Plant Prod. Sci. 3(2) : 114—123 (2000)

Improvement of Direct Somatic Embryogenesis in Rice by Selecting the Optimal Developmental Stage of Explant and Applying Desiccation Treatment

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Abstract : By selecting the optimal developmental stage of zygotic embryos used as explants and applying desiccation treatment, we improved direct somatic embryogenesis in rice scutellum from two cultivars, Nipponbare and Sasanishiki. Zygotic embryos isolated 14-17, 21, 28-30 and 35-40 d after anthesis (DAA) from Nipponbare and 14-17, 18-21, 28-30 and 40-42 DAA from Sasanishiki were cultured on the embryo induction medium (EIM). Then they were transferred to embryo maturation medium (EMM) and germinated on the embryo germination medium (EGM). Only immature zygotic embryo isolated 14-17 DAA from Nipponbare and Sasanishiki could develop somatic embryos that germinated. Explants from embryos at other developmental stages could develop somatic embryos only until the elongating or scutellar stage. They enlarged and formed callus without further development. The EIM and EMM consisted of N6 macronutrients, B5 micronutrients, and B5 vitamins, supplemented with 0.1 g L^{-1} casein hydrolysate, 1.5 g L^{-1} proline and 1 g L^{-1} MES buffer. EGM consisted of MS macro- and micronutrients and MS vitamins without organic supplement. In addition, 2 mg L^{-1} 2,4-D was added to EIM, 1 mg L^{-1} 2,4-D to EMM and 0.01 mg L^{-1} zeatin to EGM. Developmental processes of somatic embryos derived from the explants were observed by scanning electron microscopy. Desiccation treatment of maturing somatic embryo was proved to produce fully mature somatic embryos capable of germinating vigorously.

Key words : Desiccation treatment, Developmental stage, Immature zygotic embryo, Mature zygotic embryo, Nipponbare, Sasanishiki, Scanning electron microscopy, Somatic embryo.

Somatic embryogenesis is an amazing process because the bipolar structure possessing shoot and root resembling zygotic embryo is produced from somatic cells through an orderly series of characteristic morphological stages (Emons, 1994). Particularly, direct somatic embryogenesis is advantageous for plant propagation because there is no intervening callus stage, and, therefore somaclonal variation can be reduced (Williams and Maheswaran, 1986).

Jones and Rost (1989) reported direct somatic embryogenesis from rice scutellum using American cultivars. We previously examined the morphogenic process of direct somatic embryogenesis in rice cv. Nipponbare by scanning electron microscopy (SEM). This process commences with the initiation of proembryo leading to embryogenic nodule formation. Subsequently, the embryogenic nodule underwent proliferation and mass propagation (enlargement), histodifferentiation (determination of polarity), maturation and germination (Mariani et al., 1998). However, germination was spontaneous and the germinating somatic embryos showed abnormal structures.

The more closely the pattern of somatic embryo gene expression matches that of the zygotic embryo, the greater the chance of obtaining efficient regeneration systems. Such normalization of the gene expression pattern will be achieved through the optimalization of media and culture protocols for each individual stage of embryo development (Merkle et al., 1995).

The developmental stage of the zygotic embryo used as an explant is critical in direct somatic embryogenesis. Geneve and Kester (1990) reported that immature zygotic embryos of *Cercis canadencis* with an initial fresh weight between 4 and 12 mg exhibited competence to form somatic embryos. No somatic embryos could be induced from the mature zygotic embryos used as explants. Merkle et al. (1998) also reported that more immature seeds gave a higher frequency of somatic embryogenesis than mature seeds in sweetgum.

Desiccation tolerance is one of the most fundamental properties of seeds. It is acquired late in seed development and is considered necessary for completion of the plant's life cycle, as an adaptive strategy to enable seed survival during storage or environmental stress, and to ensure better dissemination of the species (Leprince et al., 1993). The natural development of a zygotic embryo was close to the optimal condition and therefore the somatic embryo should follow the same developmental program as the zygotic embryo in the seed (Bewley and Black, 1994). Desiccation of the somatic embryo serves two purposes. First, in some species, desicca-

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Table 1. The composition of media for producing rice somatic embryos.

Medium	Basal medium		Vitamin	Hormone	Carbon source		Gelling agent
	Macro	Micro			Sucrose	Sorbitol	
EIM	N6[1)]	B5[3)]	B5	2mgL^{-1}2,4-D	1%	2%	0.25%Gelrite
EMM	N6	B5	B5	1mgL^{-1}2,4-D	2%	4%	0.25%Gelrite
EGM	MS[2)]	MS	MS	0.01mgL^{-1}zeatin	1%	2%	0.4% Gelrite

EIM and EMM were supplemented with 0.1 g L^{-1} casein hydrolysate, 1.5 g L^{-1} proline and 1 g L^{-1} MES (2-(n-morpholino) ethanesulfonic acid).

1) Chu et al. (1975)

2) Murashige and Skoog (1962)

3) Gamborg et al. (1968)

tion promotes the accumulation of nutrients, such as storage protein, for use by the embryo during germination (Finer, 1994).

To our knowledge, there have been no reports on direct somatic embryogenesis in the zygotic embryo isolated at different developmental stages and the effect of desiccation on the development of somatic embryos in rice. The objective of this study was to improve the direct somatic embryogenesis in rice by selecting the developmental stage of zygotic embryo optimal for use as an explant and applying desiccation treatment. We utilized Nipponbare and Sasanishiki cultivars because they are the typical Japanese varieties that are widely used in agriculture and research work.

Materials and Methods

1. Materials

Zygotic embryos isolated from rice (*Oryza sativa* L.) cv. Nipponbare 14-17, 21, 28-30, 35-40 d after anthesis (DAA) and Sasanishiki 14-17, 18-21, 28-30, 40-42 DAA were used as explants. The seeds were sown in 0.02 m^2 pots in early May 1997, and cultivated in a phytotron at 30/23°C (day/night). The plants commenced heading at the end of July 1997. The seeds were refrigerated for 2 wk to 1 mon before starting experiments.

2. Methods

(1) Culture of zygotic embryo

The rice seeds of both cultivars at various developmental stages were sterilized using 1% Na-hypochlorite for 15 min and washed three times with sterilized distilled water. The sterilized seeds were placed onto the embryo induction medium (EIM) and cultured for 1 wk. Table 1 shows the composition of media. The N6 basal medium was used for its high concentration of nitrogen compared to MS basal medium according to Wernicke et al. (1981) and Tsukahara et al. (1996). Then, the embryos isolated from the seeds (explants hereafter) were cultured on EIM in a plastic petri dish for 2 wk. Four petri dishes with 10 explants each were used for each treatment. Thereafter, all the explants were transferred onto the embryo maturation medium (EMM), cultured for 3 wk and germinated on the embryo germination medium (EGM) for 2 to 3 wk. For desiccation treatment, the explants cultured on EMM for 3 wk were desiccated on two layers of sterilized filter papers in petri dish for 24 h and then transferred onto EGM. The temperature of culture room was 25°C. Culture on EIM and EMM was carried out in the dark and that on EGM under 16-h light (20 μmoles m^{-2} s^{-1} with a fluorescent lamp) and 8-h dark regime. These experiments were replicated 2 times.

Globular somatic embryo formation was observed after a 2-wk culture on EIM. Elongate and scutellar stages of somatic embryos were observed after a 2-wk culture on EMM. Coleoptilar stage and germinated somatic embryos were observed after a 2- and 3-wk culture on EGM, respectively. The percentage of explants producing somatic embryos at each developmental stage was calculated per dish. The germination rate (GR) was scored per dish as follows:

$$GR = \frac{\text{Number of explants with germinated somatic embryo}}{\text{Total number of explants}} \times 100(\%)$$

Germination in the present study means the emergence of both radicle and shoot. Routine observation was done using a stereo microscope.

Significance of the treatment effects on the percentage of explants with somatic embryos and the germination rate was determined using analysis of variance. One-way analysis was used if there was an interaction between the factors. Percentage data were subjected to arc sine transformation prior to statistical analysis.

(2) Scanning electron microscopy (SEM)

The morphogenic development of somatic embryos was examined by SEM. In each experiment, additional petri dishes of cultures were prepared for SEM. The procedures of SEM were described in detail previously (Mariani et al., 1998).

Results

1. Effects of explant stage and cultivar

Somatic embryos formed and developed on the scutellum of the explant (zygotic embryo). The ability to form somatic embryos varied with the physiological conditions of the explant and cultivar used. Fig. 1 shows the formation of somatic embryos from each explant. Only the immature explants 14-17 DAA from both Nipponbare and Sasanishiki produced somatic embryos capable of

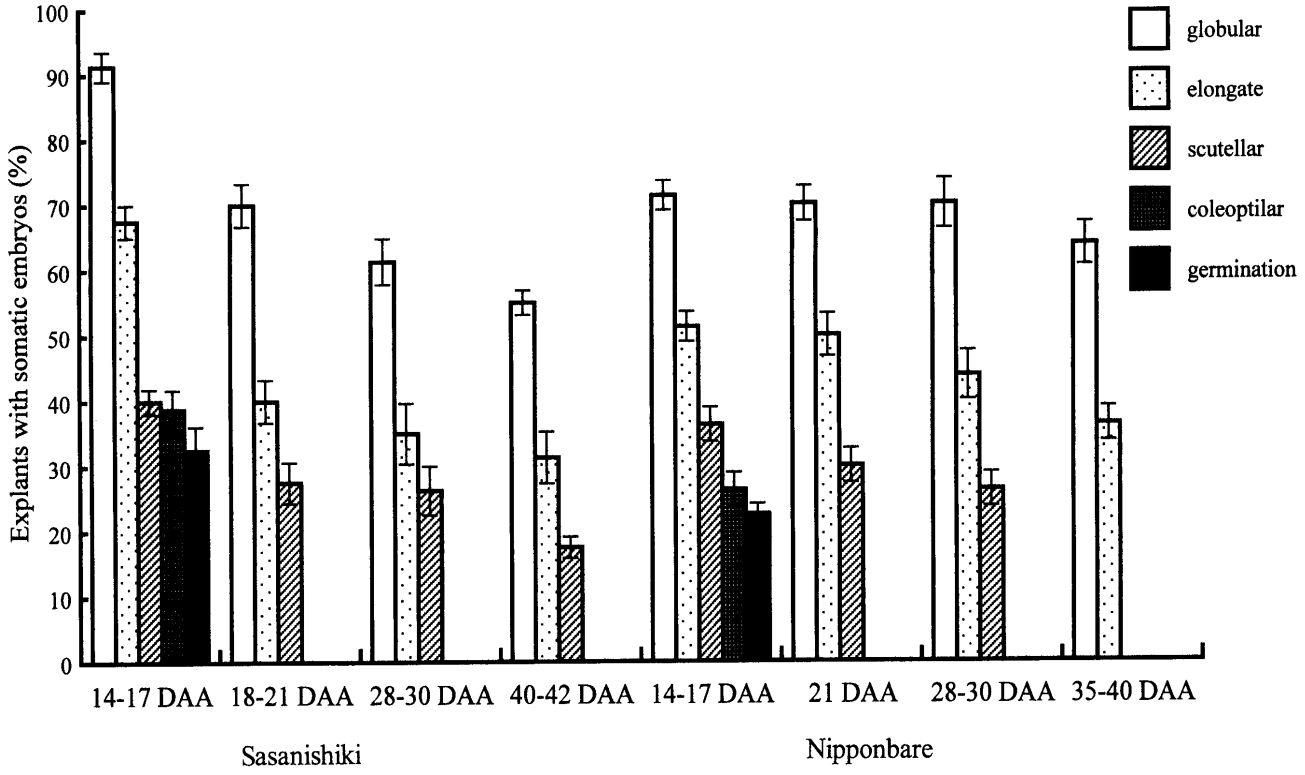


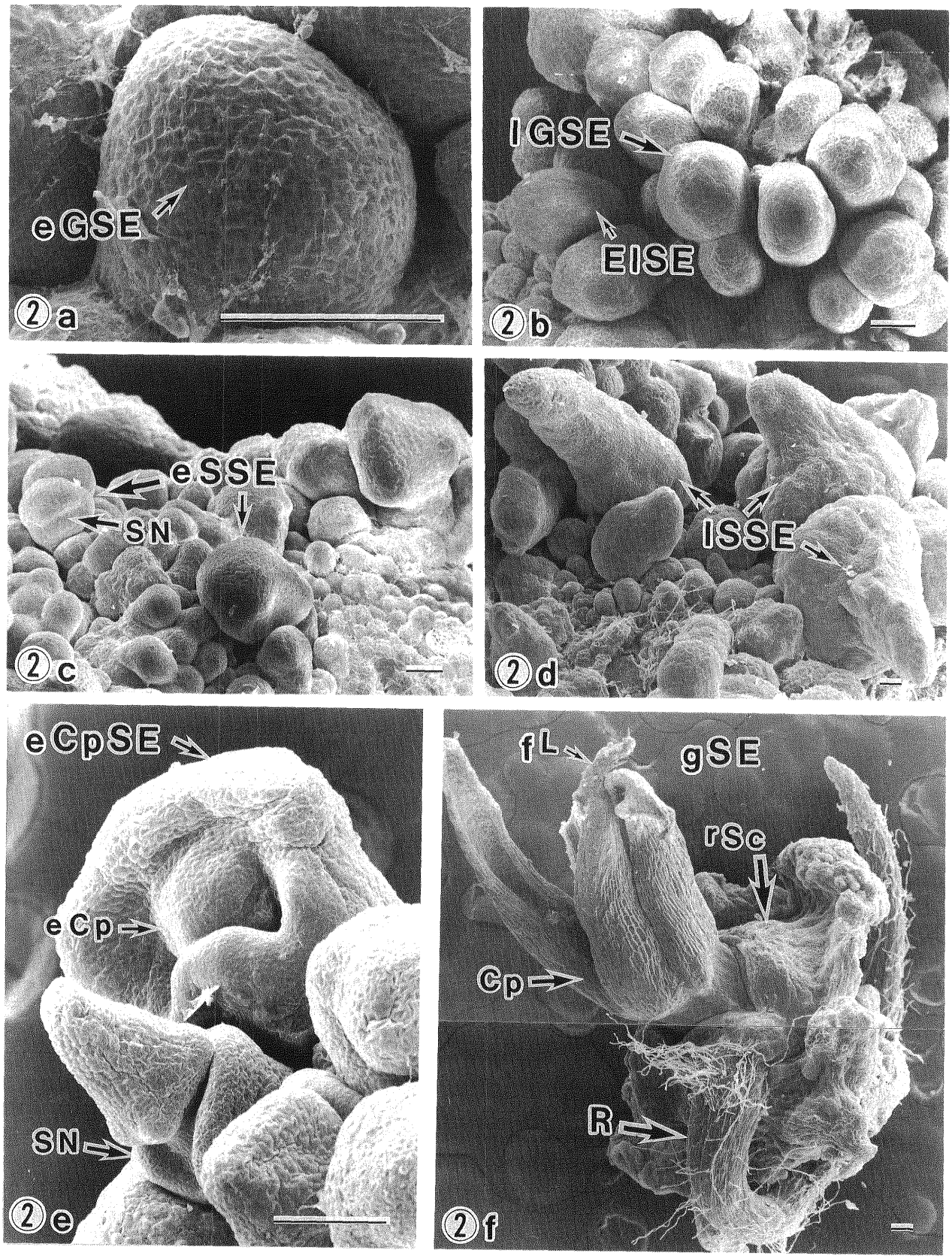
Fig. 1. Effect of the developmental stage (days after anthesis, DAA) of the explants (zygotic embryos) on the percentage of the explants with somatic embryos at various stages. Bars represent standard errors (n=8). The formation of somatic embryo was significantly influenced by the developmental stage of explant at $P=0.001$ or 0.01 except for the formation of globular embryo in cv. Nipponbare.

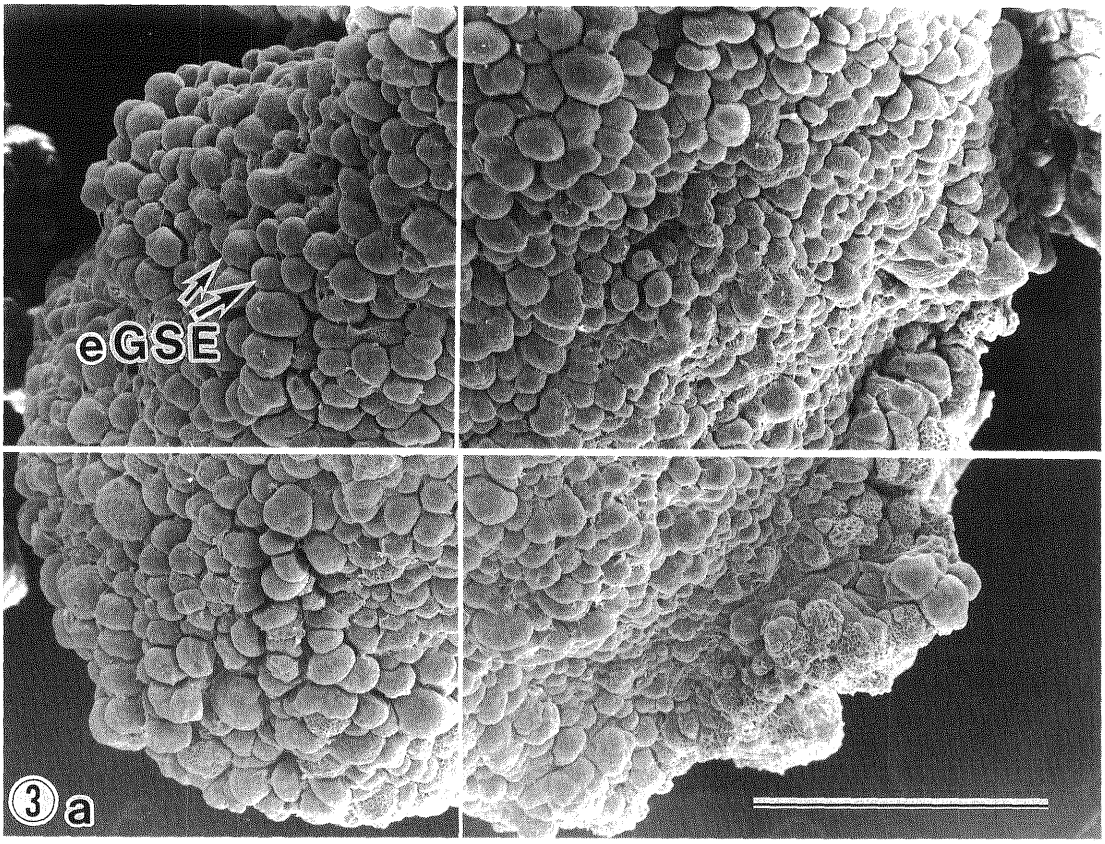
germination. The explants of Sasanishiki obtained 14-17 DAA produced globular somatic embryos on 91% of the explants, but elongate stage embryos only 68% of the explants. The percentage of the explants that produced scutellar and coleoptilar embryos was 40 and 39%, respectively. After all, the percentage of explants with germinated somatic embryos was 32%. The formation of somatic embryos in the explants of Nipponbare obtained 14-17 DAA was similar to that of Sasanishiki obtained 14-17 DAA, although the percentage of the explants with somatic embryos at each developmental stage was higher in Sasanishiki.

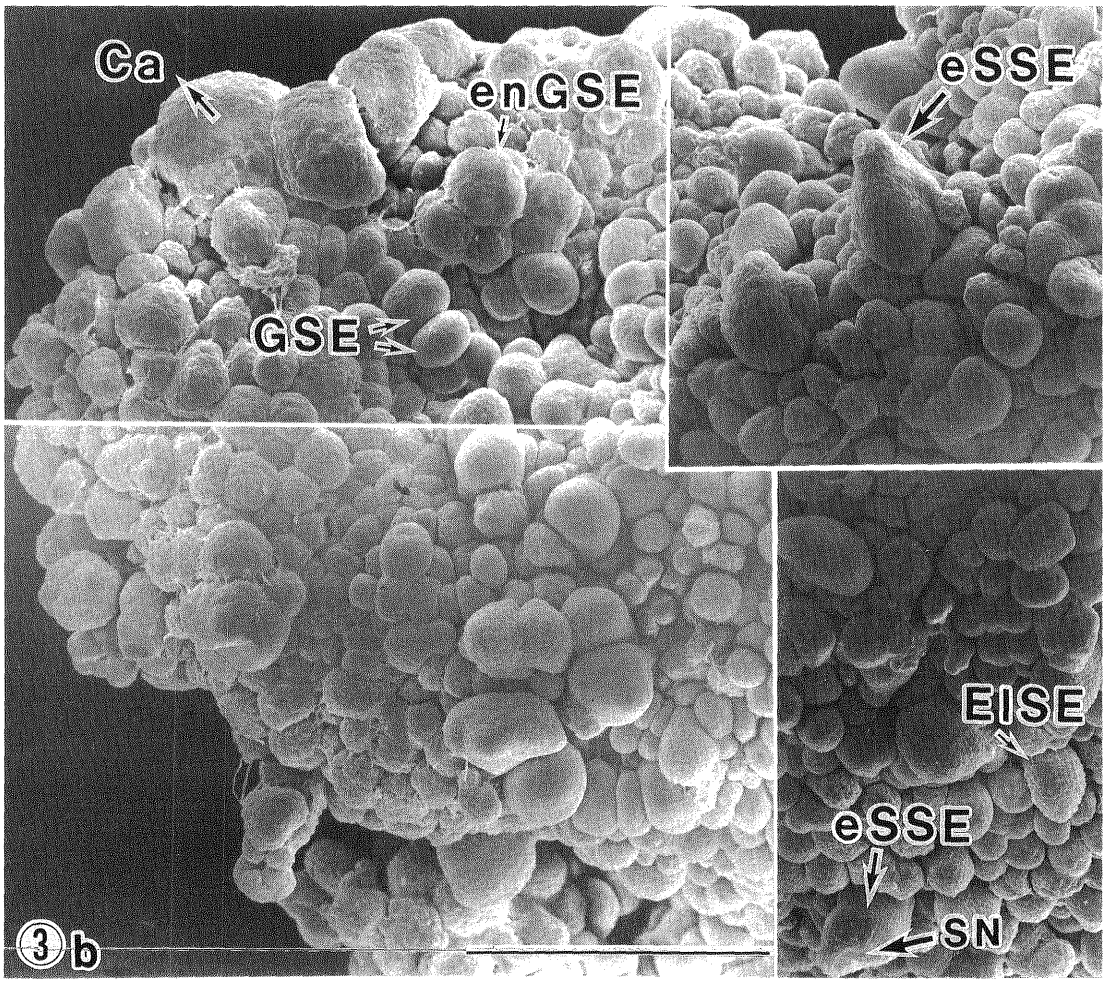
Explants of Sasanishiki obtained 18-21, 28-30 DAA and 40-42 DAA developed somatic embryos up to scutellar stage. Explants of Nipponbare obtained 21 and 28-30 DAA developed somatic embryos that reached the scutellar stage, whereas the explants obtained 35-40 DAA developed somatic embryos that reached only the elongate stage. Thereafter, the somatic embryos at the elongate and scutellar stages in both cultivars could not develop further. Based on one-way analysis of variance, somatic embryo formation was significantly influenced by the developmental stage of the explant at P=0.001 or 0.01 except for globular embryo formation in Nipponbare which was not significant. There was a strong interaction between the developmental stage of the explant and cultivar (P=0.001). Therefore, the developmental stage of the explant influenced somatic embryo formation somewhat differently in the two cultivars.

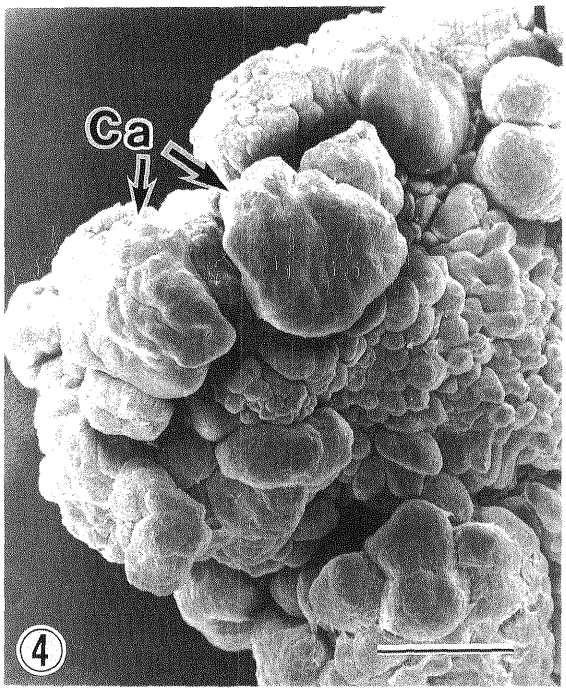
Scanning electron micrographs show morphogenic development of somatic embryos from an early globular stage to the stage at which the embryo is capable of germination. They developed from the scutellum surface of the explants obtained 14-17 DAA from Nipponbare (Figs. 2a-c) and Sasanishiki (Figs. 2d-f). Fig. 2a shows an early globular somatic embryo. The globular embryos developed into elongate somatic embryos as shown in Fig. 2b. Fig. 2c shows the formation of a notch on the scutellum of somatic embryo, which shows the development of the elongate somatic embryo to early scutellar stage. The scutellar notch is a marker of the differentiation of the shoot pole and the root pole. Fig. 2d shows the late scutellar stage and Fig. 2e shows the somatic embryo 6 days after transfer to EGM. The scutellar notch became deeper and wider and the early coleoptile developed from the inner region of the notch. The somatic embryo that had been desiccated for 2 h germinated completely 2 wk after transfer to EGM, as shown in Fig. 2f. The coleoptile, first leaf and root were observed. The coleoptile and root grew from the ventral side of the scutellum. This pattern of germination closely resembled that of the zygotic embryo as shown in Figs. 6a and b.

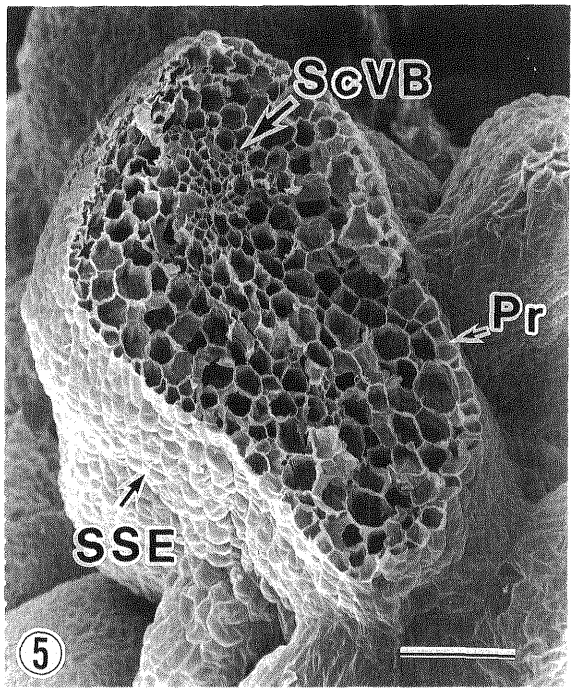
Fig. 3a shows the scutellum surface of the explant of Sasanishiki 40-42 DAA and cultured for 1 wk on EIM. Many globular somatic embryos (GSE) are observed at this stage. After 3-wk culture on EMM, a few elongate- and scutellar-stage somatic embryos developed (Fig. 3b). However, most of the globular- and elongate-stage somatic embryos enlarged and formed undifferentiated structures (callus).

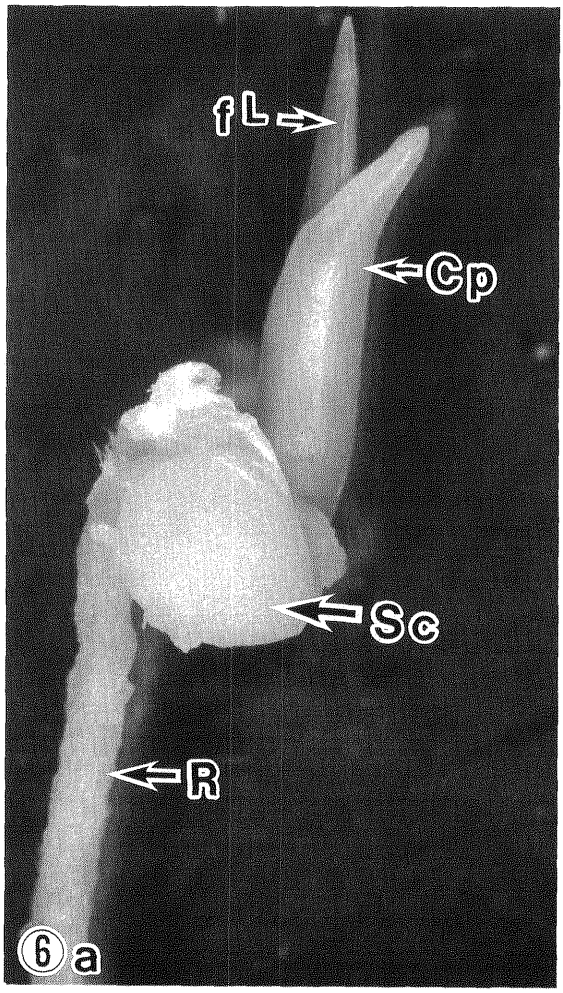


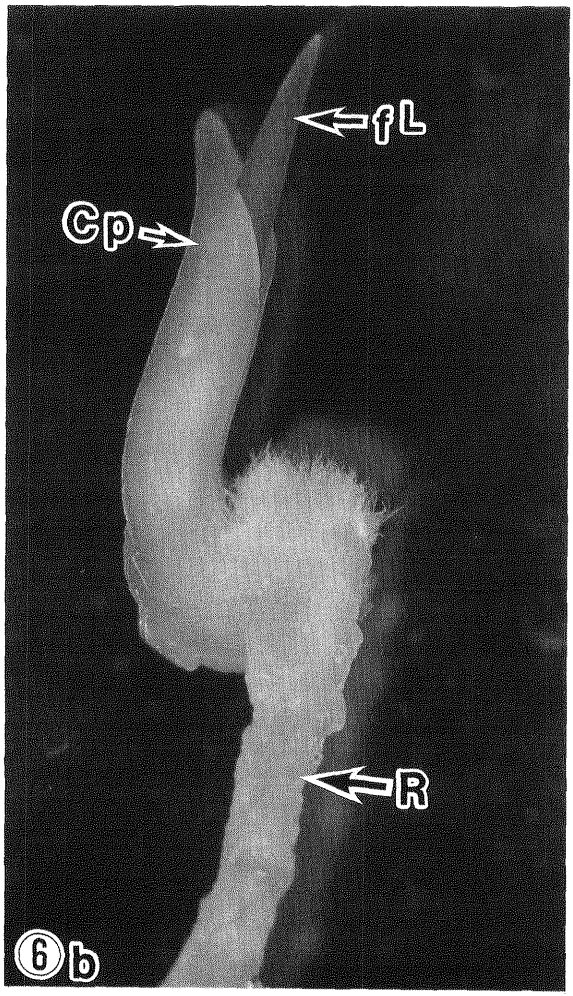












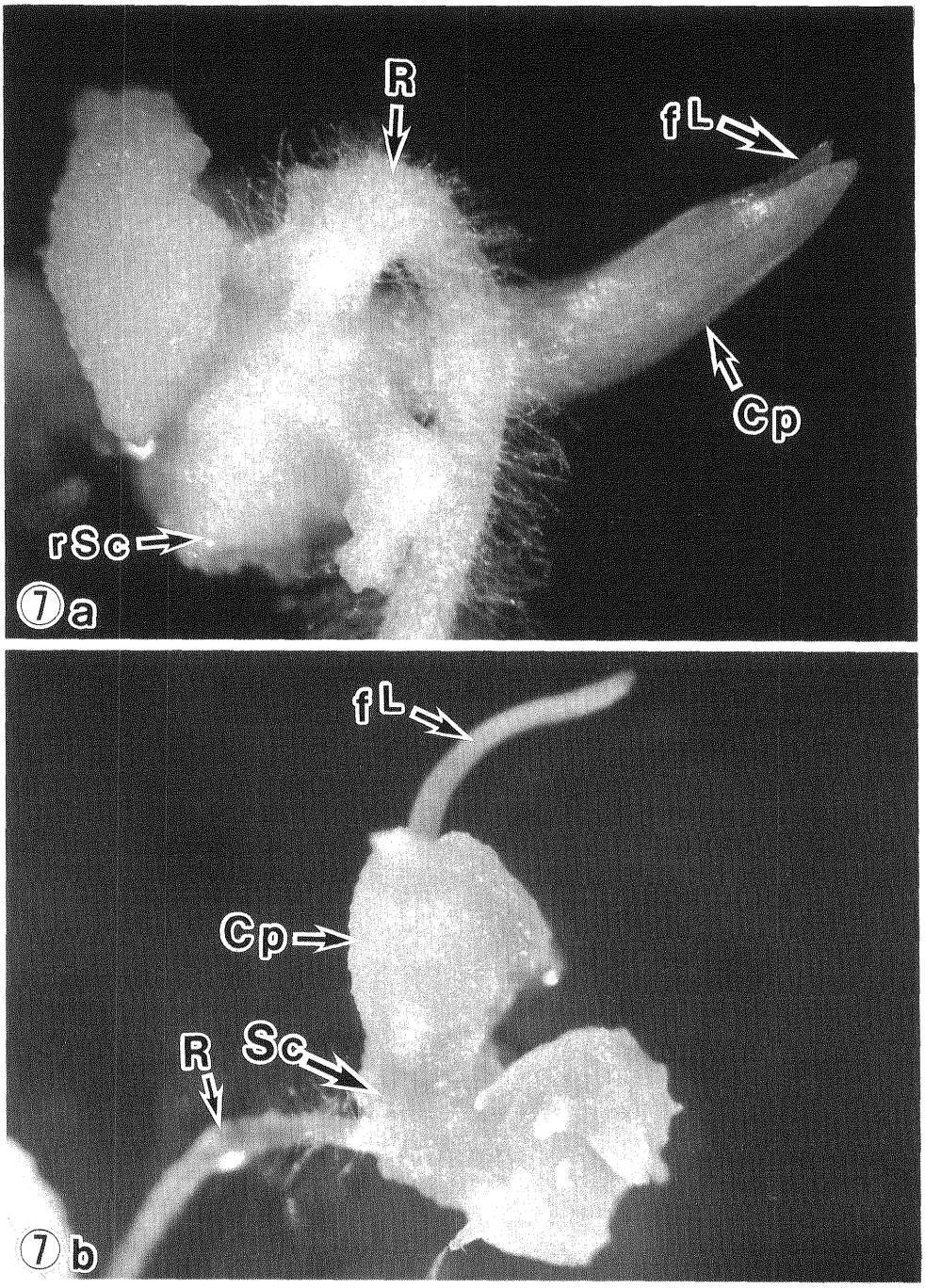


Fig. 4 shows the scutellum surface of the explant of Nipponbare obtained 35-40 DAA and cultured for 3 wk on EMM (Fig.4). The enlarged globular somatic embryo appeared to have formed a callus through the disordered growth, and this character was peculiar to Nipponbare.

Fig. 5 shows a transverse section of the scutellar-stage somatic embryo formed in the explant of Sasanishiki obtained 14-17 DAA and cultured for 2 wk on EMM. The vascular bundle of scutellum is visible in the upper region. Protoderm developed to the epidermal layer of scutellar somatic embryo.

2. Effect of desiccation treatment

Desiccation treatment applied to the maturing somatic embryos affected the germination rate and vigor of germinated somatic embryo. As a result, the somatic embryo subjected to desiccation developed plants more vigorously compared to that without desiccation treatment. Figs. 6a and b show the zygotic embryo 2 wk after germination. The coleoptile and root grew from the ventral side of the scutellum. Figs. 7a and b show the somatic embryos cultured for 2 wk on EGM, after desiccation and without desiccation treatment, respectively. The desiccated somatic embryos germinated and developed to plantlets, which was characterized by the formation of coleoptile and root from the ventral side of the scutellum (Fig. 7a). The pattern of germination of somatic embryos after desiccation treatment closely

resembled that of the zygotic embryos. The coleoptile and root developed after desiccation treatment were more vigorous than those developed without the desiccation treatment (Fig. 7b). In the plantlets developed without desiccation treatment, the coleoptile and the scutellum were swollen and the root and the first leaf were scanty. Probably, the somatic embryos that germinated without desiccation treatment underwent precocious germination.

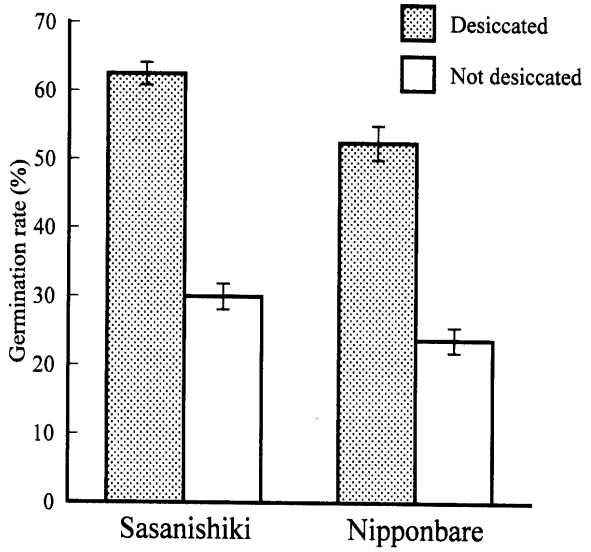


Fig. 8. Effect of desiccation treatment on the germination rate of somatic embryos. Bars represent standard errors (n=8). Effect of desiccation and varietal difference are significant at $P=0.001$.

Fig. 8 shows the effect of desiccation treatment on the percentage of explants that produced germinating somatic embryos. The percentage was 63% after desiccation treatment and 30% without desiccation treatment in Sasanishiki, and 53% after desiccation and 24% without desiccation in Nipponbare. Using two-way analysis of variance, effect of desiccation and varietal difference are significant at $P=0.001$.

Discussion

Only immature zygotic embryos isolated 14-17 DAA produced the somatic embryos capable of germination in both Nipponbare and Sasanishiki. Williams and Maheswaran (1986) described that direct somatic embryogenesis may occur only at certain developmental stages, and in particular cell types. Cells in the maturing scutellum of rice may be heterogeneous in their specialization and only limited cells may be capable of forming somatic embryos, while the cells in the immature scutellum are meristematic and easily form germinable somatic embryos.

There may be varietal difference in the development of somatic embryos from the explants at different developmental stages. Jones and Rost (1989) observed spontaneous germination of somatic embryos from mature rice scutellum of American cultivars, although they did not describe the germination rate. In the present experiment with cv. Nipponbare and Sasanishiki, development of

Explanation of figures

Figs. 2a-f. Morphogenic development of somatic embryo on the scutellum surface of the explant (immature zygotic embryo) obtained 14-17 DAA from Nipponbare (Figs. 2a-c) and Sasanishiki (Figs. 2d-f) (Bars = 0.1 mm). Fig. 2a shows the early globular somatic embryo (eGSM) after 1-wk culture on the embryo induction medium (EIM). Fig. 2b shows the late globular somatic embryo (lGSE) and elongate somatic embryo (ElSE) after 2-wk culture on EIM. Fig. 2c shows the early scutellar somatic embryo (eSSE) after 2-wk culture on embryo maturation medium (EMM). The scutelar notch (SN) is the marker of the somatic embryo polarity. Fig. 2d shows the late scutellar somatic embryo (lSSE) after 3-wk culture on EMM. Fig. 2e shows the early coleoptilar somatic embryo (eCpSE) 6 days after the transfer to EGM. The early coleoptile (eCp) grew from the scutellar notch (SN). Fig. 2f shows the germinated somatic embryo (gSE) cultured for 3 wk on EGM after desiccating for 24 h. Cp, coleoptile; fL, first leaf; R, root; rSc, rudimentary scutellum.

Figs. 3a and b. The scutellum surface of the explant (mature zygotic embryo) obtained 40-42 DAA from Sasanishiki and cultured for 1 wk on the EIM (a) and then for 3 wk on EMM (b) (Bars = 1 mm). Fig. 3a shows the early globular somatic embryos (eGSE), and Fig. 3b shows the globular somatic embryo (GSE), elongate somatic embryo (ElSE), early scutellar somatic embryo (eSSE) with its scutellar notch (SN), enlarged globular somatic embryo (enGSE) and callus (Ca).

Fig. 4. The enlargement of callus (Ca) on the scutellum surface of explant (mature zygotic embryo) obtained 35-40 DAA from Nipponbare and cultured for 3 wk on EMM. Enlarged and disordered growth of globular and elongate somatic embryos are observed (Bar = 0.5 mm).

Fig. 5. The transverse section of the scutellar somatic embryo (SSE) in the explant obtained from Sasanishiki 14-17 DAA and cultured for 2 wk on EMM. The vascular bundle of scutellum (ScVB) is observed in the upper region. The well-developed protoderm (Pr) is visible as the epidermis layer of scutellar somatic embryo (SSE) (Bar = 0.1 mm).

Figs. 6a and b. The zygotic embryo of Sasanishiki 2 wk after germination. The coleoptile (Cp) with the first leaf (fL) is observed, as well as root grown from the ventral scutellum (×12). Fig. 6a shows the dorsal side of the scutellum, but Fig. 6b shows the ventral side.

Figs. 7a and b. The somatic embryo of Sasanishiki after a 2-wk culture on EGM, preceded by desiccation treatment (7a) and without desiccation (7b) (×48). First leaf (fL), coleoptile (Cp) and root (R) developed from somatic embryo subjected to desiccation treatment look healthy, while the first leaf and root are scanty and coleoptile is swollen in the somatic embryo without desiccation. Scutellum (Sc) is swollen in the embryo without desiccation treatment, but rudimentary (rSc) in the embryo after desiccation treatment.

somatic embryos from mature scutellum was limited and the somatic embryos sometimes changed to callus, which was particularly the case in Nipponbare.

Desiccation treatment of maturing rice somatic embryo (scutellar stage) improved the germination rate of the somatic embryos and the vigor of germinated plantlet. Precocious germination was observed in the somatic embryos without desiccation whereas normal germination occurred in those after desiccation treatment. It has been reported in many species that desiccation treatment of somatic embryos enhances their subsequent germination and growth and gives a better synchronization of root and shoot growth (Merkle et al., 1995). Abscisic acid (ABA) is accumulated in both zygotic and somatic embryos during their development (Faure et al., 1998). ABA is necessary for the development of both embryos but is inhibitory for the germination of embryos. In normal seed development, the ABA content decreases during the desiccation process of the seed, and accordingly germination occurs after rehydration without the inhibitory effects of ABA in the zygotic embryo. In the same way, desiccation treatment of somatic embryos leads to the destruction of endogenous ABA, and enhances the germination of somatic embryos (Dronne et al., 1997 ; Find, 1997 ; Samoylov et al., 1998).

Merkle et al. (1995) stated that desiccation is necessary to induce the final maturation of the somatic embryo and that the embryo must reach desiccation tolerance (i.e., physiological maturity) before imposing desiccation on it. Presumably, the physiological acquisition of desiccation tolerance as in the zygotic embryo promotes the final maturation process of somatic embryo, which leads to the normal germination. In fact, Samoylov et al. (1998) reported that the somatic embryos of soybean at the cotyledon stage were ready for desiccation within 4 wk in the maturation medium. Then, the desiccated somatic embryos germinated and produced roots and shoots with leaves after germination on embryo germination medium. In the present experiment, the somatic embryo of rice at the scutellar stage seems to be ready for desiccation treatment which supported the final maturation, and the desiccation decreased the ABA content and increased the germination rate of the somatic embryo.

In conclusion, we found that immature zygotic embryo of rice cv. Nipponbare and Sasanishiki, isolated 14-17 DAA is capable of producing somatic embryos that germinated. Desiccation treatment proved to prevent precocious germination, and improved the vigor of somatic embryo and increased the germination rate.

Acknowledgment

This study was financially supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Culture and Sports (Monbusho). The authors would like to thank Dr. Wayne Parrott, Department of Crop & Soil Sciences, University of Georgia, for sharing the knowledge in the discussion of this paper.

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