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Survival Dynamics of *Trichoderma longibrachiatum* Tr58 in Conidia- and Chlamydospore-Amended Soils with Different Moisture Levels

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Abstract: Two types of *Trichoderma longibrachiatum* Tr58 propagules, conidia and chlamydospores, were added to soils with different moisture levels. The survival dynamics of Tr58 in soils were determined. There are positive linear relationships between soil moisture levels and germination rates of the two propagules. In natural non-sterilized soil, the germination of more than 95% conidia and 60% chlamydospores was inhibited, while a high soil moisture content and sterilization were beneficial to spore germination. The inhibitory effect of soil with 80% moisture content on the germination of chlamydospores was almost completely eliminated after sterilization. Twelve months after the conidia inoculated to the natural soil, the Tr58 propagules decreased continuously, which was hastened in soils with lower moisture content and almost near zero 24 months later, in all soils. In chlamydospore-amended soils, the Tr58 propagules generally showed a dynamic process of decreasing in the first month, increasing in the 2nd month, and then decreasing gradually. The average Tr58 content in chlamydospore-amended soils with 5, 10, 20, 40, and 80% moisture content was 19.2 times that of conidia-amended soils at 12 months. At 24 months, the Tr58 content was about 2.2% of the initial Tr58 content and 114 times that of conidia in soils with 20% moisture content. However, for 80% moisture content, the Tr58 content in soil was 0.0038% of the initial content of Tr58. According to the results of this study, 10–20% soil moisture content was the most favorable for the long-term survival of Tr58, and the survival ability of chlamydospores was stronger than that of conidia and had greater application potential in disease control.

Keywords: fungal propagules; soil moisture level; *Trichoderma longibrachiatum* Tr58; survival dynamics; soil fungistasis

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1. Introduction

Trichoderma spp. are common in soil and root ecosystems [1–3]. Some *Trichoderma* strains, such as *T. hazianium* T22 and T39 and *T. virens* G-41, have been used as biocontrol agents because they have demonstrated significant inhibitory effects on major soil-borne fungal pathogens, including *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., and *Rhizoctonia solani* [4]. *Trichoderma* can produce two types of asexual spores: conidia and chlamydospores. Among them, conidia are easily produced in large numbers [5], while chlamydospores are only produced in small amounts under adverse conditions [6–8]. Therefore, most commercial products of *Trichoderma* are made of conidia. However, conidia have weak stress resistance and are sensitive to adverse environments, which cannot guarantee the effect of disease control. Compared with conidia, chlamydospores are large in size, with thick cell walls, and rich nutrients within the spore content. Therefore, chlamydospores are highly resistant to adverse environments [9] and are speculated to have higher biocontrol potential than conidia [10–12].

Soil fungistasis is the capability of soils to inhibit the germination and growth of soil-borne fungi [13–15], which is beneficial for inhibiting the propagation of pathogenic fungi. However, for beneficial fungi, such as *Trichoderma*, fungistasis is not conducive to their propagation and therefore weakens the effect of plant disease control. Beagle-Ristaino et al. [16] found that soil can strongly inhibit the germination of *Trichoderma* conidia depending on the species used. However, the inhibitory effect on the chlamydospores was relatively weak. Adding nutrients, such as sucrose and glucose, to the soil can partially or completely relieve the inhibitory effect of soil on *Trichoderma* conidial germination [12], while fertilizers, such as urea, can inhibit conidia germination [17]. Therefore, understanding the effect of soil fungistasis on different types of propagules and the growth of *Trichoderma* is helpful to improve the survival ability and biomass of *Trichoderma* in soil and to improve the biocontrol effect of *Trichoderma* against disease.

In our previous study, we reported the production mechanism of *Trichoderma* chlamydospores [18,19], and established a fermentation process for producing large quantities of *Trichoderma* chlamydospores. Pesticide residues in soil have different inhibitory effects on the germination and growth of *Trichoderma* spores, and chlamydospores are more tolerant of pesticide residues than conidia [20]. Chlamydospore preparation is more effective in controlling cucumber *Fusarium* wilt and cotton *Verticillium* wilt than conidia preparation, and improves the physiological activity of plants [21,22]. Soil moisture content is a key factor affecting the development of plants and microorganisms [23,24]. Studying the survival dynamics of biocontrol *Trichoderma* in different moisture soils is helpful for further understanding the population change rule of *Trichoderma* in soil and guiding the irrigation management and ensuring the safe agricultural production. In this study, the effects of soil moisture on the survival of *T. longibrachiatum* Tr58 in soils in which conidia and chlamydospores were applied were studied. The results will be helpful for further understanding the interaction between *Trichoderma* spores and soil and laying a foundation for guiding the application of *Trichoderma* preparations and improving the effect of disease prevention.

2. Materials and Methods

2.1. Strain and Soil

Trichoderma longibrachiatum Tr58 (CGMCC No. 9727) was isolated from soil in the Xinjiang region of China. The soil was collected from 10–30 cm layers in the experimental field (39°57′58.12″ N, 116°20′11.59″ E) of the Chinese Academy of Agricultural Sciences in Beijing. The soil is a sandy loam with an initial moisture content of 15.7% and a pH of 6.4, containing 20.09 g kg^{-1} organic matter, 0.78 g kg^{-1} of total N, 134.5 mg kg^{-1} of available P, and 87.49 mg kg^{-1} of available potassium. The soil was air-dried, ground, passed through a 0.5 cm sieve, and stored temporarily at 4 °C.

2.2. Preparation of Conidia Powder of T. longibrachiatum Tr58

The *Trichoderma* strain was cultured on PDA (potato dextrose agar) culture medium (200 g of potato, 20 g of dextrose, 10 g of agar, 1000 mL of distilled water, and neutral pH) for 7 days in the dark at 28 °C, and the conidia were collected by washing the agar surface with distilled water. The conidia counts were determined under a microscope (Olympus B × 53, Tokyo, Japan) [19]. The conidial suspension was diluted to a concentration of 1×10^6 conidia mL^{-1}. In 500 mL Erlenmeyer flasks, 10^6 conidia were transferred to 100 mL PD (potato dextrose) liquid medium and cultured at 26 °C on a rotary shaker (200 r/min), in the dark for 2 days. The culture was used to inoculate sterilized rice with a 30% moisture content by weight; the inoculum was cultured at 26 °C for 7 days and mixed well once a day. The inoculum was dried at 28 °C for 2 days. Conidia powder was obtained by sifting it through a 100-mesh sieve, and it was stored at 4 °C [25].

2.3. Preparation of Chlamydospore Powder of T. longibrachiatum Tr58

Three milliliters of conidial suspension (1×10^6 conidia mL^{-1}) of Tr58 were used to inoculate 120 mL of chlamydospore-inducing medium (1% glucose, 1% cornmeal, 0.5%

yeast extract powder, 1% corn steep powder, and 5% inductive agent) [19] in 500-mL Erlenmeyer flasks and incubated at 26 °C on a rotary shaker (200 rpm) in the dark for 5 days. Diatomite (3–5%) was added to the fermentation liquor and mixed well. The chlamydospore powder was obtained by filtering and drying the above mixture at room temperature.

2.4. Effect of Soil with Different Moisture Contents on Spore Germination of T. longibrachiatum Tr58

Part of the collected soil sample was sterilized at 121 °C for 3 h. Both sterilized and non-sterilized soil samples were adjusted to different moisture contents of 5, 10, 20, 40, and 80%, by adding sterile water, placing each 20 g soil sample into a 10 cm plate (90 × 90 mm), and balancing the plate for 24 h. Then, a small 2.5 × 2.5 cm sterilized cellophane (BIO-RAD#165-0963) disk was placed on the soil, and 10 µL of spore suspension (10^5 conidia or chlamydospores/mL) of Tr58 was spread on the cellophane disk and covered with a 2.4 × 2.4 cm cover glass. The cellophane was then moved to the slide after 24 h at 25 °C to check spore germination. The PDA plate was used as a positive control instead of a soil plate. The experiment was repeated three times with three replicates each. The spore germination rates and germination inhibition rates were determined using the following formulas [26]:

Spore germination rate (%) = spore number/total spore number;

Germination inhibition rate (%) = (control spore germination rate−spore germination rate)/control germination rate.

2.5. Survival Dynamics of T. longibrachiatum Tr58 in Conidia- and Chlamydospore-Amended Soils

The experiment was conducted in the same field where the soil was collected. The soil samples were adjusted to moisture contents of 5, 10, 20, 40, and 80% and mixed well with conidia or chlamydospore powder of Tr58 to about 4.5 × 10^6 CFU/g soil. One thousand grams of each soil sample was loaded into 20 µm 30 × 30 cm nylon mesh (Star Huang Screen Products Co., Ltd., Dongguan, China) bags and sealed. The bags were placed in 40 × 50 cm plastic drums and filled with soil of the same moisture content around the bags. The buckets were then sealed and buried 20 cm underground. To test the survival dynamics of Tr58 in soil, 3 g soil samples were taken each month, and the *Trichoderma* content (CFU/g) was detected using *Trichoderma*-selective medium (0.20 g $MgSO_4$, 0.90 g KH_2PO_4, 1.0 g NH_4NO_3, 0.15 g KCl, 3.0 g glucose, 0.15 g Rose Bengal, 0.25 g chloramphenicol, 0.05 g streptomycin, 15 g agar, 0.3 g pentachloronitrobenzene (PCNB), and 1 L distilled water) with reference to the method outlined by Redda et al. [27].

2.6. Experimental Design and Statistical Analysis

A completely randomized design was adopted with three replicates per treatment. Microsoft Excel 2007 was used to prepare the figures and tables. The average and standard deviation of either three or five independent replicates for each treatment were calculated. Pearson correlation analysis was used to assess the correlations between soil moisture level and the germination rate of propagule. The data were analyzed by one-way and two-way ANOVAs followed by Tukey's post-hoc test at the significance level of $p < 0.05$, using DPS7.05 software.

3. Results

3.1. Effects of Soil Moisture Content and Sterilization on the Germination of Conidia and Chlamydospores of T. longibrachiatum Tr58

The germination rates of conidia and chlamydospores of Tr58 at 24 h in sterilized and non-sterilized soils with moisture contents of 5, 10, 20, 40, and 80% were detected as shown in Figure 1. The soil had a strong inhibitory effect on the germination of the two propagule types. With an increase in soil moisture content, the germination rate of the two propagule types increased accordingly. There are positive linear relationships between soil moisture levels and germination rates of the two propagules, with Pearson correlation coefficient of r = 0.95. Soil sterilization was beneficial to spore germination; the germination rates of

chlamydospores were significantly higher than those of conidia ($p < 0.01$). For example, the germination rate of conidia in the natural soil with 20% moisture content was only 0.79%, while it increased to 30.76% in the sterilized soil, which was 38.9 times higher than that in the non-sterilized natural soil. The germination rate of chlamydospores in natural soil with 20% moisture content was 20.72%, and was 59.90% in sterilized soil, which was 2.89 times higher than that in non-sterilized natural soil. The germination rates of chlamydospores were higher than those of conidia in different soil samples. In sterilized soil and non-sterilized soil, the chlamydospore germination rates were 6.9–26.2 times and 1.7–2.3 times those of conidia, respectively. The chlamydospore germination rate in non-sterilized soil with 80% moisture content was 34.54%, and it increased to 85.26% after sterilization, which was close to that of the control (85.92%), indicating that the inhibition of soil to chlamydospore germination was almost completely eliminated after sterilization by the soil with 80% moisture content. These results indicated that the tolerance of chlamydospores to soil fungistasis was higher than that of conidia; reducing soil microbial populations by sterilization was beneficial in relieving soil fungistasis.

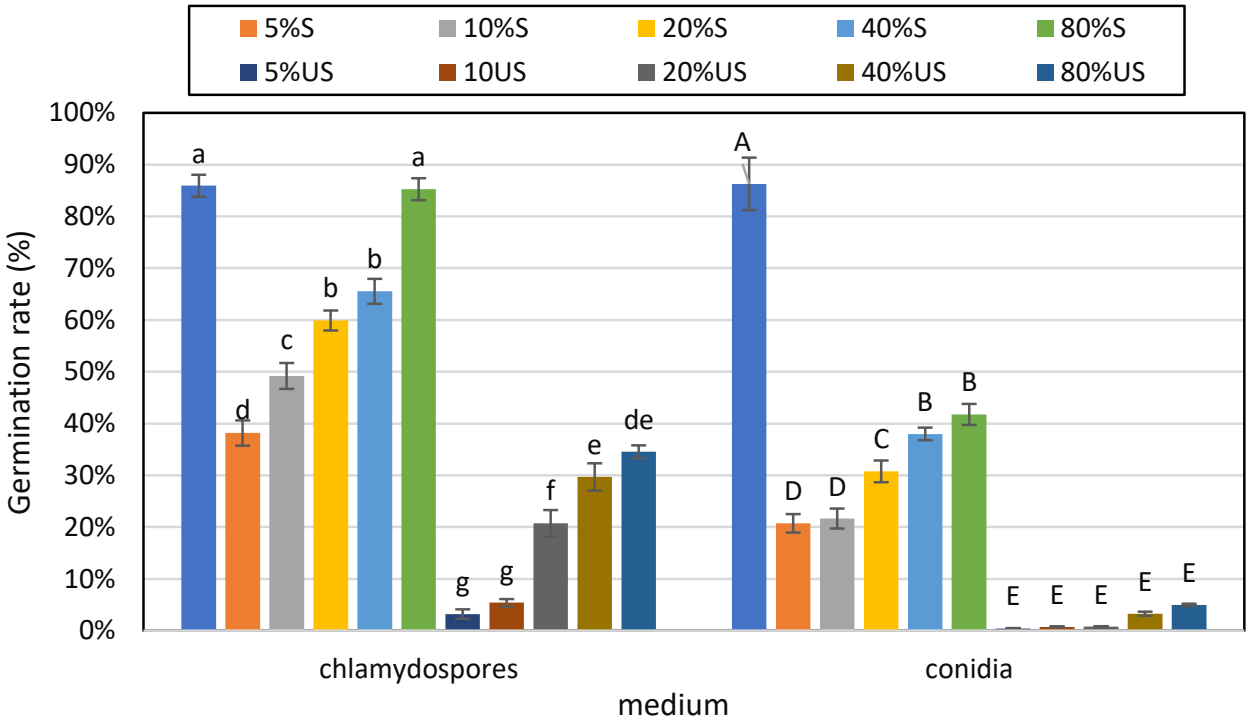

Figure 1. Germination rates of conidia and chlamydospores of *Trichoderma longibrachiatum* Tr58 in sterilized and unsterilized soils with different moisture levels. Lowercase letters and uppercase letters respectively indicate the significant differences of germination rates of chlamydospores and conidia in different mediums, the same letter above the column indicates no significant difference at $p < 0.05$. CK: PDA medium; US: unsterilized soil; S: sterilized soil, 5%, 10%, 20%, 40%, 80%: soil moistures.

3.2. Survival Dynamics of T. longibrachiatum Tr58 in Conidia-Amended Soils with Different Moisture Contents

Conidia of *T. longibrachiatum* Tr58 (about 4.5×10^6 CFU/g soil) were added to soils with moisture contents of 5, 10, 20, 40, and 80%, respectively, and the well-mixed soils were then buried underground in a natural environment for 12 months. The Tr58 content in soil was detected every month and denoted by CFUs per gram of soil. The results are shown in Figure 2. The Tr58 content in all soil samples decreased continuously over time, among which the Tr58 content in soil with a 5% moisture content decreased the most and the most quickly. The Tr58 content in 20% moisture content soil was the highest, which showed that this moisture content was relatively conducive to the survival of Tr58 and enabled disease control. In the 12th month, the Tr58 content was 0.16, 0.16, 7.38, 6.48, and 6.36% of the

initial application in soils with 5, 10, 20, 40, and 80% moisture content, respectively. A dry environment was not conducive to the long-term survival of Tr58.

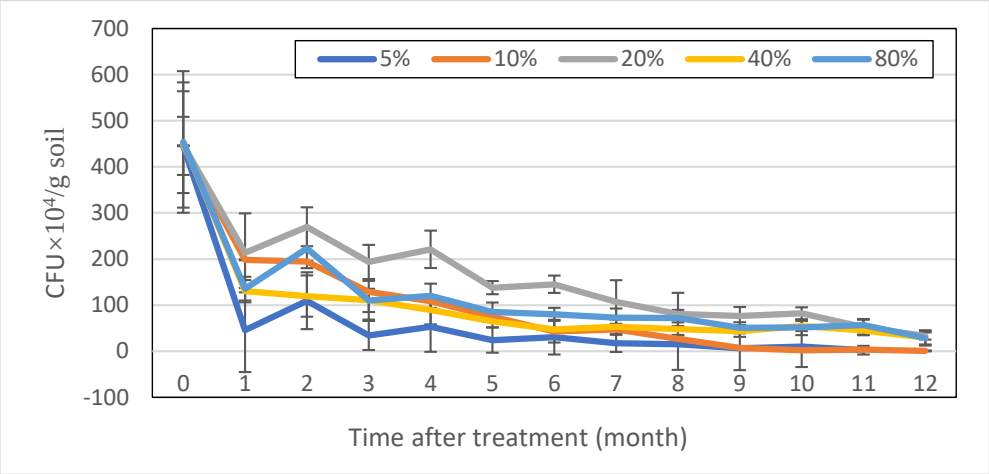

Figure 2. Survival dynamics of *Trichoderma longibrachiatum* Tr58 conidia in soils with different moisture contents.

3.3. Survival Dynamics of T. longibrachiatum Tr58 in Chlamydospore-Amended Soils with Different Moisture Contents

Chlamydospores of *T. longibrachiatum* Tr58 were applied to natural soil with different moisture contents. The Tr58 content over 12 months is shown in Figure 3. After a temporary decline in the first month, the Tr58 content increased rapidly in the 2nd month and then decreased slowly. Among the soils with different moisture contents, the Tr58 content in the soil with 5% moisture content was highest in the 3rd month, 1.90 times the initial content; it then decreased continuously to 1.40% of the initial content after 12 months. In soils with 10, 20, 40, and 80% moisture content, the Tr58 content was highest in the 2nd month and was 1.68, 2.34, 2.08, and 2.43 times the initial content, respectively, and then slowly decreased to 30.69, 102.87, 106.80, and 123.63% of the initial content, respectively, after 12 months. The Tr58 content still reached or exceeded the initial application content after 12 months in soil with a moisture content more than 10%. Tr58 chlamydospores had a strong tolerance for soil fungistasis.

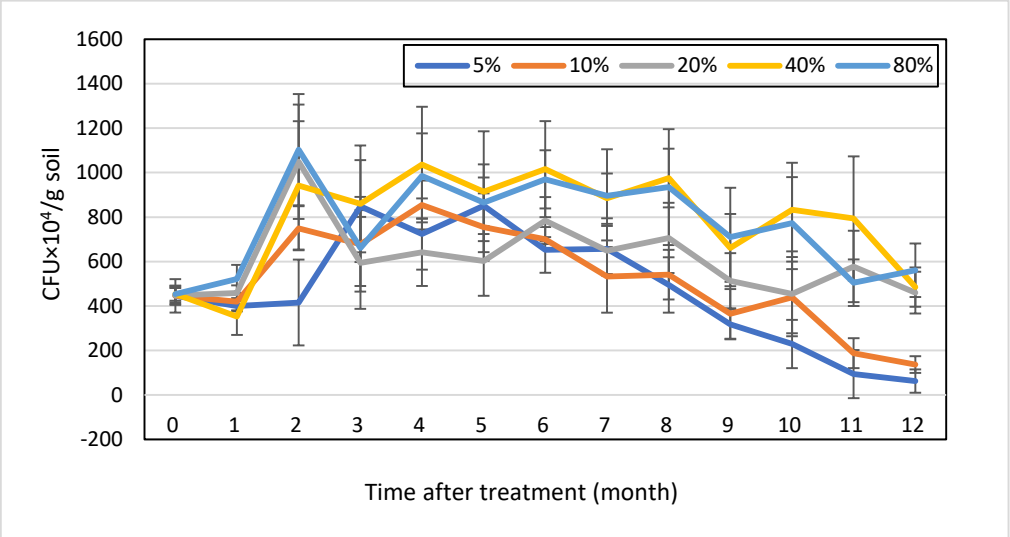

Figure 3. Survival dynamics of *Trichoderma longibrachiatum* Tr58 chlamydospores in soils with different moisture levels.

3.4. Comparison of Survival Dynamics of T. longibrachiatum Tr58 between Conidia- and Chlamydospore-Amended Soils with Different Moisture Contents

Approximately 4.5 × 10^6 CFU/g of Tr58 conidia and chlamydospores were mixed well in the soils with different moisture contents, and the dynamic changes of Tr58 contents were compared over 12 months (Figure 4). The Tr58 content in the conidia-amended soils was at a low level and continued to decrease. As for chlamydospore-amended soils, the Tr58 content showed a dynamic process of first increasing in the 2nd month and then decreasing slowly; these values were much higher than those in conidia-added soils. The average Tr58 content in chlamydospore-amended soils with 5, 10, 20, 40, and 80% moisture content was 19.2 times that of conidia-amended soils at 12 months. The results indicated that chlamydospores were more tolerant than conidia, especially in a relatively dry soil environment; therefore, they have high potential for disease control.

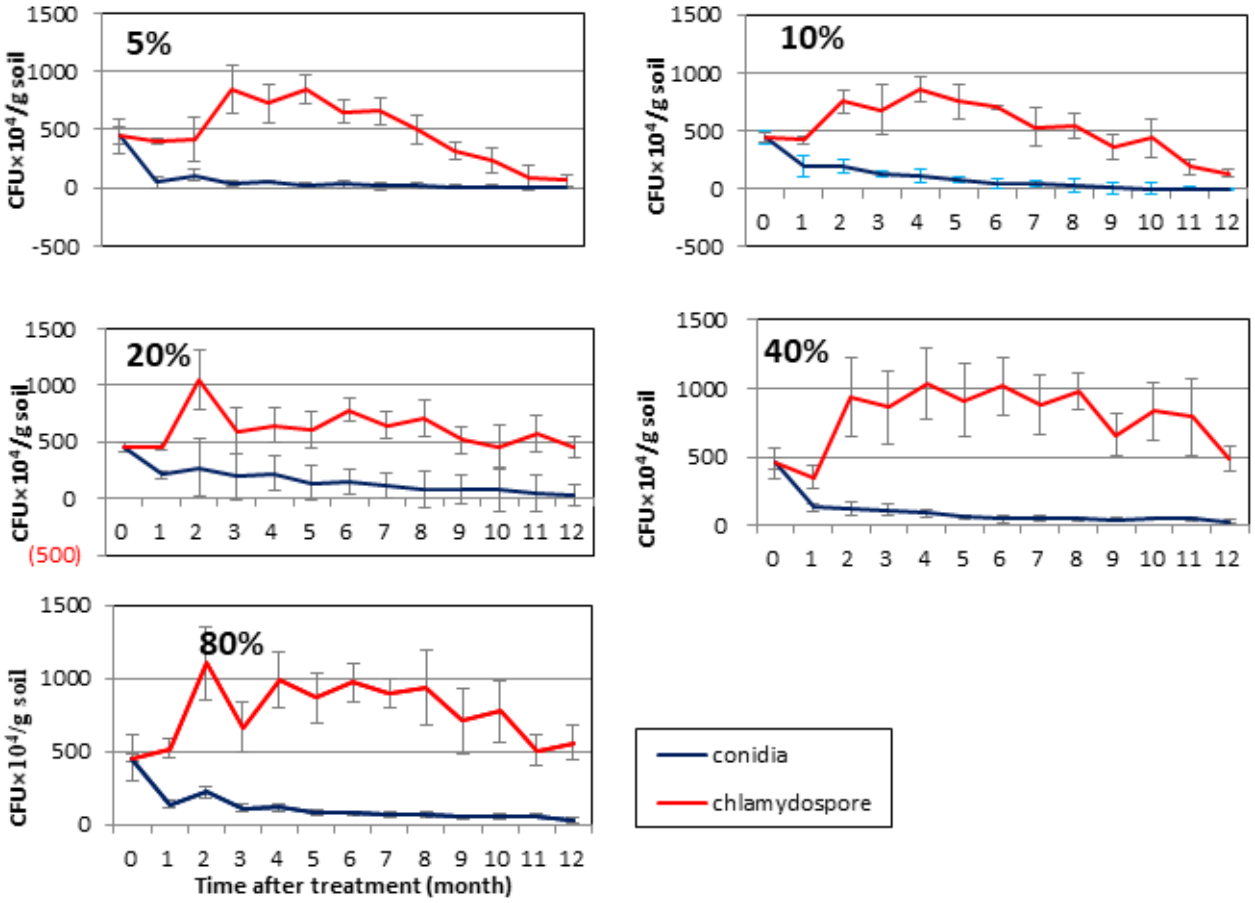

Figure 4. Comparison of *Trichoderma longibrachiatum* Tr58 content between conidia- and chlamydospore-amended soils with different moisture levels.

The effects of soil moisture content and propagules on Tr58 content in soils were analyzed statistically at 2, 7, and 12 months after inoculation of conidia and chlamydospore, as shown in Figure 5. All contents of Tr58 in conidia-amended soil were lower than those of chlamydospore. The contents of Tr58 after 2 months were generally the highest among the three detection time points. The highest content of Tr58 was found in the chlamydospore-amended soil with 80% moisture content, while the lowest content of Tr58 was in the conidia-amended soil with 5% and 10% moisture content, and the difference between them was not significant. The result showed that adding chlamydospores, a relative high moisture content of soil, and short duration in soils are beneficial for survival of *Trichoderma*.

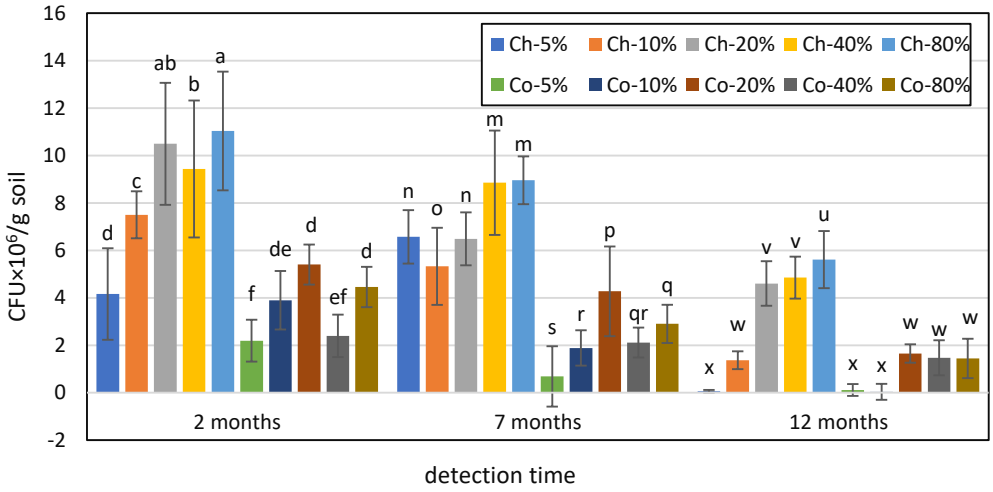

Figure 5. *Trichoderma longibrachiatum* Tr58 contents in conidia- and chlamydospore-amended soils with different moisture contents in a natural environment. Lowercase letters a–f, m–r, and u–x respectively indicate the significant difference of Tr58 contents in different moisture soils amended chlamydospores and conidia-mended soils at 2, 7, and 12 months later, and the same letter above the column indicates no significant difference at $p < 0.05$. Ch: chlamydospore; Co: conidia; 5%, 10%, 20%, 40%, 80%: soil moistures.

3.5. Comparison of the T. longibrachiatum Tr58 Content in Conidia- and Chlamydospore-amended Soils with Different Moisture Contents after 24 Months

Conidia and chlamydospores of Tr58 were added to soils with 5, 10, 20, 40, and 80% moisture content and kept in a natural environment for 24 months. The Tr58 content in soils is shown in Figure 6 and expressed as the log value of CFU/g soil. The Tr58 content in conidia-amended soils was 130–250 CFU/g, which was close to those in the natural soil (data not shown), indicating that the increase in Tr58 content in soils due to additional conidia was close to zero, 24 months later. In chlamydospore-amended soils, the Tr58 content exceeded 1000 CFU/g soil, which was significantly higher than that of conidia. The Tr58 content was $1.95 \pm 0.44 \times 10^4$ CFU/g soil with 20% moisture content, which was about 2.2% of the initial Tr5 content (4.5×10^6 CFU/g) and 114 times that of conidia in soils with the same moisture content. However, for 80% moisture content, the Tr58 content in soil was 1750 ± 677 CFU/g soil 24 months later, which was only 0.0038% of the initial content of Tr58. Chlamydospores were more suitable for long-term survival in soil than conidia, and a low soil moisture content such as 10% was conducive to the long-term survival of chlamydospores.

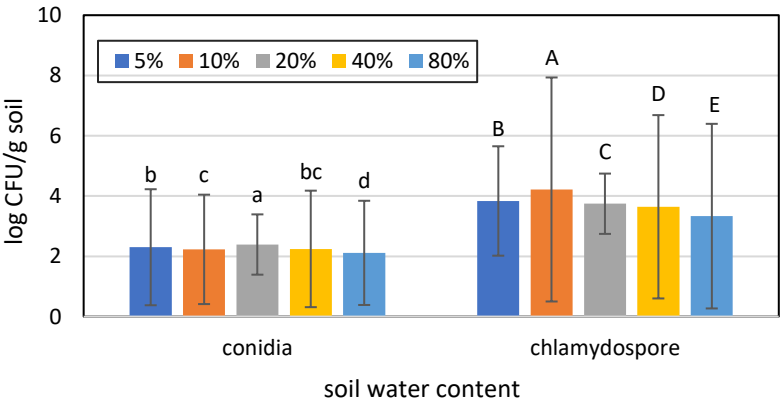

Figure 6. *Trichoderma longibrachiatum* Tr58 content in conidia- and chlamydospore-amended soils with different moisture contents after 24 months. Lowercase letters and uppercase letters respectively indicate a significant difference between Tr58 contents in different moisture soils amended chlamydospores and conidia, the same letter above the column indicates no significant difference at $p < 0.05$.

4. Discussion

Soil fungistasis was first reported by Dobbs and Hinson [28] and is an important approach to control soil-borne plant pathogens [29]; however, it also inhibits the survival of biocontrol fungi, such as *Trichoderma*. The mechanism of soil fungistasis was described as the nutrient deprivation hypothesis and the antibiosis hypothesis [30–32]. Fungal spores were susceptible to soil fungistasis due to rapid nutrient loss after entering the soil until the external environment, such as root exudates, provided nutrients to eliminate the inhibited effects. The antibiosis hypothesis considered that microbial community composition affects soil fungistasis. The more microbes in the soil, the stronger the inhibitory effect on fungi [29,32]. Some *Pseudomonas* strains were found to strongly inhibit the germination of *Fusarium culmorum* and *T. harzianum* [30]. Different strains of fungi and different propagule types and sizes of the same strain have different sensitivities to soil fungistasis [33]. Spores with larger individuals are generally thought to be more resistant to soil fungistasis than small ones [16,32]. In this study, the volume of Tr58 chlamydospores was about 40 times that of conidia (data not shown), containing more nutrients and having stronger germination and growth ability than conidia in nutrient-deficient soil environments. The germination rate of the Tr58 strain in this study was 26 times higher than that of conidia in natural soil with a 20% moisture content. In addition, the germination rates of both propagule types in sterilized soil were significantly higher than those in non-sterilized soil, which was identical to the germination characteristics of *Trichoderma viride* spores reported by Wei Pan et al. [12]. Therefore, the results of this study are consistent with the two hypotheses mentioned previously.

Trichoderma has been widely studied and applied as an important biocontrol fungus for the control of soil-borne plant disease [34,35]. It is believed that increasing the population and viability of *Trichoderma* in soil would improve the diseases control effect [36], which has always been the focus of researchers on *Trichoderma* research and application for improving its disease control effect against plant diseases. In this study, we compared the survival dynamics of two Tr58 propagule types in soils with different moisture levels to understand the interaction between different *Trichoderma* propagule types and soil and to guide the efficient utilization of *Trichoderma* biocontrol products.

Trichoderma exists in the form of hyphae and two types of spores: conidia and chlamydospores. In a complex natural soil environment, all viable spores and hyphae fragments can form colonies on the medium. It is difficult to accurately track and detect the survival of the spores and hyphae of *Trichoderma* in natural soil. Therefore, *Trichoderma*-selective medium [27,37] was used in this study to eliminate the interference of other bacteria and fungi in soil and to evaluate the *Trichoderma* content with the colony-forming numbers in soil.

According to our study, it was speculated that after a short period of adjustment, after being applied to soils, some chlamydospores began to germinate and develop hyphae. However, the germination of most conidia was inhibited and were finally inactivated. Compared to conidia, more chlamydospores germinate or remain active but dormant. They germinate when the conditions are suitable, and therefore the *Trichoderma* contents in chlamydospore-applied soils were higher than those in conidia-applied soils and had a higher disease prevention potential. Our previous study found that the control effects of *Trichoderma* chlamydospore preparations on *Fusarium* wilt of cucumber and *Verticillium* wilt of cotton are higher than those of conidial preparations with the same concentration [21,22], which can be well explained by the result of this study.

The high humidity environment was conducive to fungal spore germination and mycelial growth, while mycelia in a dry environment tended to generate conidia [38–40]. According to this study, soil water content and soil microbial community are highly correlated to the germination of *Trichoderma* conidia and chlamydospores. The content of Tr58 in soil with high moisture content was significantly higher than that in soil with low moisture content. Moreover, due to the high tolerance of chlamydospores to an adverse environment, a large amount of Tr58 was still detected in the chlamydospores-amended

soils with a moisture content of 10% or above in the 12 months. However, the content of Tr58 in conidia-amended soils was all close to zero. The environmental tolerance and biological characteristics of Tr58 chlamydospores and conidia in this paper are consistent with that of other fungi such as *Clonostachys rosea* and *Monilinia laxa* [39,40]. According to this study, soil with a 10–20% moisture content was conducive to the long-term survival of Tr58. Different *Trichoderma* strains and their spores have different reproductive characteristics and environmental tolerance. The results of this study provide guidance for the application of *Trichoderma* products for disease control.

5. Conclusions

There are positive linear relationships between soil moisture levels and germination rates of conidia and chlamydospores of *T. longibrachiatum* Tr58. Soil sterilization was beneficial in relieving soil fungistasis and helpful for germination of Tr58 spores. Tr58 content in chlamydospores-amended soils was significantly higher than that in conidia-amended soils. The average Tr58 content in chlamydospore-amended soils with 5, 10, 20, 40, and 80% moisture content was 19.2 times that of conidia-amended soils at 12 months. Tr58 content was close to zero 24 months later in all conidia-amended soils. A high moisture content of soil was conducive to the propagation of Tr58. A 10–20% soil moisture content was the most favorable for the long-term survival of Tr58. The survival ability of chlamydospores was stronger than that of conidia and had greater application potential in disease control.

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