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Callus Formation from Mature Embryos and Plant Regeneration of American Wild Rice, *Zizania palustris* L.

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Compact and nodular calli which were embryogenic emerged from mature embryos obtained from kernels of American wild rice (*Zizania palustris* L.) by culturing them on medium containing Murashige and Skoog salts, vitamins, 2, 4-D (4 mg/*l*) and BA (0. 02 mg/*l*). This nodular callus was well proliferated through subculturing on the same medium (every 4-5 weeks). Besides this callus, two other types were obtained during subculturing, although they were not embryogenic: one was friable and bright yellow and another wetty, friable and white. When the compact and nodular callus was transferred to medium with decreasing concentrations of 2, 4-D, many embryoids regenerated within 30 days. These embryoids germinated and developed into normal plants after being transferred to hormone-free media and/or soil. They flowered but did not produce seeds.

American wild rice (*Zizania palustris* L., Gramineae) in an annual, aquatic plant which grows naturally in marshes and shallow lakes in North America. American wild rice has been cultivated commercially since the 1960 s mainly in Minnesota, U. S. A. Although this plant was introduced into Japan as a health food a few years ago, it has not been commercially cultivated in this ountry. Ac high content of total protein and a richness of essential amino acids, especially lysine and methionine in seeds of this plant have attracted researchers to consider it as a gene source for improving productivity and tolerance to unfavorable environment of major cereal crops.[1,2] However, this plant has several characters unsuitable for a commercial plant and also for experimental material, such as irregular fertility, easy abscission of kernels when matured, seed dormancy and recalcitrant storage due to a low degree of tolerance to desiccation.[3-7] Nevertheless, recent progress in cell culture and molecular biology have shown a great possibility to overcome these weak points and make it feasible to utilize these advantageous characters described above.[8] A few studies have been reported on embryogenic callus formation and plant regeneration from the callus of *Zizania latifolia* (perennial grass).[9,10] In this report, we describe successful plant regeneration through embryogenesis from mature zygotic embryos of *Z. palustris* (annual grass).

Materials and Methods

Seeds of American wild rice (*Zizania palustris* L.) were harvested on July 7-14, 1988 from plants grown in the field. They were stored in a refrigerator at 4℃ soaking in water until use. A preliminary test on the suitability of mature zygotic embryos for axenic culture during storage revealed that approximately 8-month-storage was suitable for the preparation of detached embryos. Shoots and roots successfully emerged from 70-80% of these cultured embryos in hormone-free Murashige-Skoog (MS)[11] agar medium. Husked seeds were washed with a commercially available detergent and then rinsed in tap water. Subsequently their surfaces were sterilized in 2. 5% NaOCl for 20 min with

gentle stirring, followed by rinsing (3×) with sterilized redistilled water.

Mature embryos (5–8 mm long, pale yellow) were excised from sterilized kernels under a dissecting microscope and placed on the MS agar medium, supplemented with 2, 4-D (4 mg/*l*) and BA (0. 02 mg/*l*). They were incubated in a light/dark cycle of 10/14-hr at 20℃ (short-day condition), a light/dark cycle of 16/8-hr at 25℃ (long-day condition), or in darkness at 20℃ or 25℃. Proliferated calli were subcultured on the same medium under the short day condition every 4–5 weeks. The callus was cut into small pieces (*ca.* 5 mm in diameter) and cultured on MS agar medium with decreasing concentrations of 2, 4-D (0. 2 mg/*l* or 0. 02 mg/*l*). After 1–2 months, calli with embryoids were transferred into a hormone-free MS medium. The regenerated younger plants were implanted in soil and incubated at 20℃, 10-hr light/15℃, 14-hr dark cycle.

For histological observation tissues were fixed in FAA (70% ethanol : formaldehyde : glacial acetic acid=90 : 5 : 5), dehydrated in an ethanol series, embedded in paraffin, sectioned at 24 μm thickness, and stained with 1% safranin in water and then 1% gentian violet in 50% ethanol.

Results and Discussion

Callus initials were visible near the mesocotyl of the embryo after 20–30 days on the MS agar medium containing 2–4 mg/*l* 2, 4-D and 0. 02 mg/*l* BA (**Table 1**). By that time, embryos had developed slightly and had turned brown. When NAA was used as an auxin in stead of 2, 4-D (**Table 2**), younger plants with roots and leaves developed from the majority of the embryos cultured. Poorly-developed calli surrounded the base of the aerial part or root tips of these plants. The N 6[12] or B 5[13] agar medium containing 4 mg/*l* 2, 4-D and 0. 02 mg/*l* BA promoted callusing but proliferation was less than compared to the MS medium (data not shown).

Callus induction usually occurred in from 60–70% of the embryos under the short-day condition or

Table 1. Effects of varied concentrations of 2, 4-D and BA on the callus formation from detached embryos of *Zizania palustris* L.

Conc. (mg/*l*)	Appearance of embryo (2 months culture)				
2, 4-D/BA	0	0. 02	0. 2	2. 0	4. 0
0	SR	SR	SR	s	s
0. 02	SR	SR	sr	sr	s
0. 2	SR	SR+CC	SR	sr	s
2. 0	CC	CC	CC	sr	sr
4. 0	CC	CC	sr	sr	sr

Eight embryos were incubated on the MS-agar medium with various compositions of hormones under the short-day condition.

Abbreviation : SR, elongation of a shoot with roots ; sr, a small and abnormal expansion of a shoot and rooting ; s, "sr" not accompanying rooting ; CC, compact nodular calli (shoots elongated a little but browned).

Table 2. Effects of varied concentrations of NAA and BA on the callus formation from detached embryos of *Zizania palustris* L.

Conc. (mg/*l*)		Appearance of embryo (2 month culture)				
NAA	BA	0. 02	0. 1	0. 2	1. 0	2. 0
2. 0		SR+CC	SR+CC	sr	sr	sr
4. 0		SR+CC	SR+CC	SR+CC	SR+CC	sr

For abbreviations see Table 1.

in the darkness at both 20℃ and 25℃. In contrast, under the long-day condition, the rate of successful callus induction was much less (at most 20%). Jong and Chang[9] reported that a high percentage of callusing of *Z. latifolia* could be obtained under long day conditions similar to those in this study. Thus, it seems likely that the light and/or temperature conditions for callusing vary among species of *Zinania*.

During repeated subcultures, the entire callus was typically compact, nodular and whitish pale yellow but the center of the callus clump gradually turned brown only in the MS agar medium containing 4 mg/*l* of 2, 4-D (**Fig. 1 A**). Thus, callus pieces were obtained only from the whitish pale yellow part but not the brown central part. Besides the above typical callus, two other distinct types were formed, although they were not embryogenic : one was friable, bright yellow and the other was wetty, friable and white. The first, compact nodular type was the only callus from which embryoids were produced for 1-2 months after transfer into MS medium containing decreasing concentrations of 2, 4-D (**Fig. 1 B, C**). Figure 1 D demonstrates a longitudinal section of an embryoid which regenerated from the callus. As seen in Fig. 1 D, a shoot apex and a radicle developed bipolarly on the same axis but a typical scutellum was not defined. Although germination of the embryoids did not synchronously occur, they developed into normal plantlets within approximately 30 days after being transferred to the same medium without hormones (**Fig. 1 E**).

These embryoid-derived plantlets grown in test tubes were transferred to soil in pots at various growth stages and grown to mature plants in a growth chamber. When young plants developed from the plantlets in test tubes were transferred to soil before flowering, some of them successfully matured and flowered but did not produce seeds. However, the height of these mature plants was at most

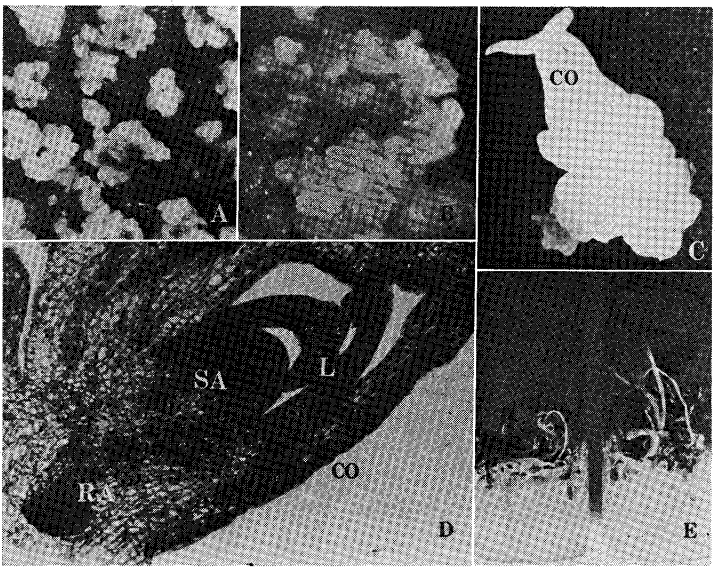

Fig. 1. Plant regeneration from callus of *Zizania palustris* L. (A) Compact nodular (pale yellow) calli, subcultured every 4-5 weeks at several times (×1). (B) Embryoids (EM) formed on the medium with decreasing concentrations of 2, 4-D (4 mg/*l* to 0. 02 mg/*l*) (×3). (C) An embryoid developed from the (B) stage callus after being transferred onto the hormone-free medium (×10). (D) A longitudinal section of an embryoid developed from callus (×100). (E) Plantlets regenerated (×1).

Abbreviations : EM, embryoid ; SA, shoot apex ; RA, radicle ; L, plumular leaf ; CO, coleoptile.

Table 3. Effects of varied concentrations of 2, 4-D and BA on regeneration from the compact nodular callus.

Conc. (mg/*l*) 2, 4-D/BA	% of callus regenerated 0	0. 02	0. 2
0	0	24	48
0. 02	0	24	56
0. 2	0	20	44
2. 0	0	8	24

Calli subcultured on the MS-agar medium, containing 2, 4-D 4 mg/*l* and BA 0. 02 mg/*l* at 25℃ in the dark, were transferred onto the MS-agar medium containing the indicated concentration of 2, 4-D and BA and incubated under the short-day condition for 2 months.

30–40 cm and was 3/4–4/5 less than that of normal plants grown from seeds in the fields. In contrast, when flowering plants in test tubes were transferred to soil, they withered within a few weeks. These results suggest that the conditions to promote vegetative growth of plantlets in test tubes before transplanting, such as temperature and light should be considered more carefully in order that the growth of these plants may approach that of normal plants.

As shown by many authors, various kinds of effectors in the medium, for instance, balances of plant hormones,[9,14,15] salts[16] or sucrose,[17] may be important in regulating embryoid formation. The frequency of regeneration through embryogenesis from the callus was 20–40% when the concentration of 2, 4-D in the medium was as low as 0. 02–0. 2 mg/*l* (**Table 3**). On the other hand, BA did not seem critical to the formation of embryoids, because embryoids were formed on the medium lacking BA in some cases. The effect of BA on embryoid formation was also variable in repeated experiments.

Plant regeneration through somatic embryogenesis has been reported on many species of monocotyledonous plants.[8] This technique is most useful for multiplication of one species by tissue culture. As far as the literature was surveyed, only one paper[9] on regeneration of genus *Zizania*, *Z. latifolia* (perennial), has been published. Thus, the present paper is the first report of successful plant regeneration of *Z. palustris* (annual). For *Z. latifolia*, Jong and Chang[9] succeeded in obtaining embryogenic calli from young inflorescence explants. On the other hand, for *Z. palustris* use of mature embryos is more advantageous than that of other tissues, since they can be readily manipulated under a dissecting microscope because of their relatively large size and they can be obtained for a long period through seasons. However, we have to consider the disadvantage of mature embryo that desirable characters of *Z. palustris* are not usually stable through generations because this is a cross-pollination plant. Thus, for developing a more efficient technique of callus formation of the present plant, use of other tissues such as young inflorescences and shoot tips is worth trying even though the growing period of these materials is limited through a year for obtaining sufficient number of explants for experiments.

Although American wild rice has several attractive points as a new cereal crop,[2,18] its domestication through conventional breeding techniques is not so easy because ⅰ) readily abscised under a natural condition, ⅱ) its seeds hardly germinate once they are dried, and ⅲ) dormancy period of seeds is about 6 months, when its seeds are harvested in Mie prefecture. Thus, application of modern techniques such as protoplast fusion and genetic engineering is required for genetical improvement of the present plant. We are planning to carry out a cell suspension culture by using the present calli as preliminary step for a future work of protoplast fusion.

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≪和文要約≫

ワイルドライスの完熟胚からのカルス誘導と植物体の再生

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4℃ で水中に貯蔵したワイルドライス (*Zizania palustris* L.) の完熟種子を滅菌処理後，これらから胚を摘出し，寒天培地に置床しカルスの誘導を試みた．カルスは，2, 4-D を 2-4 mg/*l* および BA を 0. 02 mg/*l* 添加した場合によく誘導されたが，NAA の場合には，多くの胚は植物体に成長し，カルスはわずかしか誘導されなかった．誘導されたカルスの多くは，粒状，黄白色で，他に継代培養中，白色もしくは淡黄色のずれやすいカルスなどの形態的な変異が認められた．これらのうちコンパクトな粒状のカルスを，2, 4-D を減じた培地に移植したところ，多くの胚様体を生じ，植物体に成長した．さらに再分化した幼植物を水田土壌に移植し，照明時間を 10 時間，温度を照明中 20℃・暗黒中 15℃，および湿度を 80% (Rh) に調整した培養器内で順化し，成植物を得た．これらは開花したが，種子は得られなかった．