HortScience 56(4):454–459. 2021. https://doi.org/10.21273/HORTSCI15598-20

Establishment of a Rapid Breeding System for *Bletilla striata*

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Additional index words. Direct seeding, germination system, rapid propagation

***Abstract. Bletilla striata* (Thunb. ex A. Murray) Rchb. f., a species of perennial herb of orchidaceae that has remarkable effects and high economic value, has been intensively studied by many scholars. Although this herb has many seeds, the germination rate is exceptionally low, which leads to decreased germplasm resources and increased market demand every year. To solve this problem, this study examined the aseptic germination system and the direct seeding technology system. On Murashige and Skoog (MS) medium, 2.0 mg/L 6-benzylaminopurine (6-BA) and 1.0 mg/L naphthylacetic acid (NAA) were added before seed germination, and 70 g/L banana juice and 0.5 mg/L NAA were added when rooting. Then, the seedlings were transplanted to a mixed substrate of humus, river sand, and bark (volume ratio of 3:1:1). The direct seeding system consists of substrate treatment, sowing, seedling raising, seedling growth, and transplanting. Turfy soil, Huangjiang residue, and river sand were selected as the substrate. The results revealed that the germination rate was increased to 91.8%, whereas the plantlet regeneration was increased to 82.0%. After 180 days of cultivation, the plants could be transplanted as finished seedlings. The establishment of *B. striata* seedling system provides a safe, rapid, reliable production technology route for industrial development.**

Bletilla striata, a perennial medicinal herb of the Orchidaceae family, is primarily distributed across the Hubei, Sichuan, Yunnan, Shaanxi, Guizhou, Jiangxi, and Jiangsu Provinces of China (Bai et al., 2018; Chen et al., 2019; Jiang et al., 2019). As a traditional Chinese medicine, it has been used for curing alimentary canal mucosal damage, ulcers, bruises, and burns for thousands of years (Xu et al., 2019; Zhang et al., 2019). Furthermore, it can be used to treat tuberculosis, malignant ulcers, hemorrhoids, anthrax, eye diseases, and silicosis in clinical practice, and it is widely used in the tobacco, chemical, and food industries (Chen et al., 2016; He et al., 2017a; Hirano et al., 2005).

There are many seeds in the fruits of *B. striata*, which are small, have no endosperm, and have a low germination rate under natural conditions (Guler, 2016; Wei et al., 2018). Because of the destruction and excessive development of natural habitats, wild *B. striata* resources have been severely damaged (Bai et al., 2018). Therefore, it has been listed as a rare and endangered wild medicinal plant in China (Li et al., 2018). With the decrease in germplasm resources and the increase in market demand, traditional division propagation cannot satisfy the needs of large-scale cultivation; therefore, new planting techniques are urgently required (Chen et al., 2019; Li et al., 2018).

Other plants of the Orchid family are also experiencing breeding problems, such as *Phalaenopsis Aphrodite* (Huang and Lee, 2010) and *Dendrobium officinale* (Chen et al., 2016). Unlike traditional tissue cultures, an aseptic germination system involves sexual reproduction under sterile conditions, which make it possible to achieve large-scale industrialization.

Recently, 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). It has been found that NAA can effectively help to establish high-frequency protocorm-like bodies of in vitro germinated seedlings of *Spathoglottis plicata* Blume (Haque and Ghosh, 2017).

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

Materials and Methods

Reagents and medicines. Analytically pure agar powder, NAA, sodium hydroxide, and 6-BA purine were purchased (Tianjin Tianli Chemical Reagent Co., Ltd.). Humus, river sand, crushed bark, vegetative soil, and perlite were purchased from the Xian Yanjin Road Flower Market.

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

Sterile germination. Seed viability was confirmed by 2,3,5-triphenyltetrazolium chloride (TTC) staining before sterile germination and field seeding (Briggs et al., 2009). The capsule was peeled carefully to extract the *B. striata* seeds, rinsed with sterile water, and disinfected with 75% alcohol and 0.1% corrosive sublimate.

Protocorm is an oblate globule formed during the in vitro culturing of plants that 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-BA purine, NAA, and the concentration of banana juice. Therefore, we adopted an orthogonal experimental method and selected three factors (Table 1) to optimize the seed germination medium.

Under aseptic conditions, the seeds were seeded in the preprepared medium for each experimental group and placed in a light incubator under a constant temperature and humidity. The temperature was maintained at 25 ± 2 °C and the illumination intensity was 2000 to 3000 lx. The germination of *B. striata* seeds was observed using a stereomicroscope every 5 d. After 30 d, the final germination rate was calculated.

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

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

6-BA = 6-benzylaminopurine; MS = Murashige and Skoog; NAA = naphthylacetic acid. 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		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

NAA = naphthylacetic acid.

Table 3. Transplanting matrix formulation.

Experimental group	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	Substrates	Ratio of the 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	—

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

transferred to different combinations of rooting media (Table 2). After 30 d of culturing, the changes in the seedling biomass in each experimental group were observed and counted.

According to the simulation of the original habitat combined with the unique growth habits, we created a variety of substrate formulas using the survival rate as the test standard to identify the optimal substrate for planting (Table 3). The survival rate and seedling growth of each experimental group were observed and recorded at 15 d and 30 d after transplantation.

Direct seeding of B. striata. The experiment was conducted at the Xi'an Hengfeng Biotechnology Co., Ltd. (lat. 34°08′03″ N, long. 108°37′32″ E). We selected plastic sunlit greenhouses to conduct the field sowing experiments. Humus, river sand, and bark were initially used as seedling substrates and were mixed well. We further divided the nursery pond into suitable areas by using polyvinylchloride (PVC) board dividers (10.0 cm high), sprayed insecticide, and covered the nursery pond with nonwoven fabric.

To reduce production costs, we preferentially selected local seedling-raising substrates in Shaanxi Province to conduct the experiments (Table 4). During production, different substrates were mixed and placed in a nursery pond with a thickness of 8.0 cm.

After peeling the preserved seeds with normal vigor, they were mixed with NAA and talcum powder at a weight ratio of $1:5:5.0 \times 10^4$ and sifted through a sieve (40 holes per cm^2) for sowing at a seeding density of 3.0×10^{-3} kg/m^2. After seeding, the seeds were sprayed with a nozzle and the plastic greenhouse was closed. The air temperature was maintained at 20 to 35 °C with humidity of more than 60%. Water was sprayed regularly to ensure that the substrate surface was moist.

According to the germination process of orchids, seeds can be divided into five stages: 1) the seed absorbs water, the embryo expands and breaks through the testa, and the rhizoid also appears; 2) the embryo continues to grow and the test rhizoid continues to increase; 3) protocorm and leaf primordium form; 4) true leaves elongate and the protocorm continue to swell; 5) the root appears (Fig. 1). When the seeds germinated to the fifth stage, the ventilation time of the greenhouse was gradually increased and the humidity was slowly reduced.

When the first leave sprouted, a 0.5% water solution of phosphoric acid diamine was sprayed on the seedlings every week. When the seedlings sprouted two to three leaves, the humidity in the greenhouse was reduced to 50% and the greenhouse was ventilated every night.

Data processing.

$$\text{Germination rate} = \text{seed germination}/\text{sowing number} \times 100\%$$

$$\text{Transplanting survival rate} = \text{number of viable seedlings after transplanting}/\text{number of transplanted seedlings} \times 100\%.$$

$$\text{Leaf proliferation ratio} = \text{number of new leaves}/\text{number of leaves before inoculation}.$$

All data in this study were evaluated using SPSS 17.0 software to analyze variances in the test data. The least significant difference method was used for multiple comparisons between experimental groups, and DPS software (Middlesex, UK) was used to create charts.

Results

Screening results of the germination medium. The results of TTC staining revealed that the seed viability of fresh, matured *B. striata* seeds was 93.4%, whereas the vigor of the seeds stored in the laboratory for more than 6 months was 83.4%.

After 15 d of inoculation, the seeds in experimental groups 3, 5, and 7 began to germinate successively, whereas the seeds in the other experimental groups germinated

Received for publication 2 Dec. 2020.
Published online 22 March 2021.
We are thankful for the planting site provided by Senhai Landscape Construction Co., Ltd. (lat. 34.0784°N, long. 109.1021°E), Nanjing Langchi Agricultural Science and Technology Development Co. Ltd. (lat. 31.9406°N, long. 118.9279°E), and Xi'an Hengfeng Biotechnology Co., Ltd. (lat. 34.1099°N, long. 108.5977°E) during our experiment.
This study was financially supported by the Key R&D Program of Shaanxi Province (2019SF-307 and 2018FP2-26), the Fundamental Research Funds for the Central Universities (GK201906008 and GK201806006), the National Natural Science Foundation of China (31670299), Graduate Education and Teaching Reform Research project (GERP-20-41) and the National Key Technologies R & D Program for Modernization of Traditional Chinese Medicine (2017YFC1701300 and 2017YFC1700706).
All experiments were performed within the limits of national policy of China.
The data used to support the findings of this study are available from the corresponding author upon request.
J.F.N. and Z.Z.W. conceived and designed the experiments. Z.Y.M. and D.H.L. performed the experiments. S.Q.W. and G.H.J. 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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30 d later. 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 results in Table 6, the medium type was the main factor affecting seed germination, followed by the contents of NAA and 6-BA. Through the orthogonal experiment results, we obtained the best combination of various factors ($A_1B_3C_3$), among which MS + 6-BA 2.0 mg/L + NAA 1.0 mg/L exhibited the best effects on inducing seed germination. The average germination rate of *B. striata* seeds with the optimal combination of hormones was 91.8%.

Screening results of the rooting medium. After 30 d of culturing, we observed from the statistical results (Table 7) that the average root numbers of the seedlings in experimental groups 3, 4, and 5 were significantly higher than those of the other groups. Furthermore, the proliferation multiples of leaves in experimental 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, whereas the appropriate concentration of NAA promoted the proliferation of *B. striata* leaves. Concurrently, the leaves of the plants were green and grew well in group 5. Therefore, MS + banana juice (70 g/L) + NAA (0.5/L) were selected as the rooting medium.

Screening results of the transplanting substrate. It could be observed from multiple comparisons that the survival rates of the different transplanting substrates and tube seedlings had significant effects. On day 15 after transplantation, the survival rate of the test tube seedlings in group 1 was the highest (93.183 ± 1.877) (Table 8). On day 30 after transplantation, the survival rate with the group 1 substrate was the highest (82%) and was significantly superior to that of the other substrates.

The group 5 substrates were in situ soil. Although the substrate nutrients were insufficient, the survival rate after transplantation was still 70.4%. The survival rate of the transplanted matrix in group 2 was 30.5%. The survival rate of the group 3 substrate was the lowest (7.6%).

Screening results of the direct seeding substrate. By conducting direct seeding experiments with *B. striata* on different substrates, the seeds germinated to stage 1 or 2 on day 5. After 24 d, stage 3 was attained; the seeds turned green and leaf primordia formed. On day 45, the seeds germinated to stage 4 or 5, the leaf primordium continued to grow, the first true leaf began to grow, and fibrous roots appeared (Fig. 2). During this experiment, only the process of the emergence of real leaves and fibrous roots was considered 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 the other three groups. Therefore, as shown in Table 9, we selected substrate peat soil, Huangjiang residue, and river sand as the optimal seeding substrate in production.

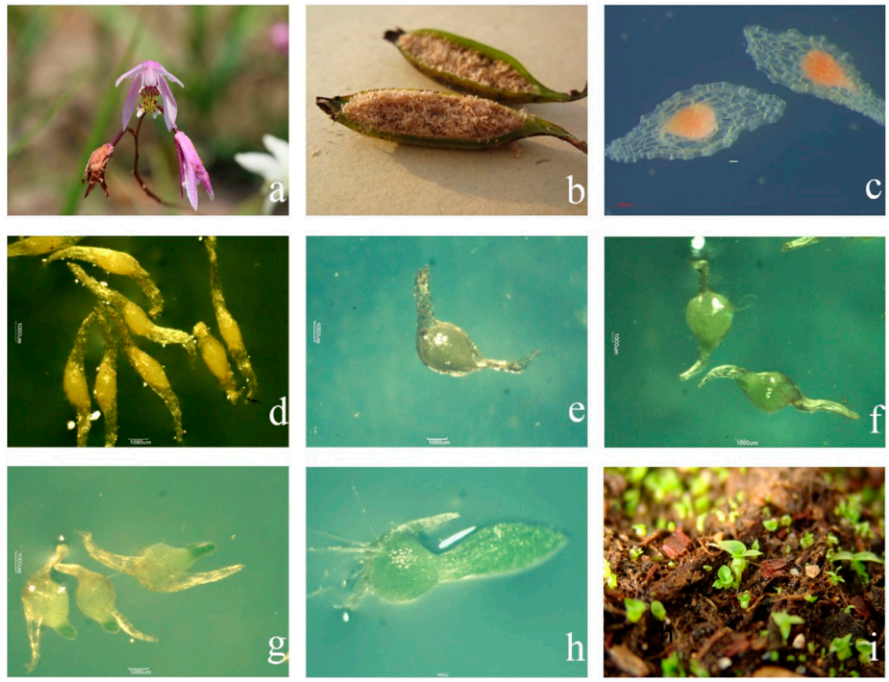

Fig. 1. Progress of *B. striata* seed germination. (**A**) Flower shape of *B. striata*. (**B**) Fruit anatomy diagram. (**C**) TTC staining of *B. striata* seeds. (**D**–**H**) Five signature stages of *B. striata* seed germination. (**I**) Seed germination in the matrix.

Table 5. Orthogonal test results.

Experimental group	Medium (type A)	6-BA B (mg/L)	NAA C (mg/L)	Germination rate (%)
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		$A_1B_3C_3$		

6-BA = 6-benzylaminopurine; MS = Murashige and Skoog; NAA = naphthylacetic acid. 1/2MS refers to half of the macroelements in MS. A = medium type; B = the concentration of 6-BA; C = the concentration of NAA.

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				

6-BA = 6-benzylaminopurine; NAA = naphthylacetic acid.

Optimum 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. When

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

Medium type	Leaf proliferation	New rooting number	Seedling development
1	3.2	2.23 ± 0.15 aA	Green leaves, thin roots, faster growth
2	2.1	2.67 ± 0.41 aA	Green leaves, thicker roots, slower growth
3	1.6	2.10 ± 0.11 aA	Green leaves, roots slender, developed
4	2.4	2.98 ± 0.27 aA	Pale green leaves, roots robust, developed
5	2.2	3.67 ± 0.24 aA	Green leaves, thicker roots, faster growth
6	1.9	2.33 ± 0.23 aA	Dark green leaves, thick and short roots, misshapen, extremely slow growth

The letters represent the significance analysis results.

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

Matrix treatment	Transplant 15-d survival rate	Transplant 30-d survival rate
1	93.183 ± 1.877 Aa	82.000 ± 1.455 aA
2	71.533 ± 2.182 bC	20.167 ± 1.093 cdC
3	65.750 ± 1.621 cD	7.600 ± 1.284 dD
4	73.267 ± 2.435 bC	30.517 ± 2.281 cC
5	90.567 ± 1.659 aB	70.450 ± 1.461 bB

The letters represent the significance analysis results.

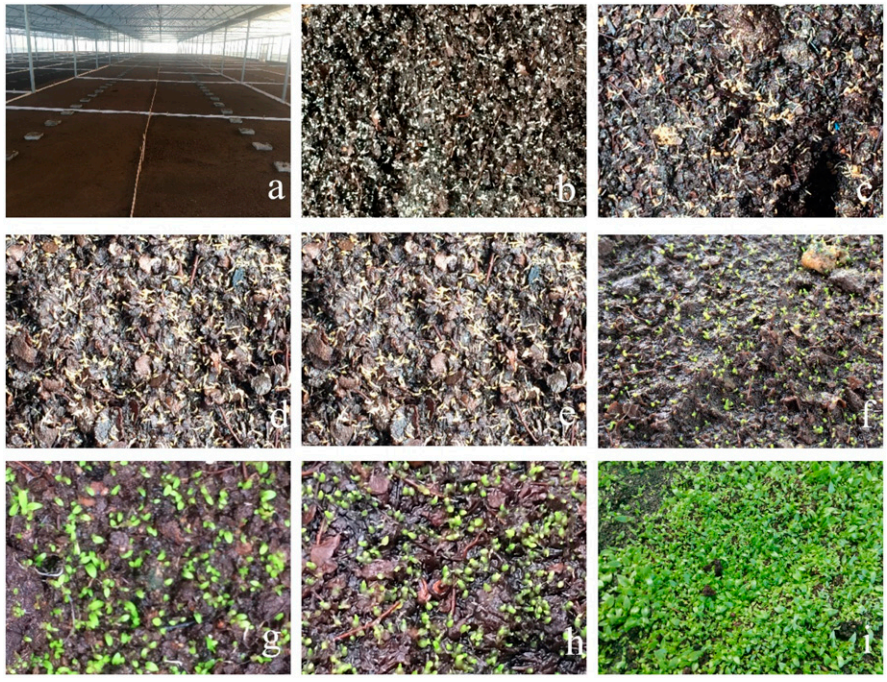

Fig. 2. Direct-seeding germination of *B. striata* seeds. (**A**) Unseeded. (**B**) The first day of sowing. The seeds were fusiform at stage 0 and did not germinate, but the embryo had activity. (**C**) At 5 d after sowing, the imbibition of seeds occurred. (**D**) At 8 d after sowing, the seeds absorbed water further and the volume of the embryo further increased. (**E**) At 15 d after sowing, the seeds reached the second stage, the embryo had broken through the seedcoat, and a large number of root-like bodies appeared. (**F**) At 24 d 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 d after sowing, the protocorm was further developed and the leaf primordium was gradually enlarged. (**H**) At 45 d after sowing, the first true leaf appeared and elongated in the fifth stage of germination. (**I**) The seeds entered the seedling growth stage at 120 d after sowing.

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

The letters in the germination rate column represent the significance analysis results between different groups.

the optimal matrix group B was broadcast, the seedlings emerged in a relatively orderly manner and the individual plants were relatively large. All of them grew two or three true leaves with heights of 6.0 cm.

After 180 d of direct seeding, all the plants had four or five true leaves. Individual plant sizes ranged from 10.0 to 15.0 cm, with well-developed roots and pseudobulbs of ≈1.0 to 1.5 cm. After reaching this stage, these seedlings can 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 successful plant tissue culturing and an important breakthrough for the rapid proliferation of plants (Nie et al., 2016). The appropriate combination and concentrations of plant growth regulators such as NAA and 6-BA have a great influence on the growth and quality of plants (Matkowski, 2008; Wang et al., 2017).

It was found that abscisic acid is a positive regulator of dormancy induction and maintenance (Kucera et al., 2005), whereas NAA could effectively help to establish high-frequency protocorm-like bodies on the in vitro germinated seedlings of *S. plicata* Blume (Haque and Ghosh, 2017). Optimizing the culture medium using the orthogonal test method is a common experimental method. Zhang selected 1/2 MS medium as the basic medium with 1% NaClO, 2% sucrose, and 0.1% activated carbon; with this, the seed germination rate of *B. striata* was 80% (Zhang et al., 2019). During our experiment, we found that the optimal media ratio for seed germination was MS + 6-BA (2.0 mg/L) + NAA (1.0 mg/L); with this, 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 used for the proliferation and differentiation of plants (Shao et al., 2017). During the process of development, the aboveground and underground plant components have a direct mutual promotion relationship; therefore, the evaluation of healthy plant growth largely depends on the development of its root system. Banana juice was found to promote root formation and growth for *D. canducum* (Couselo et al., 2012). During our experiment, we found that it was also beneficial for *B. striata*, whereas NAA was related to the proliferation of leaves (He et al., 2017b).

The direct seeding technology of *B. striata* has been reported for the first time in this article. During the screening test of transplanting substrates, the seedling survival rate was higher in the group with

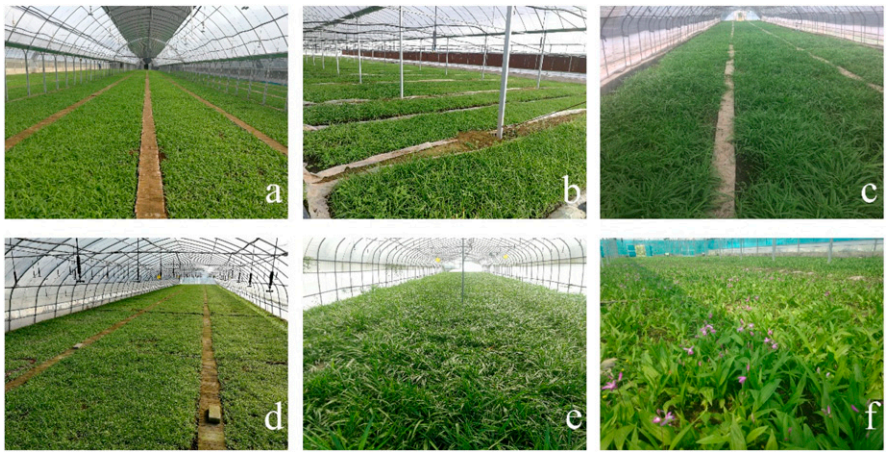

Fig. 3. Large-area planting for *B. striata*. (**A**) Yinzhen street base, Chang'an district, Xi'an, on day 200 of *B. striata* direct seeding. (**B**) Chunhua street base, Jiangning district, Nanjing, on day 205 of *B. striata* direct seeding. (**C**) Daizhao street base, Chang'an district, Xi'an, on day 220 of *B. striata* direct seeding. (**D**–**F**) Ganting street base, Huyi district, Xi'an, on days 215, 250, and 450 of *B. striata* direct seeding.

fermentative bark and a medium sediment concentration. This may be because 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 and preventing diseases and pests (Geoffroy et al., 2017). The abundant nutrients in the bark were degraded to inorganic nutrients that are more easily absorbed by plants through fermentation. Several microorganisms grew during the fermentation process, which created a rich symbiotic environment for the transplantation of seedlings (Das et al., 2016). The natural habitats of some epiphytic orchids, such as many species of *Dendrobium*, can lead to epiphytes on tree trunks (Chen et al., 2015). Through analyses and comparisons, humus, river sand, and bark were found to be the best substrates for planting. The establishment of the direct seeding system was related to the fact that we treated the seeds with hormones during the early stage and then selected a matrix based on bark and Huangjiang residue during the later stage. It is possible that these elements provided the nutrients required for seed germination. However, the specific germination kinetics need to be further studied.

Conclusions

With the discovery of the high medicinal value of *B. striata*, and because of its clinical and industrial applications, its demand is increasing significantly. The direct seeding system we have developed greatly reduces the various conditions required for seed germination under natural conditions and provides 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 in 2015 to 9000 RMB/ha in 2019) and can increase production to 24,000 kg/ha. Furthermore, it can meet the needs of large-scale commercial production (Fig. 3) and also effectively protect the resources of this important, rare, and endangered species.

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