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Improvement of Cell Culture Conditions for Rice

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Abstract
Cell culture techniques are necessary for the use of biotechnology including the production of transgenic rice plants. However, there are significant differences *in vitro* culture responses among rice varieties. These differences limit the application of biotechnology to genetic improvement of rice. In this study, it was demonstrated that nitrite reductase activity is one of the physiological factors correlated with the differences among rice varieties in cell culture. It was indicated that the modification of the culture medium was effective in overcoming the differences in cell culture: the calluses of some Indica and Japonica type varieties showed a poor growth or did not grow under the initial conditions, whereas the calluses of all the varieties tested showed good growth when the nitrogen source, carbon source and medium pH were modified. As a result, in this stud, the culture conditions applicable to a wider range of varieties, including Indica type, Javanica type and Indica-Japonica crossed varieties as well as Japonica type were identified.

Discipline: Biotechnology
Additional key words: *Oryza sativa*, abscisic acid, amino acid, genotypic difference, nitrogen metabolism

Introduction

In rice, progress is being made in the application of biotechnology, including haploid method of breeding by anther culture, the use of somaclonal variation, the production of somatic hybrids by cell fusion, and the production of transformed plants. To use these techniques, plants must be regenerated from objective cells using cell culture techniques. However, significant differences in the efficiency of callus formation, somatic embryogenesis, and plant regeneration have been detected among the varieties of many plant species, including rice[1,21]. These varietal differences limit the application of biotechnology to the genetic improvement of rice. For example, only a few Japonica varieties, such as Nipponbare and Taipei 309, can be readily cultured and maintained in the embryogenic state, and used to produce transformed rice plants[9,19,23]. So far, no reports have dealt with the improvement of culture conditions that could be applied to a wide range of varieties and genotypes in rice.

In rice cell culture, immature and mature embryos are used as materials for callus induction, because the derived calluses have a high ability of plant regeneration[19,23]. Embryogenic suspension culture can be established by continuously culturing the calluses in a liquid medium. A large amount of protoplasts, into which foreign genes have been introduced by electroporation[19,23] and polyethylene glycol[9] methods, have been isolated from the suspension cultures. And also the cultures can be used as target materials for the transfer of foreign genes by biolistic[4] and *Agrobacterium* methods[10]. Therefore, it is important to develop embryogenic suspension cultures for the application of biotechnological methods in rice.

In this study, the culture conditions for rice were improved in order to overcome the varietal differences in the culture response and to obtain embryogenic cultures from all types of rice varieties. Furthermore, the physiological factors related to varietal differences in rice cell culture using calluses cultured under the improved conditions were identified.

Materials and methods

1) Callus induction and culture

Fifty rice varieties used in this study are shown in Figs. 2 and 3. Rice seeds were provided by the Genebank

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and the Rice Breeding Laboratory, Chugoku National Agricultural Experimental Station, Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF). Seeds were sterilized in 70% ethanol for 30 s and in a sodium hypochlorite solution containing an available chlorine concentration of 1 % for 30 min, followed by rinsing with sterile water 3 times. After rinsing, the sterilized seeds were immersed in sterilized water for 6 to 7 h. Embryos were cut off from the imbibed seeds and were placed with the scutellum pointing upwards on induction media. The induction media contained constituents of Medium C (shown in Table 1) except for proline and were solidified by 0.8% agar. The pH of the medium was adjusted to 5.8. Seven-day-old calluses derived from the scutella were transferred to 20 mL of Medium A and Medium C in 100 mLflasks on a gyratory shaker at 100 rpm by subculturing every 7 days. For subculturing, about 0.4 mL cell volume of calluses were transferred to 20 mL of a fresh medium with the same composition in 100 mL flasks. The calluses were used as materials after subculture for more than 2 months. The following experiments were carried out with 3 replications.

For the measurement of the callus growth rate during the seven-day-culture period, about 250 mg fresh weight of the subcultured calluses were transferred to a fresh medium. Fresh weight of the calluses was measured after removing excess medium around calluses with pieces of sterile filter paper at 0 and 7 days after transfer, and growth rates were calculated.

2) Enzyme assays

Ten Japonica type varieties used for the enzyme assays are shown in Fig. 4. The calluses were subcultured in Medium C for more than 2 months. About 250 mg of the subcultured calluses were transferred to Medium B or C. The 2 media contained potassium nitrate at the same concentration of 20 mM. The calluses were homogenized in three-fold volumes of the extraction buffer 3 days after transfer according to the method of Ida et al.[11]. The extraction buffer contained 50 mM Tris-HCl (pH 7.9), 5 mM cysteine and 2 mM EDTA. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant (500 μL) was concentrated with Microcon 10 (Amicon Inc., USA) for reducing the amounts of nitrate ions. The concentrated supernatant was diluted by the addition of 500 mL of the buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM cysteine and 2 mM EDTA and was assayed for nitrate reductase (NR) and nitrite reductase (NiR) activities. The operations described above were carried out at 4°C. Protein content of the extract was determined by a Bio-rad protein assay kit using bovine serum albumin as standard protein.

Enzyme activity was assayed by *in vitro* method. The assay mixture for NR contained 25 mM potassium phosphate buffer (pH 7.5), 10 mM KNO_3, 0.2 mM NADH, 5 mM $NaHCO_3$ and 5 μL of extract in a final volume of 0.5 mL. The assays were conducted at 30°C for 15 min. The reaction was terminated by the addition of 50 μL of 0.5 M Zn $(CH_3COO)_2$ and excess NADH was oxidized by the addition of 50 μL of 0.15 mM phenazine methosulfate[17]. The amout of NO_2^- produced was determined colorimetrically[2].

NiR activity was assayed following the reduction of NO_2^- from the assay mixture[12]. The assay mixture contained 50 mM Tris-HCl (pH 7.5), 0.5 mM $NaNO_2$, 1 mM methyl viologen and 50 μL of extract in a final volume of 0.9 mL. The reaction was started by the addition of 100 μL of 0.12 M $Na_2S_2O_4$ dissolved in 0.2 M $NaHCO_3$ and incubated at 30°C for 60 min. The reaction was terminated by vigorous mixing on a tube mixer until the color of methyl viologen fully disappeared. After the addition of 100 mL of 1 M Zn $(CH_3COO)_2$, the mixture was centrifuged at 10,000 × g for 10 min. The amount of residual NO_2^- in the supernatant was determined colorimetrically as in the NR activity assay.

For the measurement of the NO_2^- content, the supernatant of the callus homogenate was prepared as described above 3 or 7 days after transfer. NO_2^- content of the supernatant was colorimetrically determined by the method of Aslam and Huffaker [2].

3) Measurement of abscisic acid content in calluses

The calluses of Nipponbare, Koshihikari and IR24 which were subcultured in Medium C were used for the measurement of abscisic acid. About 1 g of the calluses were collected at the end of the seven-day-culture period. The calluses were homogenized with 3 mL of the 100% methanol on ice. The homogenizer blade was washed with 16 mL of 80% methanol and the washed solution was added to the homogenate. The homogenate was kept overnight at 4°C in the dark. Polyvinylpolypyrrolidone (0.5 g) was added to the homogenate, mixed and kept for more than 10 min. The mixture was filtered and evaporated *in vacuo*. After evaporation, the residue was dissolved in 1.5 mL of 0.5 M potassium buffer (pH 8.3). The sample solution was partitioned against hexane 3 times and then against ethyl acetate 3 times. The pH of the aqueous phase was adjusted to around 2.5 and the solution was partitioned against ethyl acetate 3 times. The ethyl acetate phase was collected and evaporated *in vacuo*. The residue was dissolved in 100% methanol and loaded onto a Bondesil DEA column (Varian Associates. Inc., U.S.A.). After absorption, the column was washed with 100% methanol and eluted with methanol contain-

ing 0.5% acetic acid. The eluate was evaporated *in vacuo* and the residue was used for the determination of the ABA content by an immunoassay. The culture medium was also collected and evaporated *in vacuo* at the end of the seven-day-culture period and purified for the determination of the ABA content in the same way as for calluses except for hexan partitioning and first ethyl acetate partitioning. Butylated hydroxytoluene, an antioxidant, was added to all the solvents for homogenization and purification at the concentration of 20 mg/L. ABA content in the purified sample was determined using an abscisic acid immunoassay detection kit (Sigma-Aldrich Japan K. K., Japan).

Effects of modification of culture conditions on varietal differences in rice cell culture

In a liquid culture for suspension culture, 2 kinds of basal media, N6[5] and R2[15], are widely used in combination with 2,4-D, sucrose, proline, etc. Both media were developed for cell culture of Japonica type rice in terms of promotion of cell proliferation and callus formation from explants, and the composition of the media was characterized by low levels of ammonium sulfate, 3.5 and 2.5 mM, respectively, as reduced nitrogen sources. Fig. 1 A–C shows a comparison of callus proliferation between 'Nipponbare', 'Koshihikari' and 'IR24', when the calluses derived from mature embryos were cultured in a liquid medium containing R2 basal medium, 1 mg/L 2,4-D and 30 g/L sucrose. Japonica type variety Nipponbare is one of the varieties most frequently used for biotechnological research. Nipponbare produced yellowish-white calluses and grew vigorously in this culture medium while Japonica type variety Koshihikari produced brown calluses and grew poorly. The scutellum of the embryo of the Indica type variety IR24 enlarged in the liquid medium but ceased to proliferate. In this study, 50 varieties from 4 varietal groups, Japonica type, Indica

Table 1. Medium composition (mg/L)

Constituents	Medium A (Initial medium)	Medium B	Medium C (Modified medium)
Inorganic salts			
Macro-elements			
KNO_3	4,040	2,020	2,020
$(NH_4)_2SO_4$	330	330	–
$CaCl_2 \cdot 2H_2O$	147	147	147
$MgSO_4 \cdot 7H_2O$	245	245	245
KH_2PO_4	–	272	272
$NaH_2PO_4 \cdot 2H_2O$	312	–	–
Micro-elements			
Fe-EDTA	19	19	19
$MnSO_4 \cdot 4H_2O$	1.6	1.6	1.6
$ZnSO_4 \cdot 7H_2O$	2.2	2.2	2.2
$CuSO_4 \cdot 5H_2O$	0.20	0.20	0.20
$Na_2MoO_4 \cdot 2H_2O$	0.13	0.13	0.13
H_3BO_3	2.8	2.8	2.8
Vitamins			
Nicotinic acid	1.0	1.0	1.0
Thiamine-HCl	10.0	10.0	10.0
Pyridoxine-HCl	1.0	1.0	1.0
Myo-Inositol	100.0	100.0	100.0
Amino acids			
Alanine	–	–	445
Proline	–	–	1,151
Carbon sources			
Sucrose	30,000	30,000	–
Maltose	–	–	3,750
pH	5.8	5.8	4.5

type, Javanica type and Indica-Japonica crossed varieties were classified into the 3 types described above in terms of callus proliferation in this medium as follows: Nipponbare type, Koshihikari type and IR24 type. Fig. 2 shows that in the 50 varieties from the 4 varietal groups, the callus growth rate varied during the seven-day-culture period from 1.0 to 7.5 and that the Koshihikari and IR24 type varieties showed a lower level of growth. Based on these results, Koshihikari and IR24 were selected as recalcitrant varieties for improving the culture conditions.

In this study, it was assumed that specific constituents of the medium inhibited callus growth in some varieties, such as Koshihikari, IR24. To verify this hypothesis, experiments were conducted to remove the factors affecting varietal differences by modifying the medium composition. Improved composition by which the calluses of the 2 recalcitrant varieties and of Nipponbare could proliferate was eventually identified (Fig. 1 D–F). Ammonium sulfate and sucrose concentrations were considered to be the factors that inhibited the growth of the calluses of Koshihikari[13]. The growth rate of the Koshihikari callus was almost identical with that of the Nipponbare callus, when alanine was added to the medium instead of ammonium sulfate as reduced nitrogen source and, in addition, the sucrose concentration decreased from 3 to 0.375%. In IR24, sucrose and medium pH were considered to be responsible for the poor response in culture. IR24 could be cultured in a liquid medium when the medium used for the Koshihikari varieties was improved with the following additional modifications: (1) Maltose replaced sucrose. (2) The pH of the medium decreased from 5.8 to 4.5. Furthermore, the addition of proline at a concentration of 10 mM promoted callus growth in the 3 varieties. The composition of the modified medium (Medium C) is shown in Table 1 together with that of the initial medium (Medium A).

With the modified culture conditions, 50 varieties from 4 varietal groups were tested and the growth rates are shown in Fig 3. Koshihikari did not show any browning of calluses and all the tested IR24 varieties could be cultured under the modified conditions. The callus

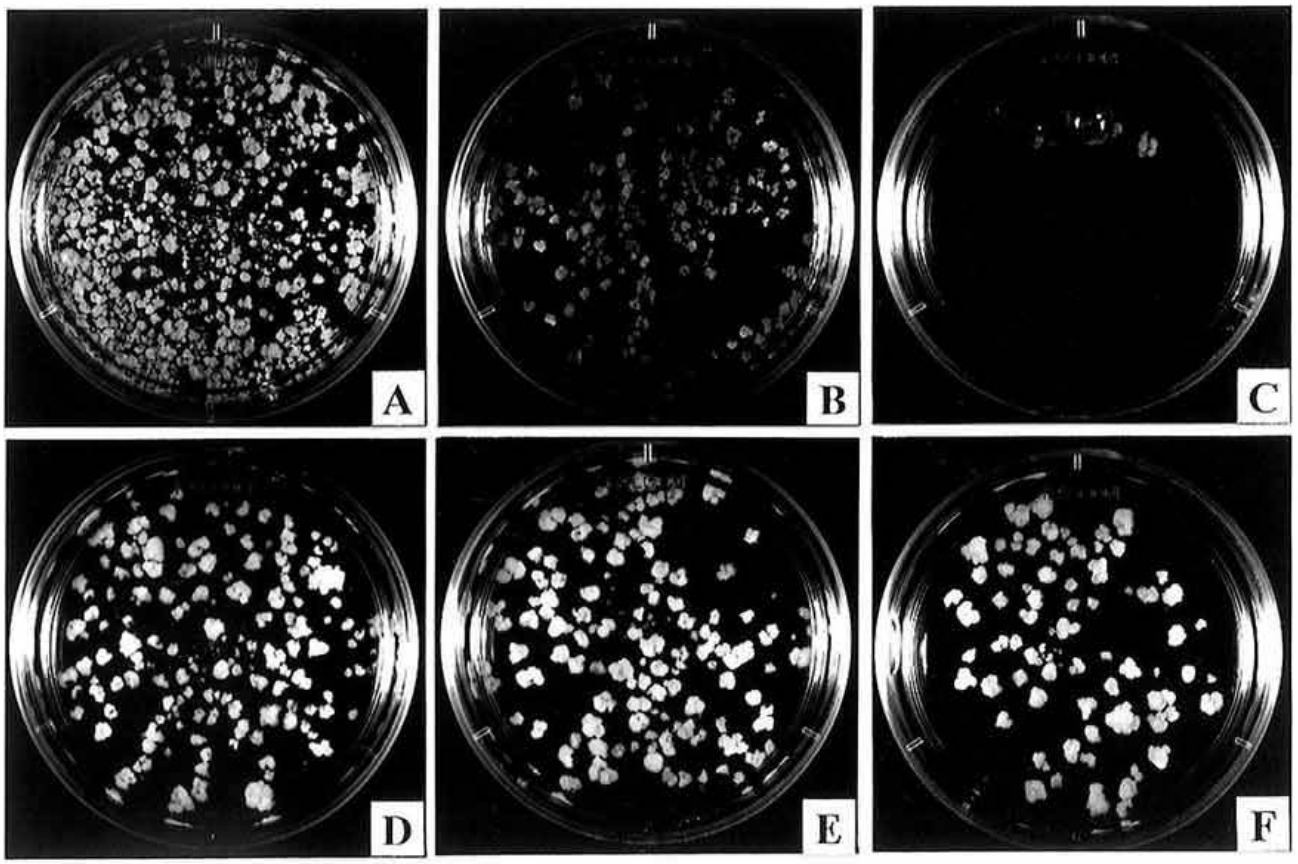

Fig. 1. Effect of modification of culture conditions on callus proliferation of 3 varieties

Variety : (A)(D) Nipponbare, (B)(E) Koshihikari, (C)(F) IR24.

Medium: (A)(B)(C) Media contain 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$ as nitrogen source and 30 g/L sucrose as carbon source. Medium pH is adjusted to 5.8. (Medium A)
(D)(E)(F) Media contain 20 mM KNO_3 and 5 mM alanine as nitrogen source, 3.75 g/L maltose as carbon source and 10 mM proline. Medium pH is adjusted to 4.5. (Medium C)

growth rates in the liquid medium ranged from 2.6 to 4.4. There were smaller differences in the callus growth rate compared to that under the initial conditions. These results indicated that the modified conditions developed in this study were applicable to a wider range of rice varieties.

Physiological factors related to differences between Nipponbare and Koshihikari in culture response

Ammonium sulfate is most commonly used as a reduced nitrogen source for preparing a basal medium in tissue and cell culture, because the addition of ammonium ion in combination with nitrate generally enhances the growth rate of cultured cells[8,15]. However, as described above, this compound was considered to be the factor that inhibited the growth of the calluses of Koshihikari type varieties. Attempts were made to investigate the metabolism of nitrogen sources in order to determine the physiological basis of the differences in callus growth between Koshihikari and Nipponbare type varieties[14]. The calluses cultured in the modified medium were considered to be suitable materials in this analysis, because no differences were observed in the growth rates and appearance of the calluses between Koshihikari and Nipponbare type varieties.

The nitrite ion content and activity of nitrate reductase and nitrite reductase were examined in the calluses of 10 varieties using the liquid medium (Medium B) with the same composition as that of the initial medium (Medium A), except that the concentration of potassium nitrate was 20 mM (Fig. 4). The calluses of the Koshihikari type varieties (No. 6–10) accumulated significantly more nitrite ions during the culture period than those of the Nipponbare type varieties (No. 1–5). The callus growth rate was negatively correlated with the nitrite ion content ($r = -0.9525$, $p < 0.001$), indicating that the calluses of the Koshihikari type varieties were injured

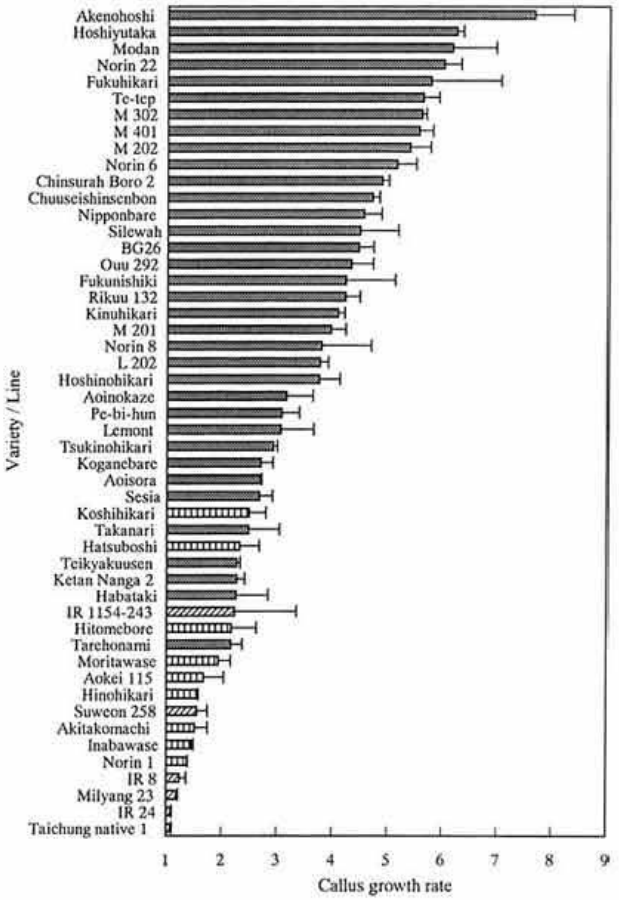

Fig. 2. Varietal differences in callus growth rate under the initial conditions (Medium A) in liquid cell culture in rice

Nipponbare type varieties or lines
Koshihikari type varieties or lines
IR24 type varieties or lines

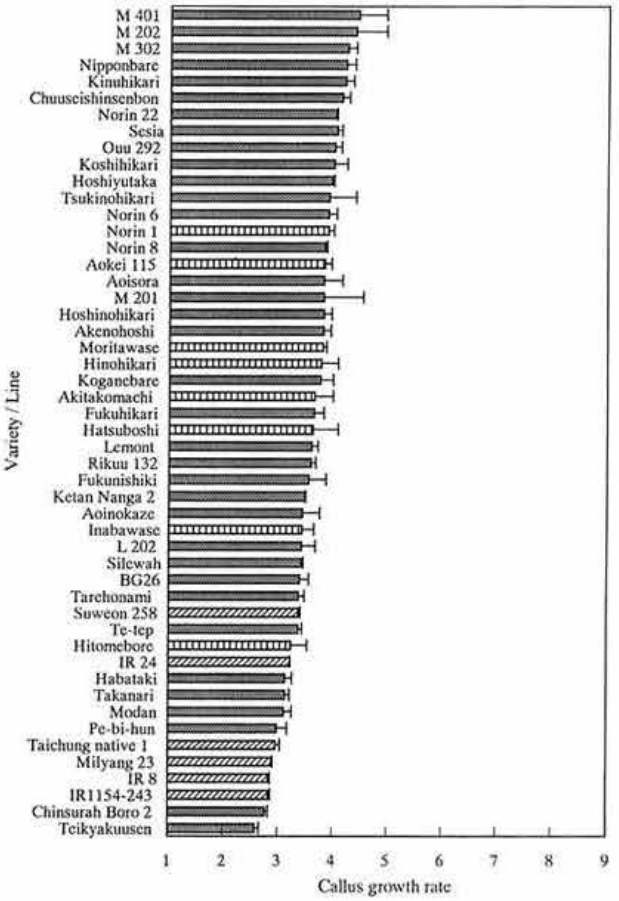

Fig. 3. Varietal differences in callus growth rate under the modified conditions (Medium C) in liquid cell culture in rice

Nipponbare type varieties or lines
Koshihikari type varieties or lines
IR24 type varieties or lines

by toxic nitrite ions, which led to browning and poor growth of the calluses.

The reduction of nitrate to ammonia is catalyzed by 2 enzymes, nitrate reductase(NR) and nitrite reductase (NiR), and nitrite ion is considered to be an intermediate in the pathway. The accumulation of nitrite ions may be attributed to either excessive production of nitrite ion by NR or slow reduction of nitrite by NiR reductase. In this study the second assumption was verified. The calluses of the Koshihikari type varieties showed significantly lower levels of NiR activity than those of the Nipponbare type varieties. On the other hand, no differences were observed in the NR activity in either group. The results suggested that the higher ion levels observed in the Koshihikari type varieties were due to a lower ability to reduce nitrite ions and that NiR activity is one of the physiological factors correlated with the differences between varieties in rice cell culture.

As shown in Fig. 5, the calluses of the Koshihikari type varieties displayed the same growth rate as those of the Nipponbare type varieties in the modified medium (Medium C), but also contained significantly lower levels of NiR. NR activity was inhibited in the calluses of both varieties in the improved medium compared to Medium B. The results suggested that the inhibition of the NR activity resulted in the accumulation of trace amounts of nitrite ions in the calluses of the Koshihikari type varieties in spite of the lower NiR activity and, as a result, callus growth improved in the modified medium.

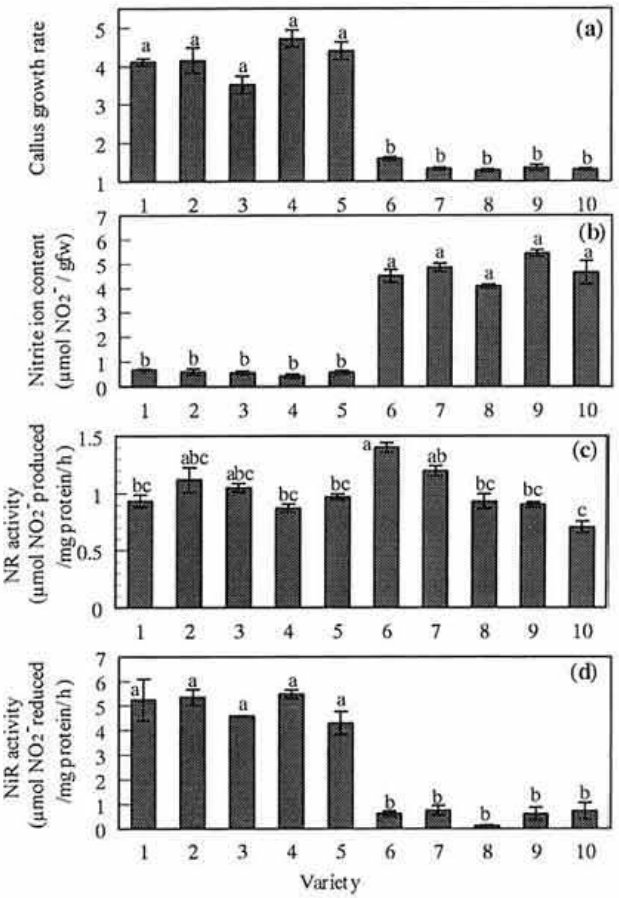

Fig. 4. Comparison of growth rate, nitrite ion content, NR activity and NiR activity of scutellum-derived calluses cultured in Medium B among 10 varieties

(a) Callus growth rate, (b) Nitrite ion content, (c) NR activity, (d) NiR activity.
Variety: (1) Nipponbare, (2) Norin 6, (3) Norin 22, (4) Norin 8, (5) Chuuseishinsenbon, (6) Norin 1, (7) Akitakomachi, (8) Moritawase, (9) Hitomebore, (10) Koshihikari.
Error bars indicate standard errors of the means in 3 replications. Differences between bars having the same letters are not significant at 1% probability level by Scheffé's multiple comparison test.

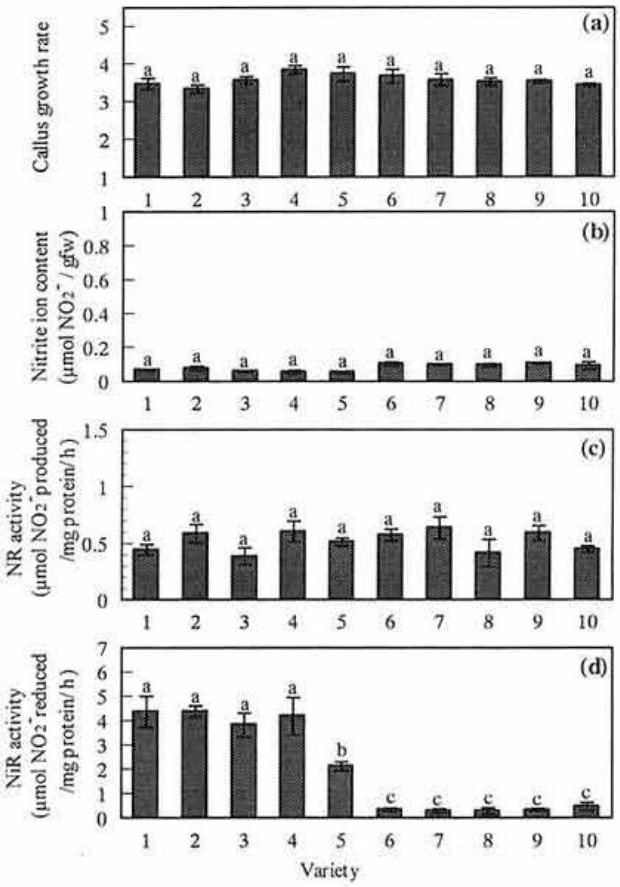

Fig. 5. Comparison of growth rate, nitrite ion content, NR activity and NiR activity of scutellum-derived calluses cultured in Medium C among 10 varieties

(a) Callus growth rate, (b) Nitrite ion content, (c) NR activity, (d) NiR activity.
Variety: the same as in Fig. 4.
Error bars indicate standard errors of the means in 3 replications. Differences between bars having the same letters are not significant at 1% probability level by Scheffé's multiple comparison test.

Table 2. Content of endogenous abscisic acid (ABA) in the calluses and concentration of abscisic acid in the media in 3 varieties

Variety	Endogenous ABA content in the calluses (ng / gfw)	Concentration of ABA in the medium (ng / mL)
Nipponbare	0.48 b	0.10 b
Koshihikari	0.67 b	0.07 b
IR24	5.78 a	0.34 a

Values within a column having different letters are significantly different at 5% level by Duncan's new multiple range test.
Values show the mean of 2 replications.

NR activity is known to be regulated by NO_3^-, light, reduced nitrogen sources, sugar, etc.[3,19]. It was reported that ammonium ions and casein hydrolysates decreased the NR activity of rice cultured cells when the nitrogen sources were added in combination with NO_3^-[16]. However, it remained to be determined whether the effect on the inhibition of the NR activity was different among the amino acids added in rice cultured cells. Filner[7] reported that the effect on the NR activity depended on the amino acids added in cultured tobacco cells: NR activity was inhibited by one group of amino acids, i. e. alanine, arginine, etc. and depressed by a second group of amino acids, i. e. asparagine, lysine. In a further study, differences in the effect of reduced nitrogen sources including alanine and proline in rice calluses will be examined. The calluses of Nipponbare type varieties showed a slightly lower growth rate in Medium C (Fig. 5) than in Medium B (Fig. 4). This decrease may be associated with the decrease in both NR and NiR activities observed in the Nipponbare type varieties. Although we replaced 30 g/L sucrose in Medium B with 3.75 g/L maltose in Medium C, differences in sugar types and concentrations may also be responsible for the decrease in the growth rate of Nipponbare type varieties in Medium C. However, it was reported that when the sucrose concentration increased, the NR activity was enhanced at least in pea seedlings[19]. Further improvement of the sugar composition for rice cell culture should be examined by monitoring the balance between NR and NiR activities to avoid the toxicity of NO_2^-.

Differences in endogenous abscisic acid content among varieties

IR24 type varieties could be cultured in the modified medium (Medium C). However, the calluses of this type showed a lower growth rate than those of Nipponbare and Koshihikari type varieties even in the modified medium: 6 varieties of IR24 type were found in the lower rank as shown in Fig. 3. In the following experiment, the regeneration ability of the calluses which were cultured in the modified medium was examined. The results showed that the calluses of IR24 type varieties exhibited a higher ability of plant regeneration than those of other types of varieties (data not shown). Based on these results, it was assumed that the IR24 varieties were characterized by both a lower ability of callus growth and high ability of plant regeneration from calluses. Although abscisic acid is a phytohormone which exerts various effects on plant growth[22], one of the effects consists of the inhibition of plant growth. In studies on plant tissue culture, the addition of abscisic acid to the medium enhanced the frequency of plant regeneration from cells[20]. The content of abscisic acid in the calluses cultured in the modified media was compared among 3 varieties, Nipponbare, Koshihikari and IR24. As shown in Table 2, in the calluses of IR24 the level of abscisic acid was 12.0 and 8.6 times higher than that in Nipponbare and Koshihikari, respectively. Similar results were obtained when the ABA content in the culture medium was compared among the 3 varieties. These results suggested that the higher content of endogenous ABA was responsible for the lower growth rate and higher regeneration ability of the IR24 calluses cultured in the modified medium.

It is assumed that the IR24 type varieties can naturally accumulate a higher level of ABA in the cells than other types of varieties. Because the initial medium contained unsuitable factors for callus growth, such as amounts of sucrose, ammonium sulfate and pH values, a high level of ABA could inhibit more strongly callus growth of the IR24 type varieties in the initial medium than in the modified medium. As a result, the calluses of IR24 type varieties may not be able to grow or display limited growth. To confirm this hypothesis, the relationships between the endogenous ABA content and differences in the culture response are being currently analyzed.

Discussion

Although varietal differences are widely observed in rice cell culture, there are few reports on the culture conditions required to overcome the differences. Daigen and Abe[6] modified a culture medium by one-fifth dilution of all of inorganic salts and obtained the conditions suitable for the culture of calluses of the Japonica type variety Koshihikari. In this study the range of tested varieties was expanded and the conditions applicable to a wider range of varieties including Indica type, Javanica type and Indica-Japonica crossed varieties as well as Japonica type were defined. For further improvement, such as enhancing the growth rate, the modified conditions developed in this study could be used as basal conditions.

For the application of biotechnology in rice, varieties and genotypes are selected based on the ability of their response to culture. The selected varieties may not necessarily belong to elite ones in rice. It is possible to transfer the traits which are genetically modified through biotechnological procedures from the manipulated plant to an elite variety by the backcrossing method. On the other hand, the backcrossing process to fix the genetic traits is time-consuming and an undesirable trait might be introduced into the derived plants if genes are closely linked to the gene for the trait. The modified culture conditions developed in this study should facilitate the application of biotechnology to rice breeding, so that elite varieties can be used directly.

There are few reports on the physiological processes related to the differences in the culture response. In most cases, no detailed analysis has been conducted to clarify the physiological aspects of varietal differences in cell culture. In this study, the nitrite ion content was found to be one of the physiological factors related to the differences in rice cell culture. It was also observed that when there is a high concentration of these ions in cells with poor growth, inhibition of the NR activity may enable to improve the growth. Physiological information related to the varietal differences in the culture response should be useful to simplify the process of improving the culture conditions as described above in rice and in other plants.

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