

## Article

# Regeneration of *Sesuvium portulacastrum* through Indirect Shoot Organogenesis and Influence of an Endophytic Fungus on Rooting of Microshoots

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**Abstract:** *Sesuvium portulacastrum* L. is a dicotyledonous halophyte belonging to the family Aizoaceae. Its young leaves are highly nutritious, and many ecotypes are used as leafy vegetable and medicinal crops. Additionally, due to their tolerance to soil salinity, flooding, and high temperatures, some ecotypes are used for the remediation of saline soils. As a result, there is an increasing need for a large number of disease-free *S. portulacastrum* propagules. This study developed an efficient protocol for the regeneration of *S. portulacastrum* through indirect shoot organogenesis. Leaf explants were cultured on Murashige and Skoog basal medium supplemented with different concentrations of zeatin (ZT) and indole-3-acetic acid (IAA). Callus was induced in all explants cultured with 1.5 mg/L ZT only or 1.5 mg/L ZT with 0.5 mg/L IAA. The callus was cut into small pieces and cultured on the same medium on which it was initially induced. ZT at 1.5 mg/L induced 73.7% of callus pieces to produce adventitious shoots, and the shoot numbers per callus piece were up to 20. To improve the in vitro rooting of adventitious shoots, commonly known as microshoots or microcuttings, an endophytic fungus, *Cladosporium* ‘BF-F’, was inoculated onto the rooting medium. ‘BF-F’ substantially enhanced rooting and plantlet growth, as the root numbers were three times more and plantlet heights were 70% greater than those without ‘BF-F’ inoculation. To detect the genes involved in the enhanced rooting and plantlet growth, qRT-PCR analysis was performed. Results showed that genes related to auxin responses and nitrogen uptake and metabolism were highly upregulated in ‘BF-F’-inoculated plantlets. Plants inoculated with ‘BF-F’ grew vigorously after being transplanted into a sand–soil substrate. Thus, this study not only established an efficient protocol for the regeneration of *S. portulacastrum* but also developed a novel method for improving the rooting of microshoots and plantlet growth. The established propagation system could be used for producing a large number of *S. portulacastrum* plantlets for commercial use and also for genetic transformation.

**Keywords:** adventitious shoots; callus induction; microshoots; plant growth-promoting fungi; plant regeneration; *Sesuvium portulacastrum* L.



**Citation:** Jiang, X.; Wang, D.; Chen, J.; He, W.; Zhou, B.; Li, Z.; Chen, L.; Peng, D.; Chen, Q.; Wei, X.

Regeneration of *Sesuvium portulacastrum* through Indirect Shoot Organogenesis and Influence of an Endophytic Fungus on Rooting of Microshoots. *Agriculture* **2024**, *14*, 1221. <https://doi.org/10.3390/agriculture14081221>

Academic Editor: Ana P. G. C. Marques

Received: 12 June 2024

Revised: 19 July 2024

Accepted: 23 July 2024

Published: 25 July 2024



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## 1. Introduction

*Sesuvium portulacastrum* L., a member of the family Aizoaceae, is a succulent annual or perennial herbaceous species distributed in tropical and subtropical regions [1–3]. It inhabits sandy soils, coastal limestone, salt flats, and marshes; thus, it is commonly known

as sea purslane or shoreline purslane [4]. Many ecotypes of *S. portulacastrum* or sea purslane plants can colonize sandy beaches by their stoloniferous, mat-forming growth habit, which can act as a barrier to stabilize sand or protect the sand from erosion [5]. Because of sea water spray and occasional flooding, sea purslane plants are tolerant to salt stress and are classified as a facultative halophyte species [2].

With the increased salinization of agricultural soils [6], there is a growing interest in pursuing a better understanding of salt tolerance mechanisms in halophytes and exploring their potentials for the remediation of saline soils. Recently, sea purslane plants have been increasingly studied and utilized [7–9]. *S. portulacastrum* plants grow well in nutrient solutions containing 100–400 mM sodium chloride (NaCl) [10] and are able to maintain a steady growth status when the salinity increases to 500 mM [11]. The tolerance is not due to avoidance, rather sodium (Na) absorption. The shoot fresh weight of plants grown at 200 mM Na was greater than those grown with potassium (K) [11]. Molecular analyses show that *S. portulacastrum* has developed an integrated mechanism for salt tolerance [11,12].

Sea purslane plants also absorb other mineral elements. In a solution culture, they quickly reduced the total nitrogen (N) from 25 mg/L to 2.0 mg/L and total phosphorus (P) from 4.5 mg/L to almost zero in 21 days [13]. When they were grown on floating beds for remediation of N and P from aquaculture water, 377 g/m<sup>2</sup> of N and 22.9 g/m<sup>2</sup> of P were removed in eight months [14]. Sea purslane plants are also tolerant to heavy metals, including cadmium [15,16], nickel [17], zinc [18], and lead [19]. In general, the absorbed metals are mainly stored in the roots [15,16].

The characteristics of sea purslane plant tolerance to the aforementioned stresses could be attributed, in part, to their rhizosphere microorganisms. Halotolerant endophytes were isolated from *S. portulacastrum* and were able to improve the salt tolerance of *Vigna mungo* L. [20]. A novel fungal strain called *Cladosporium* 'BF-F' was isolated from *S. portulacastrum* roots, and the strain was able to promote plant growth by producing indole-3-acetic acid (IAA) and increased the uptake of N under salt stress [21].

Sea purslane plants are vegetable and medicinal crops, as their leaves possess high nutritional and pharmaceutical values [22]. Young leaves contain all essential mineral elements, including iodine, selenium, and vanadium as well as amino acids and vitamins for human beings [22–24]. Young, succulent shoots have been cultivated as leafy vegetables or sea vegetables in Africa, China, and India [22]. Some ecotypes that are able to thrive in diluted sea water and contain high essential nutrients have been selected in Hainan, China and cultivated as sea vegetables [25]. Florida's Native Americans ate it raw, pickled, or cooked [22]. Researchers at Florida Atlantic University's Harbor Branch Oceanographic Institute studies sea vegetables including *S. portulacastrum* and found that the average edible portion of sea purslane plants was 55%, and they can be eaten raw, blanched, sautéed, or cooked in a dish [22]. In the Philippines, it is known as atchara after being pickled. The leaves are also rich in bioactive compounds and have been used as a traditional medicinal plant [2,23,24]. A distinct characteristic is its ability to biosynthesize ecdysteroids (ECs). Omanakuttan et al. [26] isolated ecdysterone, a representative of ECs from *S. portulacastrum*, and found that it interacts with nitric oxide synthase in an epidermal growth factor receptor-dependent manner and enhances the proliferation, spread, and migration of mouse macrophage cells, thus facilitating the wounding healing process.

Because of these characteristics, *S. portulacastrum* has been increasingly utilized [2,22]. The wide use of *S. portulacastrum* requires a large number of healthy propagules. Propagation by seeds is limited, because seed numbers per plant are low, and there is no commercial supply of seeds [2]. As a result, *S. portulacastrum* is commonly propagated by stem cuttings [2,13]. However, cutting propagation requires a large number of stock plants that may carry and spread diseases, resulting in large-scale infections and plant deaths [27,28]. Micropropagation is the most effective method for achieving the rapid propagation of disease-free propagules on a year-round basis [29]. Plantlets derived from micropropagation are healthy and can grow vigorously in plug trays as liners for transplanting.

There are two reports on in vitro shoot culture of *S. portulacastrum* [30,31]. In the study reported by Lokhande et al. [30], nodal explants were cultured on MS medium [32] supplemented with different concentrations of benzyladenine (BA), kinetin, thidiazuron (TDZ), and 2-isopentenyl adenine (2iP), respectively. The highest number of axillary shoots (5.2 per nodal explant) was induced by 40  $\mu$ M 2iP followed by 2.0  $\mu$ M BA (4.95). The percentage of axillary shoot rooting ranged from 50 to 95% depending on the concentrations of either 1-naphthaleneacetic acid (NAA) or IAA [30]. In another study conducted by He et al. [31], nodal explants cultured on MS medium supplemented with 1.0 mg/L zeatin (ZT) with 0.3 mg/L NAA produced 12 axillary shoots per explant, and 91.7% shoots or microshoots rooted on half-strength MS medium supplemented with 0.6 mg/L indole-3-butyric acid (IBA) [31]. Comparing the two shoot culture methods, ZT appeared to be more effective in inducing axillary shoots than the other cytokinins, and no rooting media achieved 100% rooting. Furthermore, there has been no report of shoot organogenesis in *S. portulacastrum*.

Shoot organogenesis is the regeneration of adventitious shoots from explants devoid of pre-existing meristems followed by rooting of the shoots [33]. In general, shoot organogenesis is more efficient than shoot culture, because more adventitious shoots can be induced through regeneration compared to limited numbers of axillary shoots induced from pre-existing meristems [34]. Additionally, shoot organogenesis is a required step for genetic transformation [29,35,36]. Thus, for increased propagation of *S. portulacastrum* and potential genetic engineering of this species, it is important to develop a shoot regeneration system. Additionally, the rooting of adventitious shoots or microshoots is critical for producing viable propagules, but the rooting of axillary shoots with the aforementioned shoot culture methods was variable. Thus, a reliable rooting method is needed. Rhizosphere microbes have been reported to enhance the rooting of microshoots [37–40]. An ericoid mycorrhizal fungal strain (*Oidiodendron maius* OM-19) has been reported to substantially enhance the rooting of rhododendron microshoots, which was attributed to its biosynthesis of IAA [41]. Recently, an endophyte fungal strain (*Cladosporium* ‘BF-F’) has been isolated from *S. portulacastrum*, and the strain has been shown to promote sea purslane growth [21]. We assumed that ‘BF-F’ could enhance the rooting of *S. portulacastrum* microshoots.

The objectives of this study were to develop an efficient shoot organogenesis method for the in vitro propagation of *S. portulacastrum*, evaluate if endophytic ‘BF-F’ could improve the rooting of microshoots, and explore likely mechanisms behind the rooting promotion mediated by ‘BF-F’. It was anticipated that a viable protocol for the efficient regeneration of adventitious shoots could meet the increasing need for *S. portulacastrum* propagules.

## 2. Materials and Methods

### 2.1. Plant Material Preparation

Mature seeds were collected from *S. portulacastrum* plants grown in Putian, a coastal city in Fujian Province, China, in October 2022, stored in a refrigerator at 4 °C for a month, and used for germination. The seeds were disinfected with 75% ethyl alcohol for 1 min and then transferred to 10% sodium hypochlorite (NaClO) with agitation for 10 min. The disinfected seeds were washed 3 times with sterile deionized water and dried with sterile filter paper. The MS basal medium (M5519, Sigma-Aldrich, St. Louis, MO, USA) was diluted to half, followed by the addition of 30 g/L sucrose and 7 g/L agar. After the pH was adjusted to 5.8, the medium was autoclaved at 121 °C for 25 min, and the autoclaved medium was aliquoted into 250 mL glass culture vessels (Shanghai Zeshine Equipment Co., Shanghai, China), at 20 mL each. Disinfected seeds were placed on the medium, and culture vessels were maintained in a culture room with a temperature of 25 °C and a light intensity of 25  $\mu$ mol/m<sup>2</sup>/s provided by fluorescent lamps with 16 lighting. The seeds germinated in 14 days, and germinated seedlings with about five true leaves (3 months old) were used for the subsequent in vitro culture.

## 2.2. Callus Induction

Callus induction media were prepared using the MS basal medium mentioned above, which was supplemented with four concentrations of ZT and four levels of IAA in a factorial design, resulting in a total of 16 media (M1-M16) based on ZT and IAA combinations (Table 1). After autoclaving at 121 °C for 25 min, the media were poured into 250 mL glass culture vessels, at 25 mL each. True leaves were cut to small pieces (0.5 cm × 0.5 cm in size) as leaf explants using the aforementioned seedlings, which were placed on the media with the adaxial surface up, with four explants per vessel. The experiment was arranged as a completely randomized design with five replications (n = 5), and each culture vessel was an experimental unit. The explants were maintained in the dark at 25 °C for 10 days in a culture room and were then transferred to a culture room at 25 °C under 16 h lighting for 20 days. Leaf explants with callus formations were counted, and the callus induction rates were calculated based on the number of leaf explants with calluses in a vessel vs. the total number of leaf explants cultured in the vessel.

**Table 1.** The list of sixteen media supplemented with four concentrations of zeatin and four levels of IAA in a factorial combination.

| Medium No. | Zeatin (mg/L) | IAA (mg/L) | Medium No. | Zeatin (mg/L) | IAA (mg/L) |
|------------|---------------|------------|------------|---------------|------------|
| 1          | 1.5           | 0.00       | 9          | 2.5           | 0.00       |
| 2          | 1.5           | 0.25       | 10         | 2.5           | 0.25       |
| 3          | 1.5           | 0.50       | 11         | 2.5           | 0.50       |
| 4          | 1.5           | 0.75       | 12         | 2.5           | 0.75       |
| 5          | 2.0           | 0.00       | 13         | 3.0           | 0.00       |
| 6          | 2.0           | 0.25       | 14         | 3.0           | 0.25       |
| 7          | 2.0           | 0.50       | 15         | 3.0           | 0.50       |
| 8          | 2.0           | 0.75       | 16         | 3.0           | 0.75       |

## 2.3. Induction of Adventitious Shoots

Calluses induced from the 16 media were, respectively, cut to small pieces (about 0.7 × 0.7 cm). The same 16 culture media mentioned above were prepared, and they were poured into 250 mL glass culture vessels, at 25 mL each. The callus pieces were cultured on fresh medium, the same as that with which they were initially induced. There was one callus piece per culture vessel. As a result, the experiment was set as a randomized complete block design: each treatment had four glass vessels in a block and a total of three blocks (n = 3). After 120 days of culture in the culture room mentioned above, callus pieces with adventitious shoots were counted, and the adventitious shoot induction rates were calculated based on the number of calluses with shoots in a block vs. the total callus pieces that were cultured in the block. Subsequently, the culture media that induced higher numbers of adventitious shoots were identified.

## 2.4. Roots Induction

Adventitious shoots induced from calluses cultured on M1 medium (1.5 mg/L ZT only) at a stage of about two axillary nodes (2–3 cm height) with 3–4 leaves (after about three months of culture) were excised as microshoots. The microshoots were transferred to a rooting medium. The rooting medium was prepared using the same MS basal medium mentioned above without any growth regulators. After autoclaving, the medium was poured into 250 mL glass culture vessels, 25 mL each. Two microshoots were inserted in the rooting medium, and a total of eighteen culture vessels were prepared. To improve the rooting efficiency, nine vessels were inoculated with the endophytic fungus *Cladosporium* ‘BF-F’ by placing a ‘BF-F’ fungal colony with a diameter of 0.5 cm between the two microshoots. The ‘BF-F’ colony was produced via culturing on PDA medium for seven days. The isolation, identification, and culture of ‘BF-F’ were previously reported [21]. The remaining nine vessels were used as controls, i.e., without ‘BF-F’ inoculation. After 30 days of culture

in the culture room, the plant height, root numbers, and root lengths were recorded. The length of each root was measured using a ruler, and the mean root length per microshoot was calculated by adding the length of each root divided by the total number of roots.

### 2.5. qRT-PCR and Gene Expression Analysis

To determine if the inoculation of 'BF-F' could affect the expression of genes related to rooting and N uptake in *S. portulacastrum*, the following genes were analyzed through qRT-PCR: the genes for auxin-induced in the root (*AIR12*) and auxin transcription factor (*ARF*), auxin responsive gene 1 (*SAUR1*), auxin signal genes (*TIR1* and *IAA8*), as well as genes for nitrate transporter (*NRT*), glutamine synthetase (*GS*), glutamate synthase (*GOGAT*), and nitrate reductase (*NR*). RNA was extracted from the roots inoculated and uninoculated with 'BF-F', respectively, using an Omega plant RNA kit (Omega, Bio-Tek Inc. Norcross, GA, USA). After the analysis of RNA quality, the first strand of cDNA was synthesized using a FastKing RT Kit (Tiangen, Beijing, China) using 1 µg of RNA. The reaction solution for the qRT-PCR included 0.8 of µL forward primer and 0.8 µL of reverse primer (Supplementary Table S1), 1 µL of cDNA, 10 µL of 2 × SYBR Premix Ex Taq (TIANGEN, Beijing), and 7.4 µL of RNA-free H<sub>2</sub>O. The *SpGAPDH* was used as an internal control [42]. Three biological replicates were used for each sample. The reaction program was as follows: 95 °C for 3 min, followed by 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s, for a total of 45 cycles. The qRT-PCR was performed on an iCycler iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA). Relative gene expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method [43].

### 2.6. Plantlet Acclimatization and Their Growth in the Greenhouse

Rooted microshoots or plantlets with a height ranging from 2 to 5 cm and leaf number varying from 8 to 14 (plantlets inoculated with 'BF-F' were larger than those uninoculated) were removed from the culture vessels, washed with water, and transplanted into plug trays filled with a sterilized substrate (at 121 °C for 90 min) consisting of soil and sand in a 2:1 ratio based on volume. The plants were watered and covered with plastic film to maintain moisture in a shaded greenhouse with a temperature range from 20 to 28 °C, a relative humidity of 50% to 80%, and a light intensity of 400 mmol/m<sup>2</sup>/s. One week later, the plastic film was removed, and plants were fertilized with a half-strength Hoagland solution [44]. Plant survival rates were recorded after 35 days of growth in the greenhouse. Subsequently, the liners (rooted plantlets in plug cells) were transplanted to 20 cm containers filled with the soil–sand substrate and grown in a greenhouse.

### 2.7. Statistical Analysis

Data were examined for normality and homogeneity of variance using SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). The data were analyzed using SPSS and presented as the mean ± standard error (S.E.). If significant differences occurred among or between treatments, the means were separated according to Fisher's Protected Least Significant Difference (LSD) at the  $p < 0.05$  level or Student's *t* test.

## 3. Results

### 3.1. Callus Induction

Seedlings germinated from the collected seeds were healthy (Figure 1A), and their leaves were used as explants for inducing calluses (Figure 1B). Calluses occurred initially at the cut edge of leaf explants in a week (Figure 1C) and then from the surface of the leaf explants (in 14 days), where they proliferated, resulting in the entire explant covered with callus mass after 30 days of culture (Figure 1D). There was no contamination in the culture vessels.

As shown in Table 2, all combinations of ZT and IAA were able to induce callus occurrence, but the percentages of callus formation differed significantly. ZT at 1.5 mg/L alone (M1) or with 0.5 mg/L IAA (M3) induced all cultured leaf explants to produce calluses. More than 90% of leaf explants cultured on M2 and M4 (1.5 mg/L ZT with 0.25

and 0.75 mg/L IAA, respectively), M7 (ZT at 2.0 mg/L with 0.5 mg/L), M9 and M10 (ZT at 2.5 mg/L only or with 0.25 mg/L IAA), as well as M14 and M15 (ZT at 3.0 mg/L with 0.25 mg/L and 0.5 mg/L IAA, respectively) produced calluses. Less than 90% of the cultured explants produced calluses when cultured on the other media (M5, 6, 8, 11, 12, 13, and 16).

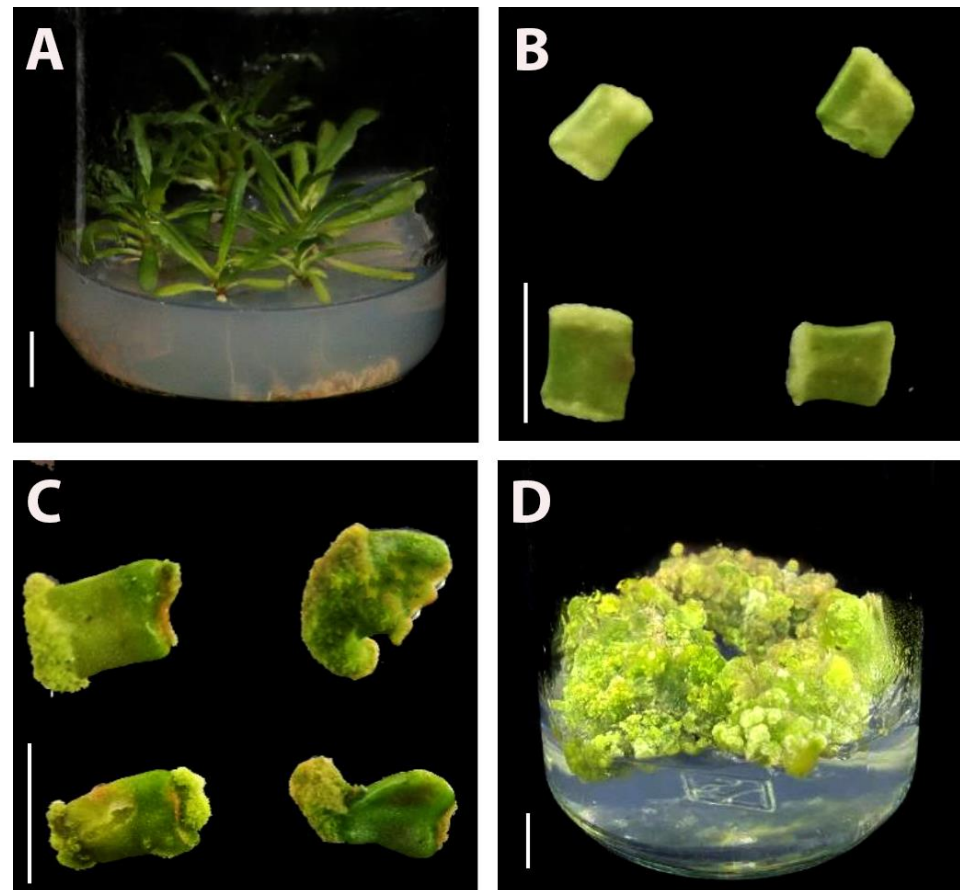


**Figure 1.** Induction of calluses from leaf explants of *S. portulacastrum*. (A) Three-month-old seedlings used as stock plants, (B) leaf explants excised from the seedlings were cultured on MS basal medium containing different concentrations of ZT with or without IAA, (C) callus appeared from the cut edge of leaf explants, and (D) proliferation of callus from leaf explants. The bar = 1 cm.

**Table 2.** The percentage of callus occurrence in leaf explants and adventitious shoot occurrence from callus pieces cultured on the 16 culture media.

| Medium No. | Callus Occurrence (%) | Bud Occurrence (%)          |
|------------|-----------------------|-----------------------------|
| 1          | 100 a <sup>z</sup>    | 73.66 ± 4.42 a <sup>y</sup> |
| 2          | 96.30 ± 3.70 ab       | 0.00                        |
| 3          | 100 a                 | 16.93 ± 2.68 c              |
| 4          | 96.30 ± 3.70 ab       | 7.12 ± 2.41 d               |
| 5          | 70.37 ± 9.80 c        | 16.98 ± 1.52 c              |
| 6          | 81.48 ± 3.70 bc       | 3.61 ± 0.07 d               |
| 7          | 96.30 ± 3.70 ab       | 26.76 ± 2.32 b              |
| 8          | 88.89 ± 6.42 b        | 5.27 ± 0.27 d               |
| 9          | 96.30 ± 3.70 ab       | 27.68 ± 1.31 b              |
| 10         | 92.59 ± 3.70 ab       | 9.06 ± 3.05 d               |
| 11         | 74.07 ± 9.80 bc       | 0.00                        |
| 12         | 88.89 ± 6.42 b        | 5.08 ± 0.15 d               |
| 13         | 85.18 ± 3.70 b        | 22.96 ± 1.39 b              |

Table 2. Cont.

| Medium No. | Callus Occurrence (%) | Bud Occurrence (%) |
|------------|-----------------------|--------------------|
| 14         | 92.59 ± 3.70 ab       | 0.00               |
| 15         | 96.29 ± 3.70 ab       | 0.00               |
| 16         | 66.67 ± 6.42 c        | 0.00               |

<sup>z</sup> Data for callus formation were taken after 30 days of culture (n = 5). <sup>y</sup> Data for adventitious shoot formation were taken after 120 days of culture (n = 3). The values in the last two columns are the means ± standard errors. Different letters after the means indicate significant differences among media in the callus or adventitious shoot inductions based on Fisher's Protected Least Significant Difference (LSD) at the  $p < 0.05$  level.

### 3.2. Induction of Adventitious Shoots

The occurrence in adventitious shoots varied substantially. Table 2 shows that 73.66% of callus pieces cultured on M1 medium (1.5 mg/L ZT) produced shoots, whereas callus pieces cultured on M2, M11, and M14–16 were unable to produce shoots. On the other hand, M7, M9, and M13 induced 26.76, 27.68, and 22.96% of cultured callus pieces to produce shoots, respectively. Callus pieces cultured on the other media had less than 17% shoot induction rates. Shoot occurrence in callus pieces cultured on M1 medium was observed: the callus mass expanded during the first 10 days of culture; small adventitious buds were sporadically dispersed within the callus mass in 20 days; and fully developed shoot buds appeared in about 40 days, followed by shoot and leaf development, and shoot elongation. The mean numbers of adventitious shoots induced from callus pieces cultured on M1, M7, M9, and M13 were 20, 2.5, 7, and 30, respectively, after 120 days of culture (Figure 2). The other media had less than two adventitious shoots induced.

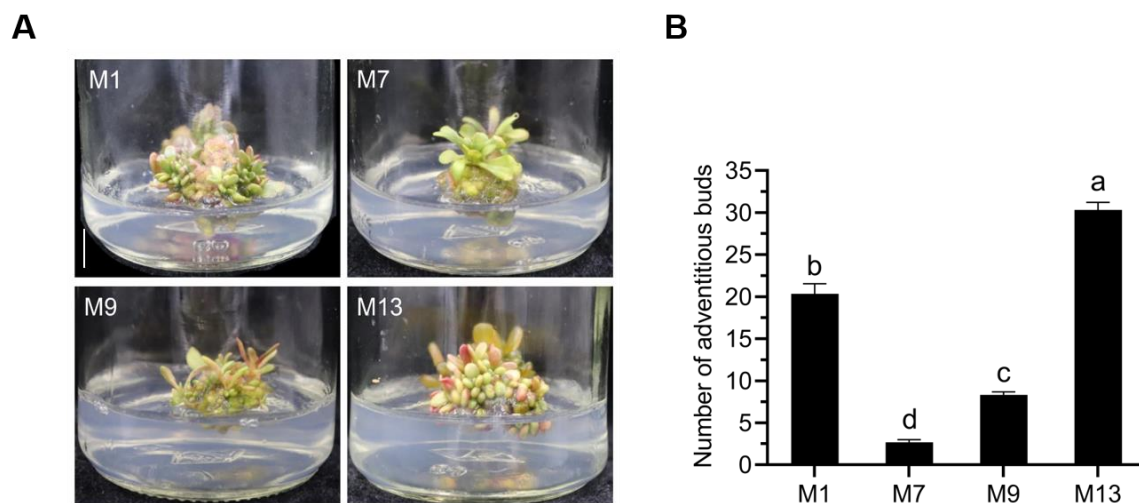


**Figure 2.** Adventitious shoot induction from callus pieces of *S. portulacastrum* cultured on MS media. (A) Shoots induced from callus pieces cultured on M1 (1.5 mg/L ZT), M7 (2.0 mg/L ZT with 0.5 mg/L IAA), M9 (2.5 mg/L ZT), and M13 (3.0 mg/L ZT) media (the bar = 1 cm) (photos were taken about three months after culture) and (B) numbers of adventitious shoots induced from M1, M7, M9, and M13 media (data were recorded 120 days after culture). Different letters above the bars indicate significant differences based on Fisher's Protected Least Significant Difference (LSD) at the  $p < 0.05$  level.

### 3.3. In Vitro Rooting of Microshoots with an Endophytic Fungal Strain 'BF-F'

The colony of endophytic fungal strain 'BF-F' (Figure 3A) was cut into small pieces (0.5 cm) and inoculated on the rooting medium between two microshoots (Figure 3B). The control microshoots were devoid of inoculation (Figure 3C). 'BF-F' quickly proliferated on the medium in less than 10 days (Figure 3D). Microshoots were rooted in the rooting medium, but there were significant differences in the root numbers, root lengths, and plantlet heights between 'BF-F' inoculated and uninoculated microshoots (Figure 4). The

differences were visible at 15 days and became substantial after 20 and 25 days (Figure 4A). On day 30, the height of the plantlets inoculated with BF-F was 3.98 cm compared to 2.29 cm for the control plants. The root number of the plants inoculated with 'BF-F' was 47 compared to 15 for the control plants. The root length of the plants inoculated with 'BF-F' was 3.26 cm compared to 1.98 cm for the control plants (Figure 4B).

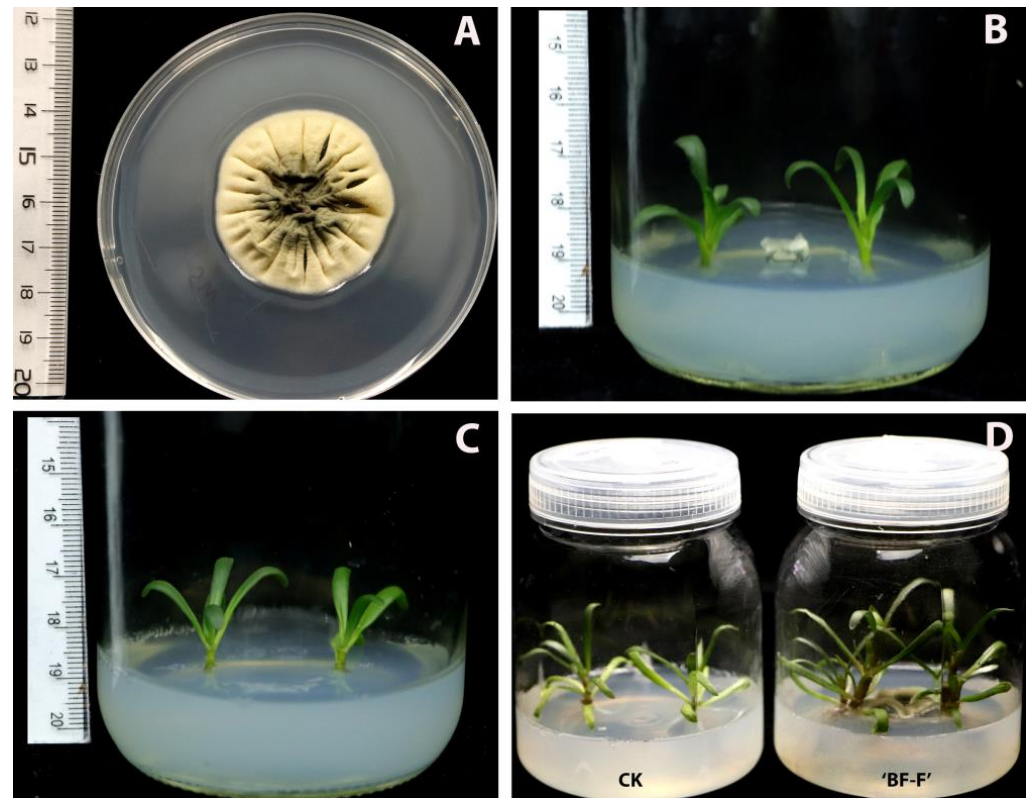


**Figure 3.** Colonial morphology of the endophytic fungus *Cladosporium* 'BF-F' and in vitro rooting of *S. portulacastrum* microshoots on MS basal medium. (A) Colony of 'BF-F' grown on PDA medium for seven days at 25 °C. (B) Two microshoots were inserted in MS basal medium, and a small piece (a diameter 0.5 cm) of the fungal colony was placed between the microshoots. (C) Microshoots inserted in the MS medium without inoculation of 'BF-F' (CK). (D) Growth of microshoots uninoculated (CK) and inoculated with 'BF-F' after two weeks of culture.

We further analyzed the effect of 'BF-F' inoculation on the expression of genes related to root formation and N uptake and metabolism in *S. Portulacastrum*. The qRT-PCR results showed that the inoculation of 'BF-F' significantly increased the expressions of *AIR12*, *ARF*, *SAUR1*, *IAA8*, and *TIR1* in the roots compared to those that were uninoculated (Figure 5). Meanwhile, 'BF-F' also enhanced the expressions of genes related to N uptake and metabolism, including *NRT*, *GS*, *GOGAT*, and *NR* (Figure 5).

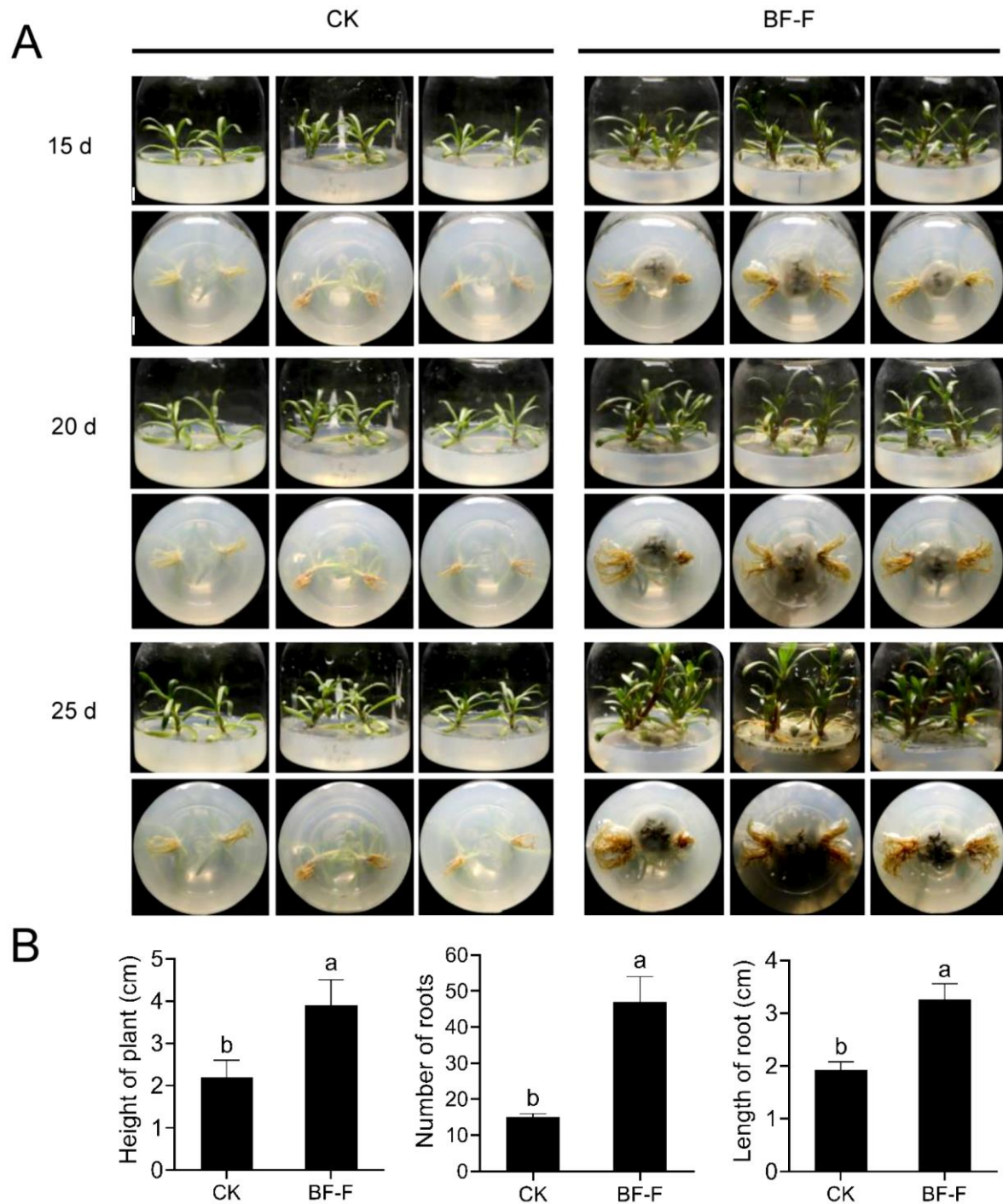


**Figure 4.** In vitro rooting of *S. portulacastrum* microshoots on MS basal medium. **(A)** Rooting of microshoots uninoculated (CK) and inoculated with 'BF-F' after 15, 20, and 25 days of culture (the bar = 1 cm). **(B)** Plantlet height, root numbers, and average root length after microshoots inserted in MS basal medium without and with the inoculation of 'BF-F' for 30 days. Different letters above the bars indicate significant differences based on Student's *t* test at the  $p < 0.05$  level.

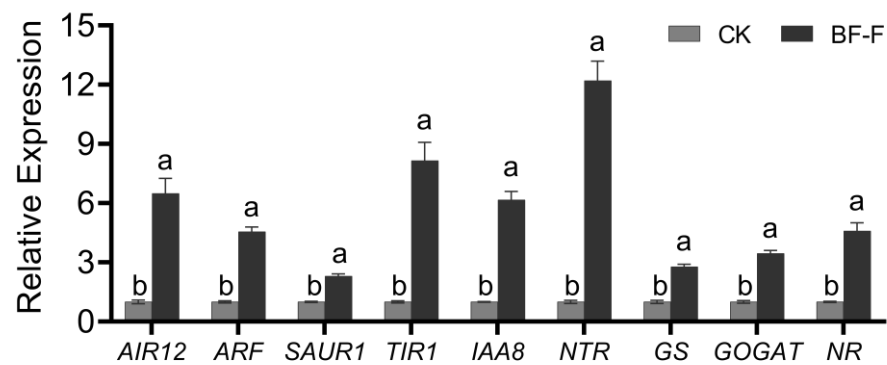


**Figure 5.** Inoculation of ‘BF-F’ enhanced the expressions of genes involved in adventitious root formation and nitrogen uptake and metabolism in roots of *S. portulacastrum*. Different letters above the bars indicate significant differences in the expression of a particular gene between the treatments analyzed by Student’s *t* test at the  $p < 0.05$  level.

### 3.4. Acclimatization and Establishment in a Shaded Greenhouse

Plantlets from in vitro culture were transplanted into plug trays filled with a mixture of soil and sand (2:1), covered with plastic film, and grown in a shaded greenhouse for acclimatization. The plantlets initially inoculated with ‘BF-F’ were larger at the beginning (Figure 6A, day 0). After one week of acclimation, the plastic film was removed, and plants were watered with half-strength Hoagland nutrient solution weekly. The plantlets fully adapted to the environment and were well grown after 35 days (Figure 6A, 35 days). All plantlets survived in plug trays, but the number of leaves and canopy height of the plants inoculated with ‘BF-F’ were higher than those of the uninoculated plants (Figure 6B,C). Three months later, the rooted plantlets of *S. portulacastrum* were transplanted into 20 cm plastic pots and grew healthily and vigorously in a shaded greenhouse.

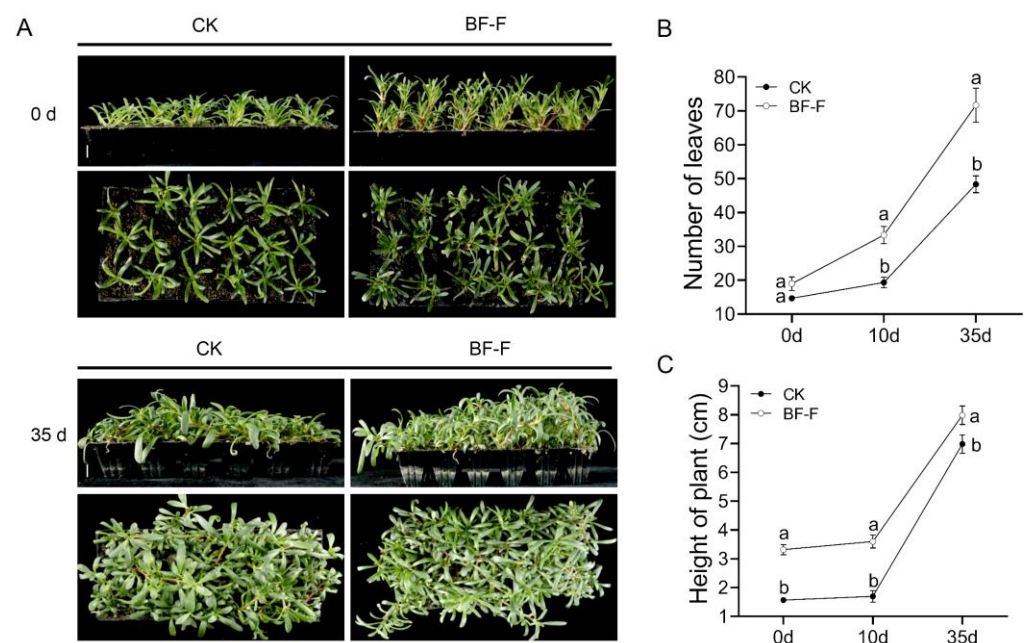


**Figure 6.** Transplanting of plantlets of *S. portulacastrum* into cell plug trays and plant growth in a shaded greenhouse. (A) ‘BF-F’ inoculated and uninoculated plantlets grew in cell plugs right after transplanting (0 d) and after 35 days of transplanting (35 d). Bar = 1 cm. Differences in leaf numbers (B) and canopy heights (C) between plants initially inoculated and uninoculated with ‘BF-F’ after 35 days of growth in a shaded greenhouse. Different letters at specific days indicate significant differences between inoculated and uninoculated plants based on Student’s *t* test at the  $p < 0.05$  level.

#### 4. Discussion

This study developed an efficient protocol for the regeneration of *S. portulacastrum* through indirect shoot organogenesis. Leaf explants were cultured on MS medium supplemented with 1.5 mg/L ZT for 30 days to induce callus formation (Figure 1). The callus was cut into a small piece (0.7 cm × 0.7 cm) and cultured on the same fresh medium for 120 days to induce adventitious shoots (Figure 2, Table 2). Microshoots derived from the adventitious shoots were rooted in MS medium devoid of growth regulator but inoculated with an endophytic fungal strain 'BF-F' for 30 days (Figure 3). Rooted microshoots or plantlets initially inoculated with 'BF-F' were larger and healthier than those without 'BF-F' inoculation. This protocol is simple and efficient, as 100% of cultured leaf explants can produce calluses (Table 2); 73.7% of cultured callus pieces produce adventitious shoots (Table 2). On average, 20 shoots can be induced per callus piece (Figure 2B). Although microshoots can root devoid of growth regulator, the root numbers, root lengths, plantlet height, and leaf numbers of plants initially inoculated with 'BF-F' are substantially greater than those without the inoculation of 'BF-F' (Figures 4 and 6).

This is the first report of indirect shoot organogenesis in *S. portulacastrum*. Previous effort on the in vitro culture of this species was focused on shoot culture using existing meristems (nodes) as explants [30,31]. In the report of Lokhande et al. [30], BA, Kin, TDZ, and 2iP were used for inducing axillary shoots from nodes; shoots were induced from all nodal explants cultured on MS medium supplemented with either BA or 2iP, and the number of shoots per nodal explant were 4.92 and 5.2, respectively. NAA and IAA were used for rooting, and the rooting percentages varied from 50% to 95%. The authors also reported that a callus was induced during shoot culture, but no adventitious shoots were induced from the callus. In our previous report on the shoot culture of *S. portulacastrum* using MS medium [31], we used BA or ZT with IBA, IAA, NAA, and 2,4-D, respectively, as well as TDZ with NAA for inducing axillary shoots, and the highest number of shoots was 7.41 when nodal explants were cultured with 2.0 mg/L ZT and 0.2 mg/L NAA. We then evaluated ZT at 0.5, 1.0, 2.0, and 3.0 with 0.05, 0.1, 0.2, 0.3, and 0.4 mg/L NAA, respectively, and found that 10 to 12 shoots were induced by the culture of nodal explants on MS medium containing 1.0 mg/L ZT with 0.2 mg/L or 0.3 mg/L NAA as well as 2.0 mg/L ZT with 0.05 mg/L NAA. A prolonged culture with ZT induced callus formation. Thus, in the present study, we used ZT directly for both callus and adventitious shoot inductions, which were successful and made regeneration through leaf explants simple. ZT is a native plant hormone, representing isoprenoid cytokinin and not aromatic cytokinin [45]. Endogenous hormones are known to play critical roles in the regulation of organogenesis and morphogenesis [46–48]. It is likely that ZT may be biologically more active for inducing calluses and adventitious shoots in *S. portulacastrum*. ZT has been extensively used for the in vitro propagation of *Vaccinium* species [49–52].

Successful rooting followed by vigorous growth into liners is critically important in plant propagation [53–55]. It was reported that 65% of microshoots of *S. portulacastrum* were rooted without rooting hormone [30]. The application of IBA at 0.6 mg/L and NAA at 1.86 mg/L achieved rooting percentages of 91.7% [30] and 95% [31], respectively, but not 100%. In the present study, we inoculated a novel endophytic fungal strain 'BF-F' for rooting. The rooting percentage elevated to 100%, and the rooting numbers, root lengths, and plantlet heights substantially increased compared to the uninoculated controls (Figure 4). 'BF-F' is an endophytic fungus and can synthesize tryptophan, IAA, and IBA [21]. It is likely that the tryptophan, IAA, and IBA synthesized by 'BF-F' could be released inside plant cells and/or into the culture medium, resulting in the increased rooting percentage and root numbers. Additionally, auxin-related genes *AIR12*, *ARF*, *SAUR1*, *IAA8*, and *TIR1* were highly upregulated in the roots of 'BF-F'-inoculated plants (Figure 5). Their expression could increase endogenous IAA production and further enhance rooting; this phenomenon was reported in *Rhododendron fortunei* colonized by an ericoid mycorrhizal fungus [41]. Furthermore, 'BF-F' induced the expressions of *NRT*, *GS*, *GOGAT*, and *NR*, which is consistent with the previous report that 'BF-F' was able to induce

the expression of genes related to N transport in *Arabidopsis thaliana* [21]. Endophytic *Bacillus* isolates have been reported to enhance rooting and plant growth in *Arabidopsis* [56], cotton [57], and tobacco [58] through the regulation of auxin metabolism. In this study, ‘BF-F’ substantially enhanced the rooting of microshoots and their subsequent growth. Thus, it should be considered a potentially important plant growth-promoting fungus. Plant growth-promoting microbes represent novel agents for sustainable agriculture and environmental remediation [59–62]. Further studies on the mechanisms underlying the ‘BF-F’-mediated growth promotion are warranted.

*Sesuvium portulacastrum* is an emerging crop species due to its nutritional and pharmaceutical values and tolerance to soil and water salinity [22,63,64]. Many ecotypes are able to grow in saline soils or on floating beds in coastal sea water [21,24,25,65]. Thus, tender shoots can be harvested as a leafy vegetable [22,63]. With the development of this new regeneration system, healthy and disease-free *S. portulacastrum* propagules could be rapidly generated for commercial production. Additionally, this regeneration method could be used for testing the functions of genes identified from previous studies [66–69] through genetic transformation.

## 5. Conclusions

This study established a regeneration protocol for the in vitro propagation of *S. portulacastrum* through indirect shoot organogenesis. Leaf explants cultured on MS basal medium supplemented with 1.5 mg/L ZT produced abundant callus mass, and callus pieces cultured on the same fresh medium induced 20 adventitious shoots. Microshoots derived from the adventitious shoots can be easily rooted in MS basal medium inoculated with an endophytic fungal strain ‘BF-F’, which substantially promoted rooting and plantlet growth. This is the first regeneration system developed for *S. portulacastrum*, and in vitro rooting using an endophytic fungal strain may represent a new approach to improve rooting and plantlet growth during in vitro culture.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture14081221/s1>, Supplementary Table S1: Primers used in this study.

**Author Contributions:** Conceptualization, X.W., D.W. and J.C.; supervision, D.P., Q.C. and X.W.; investigation, X.J., W.H. and D.W.; validation, B.Z., Z.L. and L.C.; formal analysis, X.J. and D.W.; resources, D.P., Q.C. and D.W.; data curation and writing—original draft preparation, D.W. and X.J.; writing—editing and revision, J.C.; funding acquisition, X.W. and D.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Natural Science Foundation of Fujian Province (2022J011141), University-Industry Cooperation Project of Fujian Province (2023N5016), Environmental Protection Technology Project of Fujian Province (2021R013), and Science and Technology Innovation Foundation of Fujian Province (KY-090000-04-2021-011).

**Institutional Review Board Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article.

**Acknowledgments:** The authors would like to thank Terri Mellich for critically reviewing this article.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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