Original Paper Plant Biotechnology, 19(3), 173-179 (2002)

Effect of Harvest Seasons on the Efficiency of Ovary Culture in *Panicum maximum*

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Received 22 August 2001, accepted 8 March 2002

Abstract

Cultures were made of ovaries guineagrass (*Panicum maximum*) harvested in different seasons to obtain plant regeneration via somatic embryogenesis. Callus formation and plant regeneration occurred when the ovaries harvested 0-7 days after anthesis (DAA) in summer were cultured on Murashige and Skoog medium with suitable hormones. However, most of the ovaries harvested in winter did not form calli when cultured similarly. To determine the reason for this difference, flowers at 0-7 DAA were collected during both seasons and observed under Nomarski differential interference-contrast optics. The ovaries collected at 0-7 DAA in summer showed normal development and matured seed set-up. In contrast, most of the ovaries collected at 0-7 DAA in winter degenerated gradually. This result suggests that embryo-sac analysis of plant materials provides important information on whether plant materials are suitable for the induction of calli.

Keywords: Embryo-sac analysis, facultative apomictic guineagrass (*Panicum maximum* Jacq.), harvest season, ovary culture, plant regeneration, somatic embryogenesis.

Abbreviations

DAA, day(s) after anthesis; FPA50, formalin : propionic acid : 50% ethanol = 5 : 5 : 90; MS medium, Murashige and Skoog (1962) medium; SEM, scanning electron microscope.

Introduction

Transformation experiments in plants using the *Agrobacterium* method, electroporation, and particle bombardment have been carried out not only in dicots but also in monocots in recent years. As monocot plants are not hosts of *Agrobacterium*, the transformations have been achieved mainly by using particle bombardment methods (Fromm *et al.*, 1990; Vasil *et al.*, 1992; Zhong *et al.*, 1993). Recently, a method of transformation to rice by *Agrobacterium* was reported (Hiei *et al.*, 1994). This method has the following advantages compared with direct gene transformation: no need for special instruments, simple operation, and high repeatability. This method is therefore expected to expand quickly, and to be efficient in monocot plants. Several culture systems have been used for this method, including immature embryos, mature seeds, and protoplasts (George and Eapen, 1990; Lazzeri and Lorz, 1990; Akashi and Adachi, 1991; Hiei *et al.*, 1994). These culture systems usually gave regeneration with somatic embryogenesis. When immature embryos were used, this usually meant the embryos were obtained 3-5 days after anthesis (DAA) or pollination. However, the ovaries of guineagrass at 3-5 DAA are very small, making their extraction difficult. Therefore, in the present study we investigated ovary cultures. In a primary experiment, we found that ovaries at 3-5 DAA taken in the summer gave higher regeneration, and that those taken in winter gave very low regeneration. This study describes the relationship between ovary culture efficiency and timing of collection of ovary materials using embryo-sac analysis and the regeneration system, and details how to establish a simple and efficient ovary culture system.

Materials and Methods

Plant materials

A facultative apomictic variety of guineagrass,

Table 1. Temperatures in summer (May to August, 2000) and winter (November to February, 2000 to 2001) in Miyazaki[1)]

	Summer (Average temperature/month)				Winter (Average temperature/month)			
	May	June	July	August	November	December	January	February
Maximum	25.9	27	31.4	30.8	20.3	15.4	12.5	14.3
Minimum	15.7	19.9	24.1	24.1	12.5	5.6	3	4.7
Average	20.5	23.3	27.4	27.1	16.1	10.1	7.6	9.4
Accumulative	635	700	850	839	473	217	81	135

[1)]The date collected from Miyazaki weather bureau, the Meteorological Agency of Japan.

"Petrie" (*Panicum maximum* Jacq.) was used as plant material. Ovary materials were obtained from blooming plants in fields during summer (May to August, 2000) in Miyazaki, Japan (**Table 1**). During the winter (November to February, 2000 to 2001), the plants were transferred into a greenhouse (maintained at more than 15 ℃) so that they could bloom, after which ovary materials were harvested.

Ovary culture

The flowers were collected from the two seasons at anthesis (0 DAA), and at 3, 5, and 7 DAA, and their first outside crusts were removed with tweezers and treated with ethanol for 1 min. They were then sterilized under 0.5% sodium hypochlorite solution (Antiformin) for 5 min and then washed five times with sterile water for 1 min each. The ovaries were taken out from the flowers under a dissection microscope. All the procedures were performed on a clean bench. They were then cultured in the dark at 25-27 ℃ on the callus-inducing medium: Murashige and Skoog (1962) (MS) medium supplemented with 10.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 10% coconut water and 0.8% agar. The callus formation rate was measured 20 days after the culture began. To check the position where the callus was emerging, the ovary was divided into two parts, embryo and the remaining tissue, which were cultured for callus formation under the same conditions of the ovary culture as described above for the primary experiment (**Fig. 1A, B**).

A white and compact callus formed on each ovary after 30 days of culture. The somatic embryo structures were observed with a scanning electron microscope (SEM) according to Chen *et al.* (2001). The somatic embryos were cut from the original calli and transferred onto MS medium supplemented with 1.0 mg l^{-1} kinetin, 1.0 mg l^{-1} gibberellic acid, 30 g l^{-1} sucrose, and 3 g l^{-1} Gellan gum (pH 5.6-5.8), and cultured at 25 ℃ in lighted conditions (photon flux density = 33.3 μmol $m^{-2}s^{-1}$, 16 h light/8 h dark) for plant regeneration. Regenerated plantlets were then transplanted onto 1/2 MS medium for rooting. The rooted plants were transferred onto pots containing sterilized soil and covered with a plastic bag to maintain a higher humidity for approximately 1 week. After which they were moved into the greenhouse.

Embryo-sac analysis

Over 300 flowers at anthesis (0), and at 3, 5, and 7 DAA were collected during summer and winter for embryo-sac analysis according to the methods reported by Chen and Kozono (1994a, b). The flowers collected were fixed in FPA50 (formalin:propionic acid:50% ethanol = 5:5:90) for 7 days at 4 ℃ . They were then stored in 70% ethanol until use. The ovaries were taken out from fixed flowers under a dissection microscope, dehydrated with ethanol at a series of concentrations (70%, 80%, 90%, 100%, and 100%), cleared in Herr's benzyl-benzoate-four-and-a-half fluid (Herr, 1982) for over 2 h at 0-4 ℃ , and observed under Nomarski differential interference-contrast optics.

Results

Ovary culture

The callus formation rates from ovaries collected in summer and winter were 3.3%, 9.3%, 26.2%, and 72.7% in 0, 3, 5, and 7 DAA ovaries collected in summer, while the respective values for winter ovaries were 0%, 5.7%, 0%, and 5.2% (**Table 2**). Shoots were generated at the 50-60% level from embryogenic calli obtained from the two seasons. The innovated two parts (embryo and remaining tissue) from the same ovary were cultured under the same conditions (**Fig. 1A, B**). A callus formed from the embryo (**Fig. 1C**), and the remaining tissue became brown without callus formation (**Fig. 1D**) after 10 days of culture. We therefore chose the ovary as the explant to induce calli and regenerate plants in this study.

When the ovaries collected in summer were cultured on callus-inducing medium, they became

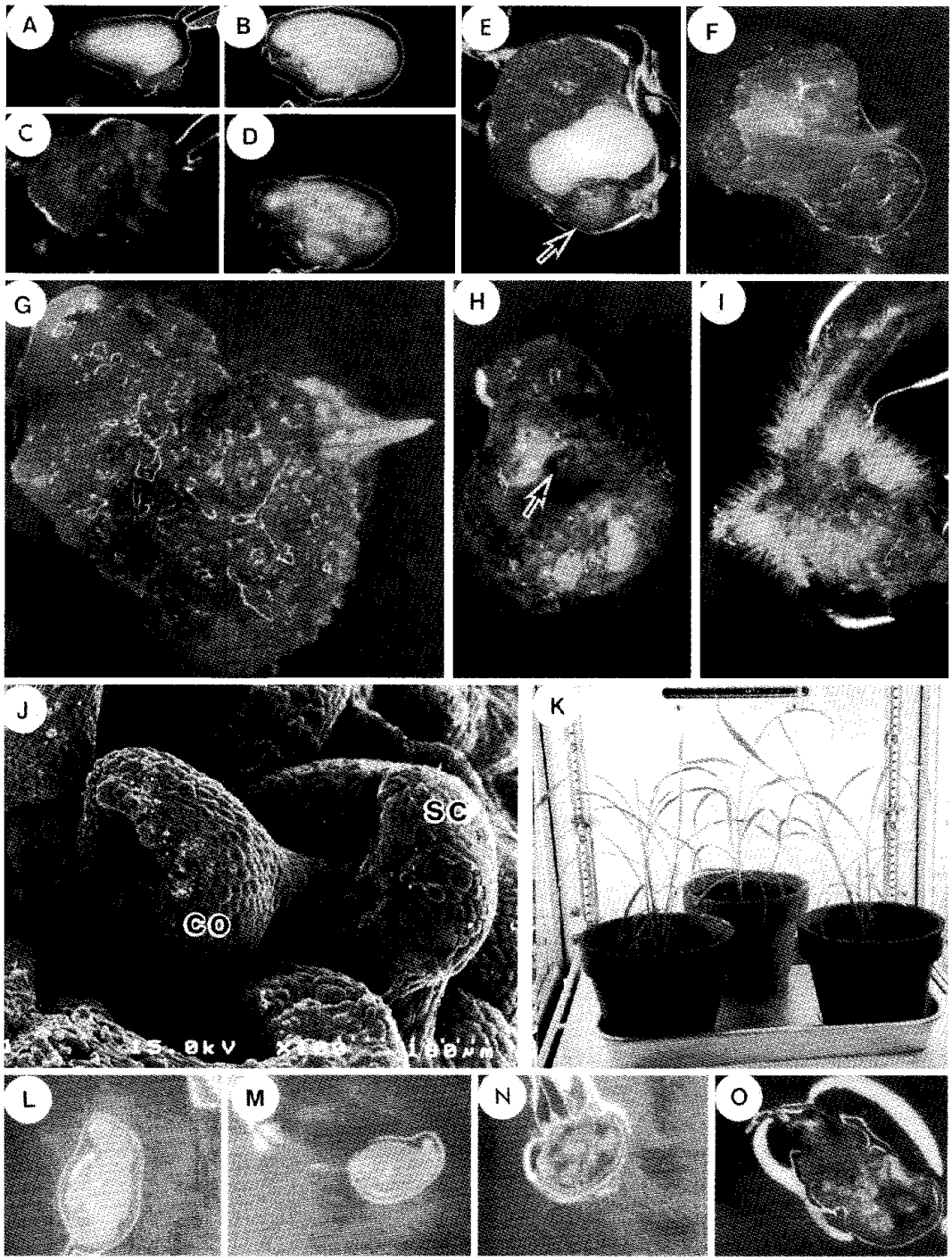

Fig. 1 Culture of ovaries collected in summer (**A**–**K**) and winter (**L**–**O**) seasons. **A**, Innovated embryo; **B**, The remaining tissue from the same ovary cultured on the same medium; **C**, Callus formation from the embryo; **D**, The remaining part browned after 10 days of culture; **E**, Callus formation from cultured ovary (arrow) after 10 days of culture; **F**, Callus of 20 days; **G**, Embryogenic callus with anthocyanin color at 40 days; **H**, Green spot formation (arrow); **I**, Multiple shoot regeneration; **J**, SEM observation of callus of **G** reveals scutellum (SC) and coleoptile (CO); **K**, Complete plants survived after acclimation; **L** and **M**, Ovary collected in winter became brown after 8 and 11 days of culture, respectively; **N** and **O**, The enlarged and browned ovary after 14 and 20 days of culture, respectively.

Table 2. Comparison of callus formation from ovary culture of guineagrass "Petrie" in summer (May to August, 2000) and winter (November to February, 2000 to 2001)[1]

	Days after anthesis (DAA)	No. ovaries cultured (A)	No. ovaries inducing callus (B)	Rates of callus formation (B/A%)	Rates fo shoot regeneration (%)
Summer	0	123	4	3.3	50(2/4)
	3	108	10	9.3	60(6/10)
	5	84	22	26.2	59(14/22)
	7	44	32	72.7	56(18/32)
Winter	0	25	0	0	0
	3	35	2	5.7	50(1/2)
	5	21	0	0	0
	7	38	2	5.2	50(1/2)

[1]The data was collected at 20 days of culture.

Table 3. Fertilization rates of guineagrass "Petrie" observed at 0, 3, 5 and 7 days after anthesis (DAA) in summer (May to August, 2000) and winter (November to February, 2000 to 2001)

DAA	No. ovaries observed (A)	No. ovaries without fertilization (B)	(B/A%)	No. ovaries degenerated (C)	(C/A%)	No. ovaries fertilized (D)	Fertilization rates (D/A%)
Summer							
0[1]	100	97	97	5	5	3	3
3	100	66	66	10	10	24	24
5	100	5	5	7	7	88	88
7	100	23	23	6	3	71	71
Winter							
0[1]	50	50	100	2	4	0	0
3	53	36	67.9	14	26.4	3	5.7
5	58	33	56.9	20	37.7	5	8.6
7	45	14	31.1	28	52.8	3	6.7

[1]At anthesis, (1) one ovary contains one more embryo sac (at a range of 1 - 7 sacs), (2) 98 - 100% of embryo sacs were matured in micropalar end, however, 80 - 100% of the sacs in the other end were matured.

elongated, and **Fig. 1E** and **F** show the transparent calli that formed after 5 and 10 days of culture, respectively. The calli formed were cut from the original ovaries, and subcultured onto fresh medium. After 30 days a white and compact callus containing anthocyanin was formed (**Fig. 1G**). When the compact callus was observed with an SEM, it showed somatic embryo structures with a scutellum (SC) and a coleoptile (CO) (**Fig. 1J**). When the calli were transferred onto regeneration medium, green spots formed on them after 40 days of culture (**Fig. 1H**). Multiple shoots regenerated form these green spots (**Fig. 1I**). The plantlets regenerated were transferred onto 1/2 MS medium for rooting (**Fig. 1I**), and 100% of the plants survived (**Fig. 1K**).

In contrast, when the ovaries were collected in winter and cultured under the same conditions as for the summer ones, the elongation of the ovary became brown (**Fig. 1L** and **M** show this after 8 and 11 days of culture, respectively). **Figure 1N** and **O** show that after 14 and 20 days of culture, respectively, most of the ovaries, were brown, and these eventually degenerated.

Embryo-sac analysis

At anthesis, each ovary usually contains one more embryo sacs (**Table 3**), and the embryo sac at the micropylar end dominantly matured (**Fig. 2A**). A typical aposporous embryo sac consists of one egg cell, two synergids, and one polar cell. The percentage of the mature embryo sacs at the micropylar end was 98 - 100%, which is higher than the 80 - 100% present at the other end at anthesis (**Table 3**).

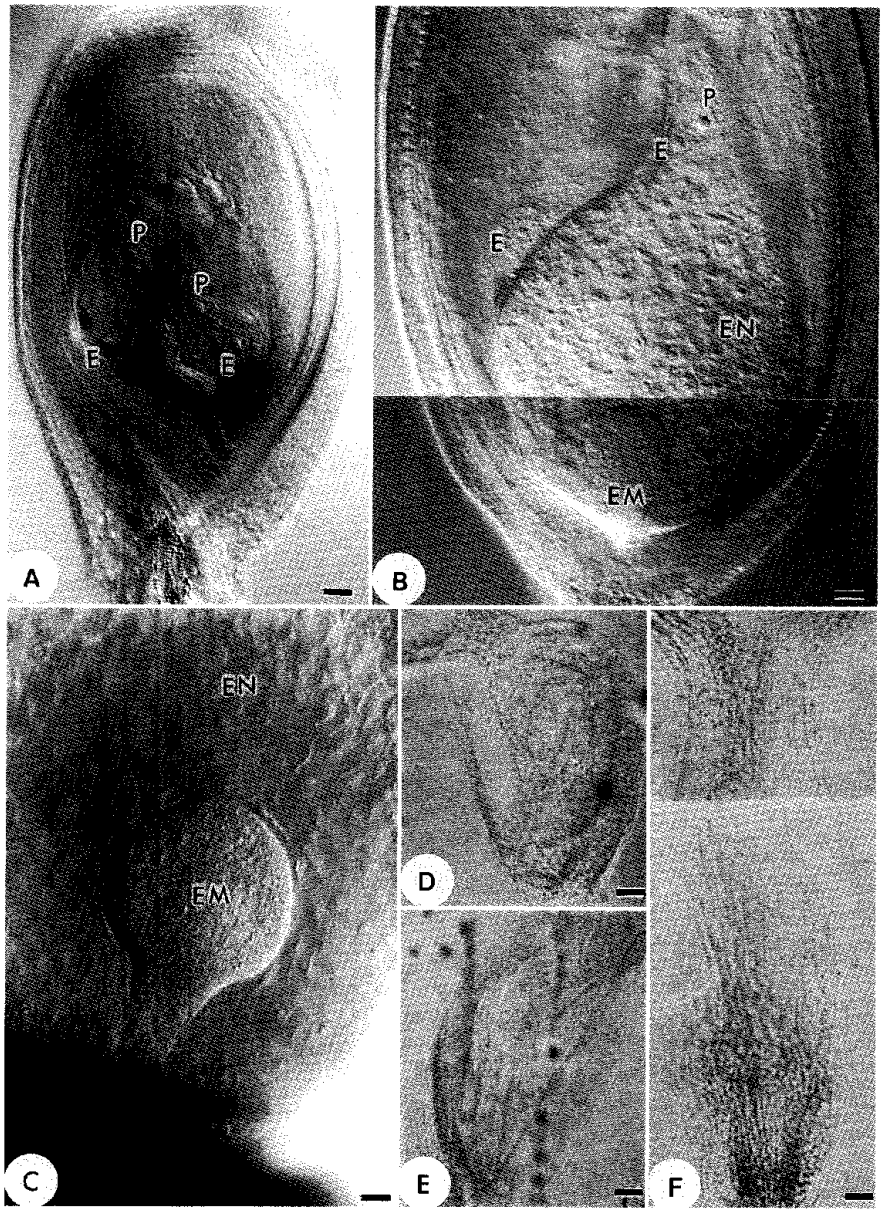

Fig. 2 Cytological observations of embryo sac development in summer (**A**–**C**) and winter (**D**–**F**) ovaries. **A**, Two embryo sacs in an ovule were observed at anthesis; **B**, The micropylar embryo sac with embryo and well-developed endosperm at 4 days after anthesis (DAA); **C**, A well-developed embryo and endosperm in micropylar sac at 7 DAA; **D** and **E**, Degenerating ovules at 3 and 5 DAA, respectively; **F**, Completely degenerated ovule at 7 DAA. E: egg cell; P: polar nucleus; EM: embryo; EN: endosperm. Bar = 45 μm in **A**, **B** and **F**; bar = 100 μm in **C**; bar = 25 μm in **D** and **E**.

There was no difference between winter and summer seasons in both the sac that dominantly matured and the percentage of mature embryo sacs at the micropylar end.

In 0 DAA ovaries, however, a different feature of embryo sac development appeared in both seasons. In summer, the polar cell firstly started to divide into 16 cells (mean value) with free nuclear endosperm, and the egg cell firstly divided into two cells in 3% ovaries (**Table 3**). In 3–5 DAA ovaries, the mean numbers of egg embryos and endosperm cells increased approximately three times per day (**Fig. 2B**). In 7 DAA ovaries, the embryo sac at the micropylar end occupied the space of the whole ovule, with well-developed embryo and endosperm (**Fig. 2C**). Ovaries containing a developed embryo sac were considered to be fertilized. The fertilization rates were 3%, 24%, 88%, and 71% in 0, 3, 5, and 7 DAA ovaries, respectively (**Table 3**).

In contrast, in winter most of the polar cells showed no division and so they were the egg cells in 0 DAA ovaries. In 3–5 DAA ovaries, the matured

embryo sac was unchanged in most ovaries and some showed degeneration, with egg cell or synergid disappearance (**Fig. 2D, E**). At 7 DAA, most of the ovaries were completely degenerated (**Fig. 2F**). The percentages of fertilized ovaries were 0%, 5.7%, 8.6%, and 6.7% at 0, 3, 5, and 7 DAA, respectively (**Table 3**).

Discussion

In this study, it was clear that the season of ovary harvest affected the efficiency of ovary culture in guineagrass: the warmer summer season from May to August in Miyazaki provides better conditions not only for blooming but also for seed setting of the warm land variety "Petrie". When the ovaries collected from summer were cultured, they exhibited good callus formation, and these calli were formed from the embryo part of the ovary. This demonstrated that ovary cultures are preferable, since they are easier to perform than immature embryo cultures (Akashi and Adachi, 1991). The SEM observations revealed that the characterizations of the somatic embryos with SC and CO were similar to those of *Paspalum notatum* (Shatters *et al.*, 1994; Chen *et al.*, 2001) and *Panicum maximum* (Lu and Vasil, 1985; Marousky and West, 1990; Akashi and Adachi, 1991). Multiple shoot regeneration was obtained from somatic embryos cultured on regeneration medium, with 100% of regenerated plants surviving after the acclimation. We are currently performing transformation experiments of the apomixis candidate gene (Chen *et al.*, 1999a, b) using an ovary culture system in *Panicum maximum*.

The guineagrass variety "Petrie" is a warm land facultative apomictic grass that originates from Tanzania, with a favorite temperature of 30-40 ℃ for good growth. The summer season in Miyazaki (**Table 1**) provides good climate conditions for its growth and seed setting, as demonstrated by the normal fertilization rate of approximately 88% being obtained at 5 DAA. This result agrees with that observed at 4 DAA in guineagrass accessions (33-91%; Chen and Kozono, 1994b). And a higher rate (72.7% at 7 DAA) of callus formation from the summer ovary culture coincided with the summer fertilization rates (71% at 7 DAA; **Table 2, 3**). In contrast, during winter a lower fertilization rate (6.7% at 7 DAA) was observed, and a lower rate (5.2% at 7 DAA) of callus formation was also obtained (**Table 2, 3**). Moreover, the 0% callus formation and 8.6% fertilization rate obtained in ovary culture and embryo-sac analysis at 5 DAA in winter (**Table 2, 3**, respectively), may be attributable to sampling errors. From the results of cytological observations and ovary culture in both seasons, we found that the rates of callus formation in ovary culture were directly proportional to the ovary's fertilization rates, and these fertilization rates were affected by the ovary harvest season. Even though the minimum temperature (17.0-24.3 ℃) in summer (**Table 1**) is similar to that of the greenhouse in winter (not less than 15 ℃), the fertilization rates were lower in winter than in summer. The maximum temperatures in summer (24.0-30.4 ℃) and winter (21.3-14.7 ℃) were clearly different. Thus the low fertilization rates in winter may be attributable to the minimum temperature for seed setting (development of embryo and endosperm) not being maintained during winter in Miyazaki.

Apomixis rates are also affected by physiological conditions, such as temperatures (Nakajima and Mochizuki, 1983). According to the weather data, accumulation temperatures (**Table 1**) in the winter season, from November to February, were 81-473, and lower than the 635-850 for May to August. Even when the culture temperature of guineagrass was set at over 15 ℃ in the greenhouse during winter, the accumulation temperatures would be lower than that of November (473), in which the average monthly temperature is 16.1 ℃ (**Table 1**). That also explains why a low fertilization rate was obtained in winter.

In addition, embryo-sac analysis of ovaries revealed evidence of low fertilization rates (**Table 3**), and similar results were obtained in the following year (data not shown). At anthesis, the same rates of maturity (98-100%) of embryo sacs were observed in both seasons. This result indicates that up to anthesis there were no differences between the developments of ovaries collected during the two seasons. It also means that the greenhouse condition in this study allows normal plant blooming and embryo-sac formation. However, the ovaries after anthesis collected during winter showed no active development of the embryo sac, and during the winter fertilization events such as pollen tube elongation and endosperm formation were only observed in a few ovaries. Furthermore, some embryo sacs were completely degenerated by 7 DAA. These results suggest that the lower temperature during winter in this study inhibited the fertilization process and caused higher rates of degenerated ovaries, which resulted in the low culture efficiency of ovaries collected in winter as the callus emerged from the embryo.

Acknowledgement

This study was supported in part by Grants-in-Aid for Scientific Research (A) No. 13306003 from the Ministry of Education, Science, Sports and Culture of Japan.

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