

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

### 1. "Tissue-cultured" shoot primordial

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#### Abstract

An in vitro propagation system was developed for mass-production of hybrid rice by the multiplication of shoot primordial subjected to tissue culture (hereafter referred to as "tissue-cultured" shoot primordial). Shoot primordial were generated and maintained from mature embryos of hybrid rice (*Oryza sativa* L.). N6 modified medium (hormone:  $10^{-6}$  M 2,4-dichlorophenoxyacetic acid (2,4-D) and  $10^{-5}$  M cytokinin) was used. Shoot primordia maintained a high level of regeneration ability for more than one year. White bodies were formed synchronously from shoot primordia by keeping them in stationary culture at high temperature (30–35°C) in the dark. When shoot primordia and white bodies encapsulated in calcium alginate gel containing N6 modified media without hormones were sowed in soil, they germinated but did not grow. However they germinated and grew on a sterilized medium and ripened when transplanted to pots. Compared with plants grown from seeds, the majority of the plants derived from shoot primordia, which headed earlier, showed a shorter plant height, lighter grain weight and 1000 grain weight, and the percentage of fertility of some individuals was lower.

**Discipline:** Biotechnology

**Additional key words:** artificial seed, cultured seedling, micropropagation, tissue culture

#### Introduction

It is generally recognized that the yield of hybrid rice (*Oryza sativa* L.) cultivars is more than 20% higher than that of pure-bred cultivars. The former cultivars are planted over one-third of the total rice-cultivated area in China. However, since seed production is laborious, hybrid rice cultivars are not used in commercial farming in Japan<sup>7)</sup>. Various studies have been carried out to address this problem, including mass-production of F<sub>1</sub> seedlings by tissue culture. A large number of culture methods can be applied to the mass-production of rice: adventitious shoot method<sup>10)</sup>, somatic embryo method<sup>9)</sup>, shoot primordia method<sup>11,12)</sup>, and multiple shoot method<sup>4,6,14)</sup>. It was pointed out that adventitious shoot method and somatic embryo method may result in genetic variation of regenerated plants, because

adventitious shoots and somatic embryos are usually regenerated from callus cultures with genetic variation. On the other hand, shoot primordia and multiple shoots are generally considered to show a high level of genetic stability when they are not derived from callus. However, in the case of multiple shoots, the use of agar culture may result in slow multiplication<sup>4)</sup> and the culture system may be inefficient<sup>3)</sup>.

"Shoot primordia" consist of aggregates of dome-shaped tissues formed during culture under bright visible light and shifting gravitational orientation, generally initiated from a shoot apex<sup>11,12)</sup>. Shoot primordia obtained by the multiplication of shoot meristematic tissues display a high genetic stability and regeneration ability<sup>13)</sup>. To avoid confusion in the use of the term shoot primordium in *in vitro* propagation with that in plant morphology, shoot primordia in propagation have been referred as

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"tissue-cultured" shoot primordia by Tanaka et al.<sup>13)</sup>.

One way to commercialize multiplication by tissue culture (*in vitro* propagation, micro-propagation) is to convert cultured products to artificial seeds; another approach is to mass-produce seedlings directly from cultured products (mass-production of cultured seedlings). Research has been initiated for the preparation of capsules of carrot (*Daucus carota*) from somatic embryos<sup>5)</sup>. Capsules of barley (*Hordeum vulgare*), a grain, have been produced from somatic embryos derived directly from pollen<sup>2)</sup>. On the other hand, the direct cultivation of cultured seedlings is another promising approach.

This paper reports on the induction and culture of shoot primordia in hybrid rice, synchronization of shoot primordia, encapsulation of shoot primordia and synchronized shoot primordia (white bodies), and the characteristics of rice plants grown from shoot primordia.

## Materials and methods

### 1) Induction and culture of shoot primordia

Six different kinds of *japonica-indica* F<sub>1</sub> hybrids (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) were used. Embryos were isolated from mature seeds with a scalpel, and sterilized with 70% ethanol for 30 s and 1% sodium hypochlorite solution for 15 min, then rinsed four times with sterile distilled water. The culture medium used was N<sub>6</sub> medium<sup>1)</sup> which was modified and contained 0.025 mg/l CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.025 mg/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.25 mg/l NaMoO<sub>4</sub>·2H<sub>2</sub>O, 100 mg/l myo-inositol, and 3% sucrose. The hormones used were 10<sup>-6</sup> M 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin and 10<sup>-5</sup> M 6-benzylaminopurine (BAP) or kinetin as cytokinin. The media used in this study were adjusted to pH 5.8 with KOH and then autoclaved at 121°C for 15 min. One to three pieces of mature embryos were immersed in 12 ml medium in a test tube, 25 mm in diameter, 200 mm in height, and grown using a vertical, rotatory culture system (2 rpm). They were cultured at 25°C under a continuous illumination of 2–4  $\mu\text{Em}^{-2}\text{s}^{-1}$  provided by cool white fluorescent tubes or under a 12 h photoperiod and illumination of 300  $\mu\text{Em}^{-2}\text{s}^{-1}$  provided by metalhalide lamps. The shoot primordia formed were maintained and multiplied under the same set of conditions at intervals of 3 weeks.

### 2) Synchronization of shoot primordia

Shoot primordia of msAkihikari/H87-36, msAkihikari/H87-37, Kanto Kou 1 and msNekken 2/H87-56 were floated on the surface of 12 ml of the same liquid medium as that used for the induction of shoot primordia in a petri dish (9 cm in diameter). They were subjected to stationary culture in the dark at 30–35°C for 7–10 days.

### 3) Preparation of calcium alginate capsules

Shoot primordia or white bodies of msAkihikari/H87-37 and Kanto Kou 1 were isolated and placed on N<sub>6</sub> modified medium containing 1% sodium alginate but lacking hormones. They were then dropped with a pipette into a 50 mM CaCl<sub>2</sub> solution to form calcium alginate capsules. The capsules were tested for the regeneration of plants under non-sterile and sterile conditions. For the non-sterile test, capsules were sowed on soil, submerged in water, and germinated under an illumination of 300  $\mu\text{Em}^{-2}\text{s}^{-1}$  (metalhalide lamps, 12 h light/12 h dark). Capsule preparation and germination were not carried out under sterile conditions. As for the sterile test, 7–10 capsules were placed on 12 ml of N<sub>6</sub> modified agar medium (3% sucrose, 0.8% agar) without hormones in a petri dish (9 cm in diameter). The culture conditions were 25–27°C, 10–30  $\mu\text{Em}^{-2}\text{s}^{-1}$  (cool white fluorescent lights), and sterility all the way through. When the seedlings reached a 5 cm height, they were transplanted to soil in 1/5,000 a pots and grown in a greenhouse.

### 4) Growth of shoot primordia

Shoot primordia of msNekken 2/H87-50 and Kanto Kou 1 cultured since July 29, 1988 were used. The hormones used were 10<sup>-6</sup> M 2,4-D and 10<sup>-5</sup> M kinetin. Subcultures were maintained under an illumination of 2–4  $\mu\text{Em}^{-2}\text{s}^{-1}$ . The shoot primordia were placed on 12 ml of N<sub>6</sub> modified agar medium without hormones (3% sucrose, 0.8% agar) in a petri dish (9 cm diameter) on April 17, 1989, germinated and rooted at 25°C under an illumination of 10–30  $\mu\text{Em}^{-2}\text{s}^{-1}$ . The seedlings were then transplanted to soil in 1/5,000 a pots (1 seedling per pot) on May 30. They were conditioned to prevent leaf withering by lowering the humidity level gradually. Seeds for control were germinated at 25°C under an illumination of 10–30  $\mu\text{Em}^{-2}\text{s}^{-1}$  on May 24, and the seedlings were transplanted on May 30.

## Results and discussion

### 1) Rice culture system

The explant of rice embedded in a medium reacts differently to different combinations of concentrations and kinds of hormones. The reactions vary depending on the rice variety, the part used, the medium, and illumination conditions. Table 1 shows as an example, the seeds of Te-tep, an *indica* variety, subjected to vertical, rotatory culture in  $N_6$  modified

media under an illumination of  $2 \mu\text{Em}^{-2}\text{s}^{-1}$ . Shoot primordia developed in the medium with  $10^{-6}$  M 2,4-D and  $10^{-5}$  M cytokinin. In the case of *indica* variety seeds, shoot primordia were secondarily formed from the surface of the callus that originally developed at the scutellum. The seeds of *japonica* varieties seldom developed shoot primordia. On the other hand, with a few exceptions, shoot primordia were formed directly from shoot apices when isolated mature embryos were used in both *japonica* and *indica* varieties (Plate 1). They were formed more

Table 1. Effects of 2,4-D and cytokinin (BAP or kinetin) on culture responses of seeds of Te-tep, rice *indica* variety, cultured in  $N_6$  modified medium by vertical rotation at 2 rpm under an illumination of  $2 \mu\text{Em}^{-2}\text{s}^{-1}$

		Cytokinin (M)			
		0	$10^{-6}$	$10^{-5}$	$10^{-4}$
2,4-D (M)	0	P <sup>a)</sup>	P, MS	MS	TMS
	$10^{-6}$	C	C, SP	SP, C	D, SP, TMS
	$10^{-5}$	C	C	D	D

a): P; Plant or shoot, MS; Multiple shoots, TMS; Tiny multiple shoots, SP; Shoot primordia, C; Callus, D; Death.

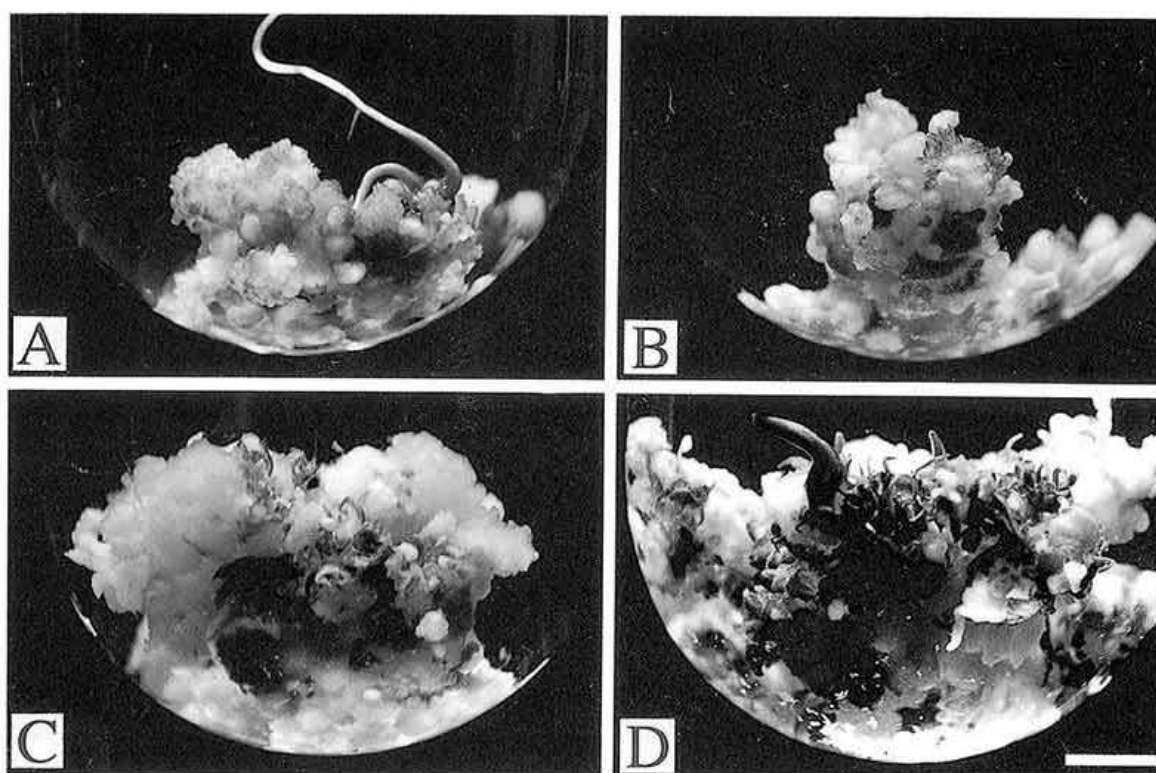


Plate 1. Mass of shoot primordia of IR 8 (A), Kannon-sen (B), Aichi-asahi (C) and Kamenoo (D) initiated from mature embryos in  $N_6$  modified medium with  $10^{-6}$  M 2,4-D and  $10^{-5}$  M kinetin

They were subjected to vertical, rotatory culture (2 rpm) at  $25^\circ\text{C}$  under an illumination of  $2 \mu\text{Em}^{-2}\text{s}^{-1}$ .

Bar indicates 5 mm.



Plate 2. Shoot primordia derived from mature embryo of msNekken 2/Milyang 23 in 6 months' culture  
Bar indicates 5 mm.

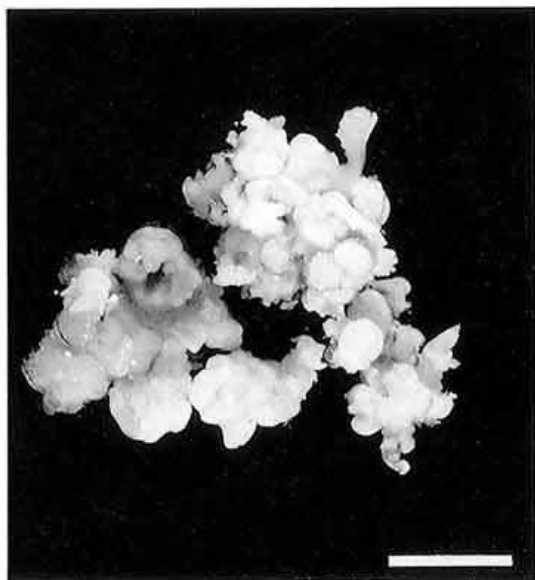


Plate 3. White bodies of msAkihikari/H87-36 induced by culture for 10 days at 30°C, in a dark and stationary environment  
Bar indicates 5 mm.

readily in  $N_6$  medium than in MS medium<sup>8)</sup>. Shoot primordia began to grow when they were cultured under intense illumination.

## 2) Multiplication of shoot primordia

A culture system was established from each of the six  $F_1$  combinations tested (Table 2). Shoot primordia were developed from shoot apices under an illumination of  $2-4 \mu\text{Em}^{-2}\text{s}^{-1}$ . The mass of shoot primordia contained green spots and compact, or friable calli (Plate 2). The systems were also established under an illumination of  $300 \mu\text{Em}^{-2}\text{s}^{-1}$  (12 h

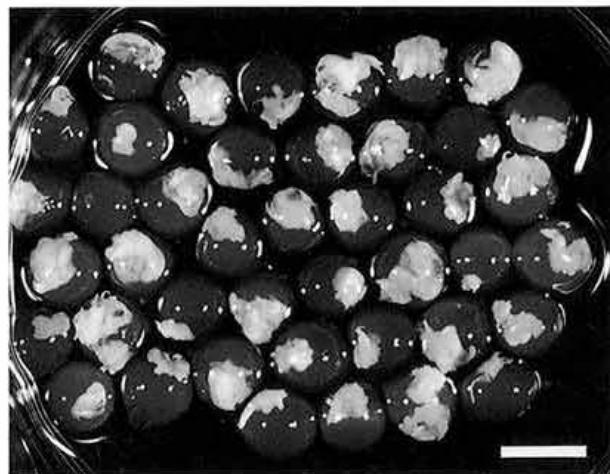


Plate 4. Encapsulated white bodies of msAkihikari/H87-37  
Bar indicates 1 cm.

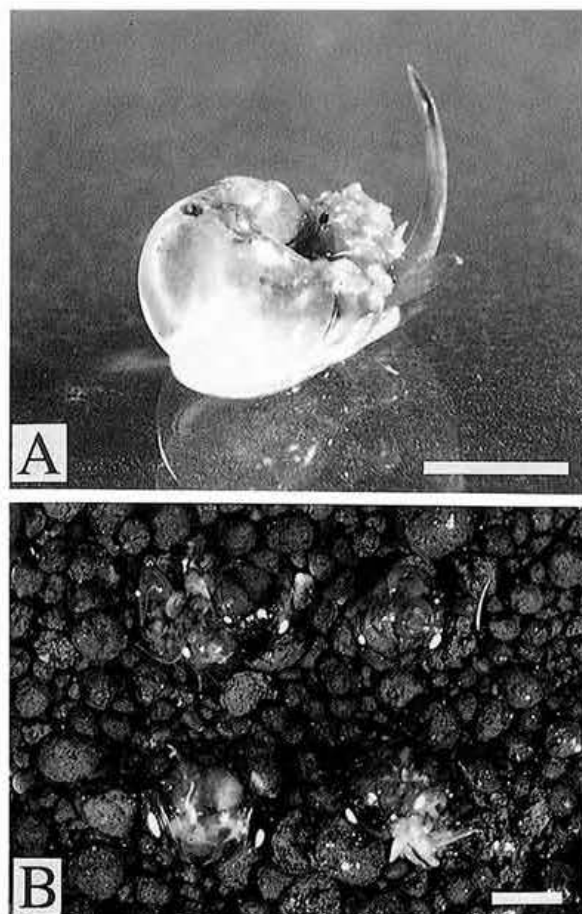


Plate 5. Germination of encapsulated white body (A) and encapsulated shoot primordia (B) of msAkihikari/H87-37 in non-sterile system 20 days after the production  
A, B: Bar indicates 5 mm.



**Table 2.** Culture of hybrid rice, developed and maintained in  $N_6$  modified media with  $10^{-6}$  M 2,4-D and  $10^{-5}$  M BAP or kinetin under illumination of  $2 \mu\text{Em}^{-2}\text{s}^{-1}$  or  $300 \mu\text{Em}^{-2}\text{s}^{-1}$  (12 h light/12 h dark)

	$2 \mu\text{Em}^{-2}\text{s}^{-1}$								$300 \mu\text{Em}^{-2}\text{s}^{-1}$							
	BAP				Kinetin				BAP				Kinetin			
	SP <sup>a)</sup>	MS	S	N	SP	MS	S	N	SP	MS	S	N	SP	MS	S	N
msAkihikari/H87-36	2	0	3	1	1	0	5	0	2	0	2	2	0	5	1	0
msAkihikari/H87-37	3	3	0	0	4	0	2	0	2	0	4	0	3	0	3	0
msNekken 2/Milyang 23	2(1) <sup>b)</sup>	0	2	2	4	0	2	0	2	1	3	0	2	2	2	0
msNekken 2/H87-50	1(1)	0	3	2	3(1)	0	2	1	2	3	1	0	1	1	4	0
msNekken 2/H87-53 (Kanto Kou 1)	1(1)	0	4	1	0	1	3	2	0	3	2	1	3	2	1	0
msNekken 2/H87-56	2	0	2	2	3(1)	0	2	1	0	1	5	0	4	2	0	0

Six mature embryos were tested for each treatment.

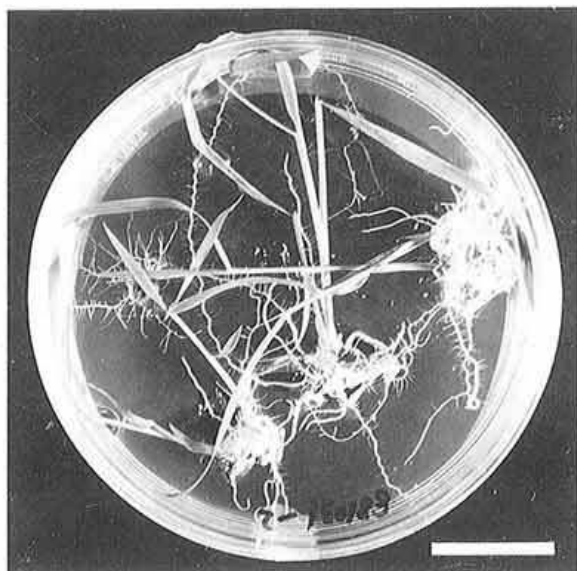
Numerals indicate the number of cultures formed.

a): SP; Shoot primordia, MS; Multiple shoots, S; Shoot, N; No response.

b): ( ); Number of shoot primordia which were maintained for more than 1 year.

light/12 h dark) as in the case of  $2-4 \mu\text{Em}^{-2}\text{s}^{-1}$ , but multiple shoots were often produced (Table 2). Although some intermediate forms were also observed, multiple shoots and shoot primordia seldom developed simultaneously.

The aggregates of shoot primordia weighed 0.75 g after being cultured for 6 weeks at  $25^\circ\text{C}$ , and then the weight tripled every 2 weeks. The number of regenerated plants was proportional to that of shoot primordia in an aggregate, and was 17–24 per gram of randomly selected shoot primordia aggregates. Four of the  $F_1$  combinations maintained a high level of regeneration after 1 year of culture (Table 2).



**Plate 6.** Plantlets under sterile conditions from encapsulated white bodies of msAkihikari/H87-37 25 days after the production  
Bar indicates 2 cm.

### 3) Synchronization of shoot primordia

White bodies were formed with all four  $F_1$  combinations tested. The white bodies were smooth,



**Plate 7.** Adult plants grown from encapsulated white bodies (a) and seeds produced by sexual propagation (b) in msAkihikari/H87-37

Both types of plants were grown under the same environmental conditions.

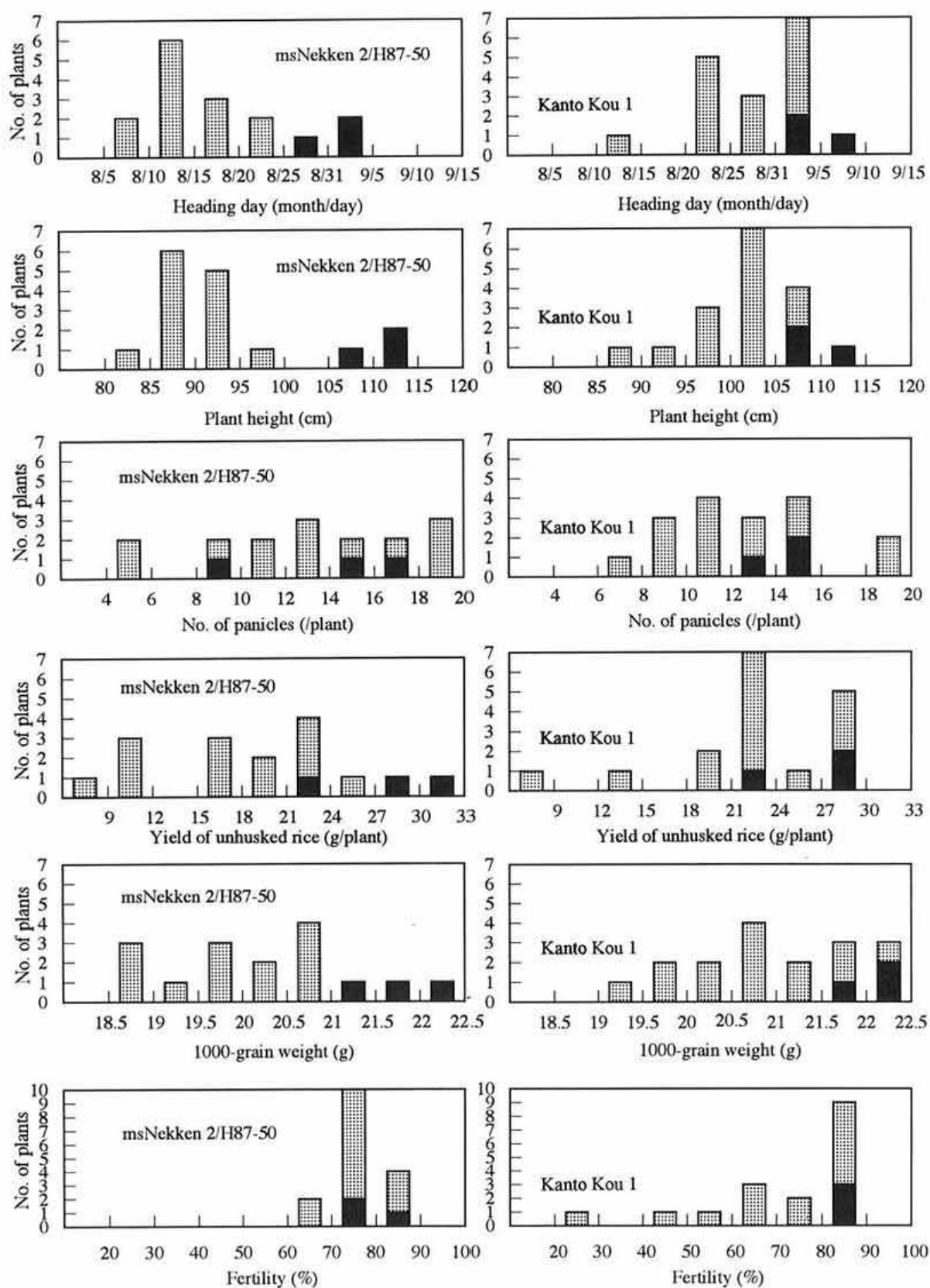


Fig. 1. Distribution of growth parameters of plants grown from shoot primordia (dotted bars) and seed (dark bars) in msNekken 2/H87-50 and Kanto Kou 1

white, and amorphous (Plate 3). One g of the cultures led to about 18 white bodies that were able to regenerate plants. The ability did not decrease after 1 year.

On a theoretical basis, after 6 months (182 days) of culture,  $13.1 \times 10^6$  seedlings could be produced from 1 g of the cultures of shoot primordia ( $3^{172/14} \times 18$ , tripled every 2 weeks, propagation for 172 days and synchronization for 10 days, 18 plants/1 g callus).

Chemical stresses or environmental stresses often lead to the formation of somatic embryos. In the current study, white bodies were formed under environmental stresses: high temperature, dark, and stationary culture. The white bodies were similar to somatic embryos in that they produced shoots and roots nearly simultaneously.

#### 4) Germination of materials in calcium alginate capsules

Growth of both shoot primordia and white bodies ceased at a juvenile stage under non-sterile conditions (Plates 4, 5). The submersion of the capsules prevented detectable contamination under non-sterile conditions. Under sterile conditions, encapsulated materials germinated readily (Plate 6) and those that germinated were all able to grow (Plate 7). There was no difference in the rate of germination and growth between the shoot primordia and white bodies.

#### 5) Characteristics of plants grown from shoot primordia

Fig. 1 and Table 3 show the growth and yield characteristics of plants grown from shoot primordia and seeds. Compared with plants grown from seeds, the majority of the plants regenerated from shoot primordia headed earlier (16 days earlier in the case of msNekken 2/H87-50 and 9 days earlier in the case of Kanto Kou 1, on the average), showed

a shorter plant height (23.7 cm and 8.7 cm shorter, respectively, on the average) as well as a lower yield of unhusked grains (11.2 g/plant and 7.3 g/plant less, respectively, on the average) and lower 1000 grain weight (2.0 and 1.6 g less, respectively, on the average). Also, some individuals of Kanto Kou 1 showed a lower percentage of fertility (15.4% lower, on the average). On the other hand, no differences in the panicle number in both combinations were observed.

The growth of the plants derived from shoot primordia was not satisfactory, presumably due to

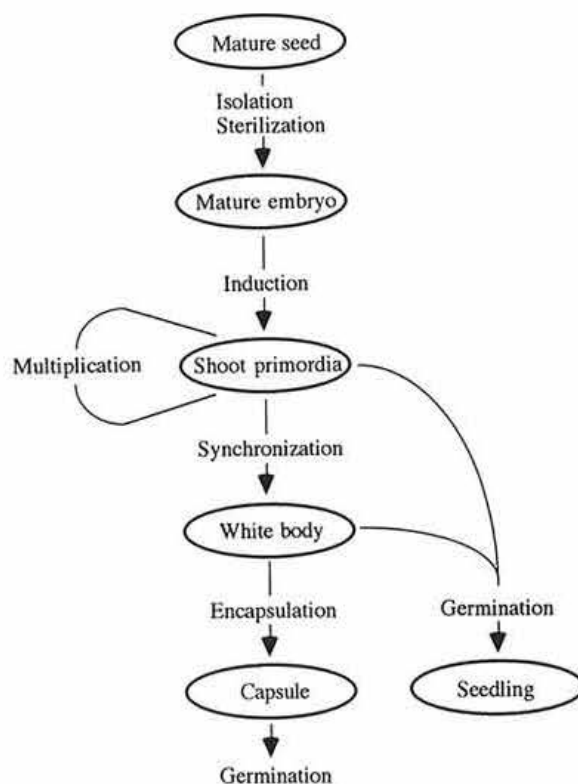


Fig. 2. Protocol for *in vitro* propagation of rice by shoot primordia multiplication

Table 3. Average values of growth and yield parameters of plants grown from shoot primordia and seeds in msNekken 2/H87-50 and Kanto Kou 1

		Duration <sup>a)</sup> (day)	Plant height (cm)	No. of panicles (/plant)	Yield (g/plant)	1000 grain weight (g)	Fertility (%)
msNekken 2/H87-50	SP <sup>b)</sup>	77 ± 5	90.0 ± 2.8	13 ± 5	16.7 ± 5.6	19.9 ± 0.9	75.5 ± 8.3
	Seed	93 ± 5	113.7 ± 2.4	14 ± 5	27.9 ± 5.3	21.9 ± 0.7	75.1 ± 9.0
Kanto Kou 1	SP	90 ± 7	101.6 ± 5.5	13 ± 4	20.4 ± 8.0	20.7 ± 0.9	68.5 ± 19.5
	Seed	99 ± 2	110.3 ± 0.6	15 ± 1	27.7 ± 3.3	22.3 ± 0.4	83.9 ± 1.3

Numerals indicate mean values ± standard deviations.

a): Duration from transplanting day to heading day.

b): SP; Shoot primordia.

the occurrence of heading before adequate growth (growth stages were advanced because the shoot primordia were cultured in the rooting medium for more than 1 month). The very low percentage of fertility observed in some individuals suggests the possible occurrence of genetic variation and indicates that genetic variation may have occurred in the shoot primordia derived from calli.

#### 6) Prospects for shoot primordia culture system

The protocol for *in vitro* propagation of rice by shoot primordia multiplication is described in Fig. 2.

The possibility of producing commercially artificial rice seeds using "tissue-cultured" shoot primordia was demonstrated. Future commercialization of artificial seeds requires the establishment of a stable liquid culture system, efficient production of shoot primordia or somatic embryos, synchronization of growth stages, improvement of regeneration ability, and the development of a culture medium comparable to endosperm. Furthermore, the preservation of artificial seeds requires a technology for the production of dried artificial seeds, which may be achieved through advances in research on dried shoot primordia or somatic embryos.

The use of cultured seedlings obtained through rooting of shoot primordia is also a suitable approach for commercialization in Japan, where technology for mechanized transplanting is well developed.

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