Establishment of a Rapid Breeding System for Bletilla Striata

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Abstract

Background

Bletilla striata (Thunb. ex A. Murray) Rchb. f., a species of perennial herb of orchidaceae has remarkable effects and high economic value; thus it has been intensively studied by many scholars. Although this herb has numerous seeds, the germination rate is exceptionally low, which leads to decreased of germplasm resources and increased market demands year by year.

Results

With the aim of addressing this issue, the present study included the aseptic germination system and the direct seeding technology system. On the basis of Murashige and Skoog (MS) medium, 2.0 mg/L 6-benzylaminopurine (6-BA) and 1.0 mg/L naphthylacetic acid (NAA) were added prior to seed germination, 70 g/L banana juice and 0.5 mg/L NAA were added when rooting, after which the seedlings were transplanted into a mixed substrate of humus, river sand and bark (3:1:1). The direct seeding system consists of a seedling base and substrate treatment, sowing and seedling raising, seedling growth and transplanting, etc. Turfy soil, huangjiang residue and river sand were selected as the substrate. The results revealed that the germination rate was increased to 91.8%, while the plantlet regeneration was increased to 82.0%. Following 180 days of cultivation, the plants could be transplanted as finished seedlings.

Conclusions

The establishment of this *B. striata* seedling system provides a safe, rapid, reliable production technology route for its industrial development.

Background

Bletilla striata, a perennial medicinal herb of the Orchidaceae family, is primarily distributed across Hubei, Sichuan, Yunnan, Shaanxi, Guizhou, Jiangxi, and Jiangsu Provinces of China (Chen *et al.* 2019, Jiang *et al.* 2019). As a traditional Chinese medicine, it has been used in curing alimentary canal mucosal damage, ulcers, bruises, and burns for thousands of years (Xu *et al.* 2019, Zhang *et al.* 2019). It can also be employed to treat tuberculosis, malignant ulcers, hemorrhoids, anthrax, eye diseases, and silicosis in clinical practice, and is also widely used in the tobacco, chemical, and food industries (He *et al.* 2017).

There are tens of thousands of seeds in the fruits of *B. striata*, which are small and have no endosperm, with a low germination rate under natural conditions (Guler, 2016, Wei *et al.* 2018). Due to the destruction and excessive development of natural habitats, wild *B. striata* resources have been severely damaged (Bai *et al.* 2019); thus, it has been listed as a rare and endangered wild medicinal plant in China (Li *et al.* 2012). Meanwhile, with decreased germplasm resources and increased market demands, traditional

division propagation cannot satisfy the demands of large-scale cultivation; thus, new planting techniques are urgently required (Li *et al.* 2018, Chen *et al.* 2019).

Other plants of the orchid family also face breeding problems; tissue-cultured *Dendrobiums officinale* have already dominated the market; however, their offspring tend to mutate and contain no original medicinal ingredients, which requires the establishment of a quality identification method (Chen *et al.* 2016). *Phalaenopsis aphrodite* is a monopodial epiphytic orchid that also depends on tissue cultures (Huang and Lee, 2010). Aseptic germination system is an effective strategy for solving the above problems and make it possible to achieve large-scale industrialization. Unlike traditional tissue cultures, aseptic germination involves sexual reproduction under sterile conditions.

In recent years, several researchers have used the seeds directly for axenic culture, by adding outer nutrients and hormones to promote their normal germination (Sarker *et al.* 2010, Sobolev *et al.* 2013, Wang *et al.* 2017), which can offer good options for plant germination with abundant seeds but no endosperm. It has been found that NAA can effectively help to establish high-frequency protocorm-like bodies (PLBs) of in vitro germinated seedlings of *Spathoglottis plicata* Blume (Haque and Ghosh, 2017).

To solve the above challenges of *B. striata*, we conducted relevant research on the characteristics of seeds, and gradually solved the germination issue of *B. striata* seeds in a sterile environment, which makes possible the generation of large numbers of seedlings in a short time. Simultaneously, we attempted to breed the seeds of *B. striata* in a natural environment. For this study, an optimum tissue culture matrix and transplanting matrix were screened through an orthogonal experiment, and a new model of large-scale standardized breeding was explored through direct seeding.

Materials And Methods

2.1 Reagents and medicines

AGAR powder, naphthalene acetic acid, sodium hydroxide, 6 - benzyl amino purine, etc. were purchased from Tianjin tianli chemical reagent co. Ltd.. The above reagents were analytically pure.

Humus, river sand, crushed bark, vegetative soil, and perlite were purchased at the Xian Yanjin Road Flower Market.

2.2 Plant Materials

Germplasm resources were planted at the National Engineering Laboratory for Resource Development of Endangered Chinese Crude Drugs, Northwest China. Pollination was completed via artificial pollination technology after flowering (from the end of April to the beginning of May, 2016). The fruits were collected at the mature stage, placed into the cowhide bag to dry in the shade, and stored in a refrigerator for subsequent experiments.

2.3 Sterile germination

Seed viability was confirmed by TTC staining prior to sterile germination and field seeding (Briggs *et al.* 2009), after which the capsule was peeled to carefully extract the *B. striata* seeds. The seeds were then rinsed with sterile water 2-3 times, disinfected with 75% alcohol for 1 min, rinsed again 3-5 times, disinfected with a 0.1% corrosive sublimate for 8 min, rinsed with sterile water five times and absorbed to the surface of sterile filter paper.

Protocorm is an oblate globule formed during the *in vitro* culturing of plants, with roots at the base, which can form a complete plant through further germination and rooting. According to the preliminary experimental results, the main factors that influenced the germination of *B. striata* seeds were the type of medium, 6 - benzyl amino purine, naphthalic acid, and the concentration of banana juice. Therefore, we adopted an orthogonal experimental method and selected three factors, including 6-BA, NAA, and medium type were (Table 1) to optimize the seed germination medium.

Under aseptic conditions, the seeds were seeded into the pre-prepared medium for each group. Groups of six bottles, each bottle containing 100 seeds, were capped and placed in a light incubator under a constant temperature and humidity. The temperature in the incubator was maintained at 25±2ºC, the illumination time was 12 h/d, and the illumination intensity was 2000-3000 lx. The germination of *B. striata* seeds was observed using a stereomicroscope every five days, and the final germination rate was calculated after 30 days.

The growth of a plant is largely determined by the development of its roots. We used MS basic medium to investigate the effects of banana juice and NAA on root proliferation of *B. striata* tissue culture seedlings. Sterile seedlings 3.0 cm high were transferred to different combinations of rooting media (Table 2). Each bottle was inoculated with eight seedlings and each medium was inoculated with nine bottles. Following 30 days of culturing, the changes of seedling biomass in each group were observed and counted.

According to the simulation of the original habitat combined with the unique growth habits, we set up a variety of substrate formula, with the survival rate of tissue culture seedlings as the test standard, to identify the optimal substrate for planting (the volume ratio of the matrix is shown in Table 3). Once the substrate was mixed evenly, 50% carbendazim and a soil pest elimination solution were used for sterilization and stood for 4 d. A total of 30 seedlings were transplanted from different substrates of each group and randomly divided into five flowerpots for repetition. The survival rate and seedling growth of each group were observed and recorded at 15 d and 30 d following transplantation.

2.4Media components

A protocorm is an oblate globule formed during the *in vitro* culturing of plants, with roots at the base, which can form a complete plant through further germination and rooting. According to the preliminary experimental results, the main factors that influenced the germination of *B. striata* seeds were the type of medium, 6 - benzyl amino purine, naphthalic acid, and the concentration of banana juice. Therefore, we adopted an orthogonal experimental method and selected three factors including 6-BA, NAA, and the medium type (Table 1) to optimize the seed germination medium.

2.5 Direct seeding of B. striata

The experiment was conducted at the Xi'an Hengfeng Biotechnology Co. Ltd. (E 108°37'32", N 34°08'03"). We selected plastic sunlit greenhouses or greenhouses with good infrastructures to conduct the field sowing experiments. Humus, river sand, and bark were initially used as seedling substrates, which were mixed well. We further divided the nursery pond into suitable areas by using wood or PVC board dividers (10.0 cm high), and finally sprayed insecticide and covered the nursery pond with non-woven fabric.

To reduce production costs, we preferentially selected local seedling raising substrates in Shaanxi Province to conduct the experiments (Table 4). In production, after mixing the different substrates, they were placed in a nursery pond with a thickness of 8.0 cm. Finally, this was flattened with a wooden board, which was sprayed thoroughly with an atomizing nozzle.

After peeling the preserved seeds with normal vigor, they were mixed with NAA and talcum powder at a weight ratio of 1:5:50,000, and sifted through a sieve (40 holes per sq cm) for sowing at a seeding density of 3.0 g/m^2. Following seeding, the seeds were sprayed with an atomizing nozzle and the plastic greenhouse was closed. The air temperature was maintained at from 20-35℃, with the humidity at more than 60%.

Water was sprayed regularly in the greenhouse to ensure that the substrate surface was moist. When the seeds germinated to the fifth stage, the ventilation time of the greenhouse was gradually increased and the humidity was slowly reduced.

Once the first leaves sprouted, a 0.5% water solution of phosphoric acid diamine was sprayed on the seedlings every week. Depending on the mildew in the seedling pond, a penicillin solution was also sprayed regularly. When the seedlings sprouted 2-3 leaves, the humidity in the greenhouse was reduced to 50%, and the greenhouse was ventilated every night.

2.6 Data processing

Germination rate = seed germination/sowing number × 100%.

Transplanting survival rate = number of viable seedlings after transplanting/number of transplanted seedlings ×100%.

Leaf proliferation ratio = number of new leaves/number of leaves before inoculation.

All data in this study used SPSS17.0 software to analyze variances in the test data, the LSD method was used for multiple comparisons between groups, and EXCEL software was used to create charts.

Results

3.1 Screening results of germination medium

The results of TTC staining revealed that the average germination rate of newly matured *B. striata* seeds was 93.4%, while the vigor of the seeds stored in the laboratory for more than six months was 83.4%.

Following 15 days of inoculation, the seeds in groups 3, 5, and 7 began to germinate successively, and 30 days later the seeds in all groups germinated. The germination rates of the seeds in each experimental group were recorded and statistically analyzed. It can be seen from the experimental results (Table 5) that the type of growth media had a significant influence on the germination of the *B. striata* seeds.

According to the range analysis results in Table 6, the order of influence of each factor on seed germination was from large to small: medium type > 6-BA > NAA. Through the orthogonal experiment analysis we obtained the best level combination of various factors (), among which MS + 6-BA 2.0mg/L + NAA 1.0 mg/L exhibited the best effect for inducing seed germination. Subsequent to the obtained optimal conditions, six experiments were conducted to verify the validity of the results of orthogonal screening. The average germination rate of *B. striata* seeds with the optimal combination of hormones was 91.8%.

Following 30 days of culturing, we observed from the statistical results (Table 7) that the average root numbers of the seedlings in the 3, 4, and 5 groups were significantly higher than that of the other groups. Further, the proliferation multiples of leaves in groups 1, 4, and 5 were 3.1, 2.4, and 2.2, which indicated that banana juice promoted the advanced-root induction of seedlings, while the appropriate concentration of NAA promoted the proliferation of *B. striata* leaves. Concurrently, we clearly observed that in group 5, the leaves of the plants are green and grew well. Therefore, MS + banana juice (70 g/L) + NAA (0.5/L) was selected as the root medium.

3.2 Screening results of transplanting substrate

It could be seen from multiple comparisons that the survival rates of the different transplanting substrates and tube seedlings had significant effects. On the 15th day following transplantation, the survival rate of the test tube seedlings in group 1 (93.183±) and 5(90.567±) was the highest, while those in group 2 (71.533±), 3 (65.750±), and 4 (73.267±) were lower (Table 8). At the 30th day following transplantation, the survival rate with the group 1 substrate was the highest (82%), which was significantly superior to the other substrates.

The group 5 substrates were in *in situ* soil, and although the substrate nutrients were insufficient, the survival rate following transplantation still attained 70.4%, which meant that some microorganisms may have existed in the original soil that possessed obvious growth promoting effects. The survival rate of the transplanted matrix in group 2 was 30.5%, while the survival rate of the group 3 substrate was the lowest (7.6%).

3.3 Screening results of direct-seeding substrate

By conducting direct-seeding experiments with *B. striata* on different substrates, the seeds germinated to stage 1-2 at 5 d. After 24 days, stage 3 was attained where the seeds turned green and leaf primordia

formed. At 45 days, the seeds germinated to stage 4-5, the leaf primordium continued to grow, the first true leaf began to grow, and fibrous roots appeared. In this experiment, only the process of the emergence of real leaves and fibrous roots was considered as normal germination, otherwise it was regarded as abnormal germination. According to the germination of *B. striata* seeds, the germination rate of substrate B was significantly higher than that of A, C, and D. Therefore, as shown in Table 10, we selected substrate B as the optimal seeding substrate in production.

3.4Optimum results of the best substrate

To better reflect the direct seeding technology, the plant growth diagram of 120 d direct seeding was selected for this study. Once the optimal matrix B was broadcast, the seedlings emerged in a relatively orderly manner and the individual plants were relatively large, all of which grew 2-3 true leaves with heights of 6 cm. At this juncture, the plants grew rapidly and were robust.

After 180 days of direct seeding, all of the plants had 4-5 true leaves, with individual plant sizes of from 10-15 cm, well-developed roots, and pseudobulbs of about 1-1.5 cm. After reaching this stage, these seedlings can already be transplanted to the field as finished seedlings.

Discussion

The basic ingredients of the growth media can provide plants with the nutrients they require for survival; however, the plants grow robustly only when these nutrients work in conjunction with the appropriate plant hormones (Franceschi *et al.* 2019). Therefore, the selection of hormones and the combination of appropriate concentrations were key factors for the success of plant tissue culturing, and also an important breakthrough for the rapid proliferation of plants (Nie *et al.* 2016). The appropriate combination and concentrations of plant growth regulators (e.g., NAA and 6-BA) had a great influence on the growth and quality of plants (Matkowski, 2008, Wang *et al.* 2017).

It was found that ABA is a positive regulator of dormancy induction and maintenance (Kucera *et al.* 2005), while NAA could effectively help to establish high-frequency protocorm-like bodies (PLBs) on the *in vitro* germinated seedlings of *Spathoglottis plicata* Blume (Haque and Ghosh, 2017). It is a common experimental method to optimize the culture medium using the orthogonal test method. Zhang selected 1/2 MS medium as the basic medium, with 1% NaClO, 2% sucrose, and 0.1% activated carbon, with which the seed germination rate of *B. striata* attained 80% (Zhang *et al.* 2019). In our experiment, we found that the optimal media ratio for seed germination was MS + 6-BA (2.0 mg/L) + NAA (1.0mg/L), under which the germination rate was effectively improved up to 91.8%.

The growth and development of plants have their own inherent genetic patterns and sequences. In certain ambient environments, external materials and energy are employed for the proliferation and differentiation of plants (Shao *et al.* 2017). In the process of development, the aboveground and underground plant components have a direct mutual promotion relationship, therefore, the evaluation of the health of a plant's growth largely depends on the development of its root system. Banana juice was

found to promote root formation and growth for *Dendrobium canducum* (Jiang *et al.* 2003). In our experiment, we found that it was also beneficial for *B. striata*, while NAA was related to the proliferation of leaves (He *et al.* 2017). Through the comparison of different media, it was found that the best media for the rooting and growth of *B. striata* seedlings was MS+ banana juice (70g/L) + NAA (0.5mg/L).

We report here, for the first time, a direct seeding technology for *B. striata.* In the screening test of transplanting substrates, the seedling survival rate was higher in the group with fermentative bark and a medium sediment concentration. This may be due to the fact that river sand promoted drainage and increased the permeability of the soil to facilitate root respiration. Simultaneously, as the external protective structure of plants, bark is primarily used for the transport of nutrients in addition to preventing diseases and pests. The abundant nutrients in the bark were degraded to inorganic nutrients that are more easily absorbed by plants through fermentation. Meanwhile, a large number of microorganisms grew during the fermentation process, which created a rich symbiotic environment for the transplantation of seedlings. The natural habitats of some epiphytic orchids, such as many species of *Dendrobium*, can have parasitic bark (Chen *et al.* 2015). Through analysis and comparison, humus, river sand, and bark were found to be the best substrate for planting. The establishment of the direct seeding system was related to the fact that we treated the seeds with hormones in the early stage, and then selected a matrix based on bark and Huangjiang residue in the later stage. It is possible that both of these elements provided the nutrients required for seed germination. However, the specific germination kinetics remain to be further studied.

Conclusion

With the discovery of the high medicinal value of *B. striata*, its demand is increasing significantly for clinical and industrial applications. The direct seeding system we have developed greatly reduces the various conditions required for seed germination under natural conditions, while providing a new artificial planting technique for the protection of excellent germplasm resources. This technology system adopts the method of sexual reproduction, which not only makes the offspring more viable and more adaptable to the environment, but also significantly reduces the planting costs, from 300,000 RMB/ha (2015) to 9000 RMB/ha (2019), and can increase production to 24,000 kg/ha. It cannot only meet the needs of large-scale commercial production, but also effectively protect the resources of this important, but rare and endangered species.

Declarations

Ethics approval and consent to participate

All experiments were carried out within the limits of national policy of China.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

J.F.N. and Z.Z.W. conceived and designed the experiments. Z.Y.M, D.H.L., G.M.Z. performed the experiments. S.Q.W. analyzed the data. Z.Y.M. drafted the manuscript, J.F.N. and Z.Z.W. revised it. All authors have discussed and commented on the manuscript.

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Tables

Table 1 - L9 (3^3) orthogonal experiment design table of *B. striata* seed germination.

group	factors		
	medium(A)	6-BA□B□	NAA(C)
1	MS	0.0 mg/L	0.0 mg/L
2	1/2MS	1.0 mg/L	0.5 mg/L
3	AGAR	2.0 mg/L	1.0 mg/L

Note: 1/2MS refers to half of the macroelements in MS.

Table 2 - Different hormone combinations for *B. striata* seedling growth.

Medium	Combination of hormone levels		Number of vaccinations
	Banana juice (g/L)	NAA (mg/L)	
1	50	0.3	9
2	50	0.5	9
3	50	0.7	9
4	70	0.3	9
5	70	0.5	9
6	70	0.7	9

Table 3 - Transplanting matrix formulation.

Experimental group	The volume ratio of the substrate
Group 1	Humus: sand: bark (scrap) = 3: 1: 1
Group 2	Humus: sand: bark (powder) = 5: 3: 2
Group 3	Humus: sand: pine needles = 4: 1: 2
Group 4	Nutrient soil: perlite: bark (powder) = 5: 2: 1
Group 5	Native soil

Table 4 - Different seeding substrate ratios.

Serial number	Different substrates	The ratio of seeding substrate
A	Bark powder: humus: nutrient soil: chicken powder: peat soil	15: 20: 8: 1:5
B	peat soil: Huangjiang residue: river sand	3: 1: 1
C	peat soil: river sand	3: 1
D	huangjiang residue	-

Note: Huangjiang residue is the plant residue of *Dioscorea zingiberensis.*

Table 5 - Orthogonal test results.

The experimental group	Medium type	6-BA	NAA	Germination rate (%)
	A	B (mg/L)	C (mg/L)	
1	MS	0.0	0.0	52.00
2	MS	1.0	0.5	71.00
3	MS	2.0	1.0	87.00
4	1/2MS	0.0	0.5	65.00
5	1/2MS	1.0	1.0	75.00
6	1/2MS	2.0	0.0	68.00
7	Water Agar	0.0	1.0	22.00
8	Water Agar	1.0	0.0	33.00
9	Water Agar	2.0	0.5	32.00
K1	210.00	138.99	147.00	
K2	204.00	179.01	168.00	
K3	87.00	183.00	183.99	
k1	70.00	46.33	49.00	
k2	68.00	59.67	56.00	
k3	29.00	61.00	61.33	
R	41	14.67	12.33	
Primary and secondary order			A>B>C	
Optimal levels	A_1	B_3	C_3	
Optimal combination			$A_1B_3C_3$	

Table 6 - Orthogonal test variance analysis.

Sources of variation	Sum of squares	Degrees of freedom	mean square	F value	P value	significance
Medium type	3206.000	2	1603.000	19.709	0.048	*
6-BA	394.667	2	197.333	2.426	0.292	
NAA	204.667	2	102.333	1.258	0.443	
error	162.667	2	81.333			
sum	3968.000	8				

Table 7 - Different hormone combinations for *B. striata* seedling growth.

Medium type	Leaf proliferation	New rooting number	Seedling development
1	3.2	2.23±	Green leaves, thin roots, faster growth
2	2.1	2.67±	Green leaves, thicker roots,slower growth
3	1.6	2.10±	Green leaves, roots slender and developed
4	2.4	2.98±	Pale green leaves, roots robust, developed
5	2.2	3.67±	Green leaves, thicker roots,faster growth
6	1.9	2.33±	Dark green leaves, thick and short roots, misshapen, extremely slow growth

Table 8 - Multiple comparisons of survival under different substrates treated after 15 and 30 days.

Matrix treatment	Transplant 15 days survival rate	Transplant 30 days survival rate
1	93.183±	82.000±
2	71.533±	20.167±
3	65.750±	7.6±
4	73.267±	30.517±
5	90.567±	70.450±

Table 9 - Germination rates of different substrates.

Serial number	Different substrates	Germination rate
A	Bark powder: humus: nutrient soil: chicken powder: peat soil	0.6972±0.0313 B
B	peat soil：huangjiang residue：river sand	0.7415±0.0238 A
C	peat soil：river sand	0.6465±0.0228 C
D	huangjiang residue	0.5662±0.0271 D

Figures

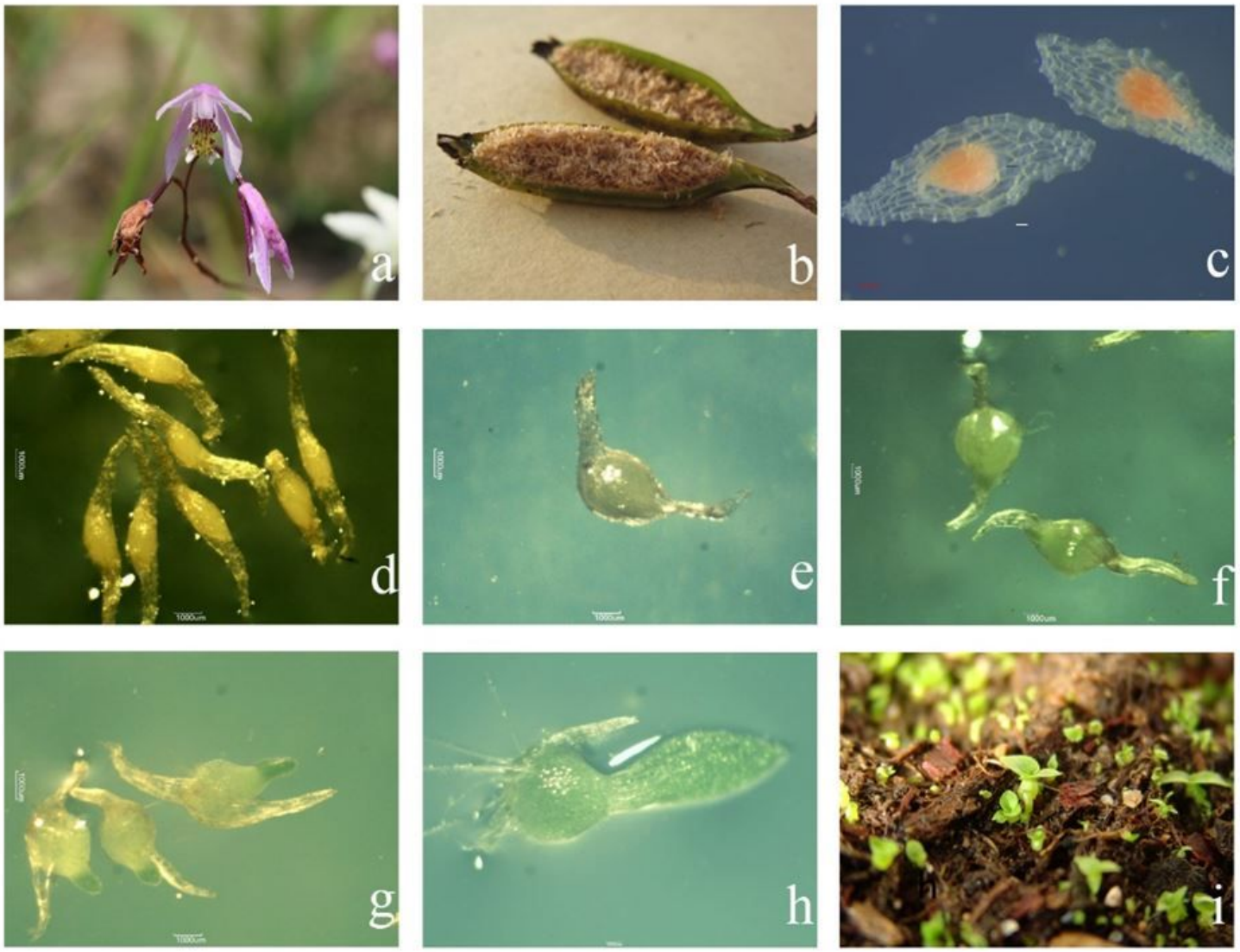

Figure 1

Progress of B. striata seed germination (a. flower shape of B. striata, b. fruit anatomy diagram, c. TTC staining of B. striata seeds, d-h. 5 signature stages of B. striata seed germination, i. seed germination in

matrix)9.22 (H) ×11.98 (W).

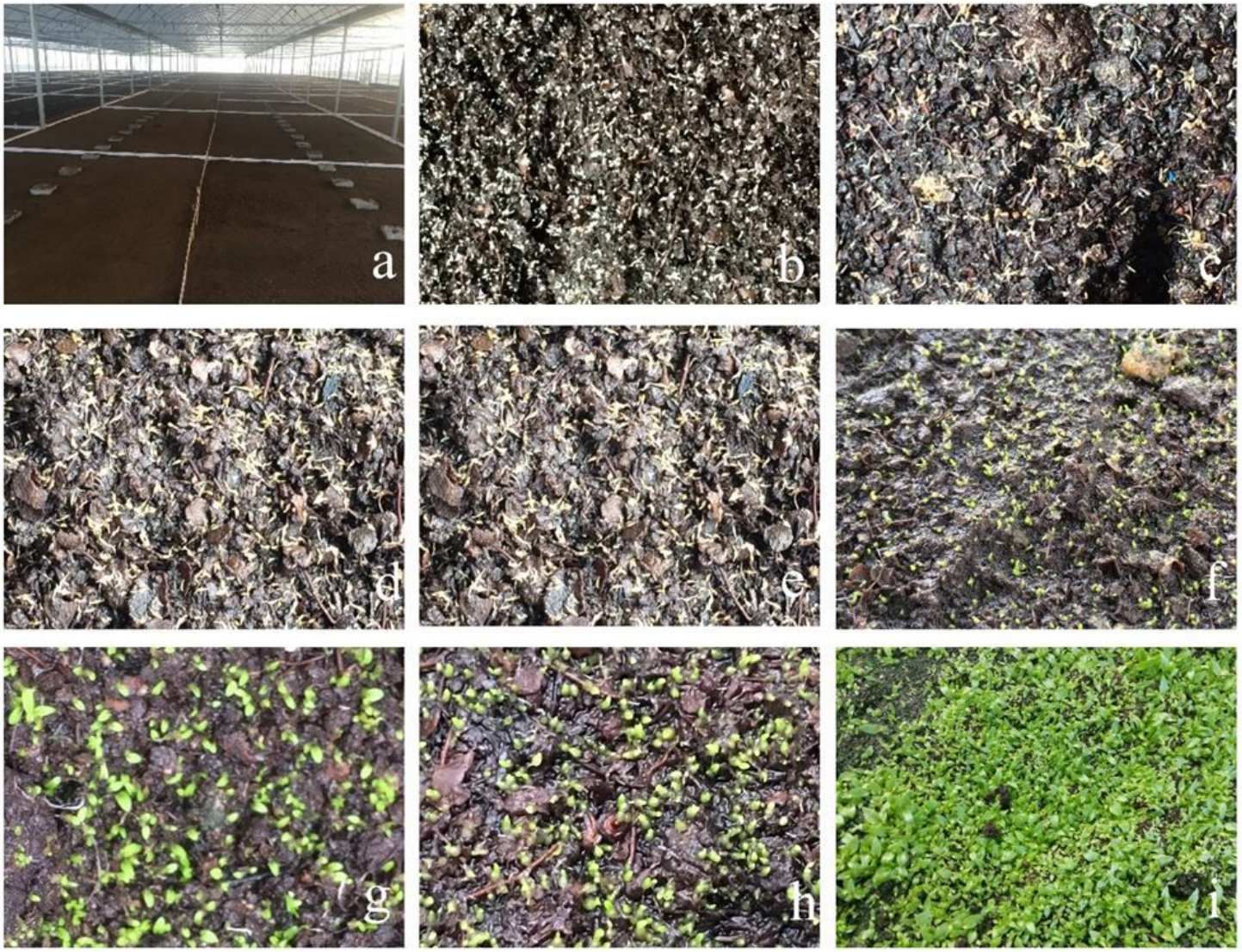

Figure 2

Direct-seeding germination of B. striata seeds (a. sowing for 0 days; b. the first day of sowing, the seeds were fusiform at stage 0, did not germinate, the embryo had activity; c. At five days after sowing, the imbibition of seeds occurred; d. At eight days after sowing, the seeds absorbed water further, and the volume of the embryo further increased; e. At 15 days after sowing, the seeds reached the first to second stage, the embryo had broken through the testa, and a large number of root-like bodies appeared; f. At 24 days after sowing, the seeds were germinated to the third stage, the primary meristem appeared and the primary base of the middle lobe was gradually formed; g. At 35 days after sowing, the protocorm was further developed and the leaf primordium was gradually enlarged; h. At 45 days after sowing, the first true leaf appeared and elongated in the fourth to fifth stage of germination; i. At 120 days after sowing, the seeds entered the seedling growth stage) 11.17 (H) ×14.64 (W).

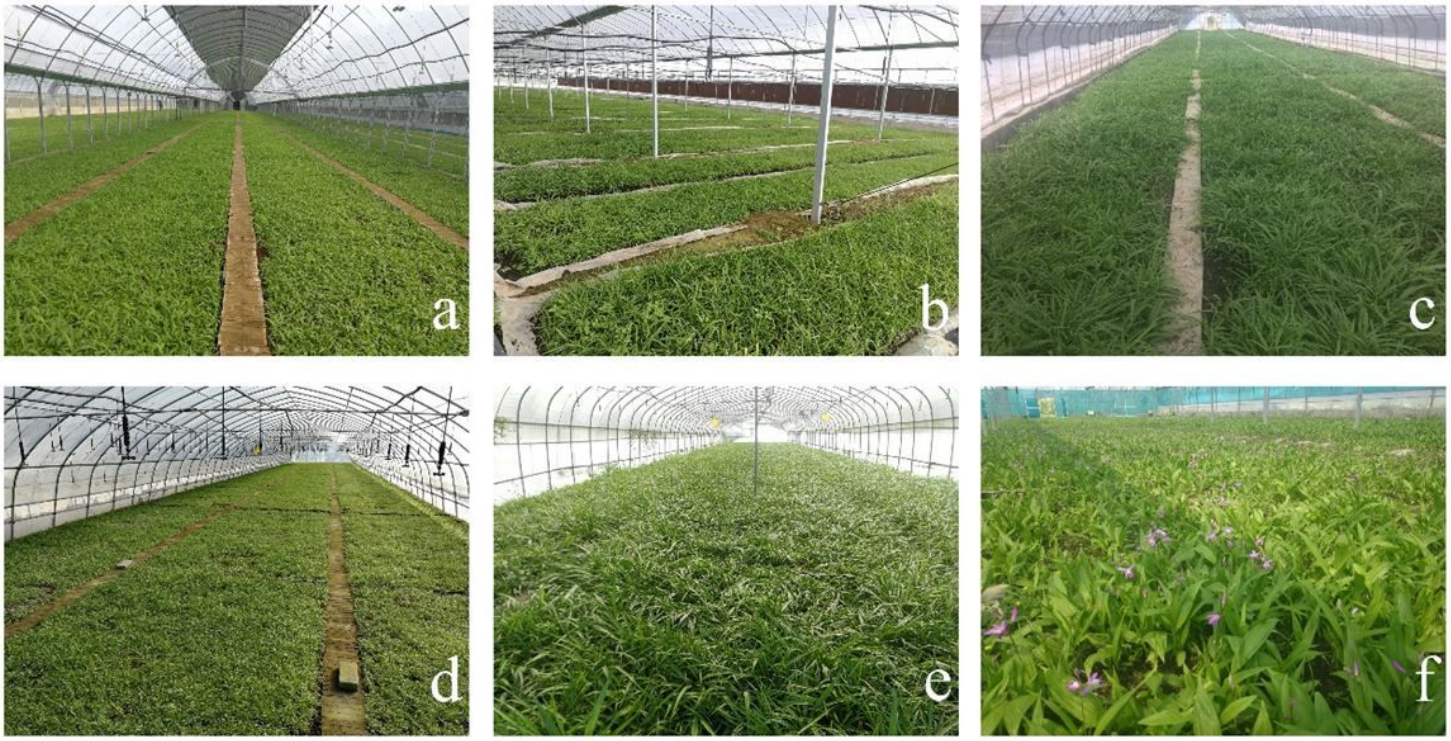

Figure 3

Large-area planting for B. striata (a. Yinzhen street base, chang 'an district, Xi 'an, 200 d of B. striata direct seeding; b. Chunhua street base, jiangning district, Nanjing, 205 d; c. Daizhao street base, chang 'an district, Xi 'an, 220 d; d-f. Ganting street base, huyi district, Xi 'an, 215 d, 250 d, and 450 days of B. striata direct seeding) 7.55□ (H) ×14.64□ (W)