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Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.)

T. Abe and Y. Futsuhara

Laboratory of Theory of Agronomy and Plant Breeding, Faculty of Agriculture, Nagoya University, Furocho, Chikusaku, Nagoya 464, Japan

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Summary. Sixty rice varieties (*Oryza sativa* L.), belonging to three subspecies, *japonica, indica* and *javanica* (some *japonica* × *indica* hybrids were included), were compared for their capacity for callus growth and plant regeneration. Tissue cultures initiated from mature seeds on Murashige and Skoog's (1962) medium with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) were transferred to a medium containing 0.02 mg/l 2,4-D and 10 mg/l kinetin, from which plantlets were regenerated. Large variabilities in callus growth and plant regeneration potentials were revealed among the varieties tested. Most *japonica* varieties formed a callus that weighed more than 100 mg per seed 30 days after inoculation, and showed a relatively high regenerative potential, whereas *indica* varieties, *japonica-indica* hybrids and *javanica* varieties showed poor callus growth and plant regeneration, although considerable varietal variation was observed in each subspecies. The callus growth potential was not correlated with the plant regeneration potential. Histological observations revealed that the epithelium cells of the scutellum mainly proliferated to form a callus, from which shoot and root primordia were differentiated independently from each other. The shoot primordia developed into plantlets when roots were formed adventitiously. In a few cases, shoots and roots were bilaterally initiated from a single primordium.

Key words: *Oryza sativa* L. – Callus growth – Genotypic variability – Organogenesis – Plant regeneration

Introduction

The application of advanced tissue culture techniques may lead to new avenues in crop improvement. Protoplast fusion, gene transfer, induction of somaclonal mutation, cell or callus culture, and subsequent plant regeneration, may become routine procedures for crop breeders in future. For successful application of the tissue culture technique to crop breeding, the callus growth and plant regeneration potential of each crop must be determined.

A number of studies on rice tissue culture have been conducted with special reference to the effect of exogenous phytohormones (Saka and Maeda 1969; Nishi et al. 1973; Henke et al. 1978; Cornejo-Martin et al. 1979). These studies involved a limited number of genotypes, though in some of them differences in callus formation or plant regeneration potential among rice genotypes were reported (Maeda 1967; Abe and Sasahara 1982; Abe and Futsuhara 1984). As yet, genotypic differences in both callus growth and plant regeneration potential, their relationship, and histological processes leading to callus induction and organ differentiation, have not been fully understood. The objective of the present work is to clarify these aspects.

Materials and methods

Sixty rice varieties including three subspecies, namely *japonica, indica* and *javanica* varieties, and *japonica-indica* hybrids (Table 1) were used in this study. Mature fine seeds were selected and dehusked as experimental material. The seeds were sterilized in 70% ethanol for 30 s and in 1% sodium hypochlorite solution fo 40 min, followed by three rinses with sterile distilled water in a 1.5 × 6.0 cm petri dish. The sterilized seeds were placed on the surface of an agar medium containing salts as in Murashige and Skoog's medium (1962), 200 mg/l myo-inositol, 1 mg/l thiamine-HCl, 2 mg/l 2,4-D, 5 g/l yeast extract, and 30 g/l sucrose. The medium was adjusted to pH 5.8 with NaOH and HCl before adding 0.8% agar. Fifty ml of the medium solution were dispersed in a 100 ml flask and autoclaved at 1.2 kg/cm² and 120 °C for 15 min.

Seeds were incubated in the medium at 28 °C in darkness. Calli formed from seeds 30 days after inoculation were isolated from the original seeds. The capacity for callus formation was evaluated by weighing 16 calli in each variety. Calli used for plant regeneration were allowed to grow for 40 days on the initial medium and subcultured at 26 °C in darkness for a further period of 40 days on a similar medium which contained 2 g/l casein hydrolysate but in which yeast extract had been removed. Twenty-five such cultures of each variety were transferred to a regeneration medium supplemented with 0.02 mg/l 2,4-D and 10 mg/l kinetin, and cultured at 26 °C at about 3000 lux continuous light intensity from a cool-white fluorescent lamp. After 30 days, the capacity for plant regeneration, i.e. shoot, root, and green spot formation, was recorded.

Morphological observations were carried out under a stereoscopic and scanning electron microscope on the differentiating cultures 20 and 30 days after transfer. Samples for scanning electron microscopy were fixed in modified Karnovsky's (1965) solution containing 2% glutaraldehyde, for 24 h at 5 °C. After dehydration through an acetone series, the samples were dried at the critical point and coated with gold before observation. For histological observation, seeds cultured on the callus initiation medium for 1 and 5 days and differentiated cultures 20 days after transfer onto the regeneration medium were fixed with FAA (70% ethanol 18: formalin 1: glacial acetic acid 1), dehydrated through a butanol series, and embedded in paraplast. Ten μm sections were stained by Delafield Haematoxylin.

Results

Callus initiation and growth

Rice seeds cultured on the callus initiation medium with 2 mg/l 2,4-D developed a coleoptile from one day onward (Fig. 2a). The scutelum region in the seed embryo began to swell and the volume of the embryo cultured for 5 days increased more than 5 fold (Fig. 2b). Further coleoptile development ceased at this time. Root initiation and development from the plated seed was not observed at all. Callus formation frequency was almost 100% in all varieties. Initiated callus grew to large masses and attained about 200 mg fresh weight in some *japonica* superior varieties after 30 days (Fig. 1a).

A comparison between sections of seed embryo cultured for one day (Fig. 2a) and 5 days (Fig. 2b) showed that the scutelum tissues in the embryo specialized to the densely staining meristematic cells of the epithelium and to the parenchymatous cells of the inner scutellum. Epithelial cells divided up to more than 15 cells, and were stratified with a thickness of 0.1 to 0.2 mm 5 days after inoculation (Figs. 2b and c). Mesocotyle and radicle regions also changed into meristematic tissues. Thus, the epithelium of the scutellum, mesocotyle, and radicle regions was assumed to participate in the formation of the callus tissues. In the following culture period, however, scutellum calli proliferated actively. Areas of cells in the callus were arranged radially, and meristematic cells were deposited at the periphery and inner parts of the callus (Fig. 2d). On the other hand, mesocotyle and radicle regions were nonprolific, retaining almost the same size even at 10 or 20 days after inoculation.

Callus growth varied widely with the subspecies (Table 1). *Japonica* varieties generally showed a high capacity for callus growth, with a yield of over 100 mg per seed. *Indica* varieties and *japonica-indica* hybrids showed a relatively low capacity for callus growth. Half of their varieties produced calli weighing more than 100 mg, and another half produced calli weighing less than 100 mg. *Javanica* varieties showed a capacity for callus growth similar to that of *japonica*.

Variability in callus growth was observed among genotypes in each subspecies (Table 1). In the *japonica*

Table 1. Comparison of capacities for callus formation and plant regeneration among rice varieties

Variety	Weight of callus/seed[a]	No. of calli[b]		
		Shoot initiation	Shoot or green spot initiation	Root initiation
japonica				
'Aikoku'	127±10	5	5	7
'Asahi'	170±15	8	10	15
'Calrose'	180±20	5 (2)	6	9
'Daikoku' 1'	17± 4	10	2	13
'Fujisaka 5'	118±25	7 (2)	13	12
'Fujiminori'	119±18	14 (2)	14	16
'Hoyoku'	226±16	7	7	8
'Ishikari'	206±23	9 (1)	9	10
'Joshu'	169–23	10	14	11
'Kamenoo'	145±13	6	8	11
'Koshihikari'	124±10	6	7	11

Table 1 (continued)

Variety	Weight of callus/seed[a]	No. of calli[b]		
		Shoot initiation	Shoot or green spot initiation	Root initiation
japonica				
'Kotake-tamanishiki'	172±20	8	10	11
'Kuju'	268–21	1	2	5
'Morita-wase'	155–25	0	0	2
'Murasaki-ine'	194±15	6 (2)	14	11
'Murasaki-daikoku'	182±13	8	11	12
'Nipponbare'	158±18	5 (1)	5	6
'Norin 1'	87±13	1	1	0
'Norin 11'	118–13	6	12	12
'Norin 22'	160±12	5	5	5
'Reiho'	295±13	6 (1)	7	8
'Reimei'	151±20	9	12	18
'Rikuu 132'	105± 8	8 (1)	8	12
'Sasanishiki'	164±18	9 (1)	10	12
'Sen-ichi'	296±16	10 (3)	10	17
'Senshyo'	138±13	0	11	5
'Somewake'	57±7	11	15	18
'Taichung 65'	101±17	4 (1)	5	4
'Tamanishiki'	154±21	8	12	15
'Waito-C'	139±12	11 (3)	12	18
japonica × *indica*				
'Tongil'	109±16	0	0	1
'Josaeng Tongil'	100±13	0	0	4
'Milyang 21'	64± 8	3	3	7
'Milyang 23'	58±11	0	0	0
'Suweon 256'	174±19	1	2	3
'Yushin'	157±25	0	2	1
indica				
'Akamai'	57± 8	3	5	7
'Amber'	137±11	0	0	0
'B H C'	107±16	0	8	15
'Chyokoto'	104±15	3 (1)	3	12
'Dojinkyo'	102± 7	1	1	5
'Dee-geo-woo-gen'	81±16	1	1	2
'Gaiya Dhan Tosar'	86± 7	2	2	9
'Kannonsen'	206±24	0	2	2
'Kinandang'	80±14	1 (1)	1	4
'IR-8'	77±15	0	0	3
'Leng-kwang'	88±13	0	0	0
'Panbila'	112±13	1	2	11
'Tadkan'	93±14	2 (1)	2	3
'Taichung Native 1'	111±24	2	2	4
'Te Tep'	134±20	3 (1)	3	1
'Zenith'	82± 8	0	1	3
javanica				
'Allorio'	117±11	15 (2)	17	22
'Anthocyane'	95±10	2	2	7
'Arditane'	210±15	0	0	5
'Lady Wright'	175±10	0	0	5
'Lomelto'	168±14	5 (1)	10	11
'Rinaldo Belsano'	84± 8	2	2	4
'Secia'	97±13	0	0	2
'Stripe 136'	110±11	0	0	0

[a] Sixteen seeds were tested per variety. Callus weight was determined 30 days after inoculation

[b] Twenty-five cultures were examined in each variety. Number of calli forming albino shoots only is shown in parentheses

varieties, 'Sen-ichi', 'Ishikari', 'Hoyoku', 'Kuju', and 'Reiho' produced the most prolific calli, with a weight of more than 200 mg, while 'Norin 1', 'Daikoku 1', and 'Somewake' produced nonprolific ones with a weight of less than 100 mg. Among them, the calli of the latter two varieties were slightly necrotic. Many *japonica* varieties, 27 out of 30 produced calli of more than 100 mg. 'Konansen' (*indica*) and 'Arditane' (*javanica*) showed relatively high callus growth, with a weight of over 200 mg.

Plant regeneration

When the initiated calli were subcultured on the maintenance medium supplemented with 2 mg/l 2,4-D and 2 g/l casein hydrolysate instead of yeast extract, they grew about 5 to 15 fold in volume 40 days after the transfer. In some cultures, organized root-like structures were initiated on the callus tissue even at a high 2,4-D (2 mg/l) concentration, but no shoots were differentiated. Within 20 days after the calli were transferred to plant regeneration medium with a reduced amount of 2,4-D and a high kinetin concentration, green pigmented spots were first observed at the lower part of the callus tissues, which developed into tiny shoots (Fig. 1 b). Scanning electron micrographs clearly revealed shoot initiation from callus tissues (Fig. 2 h). Longitudinal sections of the differentiated cultures showed that shoot primordia were initiated de novo from the callus tissues with vascular bundles being differentiated under the primordia (Fig. 2 e, f). The primordium, which was pigmented green, was surrounded by a highly organized leaf-like structure. The diameter of the primordium was 0.5–1.0 mm (Fig. 2 e). Generally more than two primordia were seen in a differentiated culture. In a few cases, only root primordia were differentiated with vascular tissues, apart from the shoot primordium (Fig. 2 g). More than 20 days after the tansfer, roots were adventitiously formed at the base of

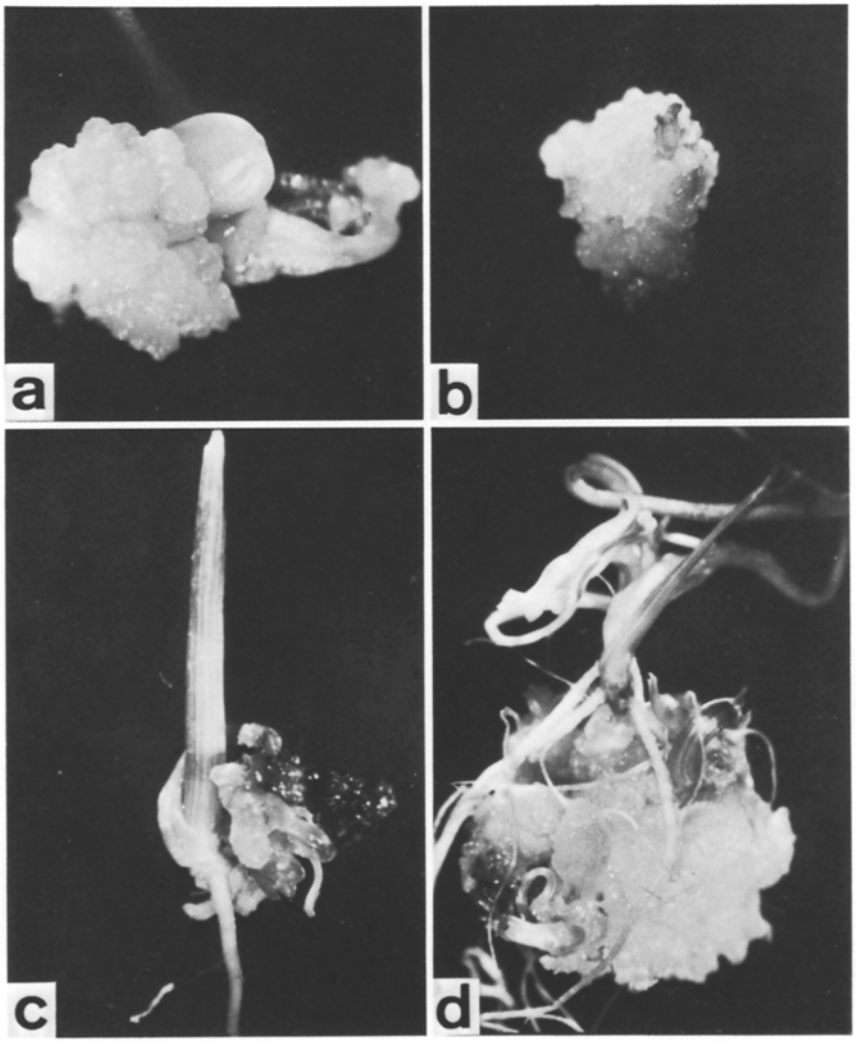

Fig. 1. **a** Callus formed from a rice seed 20 days after inoculation (×4.3). **b** A tiny shoot differentiated from callus tissue of 'Murasaki-daikoku' 20 days after transfer to the regeneration medium (×4.3). **c** A plantlet formed from callus of 'Murasaki-daikoku'. Coleoptile-shoot and root developed bilaterally (×4.3). **d** A plantlet formed from callus of 'Fujiminori' (×4.3)

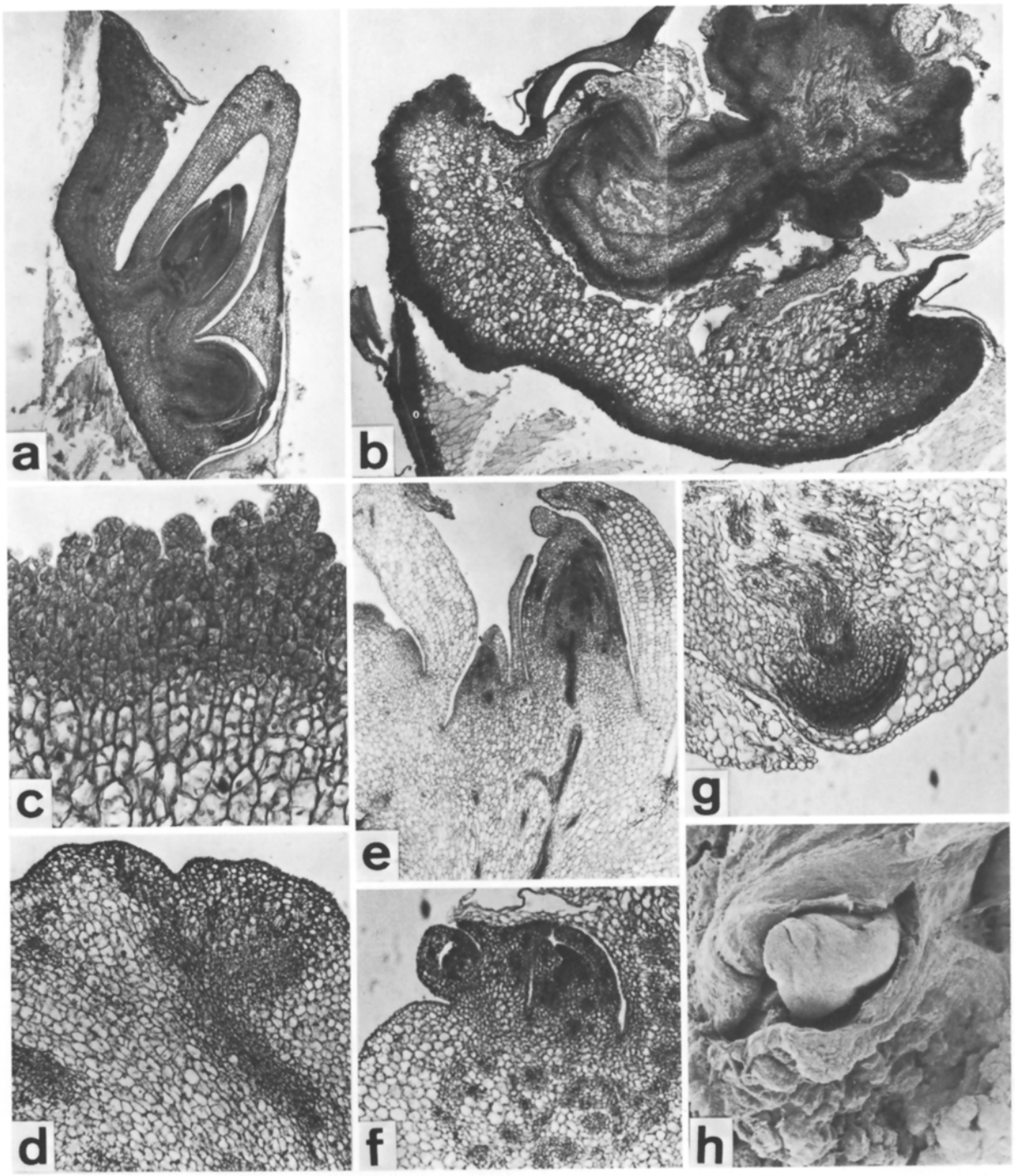

Fig. 2. a–d. Sections of rice seed embryo and callus (var 'Fujiminori') cultured on callus initiation medium: **a** slightly germinated seed embryo cultured for one day (×35); **b** seed embryo cultured for 5 days and callus derived from epithelium of scutellum (×35); **c** epithelial meristem of scutellum 5 days after inoculation (×80); **d** scutellum callus cultured for 30 days. Meristems are formed at the periphery and inner parts of callus (×80). **e–g** Sections of differentiated rice cultures 20 days after transfer to regeneration medium (**e** and **g:** var 'Chyokoto'; **f:** var 'Fujiminori'): **e** two differentiated shoot primordia with vascular tissues (×35); **f** two shoot primordia organized de novo from callus having vascular tissues (×80); **g** a differentiated root primordium with vascular tissues (×80). **h** Scanning electron micrograph of rice cultures differentiating a shoot from the original callus (×28)

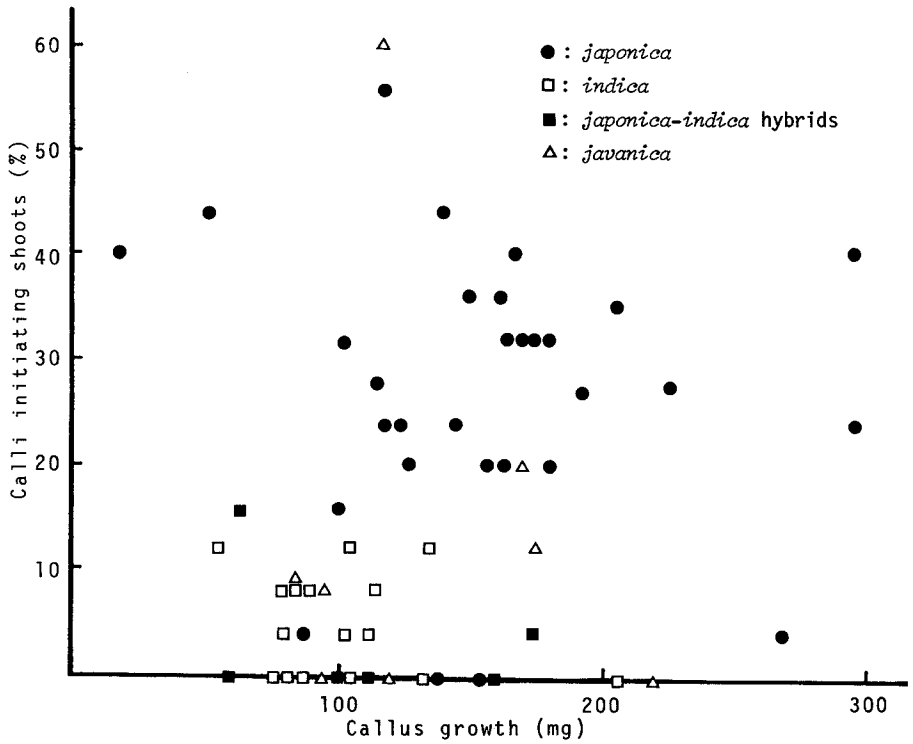

Fig. 3. Variation in the capacity for callus growth and shoot initiation in 60 rice varieties

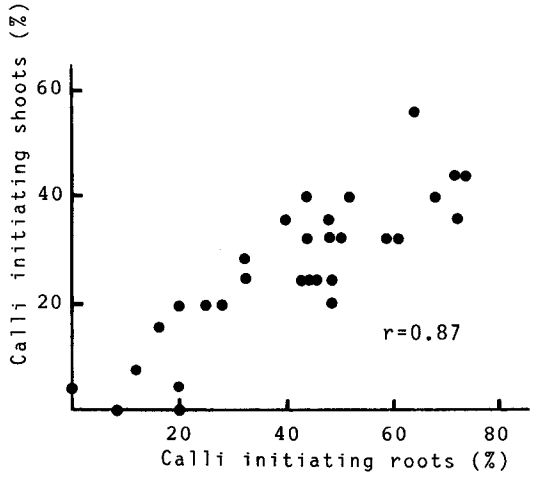

Fig. 4. Relationship between frequencies of cultures initiating shoots and roots in *japonica* varieties

shoots which were already differentiated, and grew to plantlets associated with the callus (Fig. 1 d). Occasionally, a root was initiated bilaterally and simultaneously from the base of a coleoptile-like structure (Fig. 1 c).

Remarkable genotypical variations in plant regeneration from seed calli were observed (Table 1). *Japonica* varieties showed a relatively high capacity for plant regeneration, with average initiation frequencies of 27.0% in shoots, 35.3% in shoots or green spots, and 41.9% in roots. On the other hand, *indica, japonica-indica* hybrids, and *javanica* varieties showed constantly low capacities, the frequencies being 5.4%, 10.0% and 21.8%, respectively, although one variety, 'Allorio', belonging to *javanica,* showed an extremely high capacity (having respective frequencies of 60.0%, 68.0% and 80.0%). Generally, *japonica* cultures were prolific while cultures of *indica* and *japonica-indica* hybrids were nonprolific and susceptible to necrosis whereby the tissues became brown and black in medium with decreased 2,4-D and high kinetin concentrations. Root initiation frequencies were higher than shoot or green spot initiation frequencies in almost all the genotypes. All the cultures which initiated shoots also differentiated roots, and formed plantlets. Among all the shoot-initiating cultures, the percentage of cultures inducing only albino plants was 11.2%.

Figure 3 shows the variations in the capacity for callus growth and shoot initiation among the genotypes. *Japonica* varieties showed relatively high capacities for both callus growth and plant regeneration, although some varieties showed good callus growth but low shoot formation (e.g. 'Kuju'), and the other varieties showed poor callus growth but high shoot formation (e.g. 'Daikoku 1' and 'Somewake') with one exception showing a low capacity for both traits (e.g. 'Norin 1'). *Indica* varieties and *japonica-indica* hybrids generally showed low capacities for both traits. *Javanica* varieties showed similar capacities to those of *indica,* or

intermediate between those of the *japonica* and *indica* varieties. Correlation coefficient of 0.87 revealed a closer relationship between the frequencies of cultures initiating shoots and roots in *japonica* varieties (Fig. 4).

Discussion

In relation to rice callus cultures, numerous reports about seed cultures which led to callus initiation or plant regeneration have been published (Maeda 1965; Yamada et al. 1967; Nishimura and Maeda 1977; Abe and Sasahara 1982; Heyser et al. 1983). Seed cultures have been used to compare the capacity for callus growth and plant regeneration because they are comparatively easy to establish. Rice seed calli were induced mainly from the epithelium of the scutellum, as similarly observed by Nishimura and Maeda (1977). This result, however, was different from that in the immature embryo of pearl millet in which a broad meristematic zone was found at the vascular bundle as well as the periphery of the scutellum (Vasil and Vasil 1982). In cultures of rice seed, the meristematic zone could not be found at the adaxial side of the vascular bundle.

Regeneration symptoms based on histological observations showed that caulogenesis by which only shoot primordia are differentiated independently from the root primordia, and rhizogenesis by which only root primordia are differentiated without the shoot primordia, first occurred in the differentiated cultures. Subsequently, vascular tissues may be formed under shoot primordium. Generally, roots followed with adventitious roots being formed at the base of the shoots and developed into plantlets. Thus, this type of morphogenesis led to organogenesis. Somatic embryogenesis from seed cultures as reported by Heyser et al. (1983), could not be detected in the present investigation.

Large genotypic variations in callus growth and plant regeneration potential were observed in rice. Many *japonica* varieties showed a relatively high capacity for callus growth and plant regeneration, while *indica* and *japonica-indica* hybrids, and *javanica* varieties displayed a low capacity for both traits although some exceptions were observed. These variations may be attributed to differences in the components and concentrations of endogenous phytohormones, and differences in the susceptibility to 2,4-D between *japonica* and *indica* varieties, as reported in the growth of rice plant by Sundaru et al. (1983). In the *japonica* varieties, cultures showed differences in the capacity for callus growth and plant regeneration: high capacity for both traits, high capacity for callus growth and low for plant regeneration, low capacity for callus growth and high for plant regeneration, and low capacity for both traits. Therefore, no correlation between the capacity for both traits was detected. Among the *japonica* varieties, 'Fujiminori' and 'Reimei' (the latter was induced from the former by gamma-ray irradiation, Futsuhara 1968), showed an intermediate capacity for callus growth and a high capacity for plant regeneration. 'Hoyoku' and its progeny, 'Reiho', showed a high capacity for callus growth and intermediate one for plant regeneration. 'Moritawase' and its progeny, 'Norin 1', showed low and intermediate capacity for callus growth and no shoot formation. These results suggest that the capacity is inherited from the progenitor and that at least two different genetic factors may participate in the expression of these traits independently, as suggested in barley anther culture (Foroughi-Wehr et al. 1982).

The capacity for plant regeneration from seed calli was similar to that from root calli especially in the *japonica* and *javanica* varieties, although the capacity of seed cultures for shoot formation was slightly higher (27%) than that of root cultures (16%) in the average frequency of the *japonica* varieties used (Abe and Futsuhara 1984). Among the *indica* varieties, root cultures in 'Chyokoto', 'Gaiya Dhan Tosar', and 'Te-tep' showed a considerably high capacity for plant regeneration (Abe and Futsuhara 1984), while seed cultures of the three *indica* varieties did not show such a high capacity in this experiment. Therefore, in order to obtain high totipotency, appropriate tissue sources and genotypes should be used. The identification and screening of useful genotypes for the capacity for callus growth and plant regeneration in vitro are a prerequisite for the application of tissue culture techniques to new breeding programs of rice.

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