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Self-Incompatibility and Embryo Development in *Astragali* Radix

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ABSTRACT : This study was conducted to determine the characteristics of fertilization process and embryo development of *Astragalus membranaceus* Bunge (Astragali Radix) to provide basic data needed in its breeding. *A. membranaceus* showed poor seed setting when self-pollination was induced. When artificial pollination was induced, it showed less than 5% bearing in late August, but more than 13% bearing from the beginning of September 4th. The flower size was about 17.0 ㎜ × 4.0 ㎜ and pistils and stamens had the same length of 15.0 ㎜ at flowering stage. When self-pollination or cross-pollination was induced, pollen tubes extended to an ovule. While pollen tube was extending to the ovule, reproductive cell split and formed two male generative nuclei and a vegetative nucleus. In the case of self-pollination, fertilized embryo was not observed, but was formed in the case of cross-pollination. *A. membranaceus* is noted to have zygote self-incompatibility. In the case of cross-pollination, fertilization was observed in 6 to 8 h after pollination, where apical cell derivatives split after fertilization. A spherical pro-embryo was then formed three days after fertilization. The seed attained full shape with a seed coat showing its distinctive contour 15 days after fertilization. Thus, *A. membranaceus* in Leguminosae family is found to have zygote self-incompatibility although its flower shape is shown to match the self-compatibility plant.

***Key Words* : S**elf-pollination, Cross-pollination, Zygote Self-Incompatibility, Fertilization, Embryo

INTRODUCTION

A. membranaceus belonging to Leguminosae is perennial medicinal crop reproduced by seed propagation. Dried roots are used for medicinal purpose with active ingredients such as saponin in the range of astragaloside I~NIII, isoflavonoid and γ-Aminobutyric acid (GABA), a kind of amino acid (Masaki *et al.*, 1994). *A. membranaceus* belonging to Leguminosae is perennial medicinal herb reproduced by seminal propagation. Pharmacologically it stimulates heart contraction and cardiotonic action as well as diuresis, sedative and uterine contraction function. It's also known to lower blood pressure by expanding coronary, kidney and peripheral vessels (Kim *et al.*, 1998).

Although *A. membranaceus* is one of the important medicinal herbs an oriental medicine as described above and significant income source for farmers, its importance only began to be recognized just several years ago. In the latest, there were researched about the cytogenetic analyses of *Astragalus* species (Kim *et al.*, 2006), and discrimination of *A. membranaceus* (Fish) Bunge from *A. membranaceus* (Fish) Bunge var. *mongholicus* (Bunge) with SCAR marker (Lim *et al.*, 2007). Thus many important aspects of the plant are left open to be discovered.

In order to develop a new varietal improvement, basic physiological research such as flowering, reproduction and genetics must be done beforehand. Moreover *A. membranaceus*, a seed propagation plant, shows sharp decrease in germination when seeds are over a year old. Utilized as a medicine, its tap roots are highly susceptible to rotting from excess moist during the summer rainy season, which results in sharp drop of the yield. Therefore to improve the cultivar it is required to develop one with excess

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moisture tolerance. While the existing research on *A. membranaceus* has some results on the medicinal effect and pharmacological action, no research data are established on its pollination physiology, fertilization and seed setting characteristics.

The purpose of this study is to provide the basic information for breed development by investigating the pollination and embryo development process of *A. membranaceus* flowers.

MATERIALS AND METHODS

This research was carried out in the laboratory, microscope room and experimental field, National Institute of Crop Science with the purpose of histologically observing the pollination mode, fertilization process and embryo development of *A. membranaceus* flowers. Three modes of pollination (open pollination, artificial pollination and bag wrapping) were treated to 200 flowers and the seed setting rates were investigated. In open pollination seed setting rates were examined in natural state. In artificial pollination flower buds had been wrapped with oil paper at the beginning of their formation, then three days before blossoming anthers were removed around 3-5 p.m., then artificial pollination was performed around 10 a.m. the next day, and then seed setting was surveyed after 20 days. In Bag wrapping the flower buds were wrapped with bags at the beginning of their formation, and seed setting rate was surveyed 15 days after blossoming. In order to investigate fertilization process and embryo development, self-pollination (bag wrapping) wrapped flower buds with bags when they began to form while cross-pollination did castration in the afternoon 3 days before blossoming, and pollination around 9-10 a.m. the next day. In the aspect of the investigation time, self-pollinated peduncles were collected when the third floret opened while cross-pollinated peduncles were collected 0.5, 1, 3, 6, 24 h and 2, 3, 5, 10, 15 days after pollination.

Samples were treated with fixing solution, stored in hypotonic solution and utilized in specimen production. They were fixed in fixing solution (95% alcohol : glacial acetic acid = 3 : 1) for 24 hours and then treated 2 hours in 95% alcohol and 85% alcohol respectively, and finally stored in hypotonic solution (75% alcohol). After pollination, elongation of pollen tube within the ovule was investigated by putting pistil in 1N NaOH solution and keeping the solution tube at 60°C for 1 hour, and then in aniline blue solution and keeping the solution tube at 60°C for 1 hour. Next, after placing the ovule on the slide glass, applying a drop of glycerol, putting on the cover glass and pressing lightly with a filter paper, we observed it under a fluorescent microscope (ZEISS-Axioplan). The formation of pistil and stamen and the embryo development process were investigated by producing specimens. Specimens were produced by paraffin method (Senri, 1958), stained with 1% Iron aceto-carmine, and investigated under a optical microscope (NIKON Diaphot).

RESULTS AND DISCUSSION

1. Seed setting characteristics according to pollination mode

In order to study the pollination mode of *A. membranaceus* by inducing self-pollination and cross-pollination, we found out as shown in Table 1 that almost no seed setting was observed in self-pollination after wrapping or castration. Cross or artificial-pollination showed 13% seed setting and open pollination 43%.

The seed setting according to flowering times is shown in Table 2. In the case of artificial pollination the seed setting was under 5% from late July to late August, but was over 10% from early September showing the upward tendency. In the case of self-pollination the seed setting was almost nonexistent until late August but 4-6% from

Table 1. Effects of pollination treatments on seed setting in *A. membranaceus*.

Pollination treatment	Artificial pollination	Self-pollination		Open pollination
		Wrapping with oil paper	Wrapping with gauze	
No. of crossed flower	200	200	200	200
Flower shedding (%)	87	99	100	57
Seed set (%)	13	1	0	43

Table 2. Effects of seed setting on artificial pollination according to flowering times in *A. membranaceus*.

Pollination treatment		Jul.	Aug.			Sep.			Total
		L	E	M	L	E	M	L	
Artificial pollination	No. of crossed flower	250	250	254	270	232	260	245	1,761
	No. of seed set	3	5	7	14	52	49	52	164
	Seed set (%)	1	2	3	5.2	14.7	18.8	21.2	9.3
Self-pollination	No. of wrapped flower	150	150	150	150	150	150	150	1,050
	No. of seed set	0	0	0	0	7	10	9	26
	Seed set (%)	0	0	0	0	4.7	7.7	6.0	2.4

early September.

From these results we can deduce that although in the case of induced self-pollination the rates of pollination increased in early and mid-September compared to July and August, *A. membranaceus* is a cross-pollination plant rather than a self-pollination plant. Wang *et al.* (1988) reported *A. membranaceus* var. *mongholicus* also cross-pollinated by insects and it was difficult to make pure breed by induced self-pollination because bag wrapping produced only 2% seed setting.

Legume, with very similar flower organ structure to *A. membranaceus*, was reported to self-pollinate, and double fertilization was completed within 10 hours after pollen had reached stigma (Fehr, 1980). However, among the mutated legume a lack of self-pollination and partial female sterility were found because compared to a normal flower a mutated flower had longer carpel and bigger receptacle. Moreover the calyx of a mutated flower was located in an abnormal position, and anther was not near the stigma but near the base of pistle wall (Johns & Palmer, 1982).

Thus, while the majority of Leguminosae family self-pollinates and its flower organ structure is favorable to self-pollination, *A. membranaceus* cross-pollinates. The reason behind this has not been clarified yet.

2. Pollination and fertilization process

Some plants in Leguminosae family extend diadelphous upward when the pollination period draws near, and then anther can be located right above and around the stigma, which increases the self-pollination rates (Williams, 1950). *A. membranaceus* flowers belong to Leguminosae family but don't self-pollinate. To discover the underlying reason we investigated flower organ characteristics and fertilization process after pollination. The stigma tissue of *A. membranaceus* flowers is composed of nipples with lateral protuberance joining together (Fig. 1-A). Upon falling on the stigma, the pollen germinated on the surface of membrane covered with stigma secretion and then grew into the style (Fig. 1-B~C). Pollen tube first developed on the stigma before its entry into style (Fig. 1-B~C). The embryo tissue formed secreting block section on which pollen tube grew toward the ovule (Fig. 1-D~F). While the pollen tube was extending, a generative cell divided into two male generative nuclei and one vegetative nucleus (Fig. 1-G). For the entry into the ovule the pollen tube began to envelope the ovule. Up to this point no significant differences were found in the elongation of pollen tube between the bag wrapping and artificial pollination (Fig. 1-I~L). However, *A. membranaceus* of artificial pollination showed distinct shape of ovule while that of induced self-pollination by bag wrapping showed only pollen tube elongation or fluorescent substance around the ovule (Fig. 1-K~L). *A. membranaceus* of artificial pollination extended the pollen tube up to the epidermal cells of nucellus and micropyle of the ovule, and then entered the fiber device of degenerative auxiliary cell during which the end of pollen tube opened releasing two sperm nuclei. One sperm nucleus fused with egg cell and produced zygote which became the first cell of the embryo while the other fused with two polar nuclei and produced endosperm nuclei (Fig. 1-M~N). Thus self-pollinated *A. membranaceus* by bagging grew its pollen tube upward to reach stigma and ovule without any marked differences from artificially pollinated one but didn't accompany any embryo development.

Self-incompatibility (SI) systems function distinguish between pre-zygotic SI with arrest of pollen function at the stigma or style (Matton *et al.*, 1994; Franklin *et al.*, 1995; de Nettancourt, 1997) and post-zygotic SI (Seavey

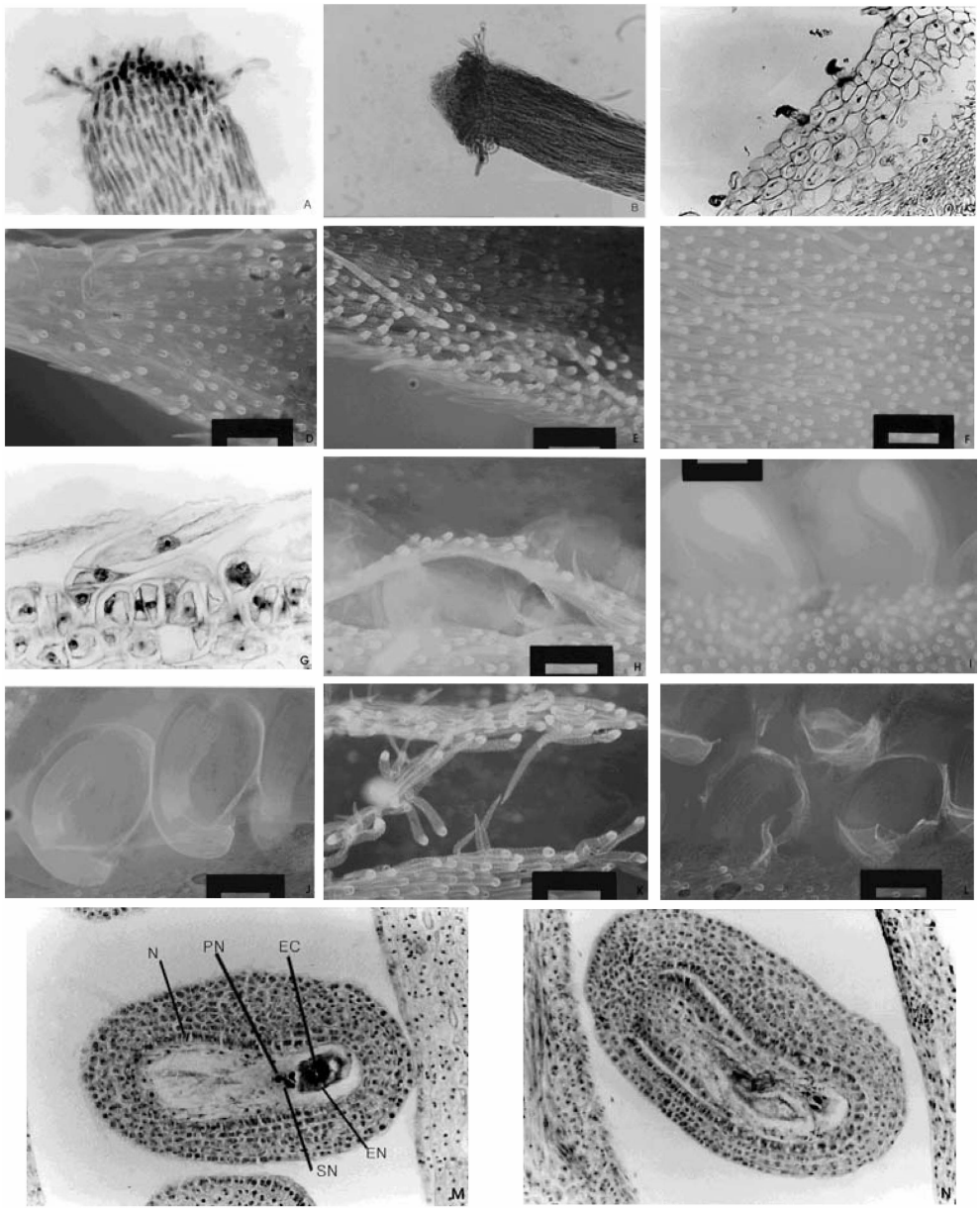

Fig. 1. Pollination and fertilization of *A. membranaceus*.
A. Stigma just prior to pollination B~C. Stigma after pollination
D~F. Pollen tube to elongate toward ovule
G. Pollen tube with two male gametes H. Pollen tube arounding ovule
I~J. Cross-pollinated ovule K~L. Self-pollinated ovule
M. Ovule after fertilization(6~8hrs after pollination)
N. Zygote and primary endosperm nucleus
Abbreviations : EC-Egg Cell, EN-Egg Nucleus, N-Nucellus, SN-Sperm Nuclei, PN-Polar Nuclei

& Bawa, 1986; Gibbs & Bianchi, 1993, 1999; Sage *et al.*, 1994; Gibbs *et al.*, 1999).

The obstruction caused by incompatibility during the fertilization process of plants (Bang, 1999) can be of three types: 1) Obstruction during the pollination stage such as dichogamy, herkogamy and diclinous flowers; 2) Physiological obstruction. In the stage before gamete union, the pollen tube cannot penetrate into stigma, which has been

observed in Cruciferae, Graminae, and the pollen tube cannot grow fully within the style, which has been observed in Liliaceae, Solanaceae; 3) Obstruction at the stage of gamete union. The gamete union cannot be established between the male and female, which has been observed in Gasteria, Hermerocallis.

A. membranaceus showed normal development up to the pollen tube elongation but no embryo development after the entry into the ovule. From these investigation we decided that *A. membranaceus* may belong to the 3rd case of Bang's reference, that is self-incompatibility at the stage of gamete union. Species within the genus *Pseudowintera* exhibit high rates of self-sterility (Tammy & Sampson 2003), and self-sterility in the genus has been previously posited-but not confirmed-to be the result of late-acting ovarian self-incompatibility functioning within nucellar tissue of the ovule to prevent self pollen tubes from entering the embryo sac.

Self-sterility during the zygotic phase of embryogeny in the ovule penetration and double fertilization rates has also been reported in other species from diverse groups of derived angiosperm families, including *Hymenaea stigonocarpa* and *Caesalpinia* spp. of Leguminosae (Lewis and Gibbs, 1999; Gibbs *et al*., 1999), *Rhododendron* spp. (Williams *et al*., 1984; Kaul *et al*., 1986), *Chorisia* spp. and *Tabebuia* spp. (Gibbs & Bianchi, 1993, 1999).

3. Embryo development characteristics

The embryo development process of *A. membranaceus* flowers after the artificial pollination is shown in Figure 2. The vacuole in the fertilized zygote was getting smaller and completely disappeared by the time of first cell division. Transverse division was observed for the first cell division of zygote (Fig. 2-A~B). Apical cell oriented toward central cell became the embryo and basal cell located in micropyle formed suspensor (Fig. 2-B). By the

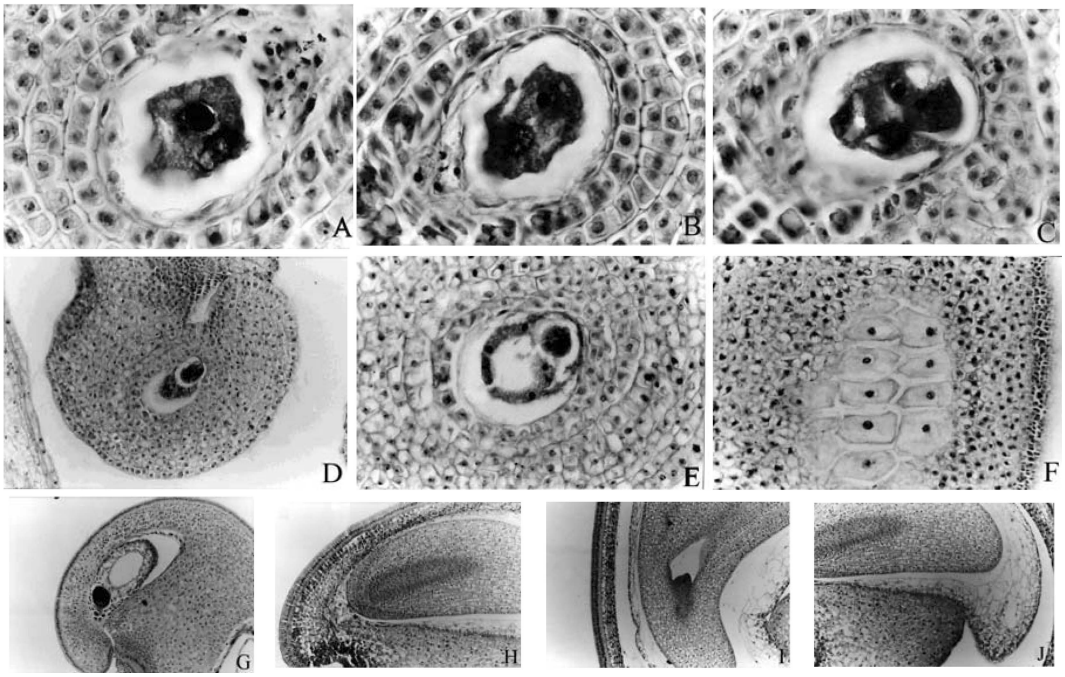

Fig. 2. The stages of embryo development of *A. membranaceus*.
A. Embryo just before division
B. Dividing embryo
C. Four-celled embryo, 2 days after fertilization
D. Club-shaped embryo, 3 days after fertilization
Endosperm surrounding embryo is acellular.
E. Nuclear and noncellular endosperm showing cytoplasm, symmetrical spacing of nuclei, 4~5 days after fertilization.
F. G. Embryo at globe stage with lightly stained suspensor, 6~10 days after fertilization
H. I. J. Late cotyledon stage with abundant cellular endosperm, 11~15 days after fertilization.

2 days from the fertilization four cell embryo formed (Fig. 2-C), and apical cell derivatives kept dividing and formed spherical pro-embryo after 2 days (Fig. 2-D). After 5 days from the fertilization contour of protoderm was evident on pro-embryo, and around 6-10 days after fertilization central vacuole of endosperm was formed and symmetrical arrangement of endosperm was established (Fig. 2-E~F). Structural organization of hypocotyl became distinct and produced protoderm, basal meristematic tissue of the cortex, and procambium. The derivatives of embryo basicyte formed primordia of root, but limited its presence within the small area right above the point where suspensor attached to, that is the end of the embryonic axis (Fig. 2-G). Around 11-15 days after fertilization first leaf primordium was formed at the spot where cotyledon of epicotyl was attached (2-H~J).

The embryo development process of both American ginseng and Korea ginseng is Chenopodium type of which the pro-embryo development process is slow.

In addition Korea ginseng shows distinctive primordium stage while American ginseng does not (Hwang & Miyazawa, 1967). Legume forms pro-embryo similar in size to suspensor 3 days after fertilization and the contour of protoderm becomes distinct in pro-embryo 5 days after fertilization, and cotyledon begins to show by partial division which happened at both poles of pro-embryo located right below the protoderm (Soueges, 1949; Kato *et al.*, 1954; Pamplin, 1963). *A. membranaceus* shows much similarity to legume belonging to the same Leguminosae family, in fertilization, embryo development, and around 15 days after fertilization seed coat of *A. membranaceus* showed distinctive division of macrosclereid and osteosclereid shaping up seed.

CONCLUSION

We aimed to find the characteristics of fertilization process and embryo development of *A. membranaceus* to provide basic data needed in its breeding.

In the case of self-pollination, fertilized embryo was not observed, but was formed in the case of cross-pollination. *A. membranaceus* is noted to have zygote self-incompatibility. Fertilization was observed in 6 to 8 h after pollination in the cross-pollination, where apical cell derivatives split after fertilization. A spherical proembryo was then formed three days after fertilization. The seed attained full shape with a seed coat showing its distinctive contour 15 days after fertilization. To conclude, *A. membranaceus* is found to have zygote self-incompatibility although its flower shape is shown to match the self-compatibility plant.

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