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Characterization of Factors Affecting Plantlet Regeneration from Rice (*Oryza sativa* L.) Callus

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Various components of culture media were tested to characterize factors affecting plantlet regeneration from rice (*Oryza sativa* L.) callus. It was found that plantlet regeneration from rice callus was affected by concentrations of gelling agents, osmoticum, and the combination of hormones in the regeneration medium. High concentrations (4–6 g/l gellan gum, 10–16 g/l agar) of gelling agents promoted regeneration frequency. However, the total number of plantlets decreased with gellan gum concentrations above 4 g/l. Addition of sorbitol (15–75 g/l) promoted plantlet regeneration. However, the addition of mannitol was inhibitory and no regeneration was observed at concentrations above 30 g/l. This difference in the effects on regeneration suggests that sorbitol had another function besides as a osmoticum. High regeneration frequency was obtained with combinations of NAA (0.05–0.5 g/l) and kinetin (0.5–2 mg/l). However, higher concentrations (2 mg/l) of NAA are preferred to increase the total number of regenerated plantlets.

Key words: Callus — Gelling agents — Phytohormones — Osmotica — Plant regeneration — *Oryza sativa* L.

The aim of this study was to characterize the factors which affect plantlet regeneration from rice callus and to establish an efficient, high-yielding culture system for the clonal propagation of rice plants. Since the early reports on successful regeneration from rice callus by several Japanese researchers (Nishi *et al.*, 1968; Tamura, 1968; Kawata and Ishihara, 1968; Niizeki and Oono, 1968; Maeda, 1968), significant improvements have been made on various factors affecting plantlet regeneration (Ling *et al.*, 1983; Chen *et al.*, 1985; Raghava-Ram and Nabors, 1985; Abe and Futsuhara, 1985, 1989; Koetje *et al.*, 1989; Hartke and Lörz, 1989). The major differences among media used in previous reports were the concentrations of macronutrients, carbon sources, and phytohormones. In addition to those major elements of media, osmolarity of media was reported to be important (Kavi Kishor and Reddy, 1986a, b; Kavi Kishor, 1987; Kavi Kishor *et al.*, 1989). Most of previous reports described regeneration frequency which was expressed as the number of callus clusters regenerated over the number of callus clusters plated. For mass propagation, however, the total number of regenerated plantlets is ultimately most important.

In the present study, we have focused on the effects of gelling agents, osmotica and combination of hormones on regeneration frequency and yield of plantlets. A highly

efficient and high-yielding regeneration system is presented and critical factors for the system's success are discussed.

Materials and Methods

Plant materials

Mature seeds of rice (*Oryza sativa* L. cv. sasanishiki) were used throughout this study.

Induction of callus and initiation of suspension culture

Induction of callus and initiation of suspension culture were performed according to Matsuno *et al.* (1990) with some minor modifications, as follows. The seeds were dehusked and surface sterilized with 10% sodium hypochlorite for 30 min and then rinsed three times with sterile distilled water. Fifteen sterilized seeds were placed on the callus induction medium (CI-medium) that contained the inorganic salts of N6 medium (Chu *et al.*, 1975) supplemented with 12 mM proline, 0.1 g/l casein acid hydrolysate (CH), 4 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 10 g/l sucrose, 30 g/l sorbitol, 5 mM 2-(N-morpholino)ethanesulfonic acid and 2 g/l gellan gum. The pH of the medium was adjusted to 5.8 with KOH, and gellan gum was added prior to autoclaving at 121°C for 20 min. The calli which formed in the scutellar region of the seeds were removed and transferred onto fresh CI-medium and incubated for an additional 14 days. Suspension cultures were initiated by transferring approximately 1 g of callus to 500 ml Erlenmeyer flasks containing 100 ml of subculture medium (CI-medium devoid of gellan gum). The cell suspension was cultured on a rotary shaker at 80 rpm in the dark and was subcultured at 7 day-intervals. At the time of subculture, callus was broken into smaller clusters and callus clusters that passed the nylon mesh with a pore size of 1 mm were subcultured. The cell suspension was subcultured at least 4 times prior to use in regeneration studies.

Plantlet regeneration

After 7 days of suspension culture in a fresh CI-medium, callus was collected by using nylon mesh with a pore size of 1 mm. The collected callus was rinsed three times with hormone-free subculture medium. The average size of the callus was approximately 1-1.5 mm in diameter. Basal regeneration medium was described by Abe and Futsuhara (1986) and contained inorganic salts and vitamins of MS medium (Murashige and Skoog, 1962) supplemented with 2 g/l CH, 0.5 mg/l kinetin, 30 g/l sucrose and 8 g/l agar. The pH of the medium was adjusted to 5.8 with KOH and agar was added prior to autoclaving at 121°C for 20 min. Sixteen callus clusters (approximately 20 mg) were plated on a 9 cm plastic Petri-dish containing 25 ml of the regeneration medium. Five replicated plates were prepared for each experimental condition. Plates were incubated under the regime of 16 hr-light at 30°C and 8 hr-dark at 25°C. After 4 weeks of culture, regeneration frequen cy was evaluated by calculating the ratios of the number of calli with plantlets over the total number of calli inoculated. Plantlet yield was computed by counting the total number of

regenerat ed plantlets on a plate.

Results

Effect of gelling agent concentration on regeneration

No significant plantlet regeneration was observed in the basal medium with 8 g/l agar. Upon increasing the agar concentration to 10-16 g/l, the regeneration frequency increased to 19-29% (Fig. 1A). Similar results were obtained in a medium

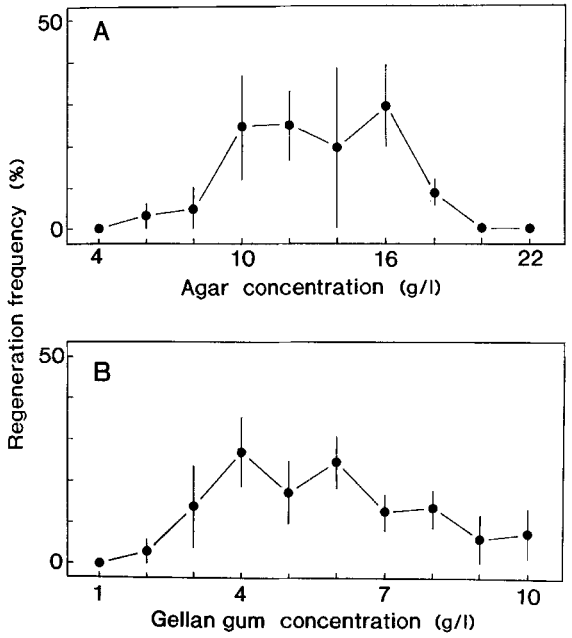

Fig. 1. Effects of concentrations of gelling agents on regeneration frequency. Basal regeneration medium used was based on that developed by Abe and Futsuhara (1986). Indicated amounts of agar(A) or gellan gum(B) were added to the basal medium. Plates were incubated for 4 weeks under the regime of 16 hr-light at 30°C and 8 hr-dark at 25°C. Each point represents the mean of 5 replicated plates. Standard errors bars reflect the range of response.

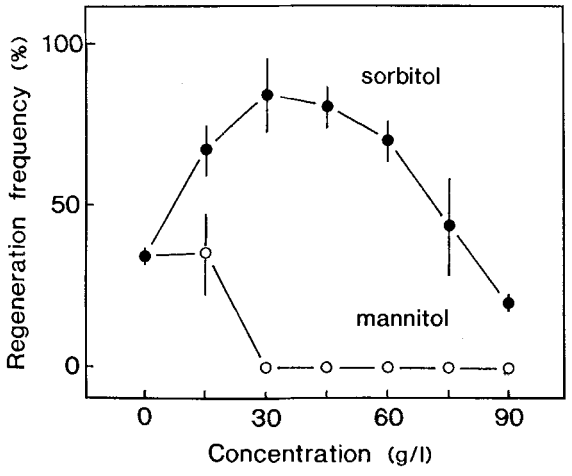

Fig. 2. Effects of concentrations of osmotica on regeneration frequency. Basal regeneration medium used was based on that developed by Abe and Futsuhara (1986) modified by supplementing 4 g/l of gellan gum instead of 8 g/l of agar. Indicated amounts of sorbitol (—●—) and mannitol (—○—) were added to the basal medium. Plates were incubated for 4 weeks under the regime of 16 hr-light at 30°C and 8 hr-dark at 25°C. Each point represents mean of 5 replicated plates. Standard errors bars reflect the range of response.

with gellan gum instead of agar. With 2 g/l gellan gum, a concentration commonly used for callus culture, regeneration frequency was less than 5%. By increasing its concentration to 4-6 g/l, regeneration frequency was increased to 18-27% (Fig. 1B). However, the total number of plantlets decreased when the gellan gum concentration was higher than 4 g/l. As the gellan gum concentration increased from 4g/l to 6 g/l, the number of regenerated plantlet decreased from 108/plate to 52/plate, while no significant change was observed in the regeneration frequency. In subsequent experiments, 4g/l of gellan gum was used since the high regeneration frequency was constantly observed in a medium with gellan gum rather than in that supplemental with agar (Fig. 1).

Effects of osmotica on regeneration

As shown in Fig. 2, the addition of sorbitol up to 75 g/l improved regeneration frequency, especially at 30 g/l where the frequency was obversed to be 84%. The addition of sorbitol at a concentration above 75 g/l proved to be inhibitory. In

Table 1. Effects of NAA and kinetin on regeneration frequency and number of regenerated plantlets.

NAA conc. (mg/l)	Kinetin conc. (mg/l)	Regeneration frequency (%)	No. of regenerated plantlet/plate
0	0	43	—
0	0.5	62	—
0	2.0	87	—
0.05	0	31	—
0.05	0.5	100	—
0.05	2.0	100	—
0.5	0	6	—
0.5	0.5	94	84
0.5	1.0	93	86
0.5	2.0	100	81
1.0	0.5	100	89
1.0	1.0	93	91
1.0	2.0	95	93
2.0	0.5	95	84
2.0	1.0	98	113
2.0	2.0	93	107
5.0	0	0	—
5.0	0.5	50	—
5.0	2.0	93	—

Basal regeneration medium used was described by Abe and Futsuhara (1986) and supplemented with 4 g/l of gellan gum and 30 g/l sorbitol. Plates were incubated for 4 weeks under the regime of 16 hr-light at 30°C and 8 hr-dark at 25°C. Data are mean of 5 replicated plates.

contrast to sorbitol, the addition of mannitol was inhibitory at concentrations above 30 g/l, even though significant callus growth was still observed at these concentrations. In subsequent experiments, basal medium supplemented with 4 g/l gellan gum and 30 g/l sorbitol was used.

Effects of auxin and cytokinin on regeneration

The addition of kinetin stimulated regeneration at concentrations up to 2 mg/l (Table 1). The addition of naphthaleneacetic acid (NAA) alone to the regeneration medium resulted in regeneration inhibition and at a concentration of 5.0 mg/l, regeneration was almost completely inhibited. When NAA was added together with kinetin, however, a substantial degree of regeneration stimulation was observed. One hundred percent regeneration frequency was observed with the combinations of 0.05 or 0.5 mg/l of NAA and 0.5 or 2.0 mg/l of kinetin. The total number of regenerated plantlets, however, increased with higher concentrations of NAA up to 2.0 mg/l. The highest number of plantlets (113 plantlets/plate) was obtained with the combination of 2.0 mg/l NAA and 1.0 mg/l kinetin (Table 1).

Discussion

In this study, regeneration frequency of rice callus has been shown to be affected by several culture parameters such as concentrations of gelling agents and osmoticum, as well as by the combination of phytohormones. Under optimum experimental conditions, it was estimated that more than 5,000 plantlets could be regenerated from 1 g of callus within 4 weeks of plating.

Gelling agents promoted regeneration frequency when it was used at concentrations higher than those commonly used. Lai and Liu (1988) showed that the water content of actively regenerating callus was approximately 10% lower than that of poorly regenerating callus. They suggested that the water status of the callus was closely related to regeneration frequency. Higher agar concentrations (16 g/l) together with mannitol were reported to reduce the water content of callus and thereby, increased regeneration frequency. It has been suggested that osmotic potentials of media utilized for both callus growth and regeneration were an essential factor in regeneration of rice callus (Kavi Kishor and Reddy, 1986a, b ; Kavi Kishor, 1987 ; Kavi Kishor *et al.*, 1989). Kavi Kishor (1987) reported that the optimum osmolarities for growth and regeneration were 300 milli osmoles and 200 milli osmoles, respectively. He used mannitol and sorbitol for adjusting osmolarity of the media, but did not report any difference between the two osmotica in terms of their effects on regeneration frequency. The striking differences between sorbitol and mannitol which were observed in this study indicate that sorbitol may not work only as a mere osmoticum. Our preliminary study showed that the sorbitol concentration in the regeneration medium decreased during the culture while mannitol concentration was constant (data not shown). It suggests that sorbitol was metabolized during regeneration. The role of sorbitol in this regeneration system remains to be clarified.

Many auxins and cytokinins in the regeneration media have been tested for their effects on regeneration frequency in rice (Bhattacharya and Sen, 1980; Inoue and Maeda, 1980; Ling *et al.*, 1983; Ozawa and Komamine 1989). In most of the reports, 2, 4-D was used for callus induction and was reported to be superior to other auxins such as indole-3-acetic acid (IAA) and NAA. In the case of the plantlet regeneration, combinations of NAA and kinetin have been widely used. Ozawa and Komamine (1989) reported the highest regeneration frequency with a combination of NAA (0.01 mg/l) and 4-pyridylurea (4-PU) (0.1 mg/l). In this study, 100% of regeneration frequency was observed in the medium with a combination of NAA (0.05 or 0.5 mg/l) and kinetin (0.5 or 2.0 mg/l) (Table 1). Other auxins such as IAA and 2, 4-D was not as effective as NAA. 2, 4-D was inhibitory for regeneration at concentrations as low as 0.01 mg/l (data not shown). Concerning the yield of plantlets, a medium with NAA (2.0 mg/l) and kinetin (1.0 mg/l) was found to be optimum.

For more effective mass propagation of rice *in vitro*, a regeneration system with liquid medium using a bioreactor is prerequisite and studies are in progress to establish a regeneration system with liquid medium.

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