Japan Jour. Crop Sci. 51(1): 70–74 (1982)

Induction and Plant Regeneration of Callus from Immature Embryos of Rice Plants (*Oryza sativa L.*)

Kwan-Long LAI and Li-Fei LIU

(*Department of Agronomy, National Taiwan University, Roosevelt Road Sec. 4 Taipei, Taiwan, ROC 107*)

Received July 1, 1981

Plant tissue culture techniques have provided a potential system for fundamental and applied research. While growth, metabolism and differentiation of tissues and cells *in vitro* have been well studied in a wide variety of dicotyledons, the progress in monocotyledons, especially in the cereals, has been much slower. This is mainly due to the low plant-regeneration ability of the callus of monocotyledons. However, one notable exception has first been reported by GREEN and PHILLIPS in 1975[6]. They have obtained high frequency of plant regeneration from callus of immature embryos of maize. Similar results have been reported in oat[2], sorghum[4,16], wheat[5] and barley[3].

During the past several years, we have constantly investigated rice protoplast culture and the possibility of using somatic hybridization as new approach to rice improvement[9,10,11]. To meet this purpose, it is of great importance to develop a culture system with high plant-regeneration ability. We have thus achieved rice callus and suspension cultures from immature embryos. The present paper describes the suitable conditions for callus induction.

Materials and Methods

The experiments were performed with two varieties of *Oryza sativa*—Taichung No. 65 and Tainan No. 5. Plants were grown in a phytotron at 30°C (day)/25°C (night). Spikelets were marked on the day of anthesis. Immature seeds were harvested from 10 to 18 days after anthesis at a 2-day interval. They were dehusked, surface-sterilized in 1% NaOCl for 20 min and washed four times with sterile water. Embryos were then excised and transferred onto agar culture medium with the rounded scutellar side up (scutellar surface side was exposed and the plumule-radicle axis side was in contact with the medium) or down (the scutellar surface side was in contact with the medium).

The basal culture medium consisted of the mineral salts of MURASHIGE and SKOOG (MS) medium[14], 30 g/l sucrose, 1mg/l thiamin-HCl, 1 mg/l pyridoxin-HCl, 10 mg/l nicotinic acid, and 8 g/l phytagar. The pH value was adjusted to 5.8 prior to autoclaving (5 min at 120°C).

For callus induction, immature embryos were inoculated onto basal media supplemented with various plant growth regulators. For organ differentiation, 4-week-old calli were transferred to basal medium supplemented with 10^{-5}M kinetin. All cultures were incubated at 26–28°C and under continuous fluorescent light with an intensity of approximately 9, 000 lx.

Results

Effects of embryo age and scutellar surface orientation

In the initial experiment, the influences of embryo age and scutellar surface orientation on callus formation and plant regeneration were studied. This experiment was conducted from October to December in 1979. Embryos were explanted onto basal medium with 10^{-5}M 2,4-dichlorophenoxyacetic acid (2,4-D). The scutellum began to enlarge and the plumule began to emerge within 24 hours of culture. After 5 days, the scutellar surface became irregular, and by 10 days, nodular yellow callus was visible. The frequency of callus formation was higher

Table 1. Effects of embryo age and scutellar surface orientation on callus growth and organ regeneration.

Variety	Days after anthesis	Scutellar surface down				Scutellar surface up			
		Callus[a] fr. wt. (mg)	Callus cultured on regeneration medium			Callus[a] fr. wt. (mg)	Callus cultured on regeneration medium		
			Total No.	With root	With shoot		Total No.	With root	With shoot
Taichung No. 65	10	87.1±14.0	18	5(28)[b]	0	104.2±34.8	18	14(78)	7(39)
	12	91.6±21.9	18	5(28)	0	125.0±40.3	18	12(67)	0
	14	97.3±27.2	15	12(80)	3(20)	114.4±33.5	18	13(72)	0
Tainan No. 5	10	96.2±24.5	31	16(51)	4(13)	129.5±37.5	31	28(90)	11(35)
	12	94.7±32.1	31	13(42)	4(13)	118.1±37.9	31	18(58)	1(3)
	14	105.2±29.5	31	8(26)	0	113.7±20.5	31	6(20)	0
	16	80.3±33.1	31	16(51)	2(6)	102.1±33.3	31	17(55)	2(6)
	18	72.3±20.0	31	13(42)	0	90.4±16.0	31	8(26)	0

a. Each mean and its standard deviation were calculated from 20 samples of 4-week-old cultures.
b. The number in parenthesis is percentage of total callus number.

than 95%; embryos failed to produce callus only when they were lifted by the germinating plumules which stuck into the medium. Growth of plumules was seriously inhibited. When embryo was placed with scutellar side down, the plumules showed slender, short (<1 cm) and white. When embryo was placed with scutellar surface up, the plumules grew stout, longer (1–2 cm) and occasionally green. In both cases, elongation of radicles was completely suppressed.

After 4 weeks, the callus enlarged to ca. 1 cm in diameter. Callus yield as showed by fresh weight was apparently affected by embryo age and scutellar surface orientation (Table 1). In the experiment ranges, callus yield was always higher when scutellar surface was placed up. Though fresh weight varied between calli derived from embryos at different age, no consistent trend could be seen.

One week after transferring callus to the differentiation medium, some portion of callus turned to green and from where roots regenerated. By 10 days, shoots started to emerge and all plantlets regenerated were green; no albino or chimeral plantlets were observed.

There were wide variations between calli in ability to regenerate roots and shoots (Table 1). The highest frequency of root and shoot regeneration was found in calli induced from 10-day-old embryo with scutellar surface up. It is consistent between two varieties. In general, calli derived from embryos with scutellar surface down showed lower regeneration ability.

Six plantlets of Taichung No. 65 were transplanted to soil and were raised to maturity. Plants were characterized by normal phenotype, but seed set was slightly variable. Five plants were fertile with seed set from 90% to 98% and one showed partial sterility with seed sed only 30%.

Effects of medium composition

The conditions for callus induction were examined in more detail from October to December in 1980. Ten-day-old embryos of Tainan No. 5 were explanted with the scutellar surface side up onto medium supplemented with various concentrations of the growth regulators. The results on callus induction are summarized in Table 2.

On medium without growth regulator, plumules developed into normal seedlings and no callus was induced. Lower concentrations of α-naphthaleneacetic acid (NAA) (10^{-6}M and 10^{-5}M) enhanced plumule and root growth; callus was only induced from a small number of embryos. Increasing the NAA level to 10^{-4}M caused inhibition of plumule growth, but promotion of callus formation. The callus proliferated very vigorously and was often light green

Table 2. Effects of growth regulators on callus induction and growth.

Growth regulator	Callus induction (%)	Callus fr.wt. (mg)	Shoot length (cm)
10^{-6}M 2,4-D	100	259.5±105.0[a]	<2
10^{-5}M 2,4-D	100	145.5± 32.8	<2
10^{-4}M 2,4-D	100	90.6± 31.0	<2
10^{-5}M 2,4-D-10^{-5}M ABA	100	111.5± 40.2	<2
10^{-5}M 2,4-D-10^{-5}M ABA-10^{-5}M Kinetin	100	59.6± 33.8	<2
10^{-6}M NAA	10	Scarce	23.2± 4.7[a]
10^{-5}M NAA	18	Scarce	23.6± 3.2
10^{-4}M NAA	100	211.5± 50.9	11.2± 8.9
10^{-5}M NAA-10^{-6}M Kinetin	24	Scarce	25.8±10.1
10^{-5}M NAA-10^{-5}M Kinetin	26	Scarce	18.7±10.1
10^{-6}M NAA-10^{-5}M Kinetin	8	Scarce	18.6± 2.8
10^{-5}M ABA	0	None	4.9± 2.2
Without growth regulator	0	None	15.4± 3.2

a. Each mean and its standard deviation were calculated from 10 samples of 4-week-old cultures.

in color with some roots.

Adding 10^{-6}M kinetin to medium with 10^{-5}M NAA caused similar effects as 10^{-5}M NAA alone. However, Increasing the kinetin level to 10^{-5}M or lowering the NAA level to 10^{-6}M led to less growth of plumules. Callus was only occasionally induced and often submerged by numerous roots.

In case of 10^{-6}M abscisic acid (ABA), plumule growth was strongly inhibited and no callus was induced at all.

The three concentrations of 2,4-D tested were all satisfactory for callus induction. An abundance of callus was produced on medium with 10^{-6}M 2,4-D. Higher levels of 2,4-D tended to reduce callus growth. On medium with 10^{-5}M 2,4-D, addition of 10^{-5}M ABA slightly decreased the yield of callus. Moreover, the combination of 10^{-5}M 2,4-D, 10^{-5}M ABA and 10^{-5}M kinetin gave the lowest callus yield. In the presence of 2,4-D, plumules grew not longer than 2 cm and elongation of radicles were completely inhibited.

Discussion

In this study, we succeeded in establishing totipotent callus cultures from immature embryos of rice. Recently, immature embryos was found to be suitable source of explant for callus induction and shoot regeneration in cereal plants[2,3,4,5,6,16].

Distinct effects of embryo age and orientation of the embryo on the culture medium on callus induction and shoot regeneration were observed. Our data showed that 10-day-old rice immature embryos with the plumule-radicle axis side placed in contact with medium gave best result in callus formation and plant regeneration. However, the conditions for other plant species varied. For examples, in maize, the optimum embryo age was 18 days post-pollination and placing the scutellar side up promoted callus formation[6]. In barley, 10- to 13-day-old embryos were suitable and placing the scutellar side down gave better results[3]. The cause of this positional effect was not understood so far. Anatomical studies are underway to compare the process of callus induction from embryo with different orientations on the medium.

Growth responses of rice immature embryo were apparently affected by the kind and level of growth regulators in the medium. Callus could be induced successfully by 2,4-D even at the level of 10^{-6}M and a high concentration of NAA at 10^{-5}M. Presently, 2,4-D is the most common and effective auxin applied for callus induction and subculturing of the cells of cereal plants. And the concentration of 2,4-D used in cereal plants tends to be higher than that in dicotyledonous plants[17]. For callus induction in rice, the conenctration of 2,4-D used was at least 0.5mg/l (ca. 0.25×10^{-5}M)[7,8,12,15]. Because 2,4-D makes callus more difficult to differentiate in subsequent organ

regeneration culture, it is of great advantage to reduce its concentration[13]. Therefore, the success in inducing prolific callus growth from rice immature embryos by very low level of 2,4-D (10^{-6}M) or by substituting 2,4-D for NAA is a very notable event. In rice anther culture, callus could be induced by NAA and kinetin, but the inducing rate was very low[1].

The induction of totipotent callus from immature embryo has no doubt to provide a very potential culture system for rice improvement, especially for the studies of protoplast culture.

Summary

Prolific callus cultures were established from immature embryos of rice (*Oryza sativa* L.). It was found that 10-day-old immature embryo with the plumule-radicle axis side placed in contact with medium gave best results in callus formation and plant regeneration. Growth responses of immature embryo were affected by the growth regulator level in the medium. Callus was successfully induced by 10^{-6}–10^{-4}M 2,4-D or 10^{-4}M NAA. Lower concentration of NAA or omitting the growth regulator enhanced the plumule growth to normal seedling and failed to induce callus.

Acknowledgements

The authors are grateful to Dr. C. C. CHEN for his helpful discussions.

References

1. CHEN, C. C. and M. H. LIN 1976. Induction of rice plantlets from anther culture. Bot. Bull. Academia Sinica **17**: 18—24.
2. CUMMINGS, D. P., C. E. GREEN and D. D. STUTHMAN 1976. Callus induction and plant regeneration in oats. Crop Sci. **16**: 465—470.
3. DALE, P. J. and E. DEAMBROGIO 1979. A comparison of callus induction and plant regeneration from different explants of *Hordeum vulgare*. Z. Pflanzenphysiol. **94**: 65—77.
4. GAMBORG, O. L., J. P. SHYLUK, D. S. BRAR and F. CONSTABEL 1977. Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci. Letters **10**: 67—74.
5. GOSCH-WACKERLE, G., L. AVIVI and E. GALUN 1979. Induction, culture and differentiation of callus from immature rachises, seeds and embryos of *Triticum*. Z. Pflanzenphysiol. **91**: 267—278.
6. GREEN, C. E. and R. L. PHILLIPS 1975. Plant regeneration from tissue cultures of maize. Crop Sci. **15**: 417—421.
7. HENKE, R. R., M. A. MANSUR and M. J. CONSTANTIN 1978. Organogenesis and plantlet formation from organ- and seedling-derived calli of rice (*Oryza sativa*). Physiol. Plant. **44**: 11—14.
8. LAI, K. L. 1971. Morphogenetical studies on the callus originated from excised roots of rice plants—The induction and anatomical observation on callus formation. Memoirs of the College of Agriculture, National Taiwan University **12**: 95—105.
9. ——— and L. F. LIU 1976. On the isolation and fusion of rice protoplasts. J. Agri. Assoc. China New Series **93**: 10—29.
10. ——— and ——— 1980. Studies on the rice protoplasts—Factors affecting the isolation and viability. Proc. Nat. Sci. Coun. ROC **4**: 183—188.
11. ——— and ——— 1981. Studies on the rice protoplasts—Growth response to medium components and cell wall regeneration during culture. Nat. Sci. Coun. Monthly ROC **9**: 23—36.
12. MAEDA, E. 1965. Callus formation and isolation of single cells from rice seedlings. Proc. Crop Sci. Soc. Japan **34**: 139—147.
13. ——— 1972. Regulation of differentiation in crop cells under aseptic conditions. Proc. Crop Sci. Soc. Japan **41**: 269—283.
14. MURASHIGE, T. and F. SKOOG 1962. A revised medium for growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**: 473—497.
15. OHIRA, K., K. OJIMA and A. FUJIWARA 1973. Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. Plant & Cell Physiol. **14**: 1113—1121.
16. THOMAS, E., P. J. KING and I. POTRYKUS 1977. Shoot and embryo-like structure formation from cultured tissues of *Sorghum bicolor*. Naturwissenschaften **64**: 587.
17. YAMADA, Y. 1977. Tissue culture studies on cereals. In Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. (Eds.) Reinert, J. and Y. P. S. Bajaj. Springer-Verlag, Berlin. 144—159.

〔和 文 摘 要〕

イネ幼胚起源のカルス誘導と分化

賴 光 隆・劉 麗 飛
(国立台湾大学農芸学系)

イネ未熟穎果より分離した幼胚を培養して，増殖が早く茎葉の分化が著しいカルスを得ることができた．開花後 10 日目の穎果から分離した幼胚を用い，胚盤側を上向きとし，幼芽・幼根側を下向きにして寒天培地に接触して培養を行った場合，カルスの誘導ならびにその後の器官分化が良好であることがわかった．幼胚からのカルス誘導は培地に加えた生長物質の種類及び濃度レベルによる影響が著しかったが，2, 4-D の 10^{-6}-10^{-4} M, 又は NAA 10^{-4} M でともにカルスの形成が顕著であった．NAA の低濃度あるいは培地に生長物質を加えない処理では，幼芽の伸長が著しかったがカルスの形成は見られなかった．