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In Vitro Propagation of Hybrid Rice (*Oryza sativa* L.)

2. Vertical, rotatory liquid culture of multiple shoots and field performance

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Abstract
A vertical, rotatory liquid culture system was developed for the mass-production of hybrid rice (Oryza sativa L.) by multiplying axillary shoots. Multiple shoots were induced by a combination of cytokinin (10-5 or 10-4 M 6-benzalaminopurine (BAP) or kinetin), which inhibits root growth and promotes tillering, and auxin (10-6 M 2,4-dicilorophenoxyacetic acid (2,4-D) or 10-5 M ?-naphthaleneacetic acid (NAA)), which inhibits the growth of shoot and root. The yield of the seedlings grown from the multiple shoots was very similar to that of seedlings grown from seeds, expect when the multiple shoots were treated with 10-4 M BAP. Treatment of multiple shoots with 10-4 M BAP resulted in a decrease of the harvest index and yield. Seedlings derived from multiple shoots that were maintained for a long period of time showed very similar yield to those from seeds when the multiple shoots were treated with 10-6 M kinetin.

Discipline: Biotechnology
Additional key words: cultured seedling, micropropagation, tissue culture

Introduction

Attempts have been made to apply tissue culture techniques for the mass-production of rice (*Oryza sativa* L.) plant. The process for producing artificial seeds from adventitious shoots[8], somatic embryos[7] and shoot primordia[9,10] and the process for producing cultured seedlings on a large scale from shoot primordia and multiple shoots[4,5,12] are considered to be applicable to micro-propagation. Though it had been suggested in the previous paper[13] that the former system could be applied, the production of artificial seeds is not necessarily required, at least in the case of rice, because rice transplanting techniques have already been established in Japan. Therefore, large scale seedling production may be more suitable. Particularly, multiple axillary shoot culture system is a relatively simple process and genetic stability can be obtained. However, some problems should be solved for the use of multiple shoots, such as the very low growth rate and difficulty in handling the materials in agar culture[2]. In this paper, the development of a new method of culture of multiple shoots was attempted using hybrid rice, in order to solve these problems. The characteristics of rice plants derived from multiple shoots were also described.

Materials and methods

1) Preparation of multiple shoots

Six F_1 combinations (msAkihikari/H87-36, msAkihikari/H87-37, msNekken 2/Milyang 23, msNekken 2/H87-50, msNekken 2/H87-53 (Kanto Kou 1) and msNekken 2/H87-56) and IR 8 were

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tested. MS[6] or N_6 modified medium[1,13] with 10^{-5} or 10^{-4} M cytokinin (6-benzylaminopurine (BAP) or kinetin) was used. In some of the experiments, 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) or 10^{-5} M α-naphthaleneacetic acid (NAA) was added to the medium. One seed or mature embryo of Kanto Kou 1 was immersed in 12 ml medium in a test tube (25 mm in diameter, 200 mm in height) which was then cultured on a gyratory shaker at a rotating speed of 2 rpm. The temperature was 25°C and the illumination ranged from 2 $\mu Em^{-2}s^{-1}$ (bottom of the rotator) to 60 $\mu Em^{-2}s^{-1}$ (top of the rotator) using cool white fluorescent tubes. Multiplication was carried out under the same conditions of medium and environment as those for the initiation of multiple shoots.

2) Field test of multiple shoots

Multiple shoots of Kanto Kou 1 subjected to short culture and prolonged culture were tested for yield in the field. Multiple shoots cultured for a short period of time were initiated from the culture of a mature embryo in 24 ml MS medium in a test tube (30 mm in diameter, 200 mm in height) under the same conditions as those described in the section "preparation of multiple shoots". The culture started in March 3, 1990. The materials were maintained at intervals of 10 days for multiplication. Multiple

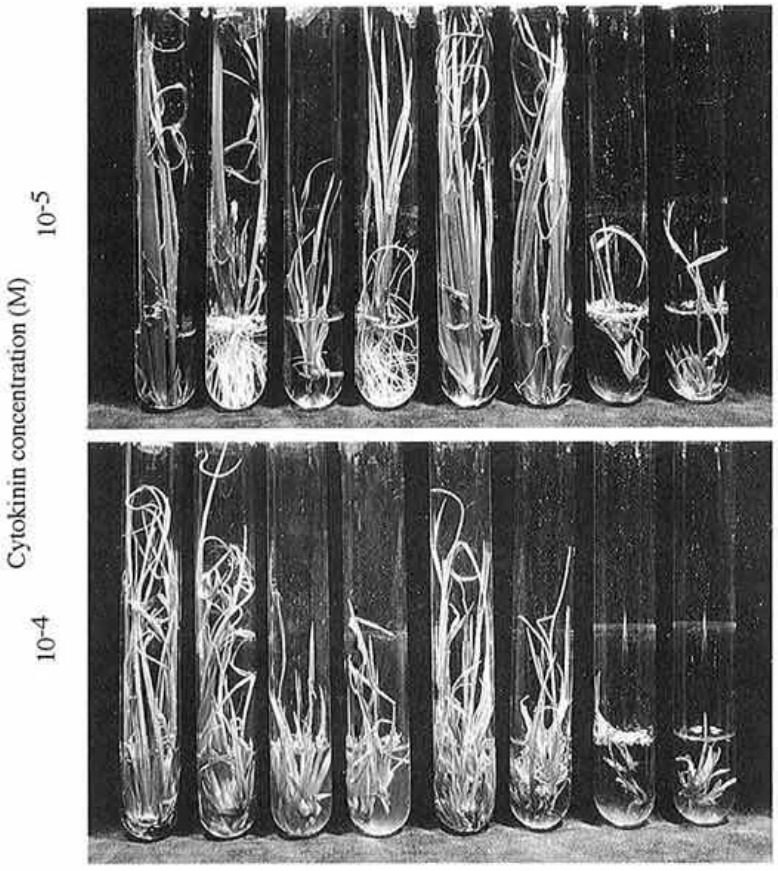

Plate 1. Effects of plant part (seed or embryo), medium (MS or N_6 modified) and hormone (10^{-5} or 10^{-4} M BAP or kinetin) on multiple shoot formation in IR 8 after 1 month of culture
Multiple shoots were initiated and cultured in 12 ml medium in a test tube (25 mm in diameter) using a vertical, rotatory culture (2 rpm) system at 25°C under an illumination of 60 $\mu Em^{-2}s^{-1}$.

shoots in prolonged culture were subjected to stationary culture using N_6 modified agar medium (0.5×10^{-4} M BAP, 3% sucrose, 0.8% agar) in a culture bottle (6 cm in diameter, 12 cm in height) at 25°C under illumination of 2 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent lights. The culture started on July 8, 1988. The multiple shoots in prolonged culture were maintained without subculture except once each in October and December 1988. They were cultured in the same manner as the multiple shoots in short culture after March 1990.

Multiple shoots were cut into pieces on May 12, 1990. Individual shoots (cultured seedlings) were grown on soil for rooting in a greenhouse. Ordinary seedlings were grown from seeds sowed in a nursery bed on May 7. The seedlings were transplanted at the planting density of 30 cm (row distance) × 20 cm (hill distance) in a field on June 7. One cultured seedling/hill or two ordinary seedlings/hill were planted. Each of the three fertilizer elements (N, P_2O_5 and K_2O) was applied at the rate of 0.5 kg/a as basal dressing, followed by 0.3 kg/a at the panicle formation stage and 0.2 kg/a at the ripening stage. Two central rows among four in a block were investigated. Other cultivation conditions and survey procedures followed standard methods.

Results and discussion

1) Effects of plant part, medium and hormone on multiple shoot formation

Plate 1 shows the effects of plant part (seed or embryo), medium (MS or N_6 modified) and hormone (10^{-5} or 10^{-4} M BAP or kinetin) on multiple shoot formation in IR 8 after 1 month of culture. In general, tillering began at a level of 10^{-6} M cytokinin, and became profuse, resulting in the formation of multiple shoots as the concentration increased. Shoot growth (elongation) was arrested at 10^{-4} M, where many shoots were formed, but frequently died, particularly when they developed from mature embryos. Shoots formed tended to become brown, frequently died at 10^{-4} M BAP, whereas browning was not significant at 10^{-4} M kinetin. Seeds, which were affected by endosperm, required a higher level of cytokinin than mature embryos for the induction of multiple shoots. Multiple shoots were formed and multiplied more readily in MS medium than in N_6 modified medium. *Indica* varieties were more sensitive to low concentrations of cytokinin than *japonica* varieties. Intense illumination promoted the formation of multiple shoots. Root development was inhibited at 10^{-5} M and 10^{-4} M cytokinin, except when seeds were cultured in a medium with 10^{-5} M kinetin.

2) Multiplication of axillary shoots

Multiple shoots developed with each of the six combinations tested through the multiplication of axillary shoots (Plate 2). Tillering was observed after 2 weeks of culture, and the number of shoots increased by 3.38–6.32 times, 4.95 times on an average, every 10 days at 10^{-5} M cytokinin in MS medium (interval for subculture: 10 days). Theoretically, 4.38×10^{12} ($4.95^{182/10}$) seedlings could be produced from a single shoot within 6 months by the application of the rotatory liquid culture of multiple shoots.

Multiple shoots shorter than 1 cm developed in the multiple shoot formation medium after the addition of a narrow range of auxin concentrations (Plate 3). In this case, adventitious shoots were sometimes initiated.

Multiple shoots of Kanto Kou 1 on N_6 modified agar medium with 0.5×10^{-4} M BAP could be maintained for more than 20 months. Multiple shoots were not subcultured during 15 months, but they

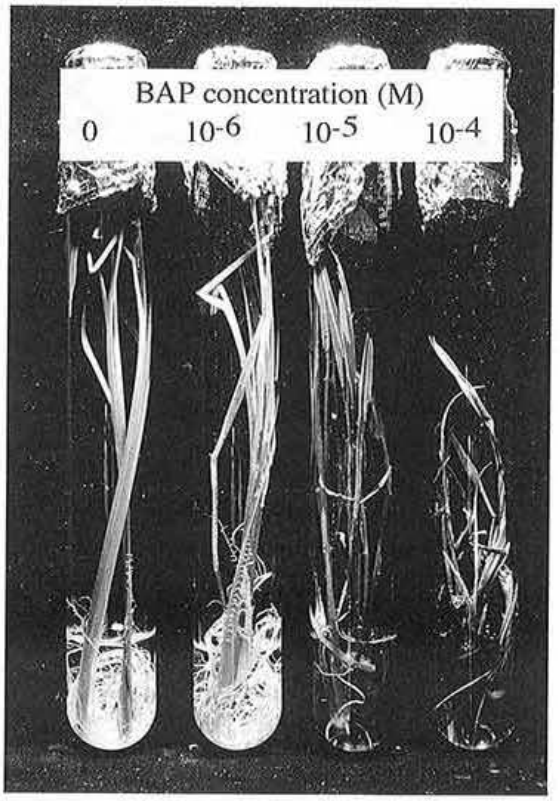

Plate 2. Effect of BAP on multiple shoot formation in N_6 modified media
Seeds of msNekken 2/H87–50 were used.

Plate 3. Multiple shoots shorter than 1 cm developed in N_6 modified medium with 10^{-4} M kinetin and 10^{-6} M 2,4-D
Seeds of msNekken 2/H87-36 were used.
Bar indicates 5 mm.

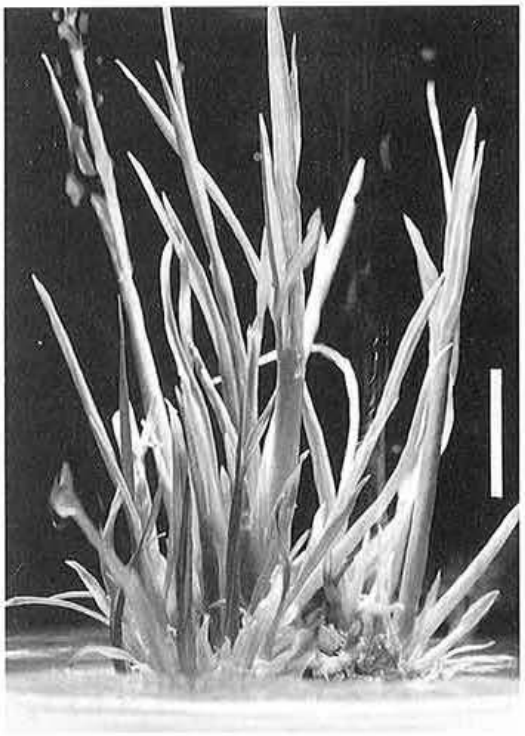

Plate 4. Multiple shoots from seed of Kanto Kou 1 on N_6 modified agar medium with 0.5×10^{-4} M BAP after 3 months of culture
They could be maintained for more than 20 months.
Bar indicates 1 cm.

were alive, though growth was arrested (Plate 4).

3) Characteristics of plants grown from multiple shoots

Although rooting of multiple shoots is often considered to be difficult in other species, the multiple shoots of rice planted in soil easily developed roots and grew (Plate 5). However, leaf withering occurred due to the delay in rooting and transpiration, and the initial growth was markedly delayed. Table 1 shows the growth and yield components of Kanto Kou 1 grown from cultured seedlings and ordinary seedlings. Compared with plants grown from seeds, the plants from multiple shoots showed a delay in heading dates (September 10-19 as compared with those of ordinary seedlings: September 8), decrease in panicle number, and increase in panicle length. The husked grain yield (58.4-71.0 kg/a) of seedlings grown from multiple shoots cultured with 10^{-5} or 10^{-4} M kinetin or 10^{-5} M BAP was very similar to that (62.8 kg/a, 65.5 kg/a) of ordinary seedlings. On the contrary, the use of 10^{-4} M BAP resulted in a lower harvest index (0.31, 0.36 compared with the values obtained in ordinary seedlings: 0.42, 0.44) and a decrease in yield (52.9, 56.7 kg/a). The yield of the seedlings grown from multiple shoots subjected to prolonged culture and treated with 10^{-5} M kinetin was very similar (64.8 kg/a) to that of seedlings grown from multiple shoots cultured for a short period of time.

The use of 10^{-4} M BAP resulted in a reduced yield compared with the use of 10^{-5} M BAP or 10^{-5} or 10^{-4} M kinetin, presumably because the former suppressed rooting and delayed the initial growth. However, genetic variations due to BAP could not be ruled out. In fact, abnormal multiple shoots caused by the application of 5 mg/l BAP have been reported in wheat[11]. Seedlings subjected to prolonged culture and treated with kinetin did not display a decrease in yield. Therefore, multiple shoots can be maintained for a long period of time by this method.

The seedlings derived from shoot primordia headed earlier than the control[13], unlike those derived from multiple shoots. The delay in growth may be due to transplanting injury, because the materials were directly converted to seedlings without aseptic rooting treatment.

Plate 5. Plants grown from multiple shoots (A) and seeds (B) of Kanto Kou 1
Multiple shoots were cultured for a short period of time in the medium with 10^{-5} M BAP.

Table 1. Growth and yield components of Kanto Kou 1 grown from cultured seedlings and ordinary seedlings

	K.C.[a]	C.C. (M)	F.C. (Y.M)[b]	H.A. (m^2)	C.L. (cm)	P.L. (cm)	P.N. (/hill)	D.W. (kg/a)	U.G. (kg/a)	H.G. (kg/a)	H.I.	Q.G.
Ordinary seedlings				2.52	86.0	22.0	18.0	143.3	79.3	62.8	0.44	5
				2.52	83.9	23.2	16.6	155.0	82.4	65.5	0.42	5
Cultured seedlings	Kinetin	10^{-5}	90.3	1.32	83.2	24.7	15.8	146.3	74.5	58.4	0.40	5
	Kinetin	10^{-5}	88.10	1.20	81.8	24.6	15.6	151.7	82.9	64.8	0.43	5
	Kinetin	10^{-4}	90.3	1.44	86.8	25.4	15.9	161.7	90.1	71.0	0.44	5
	BAP	10^{-5}	90.3	1.56	87.7	26.6	14.9	150.0	82.6	64.5	0.43	5
	BAP	10^{-4}	90.3	2.28	86.0	24.5	15.4	157.5	71.8	56.7	0.36	5
	BAP	10^{-4}	88.10	0.92	86.9	25.1	16.3	170.0	69.9	52.9	0.31	4

a): K.C.; Kind of cytokinin in the culture medium, C.C.; Concentration of cytokinin, F.C.; First day of culture, H.A.; Harvested area, C.L.; Culm length, P.L.; Panicle length, P.N.; Panicle number, D.W.; Total dry weight, U.G.; Unhusked grain yield, H.G.; Husked grain yield, H.I.; Harvest index (H.G./D.W.), Q.G.; Quality of grain (5, good; 1, poor).
b): Y.M; Year month.

4) Prospects for use of multiple shoot culture system

The multiplication rate in this culture system was considerably higher than the 3 times/4 weeks values reported by Hisajima et al.[4], 5 times/3–4 weeks values reported by Kumari et al.[5] and 25 times (maximum)/1 month values reported by Greco et al.[3]. It is thus considered that the problem of multiplication rate of multiple shoots, which was a major constraint, had been solved.

The handling of multiple shoots, which was considered to be another shortcoming, became easier by suppressing shoot growth with a narrow range of concentrations of auxin that does not allow the induction of callus or adventitious shoots. However, manual division of stocks is still required. Further studies on cost reduction, such as robotization, are necessary.

Possibility of mass-production of hybrid rice by the multiplication of axillary shoots has thus been demonstrated. This technique could be applied to hybrid rice as well as to other rice varieties.

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