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Isolation and Characterization of Endophytic Fungi from Purslane and the Effects of Isolates on the Growth of the Host

Mandlaa[1,2*], Yu Zhang[2*], Yongqing Wan[2], Ying Tie[3], Bo Zhang[4], Ruigang Wang[2], Guangxia Wang[2#]

[1]College of Food Science and Engineering, Inner Mongolia Agricultural University, Hohhot, China
[2]Inner Mongolia Key Laboratory of Plant Stress Physiology and Molecular Biology, Inner Mongolia Agricultural University, China
[3]Hesheng (Inner Mongolia) Ecological Silviculture Co., Ltd., China
[4]Research Institute of Hesheng (Inner Mongolia) Ecological Silviculture Co., Ltd., China
Email: #wgx200109@163.com

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Abstract

Purslane, a common weed, has been used as food or folk medicine in many countries. The growth, medicinal components and nutrient contents of the plant are closely associated with endophytes, especially endophytic fungi. In this study, the endophytic fungi associated with purslane were isolated, and the effects of the isolates on the host were investigated to lay a foundation for further research and development of purslane resources. The results showed that a total of eight endophytic fungi were isolated from purslane (collected from Hohhot, Inner Mongolia, China), and they belonged to the genera Penicillium (isolates K, N, P, M and I), Chaetomium (isolate J), Fusarium (isolate H) and Petriella (isolate O). Moreover, the growth of purslane was significantly influenced by its endophytic fungi. Isolate M can significantly decrease the germination rate, while J can significantly increase the germination rate of purslane. In addition, H, J and M can significantly increase the bud length of purslane, and the fermentation broth of P has a negative influence on the bud length of purslane. M and I can significantly increase the height, fresh weight and chlorophyll content of purslane due, in part, to the lower pH of the fermentation broth of I and M.

Keywords

Endophytic Fungi, Purslane, *Portulaca oleracea* L., Growth Promotion, Germination

1. Introduction

Endophytes are microorganisms that reside within the tissue of living plants

*Mandlaa and Yu Zhang contributed equally to this work.

without any symptoms [1] [2] and endophyte-free plants are currently unknown in nature [3]. It is reported that many medicinal components or the nutrient content of plants are closely associated with endophytes, especially endophytic fungi [4]. The representative example is Taxol, an anticancer drug, which is produced by *Taxomyces andreanae*, an endophytic fungus of Pacific yew [5]. Based on the potential for application to medicine or agriculture, studies of endophytes have received increasing attention [6] [7]. The diversity of functional metabolites produced by endophytes is reviewed by Tan [4], and more than ten different kinds of metabolites related to endophytes can be found in a variety of plants. In addition, it has been shown that endophytes have positive effects on the host [6], with the nutrients [8] [9], hormones [10] [11] and antibiotics [12] [13] provided by the endophyte being able to promote the growth of the host and protect against pathogens.

Purslane (*Portulaca oleracea* L.), which belongs to the Portulacaceae family [14], is a common plant throughout the world. It has been used as food and as folk medicine in many countries [15] and is also a common weed in North America [16]. It has been reported that a polysaccharide component of purslane has antitumor effects in an *in vivo* model [17] and that extracts of the aerial parts (dried leaves and stems) of purslane have analgesic and anti-inflammatory effects [18] [19]. Furthermore, five alkaloids have been isolated from purslane [15], which are the active ingredients of many medicinal plants.

It has been proven that functional natural products can be found in various endophytes [4] [20] [21]. However, the relationships between the medical functions and the endophytes of purslane are still unknown. Basic studies investigating the diversity of the endophytes in purslane are needed. In addition, there are few reports about the endophytes of purslane and the effects of those endophytes on the growth of the host. In our previous study, the endophytic bacteria in purslane were isolated and were found to belong to six genera: Enterobacter, Bacillus, Achromobacter, Paenibacillus, Leifsonia and Rhodanobacter [22]. Therefore, it is important to further study the endophytic fungi of purslane and improve upon the knowledge of the endophytes of purslane.

In this study, the diversity of culturable endophytic fungi of purslane and the effects of those endophytic fungi on the growth of their host are investigated. This study will provide some basic references for management of the plant and for expanding the potential use of purslane as a human or animal food, as a pharmacological agent in medicine and in the biocontrol of weeds.

2. Materials and Methods

2.1. Plant Material

Plants were obtained from 3 different locations in Hohhot of Inner Mongolia, China (41.44 N, 111.69 E): Saihan, Yuquan and Haorenger districts in July 2014. Plants were transported to the laboratory (Inner Mongolia Key Laboratory of Plant Stress Physiology and Molecular Biology, Inner Mongolia Agricultural

University, China), where they were processed for endophyte isolation in less than 24 h.

2.2. Endophytic Fungus Isolation

Surface-sterilization of the stem, root and leaf of purslane was conducted using the following procedure. First, after washing with tap water to remove any attachments, the stems, roots and leaves of purslane were surface-sterilized in an ethanol solution (75%, v/v) for 2 min and then washed 2 - 3 times with sterilized water. Second, a sodium hypochlorite solution (2%, w/v) was applied for 1 min to surface-sterilize the tissue. The sodium hypochlorite residue on the tissues and leaves was removed with sterilized water. Finally, the tissues and leaves were dried on sterilized filter-paper and segmented to 0.5 cm. The sterile water from the last wash was inoculated onto a fresh PDA plate to test the results of the surface-sterilization. After surface-sterilization, the stem and root tissues were placed onto a PDA plate. The leaves were homogenized after surface-sterilized, and the homogenate was diluted in different ratios with sterile water and spread onto a PDA plate. All PDA plates were cultured at 28˚C in the dark.

2.3. Endophytic Fungi Characterization

After purification, a three-point inoculation method was used to culture the endophytes, and the colony morphology was visually observed. The hyphae of endophytes were observed under a microscope. In addition, the isolates were identified with nucleotide sequencing. The genomic DNA of the isolates was extracted using a fungal DNA extraction kit (Omega) according to the manufacturer's protocol, and polymerase chain reaction (PCR) was performed according to an established protocol [23]. The ITS region of the genomic DNA of the isolates was amplified with the primers ITS1 and ITS4 (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', ITS4: 5'-TCCTCCGCTTATTGATATGC-3'). All amplified sequences (commercial sequencing services) were aligned using BLAST in NCBI, and alignments that were more than 97% identical were selected to identify the species of the isolates. Neighbor-joining (NJ) phylogenetic trees were constructed in MEGA7 using 1 000 bootstrap replicates.

2.4. Surface-Sterilization of Seed

To determine the optimal sterilization time, chlorine gas was used to sterilize the purslane seeds. First, purslane seeds in open centrifuge tubes were placed in a beaker. Second, chlorine gas was generated with a saturated solution of sodium hypochlorite and chlorane in a dryer, and the beaker was placed in the dryer. Finally, the chlorane was added to the beaker after two hours to produce chlorine gas. After different times of sterilization (0, 2, 4, 6, 10, 12, 16, 18, 20 and 24 h), 100 purslane seeds were inoculated into 1/2 MS medium, and the rates of germination and survival were calculated to evaluate the effect of sterilization after 48 h of cultivation. The rates of germination and survival were calculated as

follows: Germinated seeds divided by the total number of seeds and seeds forming colonies in 1/2 MS medium divided by the total number of seeds, respectively.

2.5. Preparation of Fermentation Broth

The isolates were inoculated into liquid PDA medium and cultured at 28˚C and 180 rpm. After 6 d of cultivation, the supernatants of the isolates were obtained by centrifugation and then filtered through gauze filters (4 layers) to remove the hyphae and stored at 4˚C. The pH of the fermentation broth of the isolates was determined using a pH meter. The fermentation broth was diluted 10-fold with sterile water to investigate the effect of fermentation broth on the seed germination rate, the bud length and the growth of purslane.

The sterilized purslane seeds were planted in sterile soil (soil:vermiculite = 1:2) with 5 seeds per pot. Then, 100 ml of fermentation broth (diluted 10-fold with sterile water) was added to the treatment group, and 100 ml of PDA medium (diluted 10-fold with sterile water) was added to the control group once a week. The plants were cultured under greenhouse conditions (16-hlight/8-h-dark cycle, 22˚C and Humidity 50%). The plant height, fresh weight and concentration of chlorophyll were determined on the 21st and 60th days. Chlorophyll determination was performed as reported by Porra *et al.* [24] and Wu *et al.* [22]

2.6. Statistical Analysis

Three replicates were performed for all treatments. Differences between two groups were regarded as statistically significant if $P < 0.05$. Microsoft Office Excel 2007 was used to analyze the data and draw the figures.

3. Results

3.1. Identification of Endophytic Fungi

After cultivation, no microbial colonies were observed on the PDA (potato dextrose agar) plates inoculated with the final rinse solution of surface-sterilization, proving that the surface-sterilization method was feasible and that the isolates were endophytic fungi of purslane. After several rounds of purification, a total of 18 isolates were obtained. Some isolates were initially considered to be the same species based on colony morphology and color by referencing the Fungal Identification Handbook [25]. Finally, eight different isolates were obtained from the samples (three from roots, two from stems and three from leaves, respectively). The results are shown in **Figure 1** and **Table 1**.

A common method for identifying fungal species is by aligning the ITS region with known sequences in GenBank in combination with the morphological analysis. In this study, eight endophytic fungi were isolated from purslane. The results of the alignments are shown in **Figure 2**. When integrated with the results of morphological analysis (**Table 1**): H was identified as *Fusarium oxysporum*; I was identified as *Penicillium ochrochloron*; J was identified as *Chaetomium globosum*; K was identified as *Penicillium citreonigrum*; M was identified

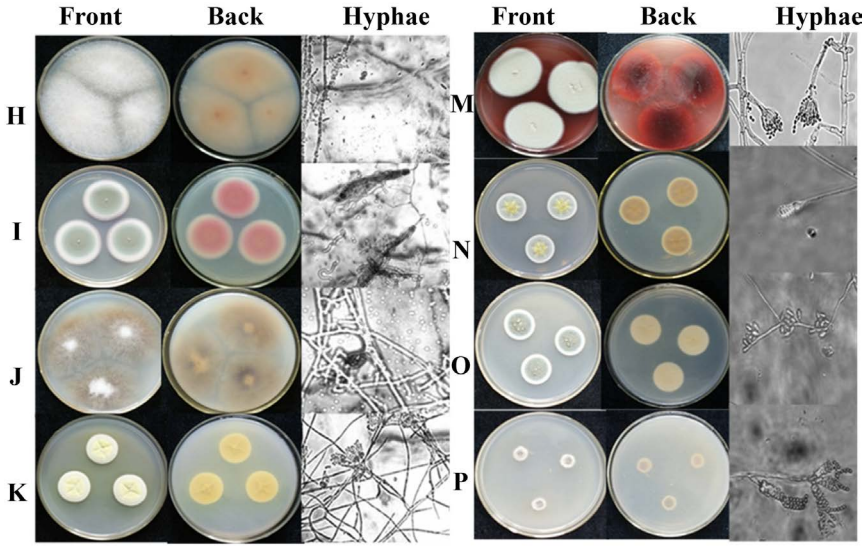

Figure 1. The colonial morphology of isolates.

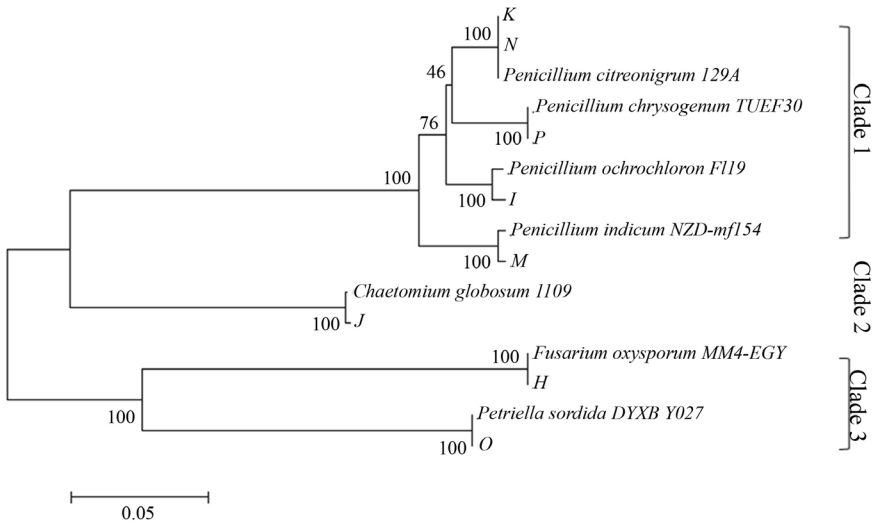

Figure 2. Phylogenetic N-J tree inferred from ITS rDNA sequences of the isolates.

Table 1. The description of colonial morphology of isolates.

Position	Strain	Description of colony	Description of hyphae
Root	H	The colony was white and structure was cotton-like. The back of colony was yellow.	The conidiophores were short without branch and had two kinds of conidia.
	J	The colony was brown and gradually generated white hyphae with irregular margins. The back of colony was and brown.	The perithecium was black and spherical. The ascus was clavate and ascospores were spindle-shaped.
	O	The colony was dark green and the edge of colony was white with regular margins. The back of colony was yellow.	There were condias on conidiophores and the conidia were oval.
Stem	K	The colony was white and gradually become light yellow with regular margins. The back of colony was yellow.	There are branches on the conidiophores and the conidia were spherical and single spore.

Continued

leaf	N	The colony had three kinds of colors: Center was yellow, middle was green and edge was white with regular margins. Water drops colored light yellow could be seen on the colony. The back of colony was yellowish-brown.	There are branches on the top of conidiophores and the conidia were spherical and single spore. Hyphae were no septum.
	M	The colony was white in initial stage and gradually become gray with regular margins. The back of colony was dark red.	The conidiophores were short with branch on the top and the conidia were spherical and single spore. Hyphae were no septum.
	P	Compare with other isolates, the colony was small. The color of colony in the center was brown and in the edge was white with irregular margins. The color of the back of colony was gray in center and white in edge respectively.	The conidiophores had short branches and the conidia were spherical, single spore. Hyphae had septum.
	I	The color of colony was white in initial stage and gradually become dark green with regular margins. The back of the colony was pink.	The conidiophores were short without branch and the conidia were spherical, single spore.

as *Penicillium indicum*; N was identified as *Penicillium citreonigrum*; O was identified as *Petriella sordid*, and P was identified as *Penicillium chrysogenum*. Furthermore, the isolates can be divided into three clades, namely: Hypocreomycetidae (H and O), Sordariomycetidae (J) and Eurotiales (K, N, P, I and M), by taxonomy.

3.2. The Effect of the Endophytic Fungi on the Host

The results of the sterilization of purslane seeds are shown in **Figure 3**. When the sterilization time was 0 h and 2 h, there were still the same number microbes on the purslane seeds. Prolonging the sterilization time can significantly reduce the survival rate of microbes on the surface of purslane seeds, and the microbes present on the seeds of purslane are completely inactivated after more than 4 h of sterilization. However, the germination rate of purslane seeds can be reduced by the increase in sterilization times. Sterilization times of more than 12 h can significantly reduce the germination rates of purslane seeds. Therefore, in order to ensure the effectiveness of seed sterilization and to minimize the harm caused by the sterilization time, we selected 6 h as the best sterilization time for purslane seeds. In addition, seeds collected from the open field are often contaminated with fungi and bacteria, which can influence the germination rate of seeds. The sterilization time (2 - 12 h) by chlorine gas has positive effects on the germination rate of purslane seed, as shown in **Figure 3**. This can probably be explained by the fact that chlorine gas can inactivate microbes that are harmful to the germination of purslane seeds.

After 6 h of sterilization, the fermentation broth of endophytic fungi was added to the purslane seeds to investigate its effects on the germination rate and bud length of the purslane seeds. The results are shown in **Figure 4**. The fermentation

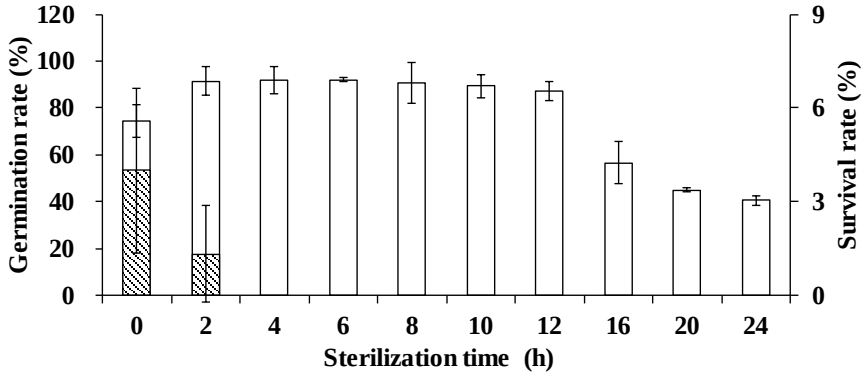

Figure 3. The effect of sterilization time on the germination rate of purslane seed and survival rate of microbial on purslane seed. The filled bar is germination rate and the open bar is survival rate of microbes on the surface of purslane seeds.

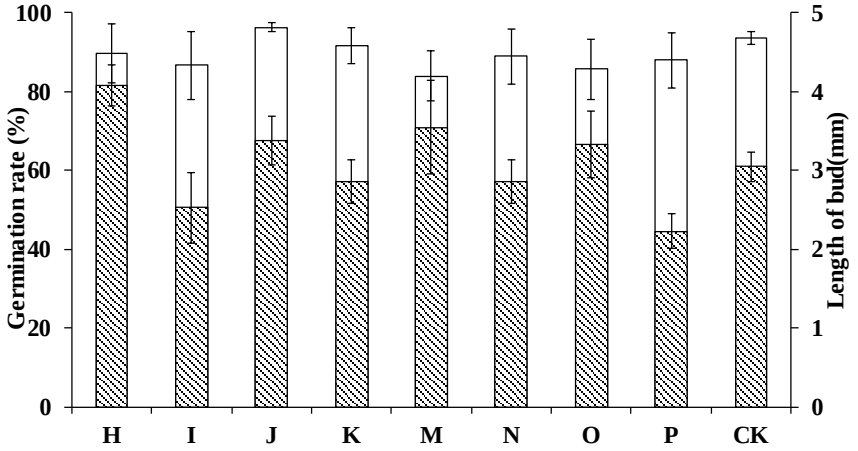

Figure 4. The effect of endophyte on the germination rate and bud length of purslane seed. The filled bar is germination rate and the open bar is length of bud.

broths of J and M significantly influenced the germination rate of purslane seeds. The germination rate was increased by 2.83% in treatment J and decreased by 9.97% in treatment M when compared with that of CK. The length of the bud was significantly influenced by the addition of the fermentation broths of H, J, M and P. The addition of the fermentation broths of H, J and M can significantly increase the bud length of purslane, while the fermentation broth of P had a negative influence on the bud length of purslane. In this study, J was identified as *F. oxysporum* and can probably produce polysaccharides to promote the growth of purslane buds, although this needs to be confirmed in future work.

The effect of endophytes on the growth of the host was investigated, and the fresh weight, height and chlorophyll content were measured at 21 and 60 d after the addition of the fermentation broths from the isolates. After 21 d of treatment, the fresh weight was increased by 47.02%, 72.64% and 58.04% with the addition of fermentation broths from I, M and O, respectively (**Figure 5(a)**). Addition of the fermentation broth of the isolates does not significantly influence the height and chlorophyll content of purslane after 21 d of cultivation

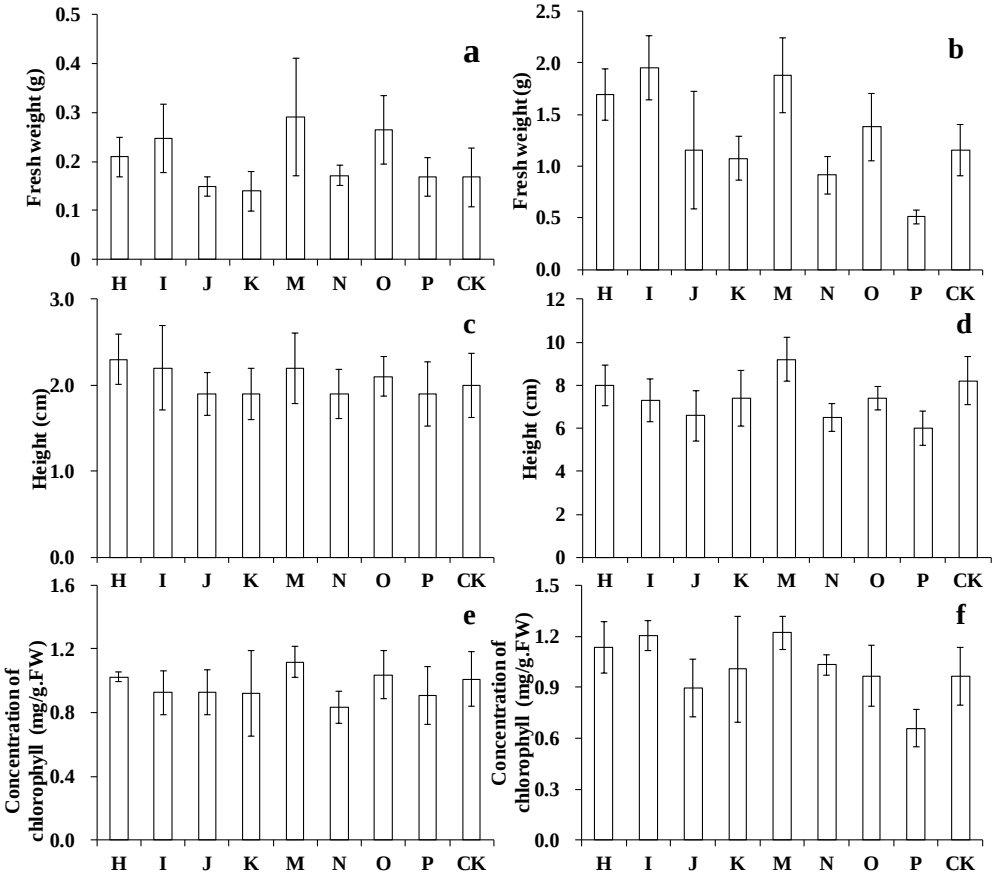

Figure 5. The effect of endophyte on the height, fresh weight and chlorophyll content of purslane.

(**Figure 5(c)** and **Figure 5(e)**). After 60 d of treatment with the fermentation broths of the isolates, there were significant differences in the fresh weight, height and chlorophyll content. The fermentation broths of H, I, and M can significantly increase the fresh weight of purslane, while the fermentation broth of P can significantly inhibit the growth of purslane when compared with that of CK. The fresh weight of purslane to which the fermentation broth of P decreased by 55.90% when compared with that of CK (**Figure 5(b)**).

Furthermore, the addition of the fermentation broth of M can significantly increase the height of purslane (by 12.19%). However, the addition of the fermentation broths of I, J, N, O and P significantly decreased the height of purslane by 11.19%, 19.85%, 20.77%, 9.56% and 26.28%, respectively, when compared with the height of CK (**Figure 5(d)**). The effects of the isolates on the appearance of purslane are shown in **Figure 6**. In this work, isolates M and I can significantly increase the height, fresh weight and chlorophyll content of purslane, as can be observed in **Figure 5**, whereas M had a significant negative influence on the germination of purslane (**Figure 4**). To obtain further information about the mechanism of promoting growth, the pH of the fermentation broths of M and I was determined, and it was found that M and I can both significantly decrease the pH of the fermentation broth (**Figure 7**).

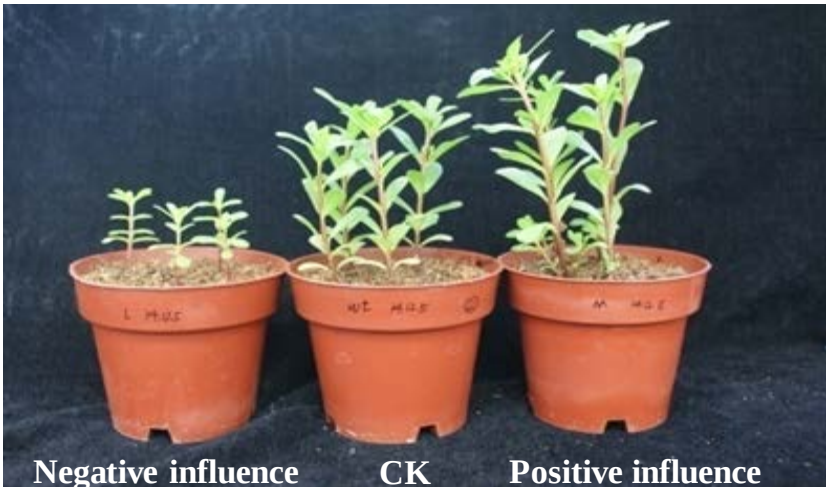

Figure 6. The effect of isolates on the appearance of purslane.

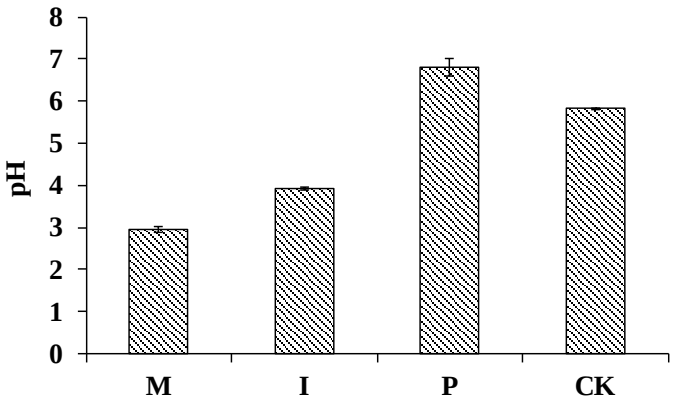

Figure 7. The effect of M and I on the pH of medium.

4. Discussions

Penicillium is a common genus of endophytic fungi found in plants, [26] and most species can inhibit the growth of other microorganisms or secrete metabolites that promote the growth of plants. Radhakrishnan and his coworker reported a strain of *Penicillium* sp. that was isolated from the rhizosphere soil of peanuts and can promote the growth and enhance the ability of sesame (*Sesamum indicum* L.) to resist salt stress by secreting various of amino acids and antagonizing pathogenic fungi [8].

In this study, four species of the *Penicillium* genus were isolated from purslane, and most of them were isolated from the stem and leaf. There was no *Penicillium* species isolated as endophytic fungi from the roots of purslane in this study. The species *P. ochrochloron*, *P. citreonigrum* and *P. chrysogenum* have been isolated as endophytic fungi in several plants and algae [27]. However, this is the first report in which *P. indicum* was isolated as an endophytic fungus. There have been few studies on *P. indicum*, especially as endophytic fungi. The first report about *P. indicum* was published in 1936, where it was isolated from sputum [28].

Some strains of *P. ochrochloron* were found to have inhibitory effects on the growth of gram-positive and gram-negative bacteria, as well as against fungi through the secretion of chitinase or other metabolites [29] [30]. *P. chrysogenum* was known as an industrial penicillin production strain [31], and it has been reported that the secondary metabolites of *P. chrysogenum*, an endophytic fungus isolated from a red alga, can inhibit the growth of the pathogen *Alternaria brassicae* [32]. *P. chrysogenum* MTCC 5108 was an endophytic fungus isolated from the mangrove plant *Porteresia coarctata*, and its culture medium showed significant activity against *Vibrio cholera*, the causative agent of cholera [33]. Some strains of *P. citreonigrum* can secrete several kinds of metabolites, which can inhibit the growth of *Staphylococcus epidermidis* and Candida strains [34]. In addition, some metabolites of *P. citreonigrum* can also inhibit the growth of the HepG2 cell line [35].

F. oxysporum was identified as an endophytic fungus in wild banana (*Musa acuminata*) roots [10] [36] and *Juniperus recurva* [37]. Some studies have shown that the polysaccharides produced by *F. oxysporum* can significantly promote the sprout growth of *Faopyrum tataricum* [38]. Strain J was identified as *Chaetomium globosum* in this study, and it has been reported that endophytic *Chaetomium* sp. isolated from wheat leaves can reduce the development of disease caused by *Pyrenophora triticirepentis* in wheat leaves [37]. Some species of endophytic *Chaetomium* sp. were confirmed to enhance the resistance to copper stress in maize seedlings [39]. The endophytic *Petriella* spp. isolated from *Pinus roxburgii* was initially identified by Altschul *et al.* [40] and some species of this genus can produce petriellin A, an antifungal depsipeptide [41].

The diversity of endophytes is closely related to the species of plant and their location. However, the physiological mechanisms that influence endophyte diversity are still unknown [42]. In this study, a total of 18 strains of endophytic fungi were isolated, belonging to four genera. However, seven genera of endophytic fungi (*Fusarium*, *Penicillium*, *Paecilomyces*, *Trichoderma*, *Cladosporium*, *Lasiodiplodia* and *Aspergillus*) were isolated from the stems of purslane grown on the shore of a reservoir containing various heavy metals [43]. Few and different endophytic fungi were isolated in this study. This was probably affected by plant species, location and selection of sterilizer and sterilization time. Some components of the same plant species from different locations or conditions are often different. This phenomenon is related to both the climate and geographical location; whether it is related to the endophytes of the plant may be a future direction for study in this area.

Seed germination is a critical life stage for plant survival and timely seedling establishment [44]. A study on *Lolium perenne* and *Festuca arundinacea* showed that endophyte-infected plants had a higher rate of germination [45]. The best conditions for the germination of common purslane were studied by Hopen [46]. However, the relationship between endophytic fungi and the germination of purslane was not examined. The fermentation broth of endophytic fungi had a significant influence on the germination of purslane, indicating that the inte-

raction between endophytic fungi and purslane began from the germination period. It has been reported that endophytes are not a strong influence on germination and that the presence of endophytes probably changes germination through an indirect effect in *L. multiflorum*. However, the presence of endophytes can influence the dormancy levels of seeds [47]. Several reports have suggested that plant growth is enhanced by endophytic fungi. The mechanism includes the fact that endophytic fungi can produce a variety of beneficial metabolites for plant growth and survival, such as gibberellins, indoleacetic and amino acids [11] [23] [48]. Additionally, they can secrete some antibiotics to defend their hosts from attack by certain pathogens [30] [33] Moreover, they can enhance nutrient uptake [9] and tolerance to environmental stress [42].

Photosynthesis is the main source of energy for plants, and its efficiency is related to photosynthetic pigments such as chlorophylls and carotenoids, the influence of the addition of the fermentation broth of isolates on the chlorophyll content of purslane leaves was significantly different after 60 d of cultivation. The addition of the fermentation broths of I and M could increase the concentration of chlorophyll by 25.10% and 26.77% respectively, while the addition of the fermentation broth of P significantly decreased the chlorophyll content of purslane (**Figure 5(f)**). It is reported that endophytic *Phomopsis liquidambari* can increase the chlorophyll content in rice shoots and the amounts of glutamate, threonine and alanine in rice roots under low-N treatment [9]. Chlorophyll synthesis begins from glutamate in plants, and sufficient glutamate is an important condition for ensuring the synthesis of chlorophyll. Therefore, the ability of an endophyte to promote nitrogen uptake may be one of the reasons for the increase in the chlorophyll content of plants, and it suggests that M and I can enhance the nitrogen uptake of purslane. The reduction in chlorophyll content as a result of treatment with endophyte P may be related to many factors, such as nutrient uptake or the secretion of harmful substances.

The growth of purslane was dependent on an adequate amount of phosphorus in the nutrient media [45] [49]. Indicating that, phosphorus was a key nutrient for purslane. In the phosphorus cycle, microbes play a crucial role in mobilizing various forms of phosphorus, and the organic acids produced by microbes can solubilize inorganic forms of phosphorus [50]. It was reported that some species of *Penicillium* increased the amount of phosphorus in solution by solubilizing inorganic phosphorus [51]. In addition, some studies have shown that over 60% of purslane seeds germinated between pH 5.0 and 10.0, while no seeds germinated at pH 4.0 [52]. The low pH probably provided more soluble phosphorus for the purslane seedlings, but it affected the germination of the purslane seeds.

5. Conclusion

In this study a total of eight species of endophytic fungi were isolated from purslane (H was identified as *F. oxysporum*; I was identified as *P. ochrochloron*; J was identified as *C. globosum*; K was identified as *P. citreonigrum*; M was iden-

tified as *P. indicum*; N was identified as *P. citreonigrum*; O was identified as *P. sordida*, and P was identified as *P. chrysogenum*) and were identified using morphological and molecular biological techniques. The effects of the isolates on the growth of the host plant were investigated, and it was determined that isolate M can significantly decrease the germination rate, while J can significantly increase the germination rate. In addition, H, J and M can significantly increase the bud length of purslane, while the fermentation broth of P had a negative influence on the bud length. However, M and I can significantly increase the height, fresh weight and chlorophyll content of purslane. The ability of M and I to decrease the pH probably was the main reason for their ability to promote growth.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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