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# *In vitro* flowering and mating system of *Eulophia graminea* Lindl.

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**ABSTRACT.** The capsules of *Eulophia graminea* Lindl. were collected from a seashore and a city of subtropical Taiwan. The seeds from the capsules sown *in vitro* and germinated, hence developed into rhizomes where they either sprouted to form flower stems or developed leaf shoots with roots. The subculture rhizomes that produced a large amount of flower stems generated flowering. The plantlets with flower stems were then cultured in a medium for fruition. Some capsules formed through the autogamous mating system were harvested and their seeds were sown *in vitro*. A total of 4 generations were cultured over a 4 year period. There are three explanations that support the findings of the *E. graminea* and its strong colonization ability: the unique storage structure of the rhizome, the brief juvenile stage and the autogamous mating system.

**Keywords:** Autogamy; *Eulophia graminea*; Flowering; *In vitro*; Mating system; Rhizome.

## INTRODUCTION

The terrestrial orchid, *Eulophia graminea* Lindl., native only to subtropical and tropical Asia, has naturalized in Miami-Dade County in southeastern Florida; and found in woodchip mulched garden beds in the Northern Territories, Australia (Pemberton et al., 2008).

Within its native range, the *E. graminea* occurs in diverse habitats. In Singapore, it is reported to be widespread on wasteland, sandy beaches, lawns, roadsides and other exposed areas, as well as in secondary forests and parks (Tan and Sin, 1993). In Taiwan, the orchid grows among lowland shrubs, sandy beaches and coastal grassland (Su, 2000).

In 1997, experiments were done which suggested that *E. graminea* is a self-compatible but pollinator-dependent out-crossing species (Sun, 1997). Pemberton et al. (2008) performed pollination treatments which determined that the flowers of *E. graminea* are self compatible, but less fruit were set in self pollinated flowers, than in out-crossed flower. No fruit set occurred while plants were in isolation from pollinators; indicating that *E. graminea* is not autogamous. Wu (2004) tested the mating system of *E. graminea* in the wild of Tainan, Taiwan. There was 3.4% (2/69) fruition occurred in isolation from pollinators in the bagging plants.

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Williamson (1984) observed a mechanism in live plants by which self-pollination may be affected in eight species of the *Eulophia* from South Central Africa. At a certain level of maturity in the flowers, the pollinium becomes active and outgrowths begin to appear from the lower border of the pollinia to the stigma.

Wild capsules from the *E. graminea* were collected from a seashore and a city in Taiwan and brought to the laboratory where the seeds were harvested from the capsules and then propagated seeds *in vitro*.

Within this culture system, we could clearly observe the conversion morphology, flowering, and mating system in the medium. This significant data facilitate the understanding of the strong colonization capability of the *E. graminea* in a feral environment.

## MATERIALS AND METHODS

### Plant material and seed germination

Capsules of *E. graminea* were collected from two locales in Taiwan: a seashore in Wuchi, (Taichung County July, 2002), and a roadside in Nankang (Taipei City, Taiwan, June, 2004). The capsules were sterilized with 2% NaOCl supplemented by 1 drop of Tween 20 for 15 min. The seed generation medium (GM) consisted of 1/4 strength MS salts (Murashige and Skoog, 1962), supplemented with (mg $l^{-1}$): niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), *myo*-inositol (100), activated charcoal (1,000), peptone (1,000), potato powder (6,000), coconut milk (150 ml/l), sucrose (20,000) and

gelrite (4,000). Medium pH was adjusted to 5.2 before autoclaving for 15 min at 121°C. The seeds were sown in 20×150 mm Pyrex tubes each with 9 ml GM medium.

Protocorms were subcultured in the same medium for rhizome development. The rhizomes were subcultured for a period between 4-6 months. All rhizome cultures were exposed to artificial light of 1000 lux (daylight fluorescent tubes FL-30D/29, 40 w, China Electric Co, Taipei, Taiwan) with an average light/dark cycle of 16/8 h at 25 ± 1°C.

### *In vitro* fruit set.

Plantlets with a 1-cm pseudobulb of *E. graminea* were transferred to the induction medium for fruit setting (FM). The medium formula was WPM basal salts (Lloyd and McCown, 1981) with (mg $l^{-1}$): *myo*-inositol (100), activated charcoal (1,000), BA (1.0), NAA (0.6), sucrose (20,000) and solidified with agar (7,000). The medium pH was adjusted to 5.2. The *in vitro* capsules were collected and seeds were sown on the GM medium.

All statistical analyses were carried out using One-way ANOVA Duncan's multiple range test at a 95% confidence level with the COSTAT statistical software.

### Scanning electron microscopy

Samples for scanning electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 4 h, then dehydrated using an ethanol series, dried in a critical-point dryer (HCP-2, Hitachi, Japan), and finally coated with gold using an ion coater (E1010, Hitachi, Japan) before viewing with a Hitachi S-3000N scanning electron microscope (Chang et al., 2005).

### *In vivo* pollen tube growth

The style of *in vitro* self-pollinated flowers were collected and fixed in FAA (50% ethanol : acetic acid : formaldehyde = 18:1:1) for 24 h, rinsed in demineralized water, and softened in 3 M NaOH for 2 h at 60°C. Afterwards, the style were rinsed in demineralized water and placed in a drop of aniline blue solution with 2% glycerol, squashed under a cover slip. After staining, it was observed by fluorescence microscopy (Peter et al., 2004).

## RESULTS

### Seed germination and plantlet establishment

Seeds of *E. graminea* (Figure 1a) were sown *in vitro*. After 2-3 months, the embryos swelled and broke out of the testa, and then formed protocorms (Figure 1b). The protocorm enlarged, and produced rhizomes with multiple buds (Figure 1c). The six month old rhizomes were subcultured in the same medium and then subcultured again for 4 months. The apex of the rhizome formed either vegetative buds (Figure 1d) or flower stems (Figure 1e). After the vegetative buds grew into a complete plantlet with expanded leaves, and were transplanted in a greenhouse where they survived and were healthy (Figure 1f). Subsequently, several plants were blooming the following year.

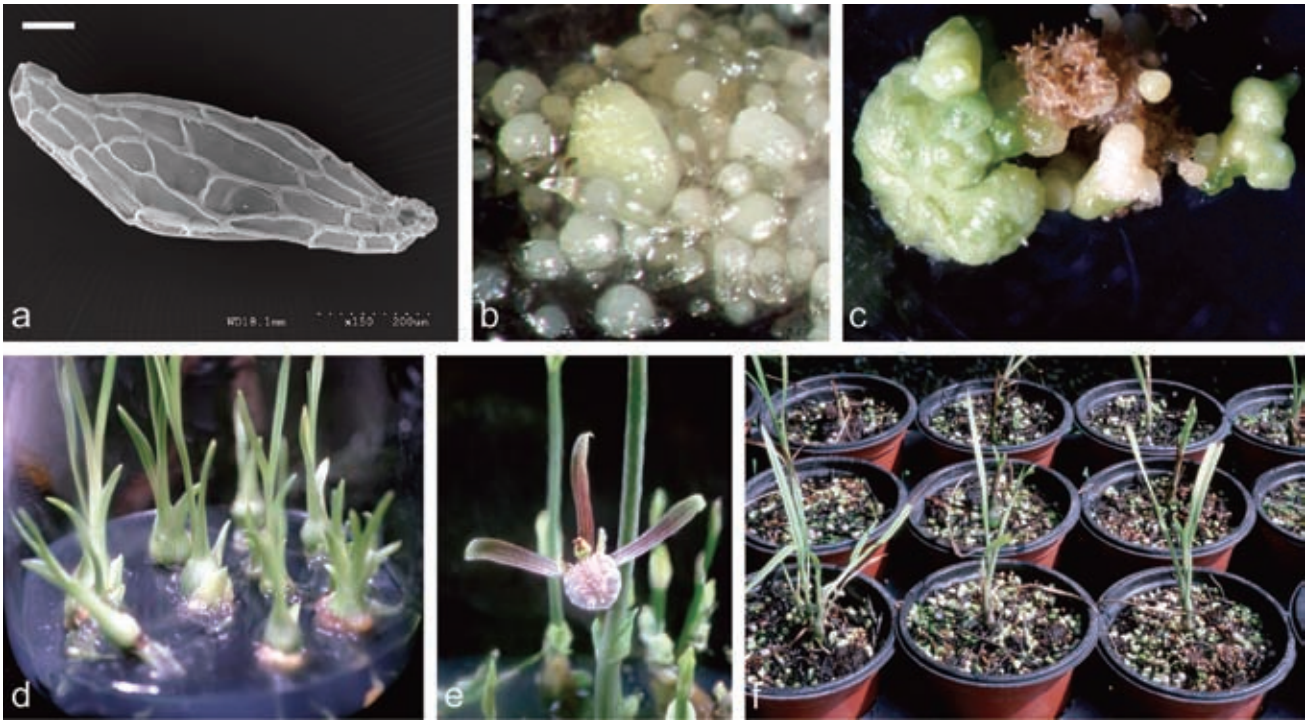


**Figure 1.** The seed germination, rhizomes development, plantlet establishment and flowering *in vitro* of *Eulophia graminea*. (a) Seed (bar = 70 μm); (b) Protocroms (bar = 100 μm); (c) Rhizomes (bar = 0.60 mm); (d) Shoots derived from rhizomes (bar = 1.3 cm); (e) Flower stems derived from rhizomes (bar = 6 mm); (f) Healthy seedling grown in greenhouse (bar = 1.9 cm).

### The ordinary phenomenon of *in vitro* flowering

12 months after being sown, the flower stems protrude and elongate from rhizomes derived from seeds collected from different locales (Wuchi, Taichung County and Nankang, Taipei City), showing 5.3% and 6.8% flowering of all the samples collected and cultivated.

### The morphology of *in vitro* flowers

Upon the investigation the third subcultures, 44-61% rhizomes spontaneously produced flower stems from the apex. Most *Eulophia graminea* rhizomes formed flower stems only, while other rhizomes formed flower stems and shoots. The rhizomes that formed flower stems or only had fewer and smaller flowers than those of the plants grown *in vivo* (the greenhouses) (Table 1). A comparison of the *in vitro* and *in vivo* flower stem indicates that 10 cm *in vitro* flower stems produced up to 2.4 flower buds, where as plants grown *in vivo* (the greenhouse) produced 11 flowers for each 30 cm flower stems. There were 23.4% *in vitro* flower buds that did not bloom.

There was 50% flowers of the *E. graminea in vitro* integrity flower parts (Figure 2a), 40% of the flowers lacked both the left petal and the right petal (Figure 2b), and 10% lacked the right flower petal only. The *in vitro* plantlets had similar flower lengths as those of the plants grown *in vivo* but the labellum and ovaries were shorter (Table 2). Moreover, not many of the *in vitro* flowers fully opened and their petals also deteriorated. All the *in vitro* flowers had a normal column, and functional anther and stigma (Figure 2c, d).

### *In vitro* fruiting

The life-spin of *in vitro* flowers was 2-3 weeks. The column apex shown- with the anther cap lifted to expose the globular pollinia following the blooming flower (Figure 2c, d). The pollinia shown below the main portion

**Table 1.** Characteristics of *in vivo* and *in vitro* panicle and flower of *Eulophia graminea*.

| | Panicle* | | Flower* | |
|---|---|---|---|---|
| | Length (cm) | Flower buds. | Length (cm) | Width (cm) |
| *In vivo* | 32.6 a | 11.2 a | 2.2 a | 2.5 a |
| *In vitro* | 10.4 b | 2.4 b | 1.7 b | 2.0 b |

*The means with 10-20 replicates with the same letters were not significantly different at $p<0.05$.

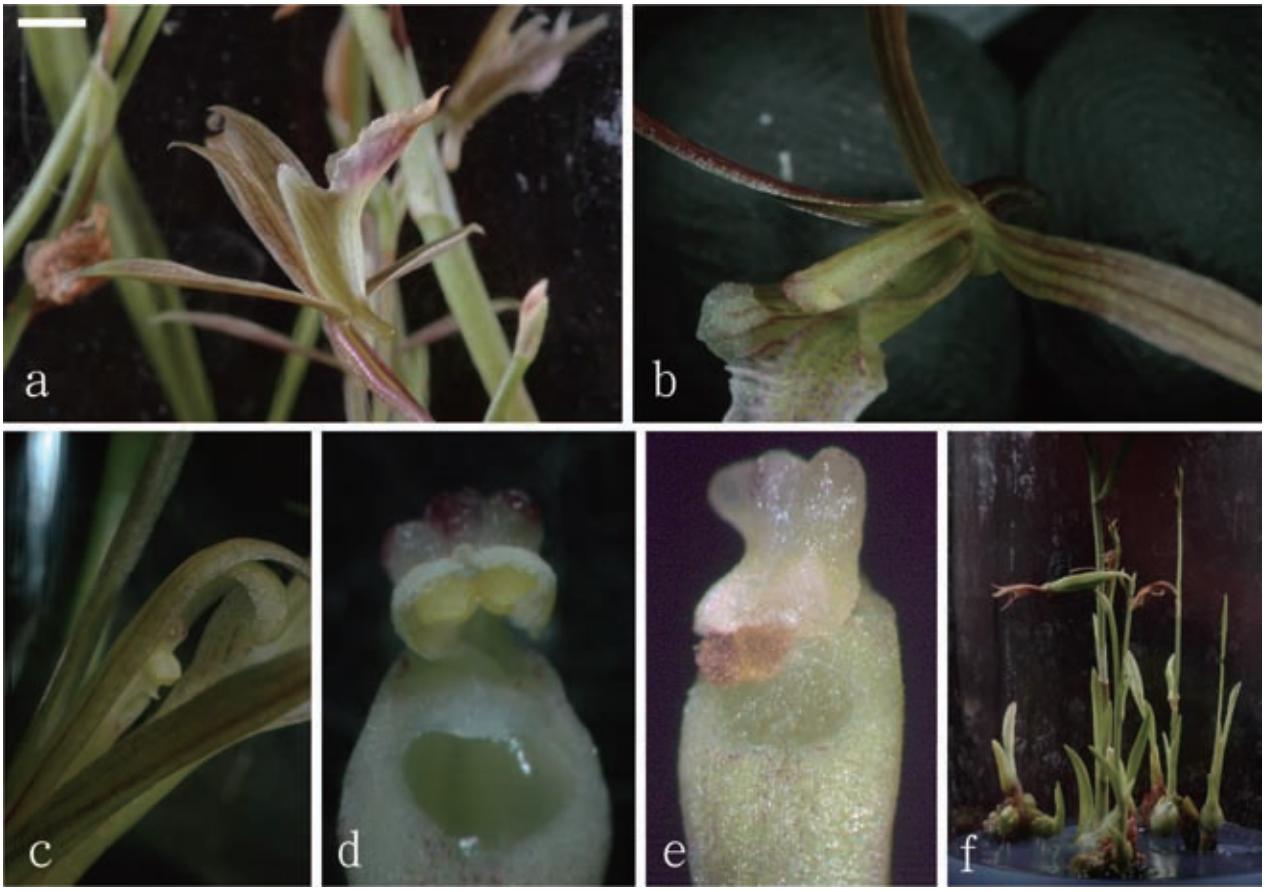


**Figure 2.** Flowering, self-pollination and fruition of *Eulophia graminea in vitro*. (a) *In vitro* flowering (bar = 4 mm); (b) *In vitro* flowering lacking two petals (bar = 2.5 mm); (c) Apex of column with anther cap lifted (bar = 2.5 mm); (d) Apex of column with anther cap lifted to exposed the pollinia (bar = 1 mm); (e) The pollinia position below the main portion of the androclinium grown touching the stigma in an early fruition stage (bar = 1 mm); (f) Fruit set (bar = 1 cm).

**Table 2.** A comparison of *in vivo* and *in vitro* flower organ of *Eulophia graminea*.

| | Flower organ (cm)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Dorsal sepal | Left sepal | Right sepal | Left petal | Right petal | Lip | Column | Ovary |
| *In vivo* | 1.34 a | 1.42 a | 1.42 a | 1.10 a | 1.12 a | 1.56 a | 0.62 a | 1.66 a |
| *In vitro* | 1.22 a | 1.08 a | 1.16 a | 1.04 a | 1.06 a | 1.24 b | 0.56 a | 1.14 a |

*The means with 5 replicates with the same letters were not significantly different at $p<0.05$.

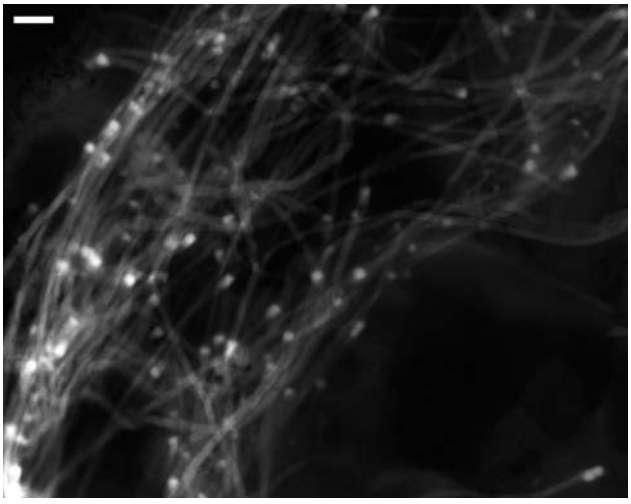

**Figure 3.** The pollen tubes growing in the style of *Eulophia graminea.* The style was examined at the time of the pollinia touched the stigma in an early fruition stage as figure 2f. (bar = 10 μM).

of the androclinium where it comes into contact with the stigma and where the ovary subsequently grew and became enlarged (Figure 2e, f). The pollen tubes were observed penetration into the style (Figure 3). Finally, there was 18% flowering and automatic fruition without artificial pollination in the isolation culture condition.

The *in vitro* cultured fruit required more than two months to mature and produced seeds with *in vitro* embryos (Figure 4a). Several hundred seeds in one *in vitro* fruit were undersized (Figure 4b). The various germination rates of *in vitro* seeds sown on the GM medium were evaluated through constant observation under a microscope after a period of 4 months (Figure 4c). The average germination percentage from three independent flask was 59.6% (16/25, 19/34, 30/51). *In vitro* flower stems protruded after 11 months of culturing in 2 subcultures, and the flowers were developed with a labellum and expanded petals and sepals (Figure 4d). After two months, the *in vitro* fruit turned yellowish and had normal seeds. Formation of second generation


**Figure 4.** Fruition, seed germination and the secondary generation flowering of *Eulophia graminea in vitro*. (a) Mature fruit *in vitro* (bar = 1 cm); (b) Seed derived from *in vitro* fruit (bar = 100 μm); (c) Seed germination and rhizome formation (bar = 2 mm); (d) *In vitro* flowering of secondary generation (bar = 5 mm).

plantlets starting from when *in vitro* seeds were sown to *in vitro* fruit maturation took 14 months. The third generation *in vitro* plantlets took 12 months from seed to fruit maturation. Subsequently, the fourth-generation seeds were harvested and sown *in vitro*. Figure 5 shows the process of the *in vitro* sexual reproduction cycle of *E. graminea*.

## DISCUSSION

*Eulophia graminea* has strong colonization abilities and is widespread throughout subtropical and tropical Asia. *Eulophia graminea* has also been detected in Florida and in Australia (Pemberton et al., 2008). *Eulophia graminea* is often found in man-made habitats; such as recently built roadside slopes, abandoned agricultural fields, secondary grassland or lawns, and open fields near construction sites (Sun, 1997). What is the reason for the widespread occurrence of this particular species?

Several characteristics were discovered in this experiment to support the pervasive nature of this unique orchid. First, the germinated seed of the orchid rapidly develop into a specific storage organ rhizome with abundant nutrient contents and multiple-buds. The seedlings established are like some terrestrial orchids of the same genus plant, the *E. cucullata*, *E. streptopetala*, *E. petersii* (McAlister and van Staden, 1998), *E. hormusjii* (Vij et al., 1989), and other genera orchids such as *Cymbidium sinense* (Chang, and Chang, 2000), *Cymbidiium ensifolium* (Chang and Chang, 2003) and *Geodorum densiflorum* (Sheelavantmath et al., 2000). We deduced that the rhizome structure would enhance seedling survival in wild habitats such as barren landscapes, drought ridden area or salty environment.

Second, the orchid has a brief juvenile stage that endures less than one year, which is a shorter span than other 82 species and cultivars orchids require. For example, the next known shortest juvenile stage is the *Spathoglottis* Penang Beauty which lasts 2 years 11 months and 13 days while the longest stage is the *Aranda* Lucy Laycock which has a juvenile stage of 13 years 3 months (Goh et al., 1982). The diminutive juvenile stage of the *E. graminea* then moves quickly into the reproduction stage, with flowering and fruiting seeds phases.

Third, the orchid has several mating systems that can help it adapt to diverse habitats, such as the self-compatible but pollinator-dependent outcrossing species in Florida and Hong Kong (Sun, 1997; Pemberton et al., 2008), and our *in vitro* culture demonstrating autogamy. Four generations of the autogamous mechanism were observed. Particular attention was focused on how the pollinia automatic growth to stigma initiating pollination and subsequent fruition. *Eulophia graminea* develops an autogamous mating system that to a non-pollinator environment as other 8 species of the *Eulophia* from South Central Africa (Williamson, 1984).

## CONCLUSION

The seeds of the orchid, *E. graminea*.- native to Taiwan- germinated *in vitro* and developed into rhizomes. They then develop shoots and root into a whole plantlet. We cultured the rhizome *in vitro* which spontaneously sprouted flower stems followed by flowering and subsequent fruition to complete a full life cycle. Overall, there were 4 years of cultures that spawned 4 generations. We deduce that there are three reasons for these findings which all support the premise that the *E. graminea* has potent colonization ability in the wild: the unique storage structure of the orchid rhizome, the brief juvenile stage of the orchid, and autogamous mating system.

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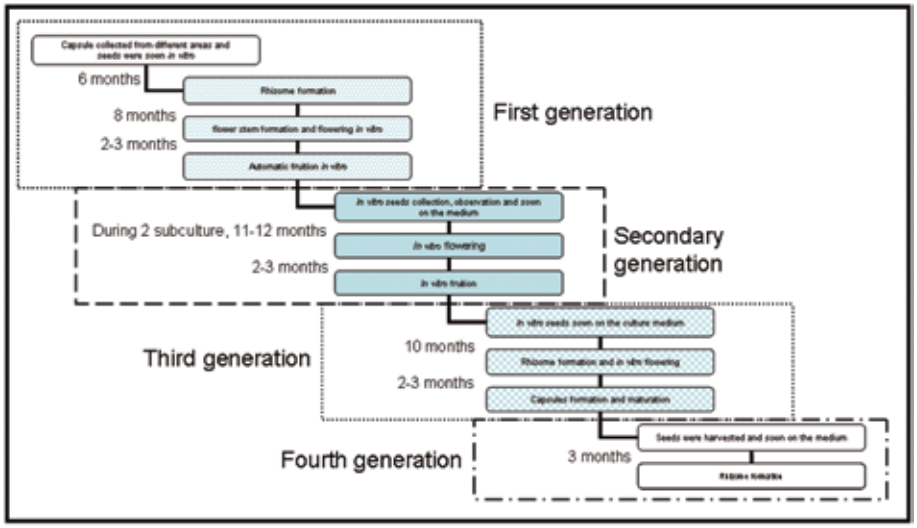


**Figure 5.** The processes of *in vitro* sexual reproduction 4 generations of *Eulophia graminea*.

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# 禾草芋蘭試管內開花與配育系統


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從臺灣的海岸及城市收集禾草芋蘭的蒴果，無菌播種後，種子發育形成根莖，再從根莖頂芽形成花莖，或形成莖葉發根後發育成為幼苗。將根莖繼代培養後，可形成更多的花莖及開花，試管內開花的禾草芋蘭，未經人工授粉可自行自花授粉後結果實，採收試管內開花的果實再行無菌播種後，可在試管內發芽、形成花莖，開花再結果，而完成完整的生命週期，在培養的四年內共完成四個有性世代的培養歷程。禾草芋蘭具有高度能力可在新的棲地生存下來，推測可能有以下三個原因，一為種子發芽後形成根莖構造，可以在各種棲地具有較高的存活率，二為禾草芋蘭具有短幼年期，可在新的棲地中較快進行有性繁殖，第三為在無授粉者的新棲地可能可以自花授粉，以繁衍後代。



**關鍵詞：**自花授粉；禾草芋蘭；開花；*In vitro*；配育系統；根莖。