INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2006/08–6–759–762
http://www.fspublishers.org

In Vitro Regeneration of Aromatic Rice *(Oryza sativa* L.)

M BELAL HOSSAIN SIKDER, PALASH KUMAR SEN†, M ABDULLAH-AL MAMUN†, M. RAIHAN ALI‡ AND S. MAHBUBUR RAHMAN†[1]
Department of Tea Technology, Shahjalal University of Science and Technology (SUST), Sylhet-3114, Bangladesh
†Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh
‡Department of Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet-3114, Bangladesh
[1]Corresponding author's e-mail: pk_bt2002@yahoo.com

ABSTRACT

This study was undertaken to find out the *in vitro* callus induction and organogenesis potential of Chiniguri variety of aromatic rice *(Oryza sativa* L.) collected from farmers of Khulna, Bangladesh. MS media supplemented with different concentrations of 2, 4- D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L^{-1}) were used for callus induction from the mature dehusked rice seeds. Callus derived from the 2.0 mg L^{-1} 2, 4-D showed the best results for plantlet regeneration. For regeneration of plantlets MS media with different concentrations of NAA+BA were employed. The highest regeneration frequencies (100%), the highest average shoot per culture (5.55±1.44) and the highest average shoot length (1.85±0.14 cm) were recorded at the MS media supplemented with 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA. The maximum shoot produced after 3-4 weeks and multiple shoots with roots were apparent in the first subculture. The highest number of shoots (10.15±1.98) per culture with average shoot length (5.26±0.91 cm) was obtained at 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA in the first subculture in same regeneration media. Callus obtained from lower concentration of 2, 4-D (1.0 mg L^{-1}) showed better performance in shoot regeneration (40%) than higher concentration (2.0, 3.0 mg L^{-1} 2, 4-D) in the same regeneration media (MS+2.0 mg L^{-1} IAA+4.0 mg L^{-1} KN). After hardening the plantlets were transferred to soil.

Key Words: *Oryza sativa*; Callus; Auxin; Cytokinin; Shoot; Root

INTRODUCTION

Rice (*Oryza sativa* L.) is the world most important cereal crop after wheat and maize. Rice has 24 species, of which 22 are wild and two viz. *Oryza sativa* and *Oryza glabrrima* are cultivated (Ray, 1985). It is grown extensively in the humid tropical and sub-tropical regions of the world. The rice grain, a caryopsis, is a dry one-seeded fruit, with its pericarp, fused with the seed coat. The main part of the seeds is the husk, the pericarp, the endosperm and the embryo. The importance of rice as staple food emphasizes its improvement undoubtedly. A considerable improvement has been done through traditional rice breeding. Traditional rice breeding has made significant progress towards higher yield, improved quality, greater disease resistance and other important characters of agricultural importance in the past and even in future, it will still play an important role (Sun *et al*., 1990).

Various tissue culture techniques are being applied for varietal development of cereal crops including rice in different countries (Dorosieve, 1996). Among these techniques, anther culture, protoplast fusion, leaf culture, root culture and dehusked seed culture are important in rice tissue culture to exploit somaclonal variation for the creation of novel rice varieties. Somaclonal variation is one aspect of biotechnology, a novel variability for crop improvement (Larkin & Scowcroft, 1981). Several high yielding rice varieties were developed through the application of anther culture in the People's Republic of China (Ying, 1983). But plant regeneration from callus obtained from somatic tissue is more successful than from that obtained by anther culture (Kucherenko, 1994).

At present, International Rice Research Institute (IRRI) is employing tissues culture technique to develop rice varieties (Bajaj, 1980). The mass regeneration of diploid plants from cultured tissue recently has provided a rich and novel source in rice (Skirvin, 1978) as the higher percentage of regenerated forms obtained by culture of somatic tissue shows desirable genetic changes such as disease resistance, salt tolerance and other physiological properties (Kucherenko, 1994). While the dehusked rice seed culture technique is used for callus induction and plant regeneration, there are many factors affecting embryogenic callus formation and subsequent shoot regeneration from rice callus (Mitsuoka *et al*., 1994; Xing *et al.*, 1995; Okamoto *et al*., 1996; Xing *et al.*, 1996).

The aromatic rice has demand in both domestic and foreign market for attractive flavor, good taste and fine grains. Country can be benefited by earning foreign exchange by production and export of aromatic rice. But there are some limitations to cultivate aromatic rice for the farmer. Such as lack of high yielding variety, fine grain quality lack of disease or pest resistant, stress and salt tolerance variety and proper cultural management. The conventional breeding techniques are time consuming and self in-compatibility act as barrier for distant hybridization and fertilization. The aromatic variety can be improved

(disease and pest resistant variety, stress and salt tolerance variety) through tissue culture techniques viz. somaclonal variation or genetic manipulation like protoplast fusion (hybrid and cybrid) and through gene transfer. Tissue culture of rice may help to get somaclone and their performance can be observed in the field. Chemical and physical mutagenic agents treated organs produce mutant callus that also help to obtain somaclones, disease, pest or insect resistant, stress or salt tolerant mutant line of aromatic rice. Somaclonal variation could be regulated through changing explants, medium especially the phytohormones in medium, culture methods and length of time spent *in vitro*. The callus obtained from the mature dehusked seed of aromatic rice variety is amenable to multiple shoot formation, and could be used for genetic transformation studies. Therefore, the experiment was undertaken considering the objectives: (i) to find out the potentiality of aromatic rice (*Oryza sativa* L.) variety Chiniguri for callus induction and plant regeneration from mature dehusked seeds, (ii) to find out the suitable concentration of 2, 4-D for callus induction and (iii) to study the single and combined effects of different cytokine and auxin on plantlet regeneration.

MATERIALS AND METHODS

This experiment was conducted at Plant Biotechnology Laboratory, Biotechnology & Genetic Engineering Discipline, Khulna University, Bangladesh. In this experiment field grown seeds of aromatic rice (*Oryza sativa* L.) variety Chiniguri were used for callus induction and plant regeneration. The explants were collected from farmer of Khulna, Bangladesh. The seeds were dehusked by hands. The dehusked seeds were then washed with 70% alcohol for one minute and then washed 4 times with double distilled water. After washing the explants were dipped in 0.1% $HgCl_2$ solution for 10 minutes. The seeds were then rinsed 5-6 times with sterile distilled water to removed $HgCl_2$ with vigorous agitation in the laminar air flow cabinet. After surface sterilization of explants, these kept onto autoclaved filter paper on the petridish. When the water removed from the seeds surface it was inoculated into the culture tubes with sterilized forceps. MS (Murashige & Skoog, 1962) basal media supplemented with different cytokinins (Kn, BA) and auxins (IAA, NAA, 2, 4-D) at varying concentrations and combination were prepared for callus induction and plantlet regeneration. The pH of the medium was adjusted to 5.8 and solidified with 0.7% agar. Test tubes containing medium were autoclaved. Inoculated shoots were then transferred immediately under light (2000 lux) provided by 40W white cool fluorescence tubes. The temperature and humidity of the culture room were 25±1ºC and 65%, respectively. The photoperiod was maintained as 14h. Visual observation of culture was made every week. Data on callus induction and plantlets regeneration were used to calculate the percentage of cultures responding per treatment. For each treatment 10 replications were used. Frequency of callus induction and shoot formation was recorded after 4-5 weeks. All the treatments were repeated thrice. Data were analyzed as means ± SE (Mian & Mian, 1984). When the regenerated plantlet produced sufficient roots the plantlets grown inside the tubes were brought out and kept in room temperature for 5-7 days. Agar attached with roots was removed and the plantlets were transferred to small pots containing soil. After hardening the pot was exposed to outer environment and finally transplanted to field.

RESULTS AND DISCUSSION

Effect of 2, 4-D on callus induction. The callus initiation was started at 7th to 8th day after transfer of the caryopses to culture tubes and their incubation. The final data on callus induction was recorded after five weeks of inoculation. It was noticed that MS media supplemented with 1.5, 2.0, 2.5 and 3.0 mg L^{-1} 2, 4-D produced 100% callus (Fig. 1). MS medium supplemented with 0.5 mg L^{-1} 2, 4-D produced the lowest percentage (40%) of callus. The color of the all callus was whitish and the texture of them was friable (Table I). Pandey *et al*. (1994) cultured the explants of 10 rice genotypes on MS medium with 5 different concentrations of 2, 4-D and found that MS medium supplemented with 2.0 mg L^{-1} 2, 4-D produced the most desired calli. Azria and Bhalla (2000) also obtained plantlets from callus, induced from embryos of mature seeds of 4 Australian varieties (Amaroo, Millin, Pelde and Langi) of rice. They found that MS medium supplemented with 0.5-2.0 mg L^{-1} of 2, 4-D is suitable for callus formation.

Table I. The effect of different concentrations of 2, 4-D on callus induction after one week

Concentrations of 2,4-D (mg/l)	Percentages of explant producing callus	*Degrees of callus
0.5	40	+
1.0	70	+
1.5	100	+++
2.0	100	+++
2.5	100	+++
3.0	100	+++

*(+ = poor callus, ++ = good callus, +++ = very good callus)

Table II. Effect of IAA and Kn on shoot formation (calluses were obtained from the different concentrations of 2, 4-D)

Calluses of different concentrations of 2,4-D (mg/l)	Concentrations of IAA+KN (mg/l)	Percentages of callus producing shoot	Average number of shoot per culture $\bar{X}$±SE	Average height of shoot (cm) $\bar{X}$±SE
1.0	2.0+4.0	40	2±0.58	3.63±0.74
2.0	2.0+4.0	20	3.5±0.50	2.5±0.65
3.0	2.0+4.0	20	1.5±0.50	2.37±0.54

Fig. 1. Callus induction on MS medium supplemented with 2.0 mg L^{-1} of 2, 4- D for aromatic rice (*Oryza sativa* L.) variety Chiniguri

Fig. 2. Plantlet regeneration on MS medium supplemented with 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA for aromatic rice (*Oryza sativa* L.) variety Chiniguri

Fig. 3. Plantlet regeneration on MS medium supplemented with 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA for aromatic rice (*Oryza sativa* L.) variety Chiniguri after first subculture

Sripichitt and Cheewasestatham (1994) also reported that the callus formation from rice cv. Khao Dawk Mali 105 embryos was optimum (96.3%, mean size 9.4 mm) when cultured on Murashige and Skoog (MS) medium supplemented with 2 mg L^{-1} 2, 4-D and 300 mg L^{-1} casein hydrolysate. Asad *et al*. (2001) also observed that N6 medium containing 2 mg L^{-1} of 2, 4-D was optimum for callus induction of four rice genotypes i.e. Swat I, Swat II, Dilrosh 97 and Pakhal.

Effect of cytokinins and auxins on plantlet regeneration. Calli derived from the different concentrations of 2, 4-D (1.0, 2.0, 3.0 mg L^{-1}) were cultured on MS medium supplemented with 2.0+4.0 mg L^{-1} of IAA+Kn and the highest percentage (40%) of callus response for shoot formation. The highest average length (3.63±0.74 cm) of shoots were observed from the callus obtained from 1.0 mg L^{-1} 2, 4-D. Besides the highest average number of shoots (3.5±0.5) was observed on the callus derived from 2.0 mg L^{-1} 2, 4-D (Table II). Pandey *et al*. (1994) reported that 2.0 and 3.0 mg L^{-1} IAA and kinetin produced the most shoots. These findings are fully consistent to the present study. Jiahua *et al.* (1995) also reported that 0.5 and 2.0 mg L^{-1} IAA and kinetin in regeneration medium were beneficial for green plantlet differentiation of japonica rice (*Oryza sativa*).

We then used the callus derived from 2.0 mg L^{-1} 2, 4-D and cultured on MS medium supplemented with fixed concentration (0.05 mg L^{-1}) of NAA and different concentrations (2.0, 3.0, 4.0 and 5.0 mg L^{-1}) of BA. Few green spots were appeared at 7^{th} to 8^{th} day after transferring of callus, which increased day by day. Regular observation was maintained during the incubation period and found that within 2-4 weeks the plantlet regenerated from the callus at various frequencies at different concentrations of the growth regulators. The highest regeneration frequency (100%) was recorded after 4 weeks at 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA. The highest average number of shoot per culture (5.55±1.44) and the highest average shoot length (1.85±0.14 cm) also observed on the same media (Fig. 2). The second highest regeneration frequency (90%) with average shoot per culture (5.11±0.99) and average shoot length per culture (1.81±0.22 cm) were observed on MS media supplemented with 0.05 mg L^{-1} NAA+4.0 mg L^{-1} BA. On the other hand, the lowest regeneration frequency (50%) with average number (4.6±1.50) and average height (1.67±0.14 cm) of shoot were obtained at 0.05 mg L^{-1} NAA+2.0 mg L^{-1} BA (Table III). Amirkhanov *et al.* (1991) reported that 5 mg L^{-1} BA to be best for regeneration. These findings are fully consistent to the present study. Marassi *et al.* (1996) found the best shoot responses on MS media supplemented with 0.1 or 1 mg NAA and 1 or 1.5 mg L^{-1} BA. Sharma *et al.* (1998) used immature embryos of three indica rice varieties (Heera, Pankaj & Basmati-370) and observed better response of callus proliferation and shoot bud regeneration on MS medium supplemented with 100 mg adenine sulfate, 2.0 mg BA and 1.0 mg NAA L^{-1}. Liu *et al.* (2002) also investigated with young embryos of grain-straw-dual-use-rice (GSDUR) 201 and found that NB supplemented with 0.05 mg NAA+2 mg BA L^{-1} was the most suitable differentiation medium for immature GSDUR embryos.

The maximum shoots produced roots after 3-4 weeks though initially it did not appear. The callus containing green spots and plantlets of the tubes were subculture to the

Table III. Effect of NAA and BA on plantlet regeneration (calluses were obtained from 2.0 mg /l 2, 4-D)

Concentrations of NAA+BA (mg/l)	Percentages of callus producing plants	Average number of shoot per culture $\overline{X}$±SE	Average height of shoot (cm) $\overline{X}$±SE
0.05+2.0	50	4.6±1.50	1.67±0.14
0.05+3.0	60	4.67±0.67	1.68±0.17
0.05+4.0	90	5.11±0.99	1.81±0.22
0.05+5.0	100	5.55±1.44	1.85±0.14

Table IV. Effect of NAA and BA on plant regeneration after first subculture

Concentrations of NAA+BA (mg/l)	Average number of shoot per culture $\overline{X}$±SE	Average height of shoot (cm) $\overline{X}$±SE
0.05 +2.0	8.5±1.77	3.0±0.87
0.05+3.0	9.2±1.90	3.11±0.76
0.05+4.0	10±1.87	5.20±0.95
0.05+5.0	10.15±1.98	5.26±0.91

same regeneration media after 4 weeks. New shoots induced from the callus and multiple shoot were appeared in the first subculture and every shoot produced roots that liked complete plantlets and suitable for hardening (Fig. 3). The average highest number of shoots (10.15±1.98) per culture with the average height of shoots (5.26±0.91 cm) was obtained at 0.05 mg L^{-1} NAA+5.0 mg L^{-1} BA. The second highest number of shoots (10.0±1.87) per culture with average height (5.20±0.95 cm) was obtained at 0.05 mg L^{-1} NAA+ 4.0 mg L^{-1} BA. On the other hand, the lowest average number of shoots (8.5±1.77) with average height of shoot (3.0±0.87 cm) was observed at 0.05 mg L^{-1} NAA+2.0 mg L^{-1} BA (Table IV).

It can be concluded that 2.0 to 3.0 mg L^{-1} 2, 4-D was suitable for callus induction from dehusked seed of aromatic rice but callus from high concentrations (3.0 mg L^{-1}) of 2, 4-D was low responded to plantlet regeneration than the lower concentrations (1.0, 2.0 mg L^{-1}) of that in the same regeneration media. These observations are consistent with earlier report of Mitsuoka *et al.* (1994) and Al-Forkan *et al.* (2005).

REFERENCES

Al-Forkan, M., M.A, Rahim, T. Chowdhury, P. Akter and L. Khaleda, 2005. Development of highly callogenesis and regeneration system for some tolerant rice (*Oryza sativa* L.) cultivars of Bangladesh. *Biotechnology*, 4: 230–4

Amirkhanov, O.N., L.A. Kucherenko and N.A. Dolotova, 1991. Morphogenesis in the culture of somatic tissues of rice, and the composition of the regeneration medium. *Doklady Vsesoyuznoi Ordena Lenina I Ordena Trudovogo Krasnogo Znameni Akademii Sel'skokhozyaistvennykh Nauk im.V.I. Lenina*. 2: 6–8

Asad, J., M.H. Qazi, F. Tahira and H. Tayyab, 2001. Tissue Culture response of Local Varieties of Rice *(Oryza sativa* L.) of NWFP. *Online J. Biol. Sci.*, 1: 387–90

Azria, D. and P.L. Bhalla, 2000. Plant regeneration from mature embryo-derived callus of Australian rice (*Oryza sativa* L.) varieties. *Australian J. Agri. Res*., 51: 305–12

Bajaj, Y.P.S., 1980. Induction and cryopreservation of genetic variability in rice. *Proc. Rice Tissue Culture Planning Conf*., Los Banos, Philippines.

Dorosieve, L., 1996. Plant cell and tissue cultures present state and future prospects. *Genet. Selektasiya*, 19: 356–62

Jiahua, X., G. Mingwei, C. Qihua, C. Xiongying, S. Yuwei and L. Zhuqing, 1995. Improved isolated microspore culture efficiency in medium with maltose and optimized growth regulator combination in japonica rice (*Oryza sativa*). *Plant Cell Tiss. Org. Cult*., 42: 245–50

Kucherenko, L.A., 1994. Some features of the of rice regeneration *in vitro*. *Sel'skokhozyaistvennaya Bio.*, 5: 74–7

Larkin, P.J. and W.R. Scowcroft, 1981. Somaclonal variation- a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet*., 60: 197–214

Liu, F., M. Xu, X.F. Wang and J.G. Zheng, 2002. A preliminary study on establishing high regeneration frequency system of grain straw dual use rice 201, an indica rice. *J. Fujian Agri. For. Uni*., 31: 146–9

Marassi, M.A., O.A. Bovo, A. Scocchi and L.A. Mroginski, 1996. Cytokinins in the callus induction medium for plant regeneration of rice (*Oryza sativa* L. indica) var. Basmati-370. *Phyton*, 59: 155–60

Mian M. A. and M. A. Mian, 1984. *An Introduction to Statistics*. 4th Ed, p. 125-9. Ideal Library, Dhaka, Bangladesh.

Mitsuok, K., H. Honda, X.H.. Xing and H. Unno, 1994. Effect of intracellular 2, 4-D concentration on plantlet regeneration of rice (*Oryza sativa* L.) callus. *Appl. Microbiol. Biotechnol*., 42: 364–6

Murashige, T and F. Skoog, 1962. A revised medium for rapid growth and Bioassays with tobacco tissue cultures. *Physiol. Plant*., 15: 473–97

Okamoto, A., S. Kishine T. Hirosawa and A. Nakazono, 1996. Effect of oxygen-enriched aeration on regeneration of rice cell culture. *Plant Cell Rep*., 15: 731–6

Pandey, S.K., B. Ramesh and P.K. Gupta, 1994. Study on effect of genotype and culture medium on callus formation and plant regeneration in rice (*Oryza sativa* L.). *Indian J. Genet. Plant Breed*, 54: 293–9

Ray, J.K., 1985. *Introduction to Botany of the Rice Plant*. 2nd Ed, p. 5. Rice Research Institute in India. Indian Council of Agricultural Research, New Delhi, India.

Sharma, J.P., B.B. Mukherjee and S. Gupta, 1998. Callus induction and plant regeneration from immature embryos of indica rice varieties. *Oryza*, 36: 32–4

Skirvin, R.M., 1978. Natural and induced variation in tissue culture. *Euphytica*, 27: 241–6

Sripichitt, P. and P. Cheewasestatham, 1994. Plant regeneration from embryo-derived callus of aromatic rice (*Oryza sativa* L.) cultivar Khao Dawk Mali 105. *Kasetsar J. Nat. Sci*., 28: 27–37

Sun, Z.R., P.C. Ni and Z.Z. Hung, 1990. Studies on the analysis of variance and major/minor factors of medium components influencing the efficiency of callus production ability. *Acta Argon. Sin*., 16: 123–30

Xing, X.H., M.Huang, and H.Unno, 1996. Shoot regeneration from rice (*Oryza sativa* L.) callus precultured anaerobically. *J. Fer. Bioeng*., 82: 164–6

Xing, X.H., M. Huang, N. Shiragami and H. Unno, 1995. Effect of abscisic acid on shoot regeneration from rice (*Oryza sativa* L.) callus. *Plant Tiss. Cult. Lett*., 12: 125–30

Ying, C., 1983. Anther and pollen culture of rice in China. *Proc. Workshop on cell and tissue culture technique for cereal crop improvement*. Sci. Press, pp. 27. Beijing.

(Received 12 July 2005; Accepted 10 August 2006)