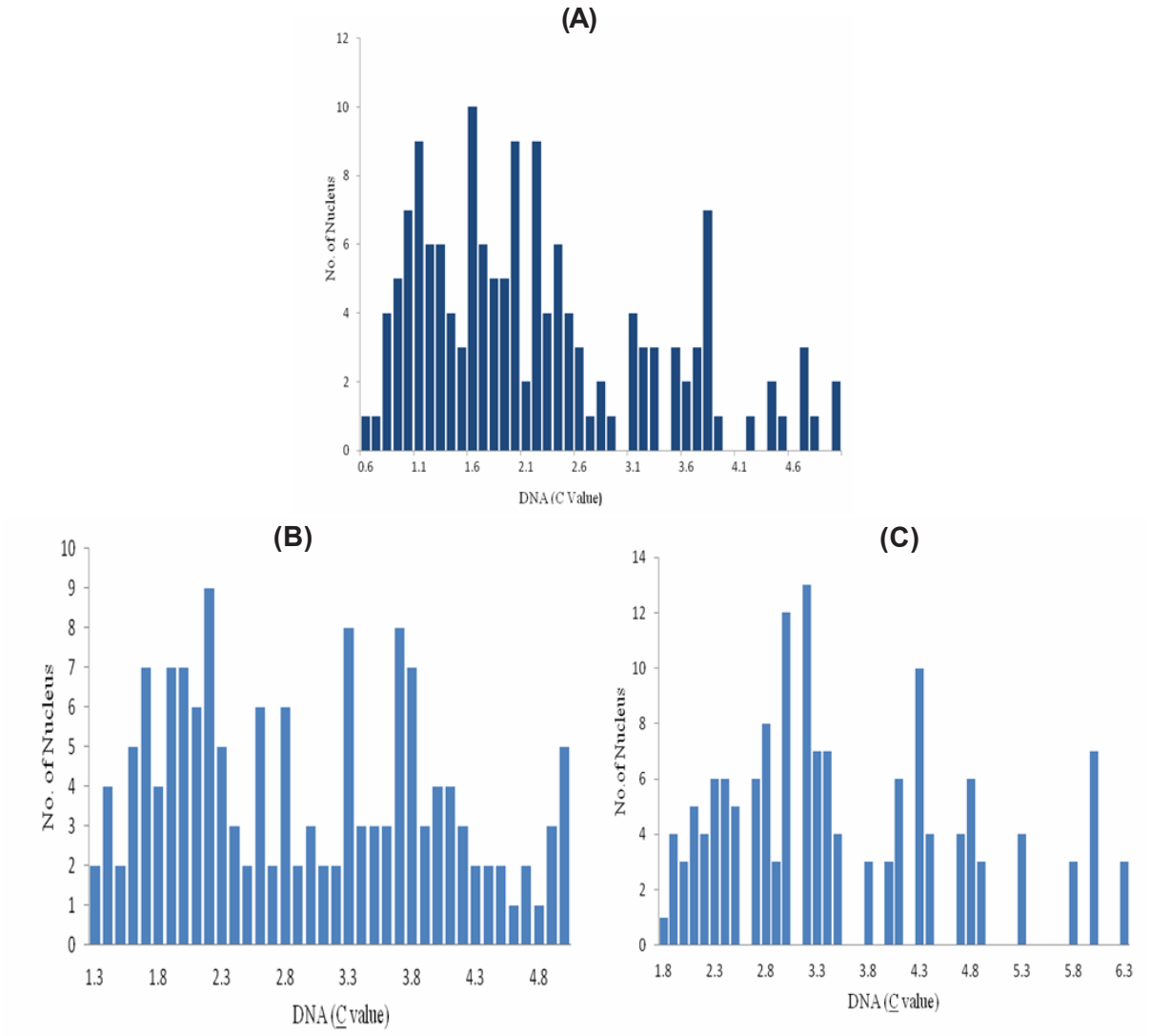


Figure 4 - The distribution of DNA C values of interphase cells from root tip meristem of *Oryza sativa* MRQ 74 grown *in vivo* (A), MS basal media (B) and MS media supplemented with 0.5 mg L-1 2,4-D (C) after 8 weeks of culture.

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