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Effects of Reduced Nitrogen Source and Sucrose Concentration on Varietal Differences in Rice Cell Culture

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Summary

Effects of changes in medium composition on callus proliferation were examined in five varieties including Koshihikari and Norin No. 1, which could not be easily handled in cell culture. Calluses were induced from mature embryos and were transferred into liquid media with various compositions. Sucrose concentration affected differences in growth rates among the calluses from the five varieties. The differences in growth rates were smaller at a concentration of 0.375 % sucrose in any of the four media with various compositions of nitrogen sources than at a concentration of 3 % sucrose. Reduced nitrogen source also affected the varietal differences in growth rates. At a concentration of 0.375 % sucrose, the growth rates of the Koshihikari and Norin No. 1 calluses were lower than those of the calluses from other varieties in the media containing 20 mM potassium nitrate (KNO_3), 20 mM KNO_3 and 2.5 mM ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) or 20 mM KNO_3 and 5mM glutamine as nitrogen source, while the growth rates of the Koshihikari and Norin No. 1 calluses were almost identical with those of the calluses from other varieties in the medium containing 20 mM KNO_3 and 5 mM alanine. In the next experiment, changes in the medium pH, which were induced by the growth of the calluses, were monitored at a concentration of 0.375 % sucrose during the culture period and were compared between Nipponbare and Koshihikari. When Nipponbare was cultured in the media containing 20 mM KNO_3 and 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ or 20 mM KNO_3 and 5 mM glutamine, the medium pH decreased to a value of about 4 and finally rose to 6.5, while it decreased to a value of about 4 but did not recover until the end of the culture in the case of Koshihikari. However, the patterns of changes in the medium pH in the two varieties were similar in the medium containing 20 mM KNO_3 and 5 mM alanine : the medium pH decreased to a value of about 5 and afterwards recovered to about 6.5. The calluses of Nipponbare and Koshihikari obtained in the medium containing 20 mM KNO_3 , 5 mM alanine and 0.375 % sucrose displayed high regeneration frequencies of 36.0 % and 26.0 %, respectively. Our results indicated that varietal differences in rice cell culture depended on the kind of reduced nitrogen source and sucrose concentration in the culture medium.

Key Words: *Oryza sativa*, cell culture, nitrogen source,

amino acid, varietal difference, pH change

Introduction

Varietal differences, which are one of the major issues in rice cell culture, limit the application of biotechnology via cell culture to rice breeding. Koshihikari is a typical variety that can not be easily handled in cell culture. The callus of this variety turns brown during the culture period and shows poor growth and a low frequency of plant regeneration.

In this study, the effect of reduced nitrogen sources at concentrations of 3 % or 0.375 % sucrose in the culture medium on the growth rate of calluses derived from mature embryos was compared among five varieties including Koshihikari.

Materials and Methods

Hulled mature seeds of Nipponbare (Japonica variety), Norin No. 1 (Japonica variety), Akenohoshi (a variety derived from Japonica-Indica crossing), Koshihikari (Japonica variety) and BG26 (Javanica variety) were surface-sterilized in a 10 % sodium hypochlorite solution (available chlorine concentration of 1 %) for 30 minutes with vigorous shaking, followed by rinsing in sterilized water three times. After rinsing, the sterilized seeds were immersed in sterilized water for 6 to 7 hours. Embryos were cut off from the imbibed seeds and were placed with the scutellum pointing upwards on induction media which contained R2 salts (Ohira *et al.* 1973), B5 vitamins (Gamborg *et al.* 1968), 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 30g/l sucrose and 8 g/l agar (pH 5.8). Seven-day-old calluses were transferred to 20 ml of liquid proliferation media which consisted of nitrogen sources with various compositions, 1 mM calcium chloride (CaCl_2), 1 mM magnesium sulfate (MgSO_4), 2 mM monopotassium phosphate (KH_2PO_4), R2 micro-salts, B5 vitamins, 1 mg/l 2,4-D and 3.75 g/l or 30 g/l sucrose (pH 5.8). Four compositions of nitrogen sources were tested in this study : 20 mM KNO_3 , 20 mM KNO_3 and 2.5 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM KNO_3 and 5 mM glutamine, 20 mM KNO_3 and 5 mM alanine. The calluses were cultured in 100ml-flasks on a gyratory shaker at 100 rpm, with subculturing every 7 days. For subculturing, about 0.4 ml cell volume of calluses were

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transferred to 20 ml of a fresh medium with the same composition in 100ml-flasks. One cell line was derived from a single embryo in each of 8 different media. Growth rate was determined using calluses (200-250 mg fresh weight) maintained for more than 49 days as materials. At the beginning and end of the culture for 7 days, the calluses were collected and the fresh weight of the calluses was determined after extra-amounts of media around calluses were removed using pasteur pipettes, and then growth rate, the ratio of fresh weight of calluses at the end of 7-day period of culture to that of calluses at the beginning of culture, were calculated. The determination of the callus growth rate in each cell line was carried out three times.

Effects of reduced nitrogen source on pH change in the medium with 0.375 % sucrose were examined using the calluses of Nipponbare and Koshihikari that were maintained in the medium containing 20 mM KNO₃, 5 mM alanine, 1 mM CaCl₂, 1 mM MgSO₄, 2 mM KH₂PO₄, R2 micro-salts, B5 vitamins, 1 mg/l 2,4-D and 3.75 g/l sucrose (pH 5.8). These calluses were transferred to 4 different kinds of liquid media containing nitrogen sources varying in composition, as well as 1 mM CaCl₂, 1 mM MgSO₄, 2 mM KH₂PO₄, R2 micro-salts, B5 vitamins, 1 mg/l 2,4-D and 3.75 g/l sucrose (pH 5.8). Composition of the nitrogen sources used in this experiment and culture conditions were the same as those described in the previous experiment. Changes in pH of the liquid media were monitored by determining the pH of 0.1 ml of aliquots of the media. Sample aliquots were collected every day after transfer and stored at -20 °C until measurement. The pH of the aliquots was measured with a Horiba Twin pH B-112.

For plant regeneration, the subcultured calluses of Nipponbare and Koshihikari were transferred to solid proliferation media consisting of 20 mM KNO₃, 5 mM alanine, 1 mM CaCl₂, 1 mM MgSO₄, 2 mM KH₂PO₄, R2 micro-salts, B5 vitamins, 1 mg/l 2,4-D, 30 g/l maltose and 8 g/l agar (pH 5.8) and lastly to regeneration media consisting of 20 mM KNO₃, 5 mM alanine, 1 mM CaCl₂, 1 mM MgSO₄, 2 mM KH₂PO₄, R2 micro-salts, B5 vitamins, 0 or 1 mg/l benzyl adenine (BA), 30 g/l maltose and 8 g/l agar (pH 5.8) 7 days after the transfer to the proliferation media.

Results

Fig. 1 and 2 show the effects of reduced nitrogen sources on callus proliferation in liquid media containing 3 % sucrose and 0.375 % sucrose, respectively. In these experiments, 3 different sources of reduced nitrogen, 2.5 mM (NH₄)₂SO₄, 5 mM glutamine and 5 mM alanine, were examined in combination with 20 mM KNO₃. At the concentration of 3 % sucrose (Fig. 1), the Koshihikari and Norin No. 1 calluses showed lower growth rates in the media containing 20 mM KNO₃ as the sole nitrogen source than the Nipponbare and Akenohoshi calluses. The BG26 calluses showed the lowest

Fig. 1. Effect of reduced nitrogen sources on callus growth rate in media with 3 % sucrose.

Callus growth rate shows the ratio of fresh weight of calluses at the end of 7-day period of culture to that of calluses at the beginning of culture. Fresh weight of initial inoculum is 200-250 mg. Bars show the mean values in three replications.

Fig. 2. Effect of reduced nitrogen sources on callus growth rate in media with 0.375 % sucrose.

Callus growth rate shows the ratio of fresh weight of calluses at the end of 7-day period of culture to that of calluses at the beginning of culture. Fresh weight of initial inoculum is 200-250 mg. Bars show the mean values in three replications.

growth rate in this medium among the calluses from the five varieties. In the media containing 20 mM KNO₃ and 2.5 mM (NH₄)₂SO₄ or 20 mM KNO₃ and 5 mM glutamine, the Koshihikari and Norin No. 1 calluses showed lower growth rates than the calluses from other varieties. The growth rates of the calluses of Koshihikari and Norin No. 1 became identical with those of the Nipponbare calluses in the medium containing 20 mM KNO₃ and 5 mM alanine, but were still lower than those of the Akenohoshi and BG26 calluses.

At the concentration of 0.375 % sucrose (Fig. 2), the growth rates of the Koshihikari and Norin No. 1 calluses were lower than those of the calluses from other varieties in the media containing 20 mM KNO₃, 20 mM KNO₃ and 2.5 mM (NH₄)₂SO₄ or 20 mM KNO₃ and 5 mM glutamine, though there were smaller differences in the growth rates among the five varieties at the concentration of 0.375 % sucrose than at the concentration of 3 % sucrose. In contrast, the growth rates of the Koshihikari and Norin No. 1 calluses became almost identical

with those of the calluses from other varieties in the medium containing 20 mM KNO_3 and 5 mM alanine.

The calluses of Nipponbare and Koshihikari, which were maintained in the medium containing 20 mM KNO_3 , 5 mM alanine and 0.375 % sucrose, were transferred to the media with different compositions of nitrogen sources at the concentration of 0.375 % sucrose and then the changes in the medium pH were monitored during the 7-day period of culture. The patterns of changes in the medium pH were reproduced in two replications. The results are shown in Fig. 3. In the medium containing KNO_3 as sole nitrogen source, the medium pH rose to a value of about 7 and then decreased in both varieties. In the medium containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$, the patterns of the changes in the medium pH were different between the two varieties. In Nipponbare, the medium pH rapidly decreased to a value of about 4, rose after the fourth day and the value exceeded 6 at the end of the 7-day period of culture. On the other hand, in Koshihikari, the medium pH initially decreased to a value of about 4 as in the case of Nipponbare but afterwards did not rise. The patterns in the medium containing KNO_3 and glutamine as nitrogen sources were similar to those in the medium containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ except for a rise on the seventh day in the case of Koshihikari. In the medium containing KNO_3 and alanine, the patterns of the two varieties were similar. The medium pH decreased to a value of about 5 by the third day and afterwards gradually rose to about 6.5 until the end of the culture.

Fig. 4 shows the extent of browning of the Koshihikari calluses during liquid culture in the media. The Nipponbare and Koshihikari calluses cultured in the medium containing 20 mM KNO_3 as the sole nitrogen source formed adventitious roots even in the presence of 2,4-D at concentrations of 3 % or 0.375 % sucrose, though they did not turn brown (data not shown). The Koshihikari calluses turned brown in the medium containing 20 mM KNO_3 , 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ and 3 % sucrose, while the Nipponbare calluses remained yellowish

white. On the other hand, the Koshihikari calluses remained healthy and yellowish white in the medium containing 20 mM KNO_3 , 5 mM alanine and 0.375 % sucrose as in the case of the Nipponbare calluses.

Regeneration ability of the Koshihikari calluses, which were maintained in the liquid medium containing 20 mM KNO_3 , 5 mM alanine and 0.375 % sucrose, was compared with that of the Nipponbare calluses using two regeneration media (Table 1). The medium containing 1 mg/l BA gave a higher frequency of regeneration for both varieties than the medium lacking BA. The regeneration ability of the Koshihikari calluses was as high as that of the Nipponbare calluses, *i. e.* 26.0 % and 36.0 %, respectively, in the regeneration media containing 1 mg/l BA.

Discussion

Ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$ is most commonly used as a reduced nitrogen source for preparing a basal medium in tissue and cell culture, because the addition of an ammonium ion in combination with nitrate generally enhances the growth rate of cultured cells (Ohira *et al.* 1973, Gamborg *et al.* 1968). However, a deleterious effect of ammonium ion has been reported in rice tissue and cell culture. Chu *et al.* (1985) and Siva Reddy *et al.* (1985) reported that the decrease in the concentration of ammonium ion in the solidified medium promoted the formation, growth and regeneration of pollen calluses of Japonica rice and Indica rice, respectively. Daigen and Abe (1993) indicated that dilution of all inorganic salts was effective in enhancing the growth rate of the Koshihikari calluses in liquid cell culture. Iwai *et al.* (1990) reported that $(\text{NH}_4)_2\text{SO}_4$ in the medium induced the browning of Koshihikari calluses in anther culture. We also observed that the addition of $(\text{NH}_4)_2\text{SO}_4$ led to poor growth of the Koshihikari and Norin No. 1 calluses. However, our results indicated that the growth rates of the Koshihikari and Norin No. 1 calluses were still lower in the medium lacking $(\text{NH}_4)_2\text{SO}_4$ even at the

Fig. 3. Effect of nitrogen source composition in the media on pH changes during the culture period.

● 20 mM KNO_3 ▲ 20 mM KNO_3 + 5 mM Glu
 ■ 20 mM KNO_3 + 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ ○ 20 mM KNO_3 + 5 mM Ala
 All four media contain 0.375 % sucrose as carbon source.

Fig. 4. Effect of changes in medium composition on callus proliferation in Nipponbare and Koshihikari. (A) and (C): Nipponbare, (B) and (D): Koshihikari. Media in (A) and (B) contain 20 mM KNO₃, 2.5 mM (NH₄)₂SO₄ and 3 % sucrose, while media in (C) and (D) contain 20 mM KNO₃, 5 mM alanine and 0.375 % sucrose. The photographs of the calluses were taken 49 days after callus initiation.

Table 1. Effect of benzyl adenine (BA) application on plant regeneration in Nipponbare and Koshihikari

Variety	BA (mg/l)	Exp. 1		Exp. 2		Total		(%)
		No. of calluses tested	No. of calluses regenerated	No. of calluses tested	No. of calluses regenerated	No. of calluses tested	No. of calluses regenerated	
Nipponbare	0	30	4	20	2	50	6	(12.0)
	1	30	8	20	10	50	18	(36.0)
Koshihikari	0	30	0	20	2	50	2	(4.0)
	1	30	6	20	7	50	13	(26.0)

Note: 124 - and 131 -day-old calluses were tested in experiments 1 and 2, respectively.

concentration of 0.375 % sucrose than those of the Nipponbare calluses in spite of the absence of browning. Abe (1992) reported that in anther culture the frequencies of callus formation and plant regeneration from pollens of Koshihikari were as high as those of Nipponbare, when (NH₄)₂SO₄ was removed from the induction medium containing casein hydrolysate, a mixture of va-

rious free amino acids and peptides (Rose *et al.* 1975). Our results indicated that the Koshihikari and Norin No. 1 calluses showed approximately the same growth rates as the calluses from other varieties, when (NH₄)₂SO₄ was replaced by alanine at the concentration of 0.375 % sucrose. We identified the single nitrogenous constituent that promotes the growth of the

Koshihikari and Norin No. 1 calluses in combination with KNO_3 in the liquid cell culture in the current study.

Changes in medium pH are known to be related to the balance between ammonium and nitrate utilizations during the period of culture (Rose and Martin, 1975). In cell suspension culture of rice, Ohira *et al.* (1976) monitored the pH changes during the culture period in the medium containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source. They reported that in the initial phase ammonium was selectively absorbed and at the same time the pH rapidly decreased to a value of about 4 and that in the second phase nitrate was utilized and the pH rose from a value of about 4 to about 7. We also observed similar changes in the medium pH when the Nipponbare calluses were cultured in the media containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 and glutamine as nitrogen sources. However, such pH changes did not occur when the Koshihikari calluses were cultured in the same media. Namely, after the initial decrease to a value of about 4, the medium pH did not recover and remained at a low level. On the other hand, when alanine was added to the medium as reduced nitrogen source, the pattern of the pH change in Koshihikari was similar to that in Nipponbare: the medium pH decreased more slowly to a value of about 5 and afterwards the pH gradually increased. It is considered that the medium pH decreased as ammonium ions were taken up by cells and increased as nitrate was taken up by cells (Kirkby and Mengel, 1967). Our results suggested that 1) since the Koshihikari calluses showed a lower ability to utilize nitrate under a pH value of about 4 than the Nipponbare calluses, the medium pH did not increase in the second phase when the Koshihikari calluses were cultured in the medium containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 and glutamine, 2) since the Koshihikari calluses could utilize nitrate at a pH value of about 5, they continued to grow in the medium containing KNO_3 and alanine as in the case of the Nipponbare calluses and accordingly 3) alanine was a reduced nitrogen source more suitable for the calluses from five varieties because the medium containing KNO_3 and alanine could maintain the medium pH at relatively higher level during the period of culture than the media containing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 and glutamine. Further studies on the assimilation and consumption of nitrogen sources should be carried out in cultured cells of rice.

The toxic effect of sucrose was observed in barley microspore culture by Scott and Lyne (1994). They reported that none of the microspores that were incubated at a concentration of 40 mM sucrose (1.36 %) for 24 hours survived within 5 days. Ghosh Biswas and Zapata (1993) reported that maltose was superior to sucrose as a carbon source for callus growth and they observed the regeneration of plants from protoplast-derived calluses of Indica rice. Our results also showed that higher concentrations of sucrose exerted a toxic effect on the growth of calluses of Koshihikari and Norin No. 1 in the

medium containing $(\text{NH}_4)_2\text{SO}_4$ or glutamine. On the other hand, the growth rates of the calluses from the other three varieties decreased as the sucrose concentration decreased from 3 % to 0.375 %, as described by Ohira *et al.* (1973). The response to sucrose concentrations appeared to depend on the genotype. A similar response to sucrose concentration was observed in the medium containing alanine, though the effect of the sucrose concentration was not distinct in Nipponbare and Norin No. 1. It is considered that varietal differences in the growth of the calluses were attenuated at the concentration of 0.375 % sucrose based on the differences in the response to sucrose concentrations.

We developed a method for the culture of embryo-derived calluses in the Japonica varieties Koshihikari and Norin No. 1, which hitherto could not be easily handled in cell culture, as well as for Nipponbare. Further studies are under way to expand the applicable range of varieties, including Indica varieties.

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