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Microspore Division and Plant Regeneration from Shed Pollen Culture in Rice

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ABSTRACT : An efficient system of rice microspore culture could contribute to the production of genetically modified rice. The microspores were isolated by mechanical or shed methods. The number of microspores per 100 anthers isolated at uninucleate stage was higher than (or similar to) those at binucleate stage in isolation method with pestle or spatular, but microspore divisions were not easily observed on both stages. On the other hand, pollen division in shed pollen culture was observed more frequently at uninuclear than at binuclear stage. Cold pretreatment at 10°C for 10 days resulted in the best multicellular division to produce microcalli at 12.5% efficiency in shed microspores. Heat shock at 33°C for one hour before or after pollen shedding enhanced cell division and callus formation. Out of twelve green regenerants, two were haploids and ten were diploids based on the chromosome analysis of root tips. The size of stoma was 12 ᵐm in haploids and 15 μm in diploids determined by scanning electron microscope (SEM).

Keywords : microspore isolation, shed pollen, cold treatment, heat treatment, ploidy

The potential of microspore-derived homozygous lines in plant improvement has long been recognized and resulted in the development of new rice cultivars (Cho & Zapata, 1988; Kim *et al.*, 1991; Lee *et al.*, 1991). Although anther culture has been successful for the production of homogeneous lines over the last decade, the benefit derived from isolated microspore culture, as compared with anther culture is important to plant breeders for genetics, mutation and biochemical studies. Recently, microspores have been raised as a natural cellular vector for transformation (Cho & Zapata, 1988).

However, the adoption of isolated pollen culture either for pure line production or for genetic manipulation is constrained by its low efficiency in terms of both callus induction and plant regeneration. In addition, isolated pollen culture is much more difficult than anther culture and involves procedures which are unreliable and inefficient. We attempted to optimize the conditions of developmental stages and temperature treatments for plant regeneration.

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MATERIALS AND METHODS

Pollen isolation and culture

A rice variety, Deacheongbyeo (japonica) was used for the experiments. The pollen was isolated at uninucleate or binucleate stages. Spikelets were surface-sterilized with 70% ethanol for 30 seconds, and then the anthers were excised aseptically from the spikelets. The pollen grains were isolated from anthers either by pressing with a spatula or by gently grinding with a pestle method. The isolated pollen grains were filtered through a nylon mesh sieve (pore size 100 μm). Another method for collecting pollens, allowing pollens shed from anthers into liquid medium and collected 14 days later. The pollens from these three kinds of methods were rinsed twice in 0.3 M mannitol by centrifuging at 800 rpm for 3 minutes. The pollen suspension was cultured at 4×10^3 pollen grains/2 ml of N6 medium (Nishi *et al.*, 1968) containing 2 mg/l NAA + 1 mg/l kinetin in petri dishes (30 × 15 mm) for callus formation. The dishes were sealed with parafilm and cultured at 27°C in the dark. Culture media were replenished at 2-week intervals. The dishes placed on a horizontal shaker at 100 rpm under dark condition for the first two weeks and thereafter remained stationary until microcalli developed. Temperature was maintained at 27°C for the whole period of culture. Plating efficiency was determined under the microscope at 10 days after collecting microspores and calculated as the number of multicellular pollen grains/number of observed pollen grains × 100.

Panicle was pretreated with either cold or heat for efficient pollen division. The cold pretreatment of panicles before isolating pollens was at 10°C for 0, 5, 10 and 15 days. The short-heat treatment was done at 33°C for one hour before or after pollen shedding. Untreated heat-shock pollen was incubated at 27°C. After 40 days in culture, microspore-derived microcalli were transferred to a solid medium composed of the basal constituents of N6 medium containing 1 mM of proline/l + 0.2 mg/l naphthalene acetic acid (NAA) + 1 mg/ kinetin for plant regeneration.

Ploidy analysis of microspore-derived plants

Regenerated rice plants were grown in pots in the screening house. The ploidy level of microspore-derived plants was determined by root tip chromosome counts (Chen *et al.*, 1978). For cytological experiments, the materials were fixed in acetic acid-ethanol (1 : 3) for one hour after treatment with 0.003 M 8-hydroxyquinoline solution.

Stomatal observation of regenerants by Scanning Electron Microscope (SEM)

The flag leaves of regenerated plants were collected after heading and observed respective stomata with SEM. Fresh leaf samples were fixed in FAA and dehydrated in a graded series of acetone concentrations. The dried samples were mounted on aluminum stubs, coated with gold using an Eiko Model IB-3 ion coater at 6 mA for three minutes, and examined and photographed using an Akashi Model SX-40 SEM. The number of stomata were counted under microscope and SEM.

RESULTS AND DISCUSSION

Mechanical pollen isolation

Pollens were isolated at either uninucleate or binucleate stage. The greater number of pollens per 100 anthers was isolated at uninucleate (1×10^5) stage than those of at binucleate (8×10^4) in pestle isolation method. However, microspores at two stages was isolated about the same in number as in a spatula isolation method (Fig. 1). Mechanical pollen isolation was efficient in terms of number of pollens,

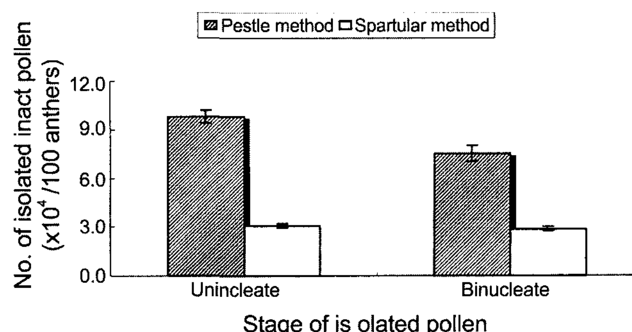

Fig. 1. Effect of developmental stages on numbers of isolated pollen.

but pollen division in culture medium was not easily observed unlike other previous reports (Cornajo-Martin, 1981; Imamura *et al.*, 1982; Polsoni *et al.*, 1988). Pollen division was observed only in shed pollen and pollen at binucleate stage was better than that at uninucleate stage in terms of microcalli production (Table 1). Similar results were reported in asparagus microspore culture. Only shed microspores showed androgenesis at 14 days after culture and resulted in the highest callus yield (Peng & Wolyn, 1999).

Tapetum cells in anther have long been thought to have a nutritive function for cell division in pollen grain development (Chen, 1983). Evidence of medium effect from anther tissue has also been mentioned in *Nicotiana tabacum* shed pollens by Sunderland & Roberts (1977), Taguchi & Mii (1982), and Chen (1983). But direct culture was accomplished with pollens isolated by a pestle isolation method in rice (Cornajo-Martin, 1981), and *Nicotiana tabacum* and *Nicotiana rustica* (Imamura *et al.*, 1982) or by mechanical homogenization of whole racemes in *Brassica napus* (Polsoni *et al.*, 1988). In our results, direct mechanical isolation method were not successful in terms of pollen division and microcalli production. Therefore, further experiments were carried out with shed pollens in culture medium and anthers removed after 14 days of floating.

Development of shed pollen at uninucleate and binucleate stage

The pollen developmental stage at the time of isolation is

Table 1. Effect of pollen developmental stage on plating efficiency in shed pollen culture after anther preculture at 10^0C for 10 days.

Pollen stage	No. of precultured anthers	Plating efficiency			No. of produced microcalli
		No. of observed pollen (A)	No. of multicellular grains(B)	B/A (%)	
Uninucleate	1,020	1,303	81	6.2	14
Binucleate	980	1,078	16	1.5	0

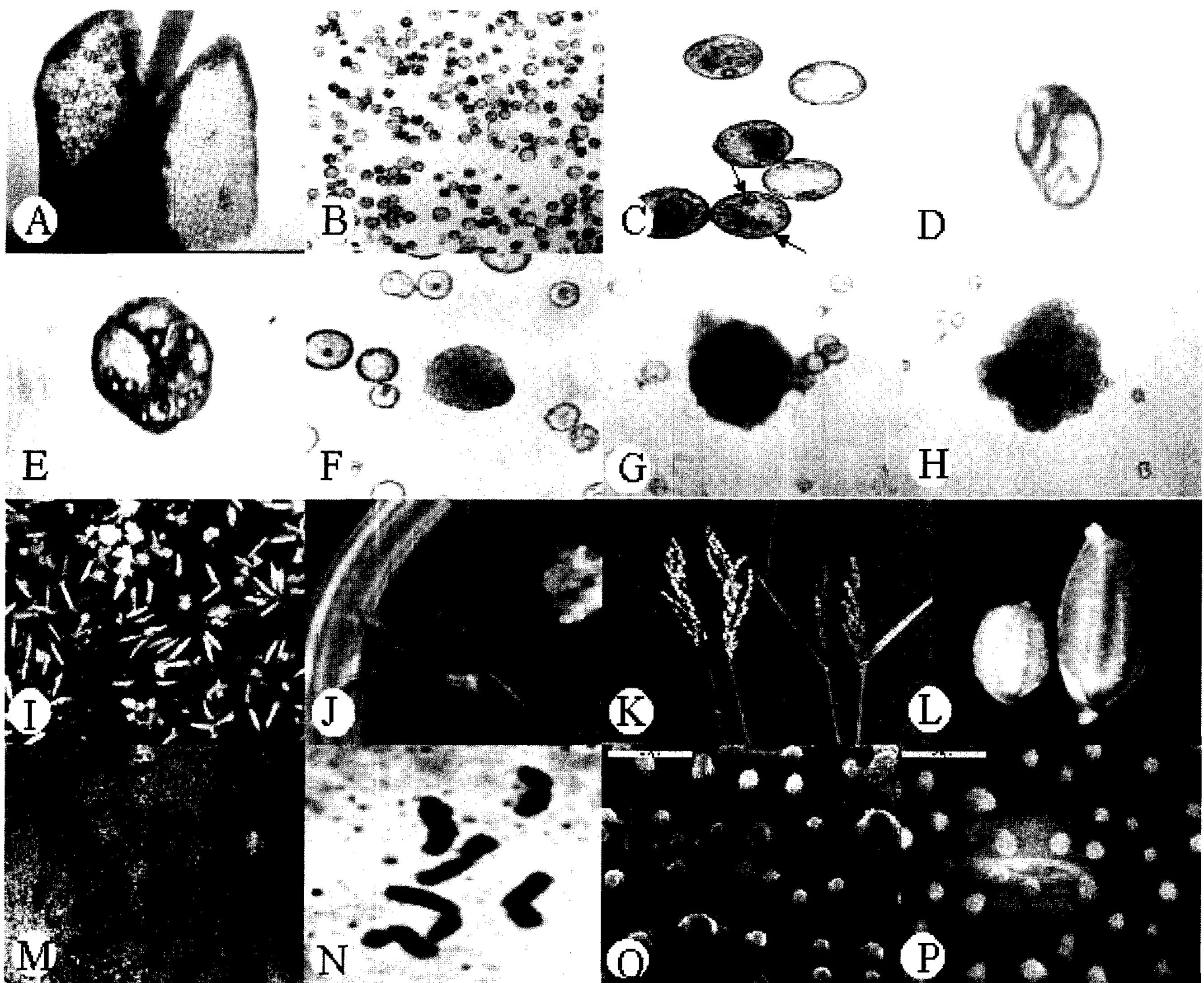

Fig. 2. A series of cell division from shed pollen and analysis of regenerants in cv. Daecheongbyeo. A: empty anther sac after shedding the pollen into the medium, B: collect the shed microspores. C: binucleate formed (arrow). D, E: multicellular stages after three weeks. F: rupture of cell extine and cell clump (G), and microcalli after four weeks (H). I. microcalli produced in culture medium without removing the anthers during the whole culture period. J: plant regeneration. K: diploid (left), and haploid (left) derived from pollen. L: grain of haploid (left), and diploid (right) M: diploid chromosome (2n=24) analysis at root tips. N: haploid chromosome (n=12). O: stoma and pacicle apparatus of the flag leaf in diploid, P: in haploid.

one of the important factors determining the success or failure of isolated pollen culture. The appropriate pollen developmental stage for plant regeneration was not entirely confirmed although most of the pollen grains were at uninucleate stage. As the culture period of floating anther got longer, anthers became empty (Fig. 2-A) due to pollen shedding (Fig. 2-B). At the initial stage of floating of anthers containing uninucleate stage microspores, anthers did not open easily and only containing a few shed pollen over the first 10 days. These shed pollens continued to grow even when the anthers were removed from the culture medium. Shed pollens showing division were mostly in the late uninucleate stage. The shed pollens at uninuclear stage showed higer percentage (6.2%) of cell division than those at binuclear stage (1.5%) (Table 1).

After about 5 days of culture, the pollen was divided into two cells and cytoplasm of some microspores completely disappeared (Fig. 2-C). After 2 weeks of culture, multicellular cells were developed from pollen grains (Figs. 2-D & E). Continued divisions resulted in the production of multicellular structures and microcalli (Figs. 2-F to H). A series of developed pollens were observed in peony (Ono & Harashima, 1981), asparagus (Peng & Wolyn, 1999) and *Nicotiana rustica* (Masaharu & Harada, 1985). Microcalli developed

Table 2. Effect of cold pretreatment (10°C) for different durations on microspore division and callus production in shed pollen culture.

Pretreatment (days)	No. of precultured anthers	Plating efficiency			No. of produced microcalli
		No. of observed pollen (A)	No. of multicellular pollen (B)	B/A (%)	
0	1,142	966	16	1.7	0
5	1,280	1,206	64	5.3	10
10	1,198	1,318	96	7.3	22
15	1,034	968	4	0.4	0

only from the shed pollens at uninucleate stage of shed pollens, but not from those at binucleate stage. Microcalli were vigorously produced when anther was not removed from the culture medium during the whole culture period (Fig. 2-I). However, we did not follow this method for the fear of microcalli developing from anther wall. After 40 days of culture, microspore-derived calli were transferred to plant regeneration medium. Some shoots developed and grew into normal plants (Fig. 2-J).

Based on our results, preculture of anther at binucleate stage of pollen increased the pollen shedding, but pollen culture at binucleate stage decreased the cell division. However, embryos or embryogenic calli were reported to have been developed from binucleate pollen grain in *Nicotiana rustica (*Masaharu & Hiroshi, 1985) and in *Nicotiana tabacum* (Imamura *et al*, 1985). On the other hand, Taguchi & Mii (1982) reported that only microspore of uninucleate and mitotic stage of *Nicotiana rustica* were responded in liquid preculture.

Pollen development in shed pollen culture is affected by many factors such as the developmental stage of microspores at the time of culture, pretreatment, genotype of donor plants, composition of the culture medium, age of the cultivars, etc, (Chen *et al.*, 1978; Ono & Harashima, 1981; Chen, 1983; Taguchi & Mii, 1982; Lee *et al.*, 1991; Ogawa *et al.*, 1992; Peng & Wolyn, 1999).

Effect of temperature treatments on plating efficiency of pollen

We tried to enhance the yield of microspore-derived plantlets through microspore divisions with temperature treatments for the microspore at uninucleate stage. Low temperature pretreatment was very effective in enhancing the cell division and microcalli formation from shed pollen at uninucleate stage. Cold pretreatment period affected microspore response in culture (Table 2).

Without cold pretreatment, mirospores were divided poorer than for 5 and 10 days in cold pretreatment culture. Cold pretreatment for 10 days resulted in the best cell division response and callus formation. About 7.3% of multicellular pollens were developed, and 12.5% of microcalli was regenerated into plants. Microspore division frequency at 10°C for 15 days was the lowest compared to other pretreatment periods. The role of cold treatment from microspore precultured in anther has been discussed in many reports (Taguchi & Mii, 1982; Chen, 1983; Cho & Zapata, 1988; Lee *et al.*, 1991; Kim *et al.*, 1991; Ogawa *et al.*, 1992). Ogawa *et al.* (1992) reported that more than 20 days of cold treatment (10°C) was required for microspores of indica rice to divide; highest colony formation (0.4%) was obtained after a cold treatment at 10°C for 25 days. These results suggest that cold pretreatment duration for increasing pollen division efficiency might be different depending on the genotypes.

Cold shock is known to initiate the normal asymmetric pollen mitosis as well as to keep the microspore alive. Thus, the pollen with two equal-sized cell contributes significantly to the population of grains that undergo embryogenesis through a sporophytic rather than gametopytic pathway (Chen, 1983). Cold treatment may promote the division of vegetative nucleus of bicellular pollens grains with differentiated

Table 3. Effect of heat shock treatment (33°C, one hour) on plating efficiency in shed pollen culture.

Heat treatment	No. of precultured anthers	Plating efficiency			No. of produced microcalli
		No. of observed pollen (A)	No. of multicellular pollen (B)	B/A (%)	
Control	1,020	1,303	24	1.8	2
Before shedding	980	1,842	18	2.1	11
After shedding	1,004	1,752	6	0.8	8

vegetative and generative nuclei. Chen *et al.* (1991) proposed that pretreatment may either shut down or inhibit the function of genes responsible for gametophytic development so that these may be more easily shifted to sporophytic development. In our experiment, the best results were obtained from preculture at 10°C for 10 days.

On the other hand, a short heat treatment was also beneficial for callus induction from pollens. The anthers were heat-shocked at 33°C for one hour before or after shedding the pollens for enhancement of shedding and pollen divisions (Table 3).

Heat shock before shedding and after shedding showed no significant influence on cell division of pollen but promoted the callus formation. Early heat shock like cold shock might have promoted asymmetric pollen division for a sporophytic pathway. By this result, changes in the physiological characteristics of the pollen grains during temperature treatment resulted in their ability to divide. Pollen division was higher when anthers were treated before shedding than after shedding; normal pollen pathway may have been already determined at early stage rather than at late stage. Similarly, Lee *et al.* (1991) reported that increased callus formation was observed when anthers were subjected to cold pretreatment at an 10°C for 8 days, cultured at 25°C for 8 days and followed by 30°C and 35°C heat shock treatment for 2 days. This series treatment was effective for callus induction and plant regeneration. Reddy *et al.* (1985) induced callus from rice anthers by pretreating them at 35°C for 5 min followed by cold pretreatment at 10°C for 7 days or at 35°C for 15 minutes prior to cold treatment at 10°C for 7 days (Zapata & Torrizo, 1986). Sathish (1995) found that a heat pretreatment by immersing the panicles in a water bath at 32°C for 2.5 or 5 hours enhanced the ability of anthers to initiate callus. Comparing cold and heat treatments, the former had greater higher efficiency of cell divisions. It seems that treatment duration is more important than treatment temperature.

In addition, sugar starvation for three days in the medium, various hormones, and amino acids were known to be involved in the enhancement of cell division frequency and cell growth in rice (Taguchi & Mii, 1982; Cho & Zapata, 1988; Ogawa *et al*, 1994; Peng & Wolyn, 1999).

Ploidy analysis

Twelve green plants with various ploidy levels were obtained by shed pollen culture from the whole experiments. The green plants grew normally and ten of twelve plants were spontaneous diploids exhibiting high fertility(Fig. K, left) (Fig. L, right) and two were haploid (Fig. 2-K, right) (Fig. 2-L, left) according to usual evaluation. The ploidy level of regenerated plants were confirmed by chromosome counts in root tips as diploid chromosome (2n=24)(Fig. 2-M) or haploid (n=12) (Fig. 2-N). Peng and Wolyn (1991) reported that haploid plants were produced by shed pollen culture but the frequency was low. All plants were regenerated through callus formation rather than direct embryogenesis. Many factors, such as developmental stage of microspores at the time of culture, pretreatments, genotype of donor plants, composition of the culture medium, and age of the cultivars may affect the ploidy of the regenerated plants.

Stomatal observation of regenerants by Scanning Electron Microscope (SEM)

Rice classification such as wild, indica, japonica, or javanica etc, can be identified based on morphology and physiology. Recently, Rha & Kim (1998) tried to classify rice using microstructures in leaves and Maeda & Miyake (1973) identified between green and albino plants by SEM based on surface structures in leaf blade. The plants containing different number of chromosomes showed difference in arrangement of pacicles with nipple-like projections around the stoma cell (Zhang, 1995).

Plant classification was related to different type of pacicles (Piperno, 1985). Pacicles were evenly distributed near stomata in haploids (Fig. 2-P), while stoma was surrounded by ten pacicles and large-tubercles (23.0 ± 5 μm) was distributed in diploids (Fig. 2-O). On the other hand, there were significant differences on the size of stoma as 12 μm in haploids and 15 μm in diploids. Formation and distribution of pacicles in higher plants are known to be affected by environmental rather than genetic factors. In addition, stoma in diploid appeared open and bigger (Fig. 2-O) than in haploid (Fig. 2-P).

For the application of the pollen culture system for gene transfer, further studies are needed to develop protocol that enhance the rate of green plant regeneration from microspore culture.

REFERENCES

Chen, C. C., H. S. Tsay, and C. R. Huang. 1991. Factors affecting androgenesis in rice (*Oryza sativa* L). In: Y. P. S. Bajai(Ed.), Biotechnology in Agriculture and Forestry, Vol. 14, pp. 193-215. Springer-verlag. Berlin.

Chen, C. H., F. T. Chen, C. H. Chien, and C. H. Wang. 1978. Obtaining pollen plants of *Hevea brasiliensis* MUEll.-ARG (In: Plant Tissue Culture, proceedings of the Beijing (Peking) Symposium. Pitman p11-22. Chen, Y. 1983. Anther and pollen culture of rice in China (In. Cell and tissue culture techniques for cereal crop improvement). Science press & IRRI. pp 11-25.

Cho, M. S. and F. J. Zapata. 1988. Callus formation and plant

regeneration in isolated pollen culture of rice (*Oryza sativa* L. cv. Taipei 309). *Plant Science*. 58 : 239-244.

Cornajo-Martin, M. J. 1981. Anther and pollen grain culture of rice (*Oryza sativa* L). *Euphytica* 30(3) : 541-546.

Imamura, J., E. Okabe., K. Masaharu, and H. Harada. 1982. Embryogenesis and plantlet formation through direct culture of isolated pollen of *Nicotiana tabacum* cv. Samsun and *Nicotiana rustica* cv. *Rustica. Plant and cell physiol.* 23(4): 713-716.

Kim, H. S., Y. T. Lee, S. Y. Lee, and T. S. Kim. 1991. Anther culture efficiency in different rice genotypes under different cold pretreatment duration and culture temperature. Res. Report (Biotec.) 33(1) : 5-13Lee, S. Y., T. L. Lee, and B. T. Jun. 1991. Effect of preculture temperatures and isolation methods on callus formation and plant regeneration in microspore culture. *Korean J. of Breeding* 23(3) : 205-210.

Maeda, E. and H. Miyake. 1973. Surface structure of rice leaf blades observed through a scanning electron microscope. *Proc. Crop Sci. Soc. Japan* 42 : 327-333.

Masaharu, K. and H. Hiroshi. 1985. Studies on conditions for cell division and embryogenesis in isolated pollen culture of *Nicotiana rustica. Plant physiol.* 79 : 90-94.

Nishi, Y., Y. Yamada and E. Takahashi. 1968. Organ redifferentiation and plant restoration in rice callus. Nature (London) 219: 508-509 Ogawa, T., H. Fukuoka, and Y. Ohkawa. 1994. Induction of cell division of isolated pollen grains by sugar starvation. Breeding Science 44: 75-77 Ogawa, T., T. Hagio and Y. Ohkawa. 1992. Plant regeneration from isolated pollen grains in indica type rice. Jpn. J. Breed. 42: 675-679Ono, K. and S. Harashima. 1981. Induction of haploid callus from isolated microspores of peony *in vitro. Plant & Cell Physiol.* 22(2) : 337-341.

Piperno, D. R. 1988. Phytolith analysis: An archaeological and geological perspective. San Diego Academic press, New York.

Peng, M. and D. J. Wolyn. 1999. Improved callus formation and plant regeneration for shed microspore culture in asparagus (*Asparagu officinalis* L.) *Plant Cell report* 18 : 954-958.

Polsoni L., L. S. Kott, and W. D. Beversdorf. 1988. Large-scale microspore culture techniques for mutation-selection studies in *Brassica napus. Can. J. Bot.* Vol. 66: 1681-1684.

Reddy, Y. S., S. Leelavathi, and S. K. Sen. 1985. In fluence of genotype and culture medium on microspore callus, induction and green plant regeneration in anthers in *Oryza sativa.* Physiol. Plant. 63: 309-314Rha, E. S. and J. K. Kim. 1998. Characteristics of phytolith on rice leaf. *Korean J. Crop Sci.* 43 : 205-208.

Sathish, P., O. L. Gamborg, and M. W. Nabors. 1995. Rice anther culture: callus initiation and androclonal variation in progenies of regenerated plants. Plant Cell Rep. 14: 432-436Sunderland, N. and M. Roberts. 1977. New approach to pollen culture. *Nature.* 270: 236-238.

Taguchi, T. and M. Mii. 1982. Effects of chilling, anther preculture and growth regulators on embryogenesis in isolated pollen culture of *Nicotiana rustica* L. *Japan J. Breed.*, 32(4) : 303-310.

Zapata, F. J. and L. B. Torrizo. 1986. Heat treatment to increase callus induction efficiency in anther culture of IR42. Intl. *Rice Res. News*. 11: 25-26.

Zhang, W. 1995. The observation of submicrostructure on leaves-back in Oryza. *Chinese J. Rice Sci.* 9 : 71-76.