Combined Use of Two *Trichoderma* Strains to Promote Growth of Pakchoi (*Brassica chinensis* L.)

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Abstract: *Trichoderma* spp., which widely exist in nature, are always used as plant growth-promoting stimulants. However, there is little reporting about the combined use of *Trichoderma* strains to promote growth of plants. We explored two different *Trichoderma* strains (*Trichoderma atroviride* LX-7 and *Trichoderma citrinoviride* HT-1) and a mixture of the two on the growth-promoting effects of pakchoi. In this study, in vitro-promoting traits of two *Trichoderma* strains were determined, and six treatments were used: *T. atroviride* LX-7, *T. citrinoviride* HT-1, different mixtures of these two (1:1, 1:2 and 2:1) and no inoculation in the seed germination and pot experiment. The results showed that *T. atroviride* LX-7 and *T. citrinoviride* HT-1 had the ability of siderophore and indol acetic acid (IAA) production, and LX-7 had the capacity for potassium solubilization. The highest seed germination percentage (GP), germination energy (GE), germination index (GI), vitality index (VI) and growth of radicles and plumules was observed in the LX-7 + HT-1 (1:1) combination, the highest biomass and quality of plants was observed in the LX-7 + HT-1 (1:2) inoculation (followed by a single LX-7 or HT-1 strain inoculation), while the lowest values were obtained in the untreated seeds or plants. On the basis of this study, combined use of two *Trichoderma* strains had greater benefits for growth and quality of pakchoi, making this formulation attractive for future field applications.

Keywords: *Trichoderma*; synergies; plant growth; biostimulant; pakchoi

1. Introduction

Environmental factors, such as water, light, temperature, nutrients, and microorganisms, always influence plant growth. Many studies have reported that some microorganisms can produce beneficial effects for plants [1–4]. *Trichoderma* is well known as a plant growth-promoting fungi that exists widely in nature and includes plant tissues, the rhizosphere, soils, etc. [5]. During the process of plant–*Trichoderma* interaction, *Trichoderma* can benefit the plant in various ways [6]. Many reports have shown that *Trichoderma* stimulated growth and increased yield in several plants including pepper [7], *Pinus sylvestris* var. *mongolica* [5], and maize [8]. Meanwhile, *Trichoderma* can also improve tolerance to biotic or abiotic stresses. Inoculating *Trichoderma* can effectively control plant diseases caused by *Botrytis cinerea* [9], *Fusarium oxysporum* [10], *Colletotrichum gloeosporioides* [11] and other pathogens. *Trichoderma* also protects plants against drought stress [12] and heavy metal (Pb and Cd) stress [13]. In addition, Kulandaivelu et al. [14] reported that *Trichoderma* can also enhance soil enzyme activity and nutrient elements as a biostimulant. Therefore, compared with chemical pesticides or fertilizer, *Trichoderma* spp. is a new environmentally friendly option for plant health management, which has the advantages of being pollution-free, residue-free, and safe for humans and animals, as well as providing environmental protection and making it difficult to produce resistance [15].

However, microorganisms do not exist alone in nature. In the process of microbial resource utilization and microbial fertilizer research, several microorganisms have become mixed together, which can adapt to a wider ecological environment and increase the
functional diversity of strains [16]. Furthermore, it may produce a better plant growth-promoting effect than a single strain, which has important implications for the development of compound microbiotic fertilizers. In recent years, most researchers, such as Esitken et al. [17], Banchio et al. [18], Karlidag et al. [19], Orhan et al. [20] and Colla et al. [21], mainly focused on mixed bacteria, AM (arbuscular mycorrhizae) fungi and \textit{Trichoderma} spp. They inoculated mixed strains into the rhizosphere of plants (vegetables, fruits, etc.) and found that this could promote plant growth and improve plant yield. It is verified that the suitable mixed bacterial could again play a synergistic role, however, there are few reports about the effects of mixed \textit{Trichoderma} strains on plant growth.

\textit{Pakchoi} (\textit{Brassica chinensis} L.) is rich in high nutritional value such as vitamin C and minerals [22], is growing rapidly, has low production input, and is an important vegetable largely consumed in China [23]. The farmers usually pursue high-yield products and effective disease control; they extensively use chemical fertilizers and high toxic pesticides, which leads to excessive heavy metal and high nitrate content and threatens human health [24]. Therefore, it is necessary to find a safe and sustainable way to improve the growth of pakchoi.

In this study, we compared combined use of two \textit{Trichoderma} strains and single strains to promote the difference in pakchoi growth and quality. The positive results of these tests lead us to highly recommend the \textit{Trichoderma} spp. to be exploited as plant growth-promoting towards pakchoi seedlings. This lays a foundation for the development of biological agents of mixed microbials.

2. Materials and Methods

2.1. Plant Samples and Fungal Strain Culture

\textit{Pakchoi} seeds (\textit{Brassica campestris} L. ssp. \textit{chinensis} Makino) were bought from Gansu Minsheng Agricultural Technology Co. LTD, Lanzhou, China.

In this study, \textit{T. citrinoviride} HT-1 (accession number: MT781604.1) and \textit{T. atroviride} LX-7 (accession number: MT781611.1) were isolated from the roots of \textit{Rheum palmatum} from HuaTing city (1746 m, 106°30′26.28″ E, 35°18′22.18″ N) and Li county, Wudu city (2282 m, 104°52′38.03″ E, 34°05′46.48″ N) in China, respectively, which was preserved in the Microbial Collection of College of Life Science, Northwest Normal University. The fungal isolates were saved on PDA medium (potato dextrose agar) at 4 °C. Each fungus was grown on PDA plates at 28 °C for 7 days with a 16 h light/8 h dark cycle. Conidial suspensions were harvested from the PDA mediums by using 2 mL sterile water, and then diluted to $10^7$ spore/mL for subsequent experiments.

2.2. In Vitro Promoting Traits of Strains

Indol acetic acid (IAA) by fungal strains was tested by using the procedure of Wani et al. [25]. Siderophores production by fungal strains was measured by using the procedure of Alexander et al. [26]. Phosphorus solubilization and potassium absorbable was tested by using the procedure of Yuan et al. [27].

2.3. Bioassay for Germination Test

The homogeneous pakchoi seeds were selected for uniformity based on size and color, and 2% (v/v) NaClO solution was used to surface-sterilize for 20 min at room temperature, then rinsed thoroughly with deionized water. Disinfected pakchoi seeds were set on the two layers of filter papers in 90 mm sterilized culture dishes, and then we used 5 mL different treatments and sterile water (control, CK) to treat seeds. There were 5 treatments in the experiment: HT-1 and LX-7 alone, and strains were mixed in 1:1, 1:2 and 2:1 ratios. Three replicates of 100 seeds were tested for germination per treatment. Seeds were incubated in a dark incubator under 25 ± 1 °C, 70–75% relative humidity for 7 d. During the 7 d germination process, the number of germinated seeds were recorded daily. Germination percentage (GP), germination index (GI), germination energy (GE) and vitality index (VI), radicle length, plumule length, fresh weight and dry weight were measured after 7 d.
2.4. Determination of Germination Parameters

When the radicle elongated at least 2 mm from the seed surface, we considered that seeds had germinated. Related indexes of seed germination were calculated according to the following formula:

Germination percentage (%) = \( \frac{\text{number of germinated seeds in 7th d}}{\text{number of all tested seeds}} \times 100 \)

Germination energy (%) = \( \frac{\text{number of germinated seeds in 3rd d}}{\text{number of all tested seeds}} \times 100 \)

Germination index = \( \sum (\frac{\text{Gnt}}{\text{Dt}}) \)

Vitality index = \( \sum (\frac{\text{Gnt}}{\text{Dt}}) \times R \)

In the formula, Gnt is the germination number at a given time, Dt is the germination day, and R is the radicle length of the seedlings.

2.5. Test for the Capacity to Promote Plant Growth (Pot Experiment)

The disinfected seeds were incubated on the two layers of filter paper in sterilized 90 mm culture dishes at 25 °C for 4 d. After seed germination, the seedlings were sowed in plastic pots (15 × 15 cm², 10 seedlings per pot) filled with soil/vermiculite (3:1, v/v) mixture, which was sterilized at 121 °C for 2 h. The half-strength Hoagland’s nutrient solution (including 2 mM KNO₃, 0.5 mM NH₄H₂PO₄, 0.1 mM Ca(NO₃)₂·4H₂O, 0.25 mM MgSO₄·7H₂O, 0.5 mM Fe citrate, 92 μM H₃BO₃, 18 μM MnCl₂·4H₂O, 1.6 μM ZnSO₄·7H₂O, 0.6 μM CuSO₄·5H₂O and 0.7 μM (NH₄)₆Mo₇O₂₄·4H₂O) was irrigated every 3 d and maintained the soil water content at 60−70%. The pots were cultured in a greenhouse under 25 ± 1 °C with 70–75% relative humidity, 14 light/10 h dark cycle with a light intensity of 120 μmol/m²/s, and watered every 2 d. When seedlings grew to the two-leaf stage, they were inoculated with 50 mL of the different treatments (CK (control, distilled water), HT-1 and LX-7 strains mixed in 1:1, 1:2 and 2:1 ratios, and single LX-7 and HT-1 strains).

2.6. Biomass and Quality Measurement

Biomass and quality of all plants were measured 45 d after sowing. After removing the attached soil from the root surface, the plant was washed with tap water and rinsed three times with deionized water. The plants were immediately divided into roots and shoots, and root length, shoot length, maximum leaf length, maximum leaf width, fresh weight and dry weight were used for the phytochemical determination.

The chlorophyll content was tested by using the procedure of Gratani et al. [28] and Song et al. [29]. The content of soluble protein was determined by using the procedure of Bradford et al. [30]. The content of soluble sugar was measured by using the procedure of Song et al. [31]. The nitrate content was tested by using the procedure of Cataldo et al. [32]. The vitamin C content was measured by using the procedure of Nomkong et al. [33].

2.7. Statistical Analysis

All data were analyzed by SPSS16.0 software for variance (one-way, ANOVA) and Duncan’s multiple range test (\( p < 0.05 \)).

3. Results

3.1. In Vitro-Promoting Traits of Strains

In our experiments, both strains LX-7 and HT-1 showed IAA production capacity because the pink color of the medium was supplemented with tryptophan. The IAA concentrations of 10.06 and 9.29 mg/L were detected respectively at in culture filtrates of the HT-1 and LX-7 strains (Table 1, Figure 1). The IAA content of mixed strains was higher than that of single strains. Among them, the highest IAA content (23.07 mg/L) was obtained by LX-7:HT-1 = 1:2 (Table 1, Figure 1).
Table 1. In Vitro-Promoting Traits of HT-1 and LX-7 strains.

| Strain       | IAA (mg/L) | Siderophore (%) | Organic Phosphate Solubilization (mg/L) | Inorganic Phosphate Solubilization (mg/L) | Potassium Absorbable (mg/L) |
|--------------|------------|-----------------|----------------------------------------|-------------------------------------------|-----------------------------|
| HT-1         | 10.06 ± 0.08 d | 39.41 ± 0.27 d  | 0                                      | 0                                         | 0.00 ± 0.00 d               |
| LX-7         | 9.29 ± 0.09 e  | 31.22 ± 0.23 e  | 0                                      | 0                                         | 2.05 ± 0.07 a               |
| LX-7:HT-1 = 1:2 | 23.07 ± 0.05 a | 52.75 ± 0.15 a  | 0                                      | 0                                         | 1.23 ± 0.06 c               |
| LX-7:HT-1 = 1:1 | 20.63 ± 0.12 b | 47.96 ± 0.32 b  | 0                                      | 0                                         | 1.46 ± 0.07 b               |
| LX-7:HT-1 = 2:1 | 16.19 ± 0.05 c | 43.77 ± 0.19 c  | 0                                      | 0                                         | 1.57 ± 0.07 b               |

Note: Different letters in the table indicate the differences are significant at \( p < 0.05 \).

Figure 1. IAA production by LX-7:HT-1 = 1:2 (a), LX-7:HT-1 = 1:1 (b), LX-7:HT-1 = 2:1 (c), LX-7 (d) and HT-1 (e).

The ability for siderophore production was exhibited by LX-7 and HT-1 strains and was evidenced by the color changes in the media from blue to yellow. HT-1 and LX-7 strains showed that the siderophore productions were 39.41% and 31.22%, respectively (Table 1, Figure 2). The siderophore production of mixed strains was higher than that of single strains. Among them, the highest siderophore production (52.75%) was obtained by LX-7:HT-1 = 1:2.

Figure 2. Siderophore production by HT-1 strain (a) and LX-7 strain (b).

The presence of transparent circles on PDA medium containing organic phosphate or inorganic phosphate indicated that the strain had the ability of phosphate solubilization. The results showed that HT-1 and LX-7 strains could not form transparent circles on the culture medium containing organic phosphate or inorganic phosphate, which indicated that HT-1 and LX-7 strains had no phosphate solubilization ability. The quantitative determination results showed that the organic phosphate or inorganic phosphate solubilization content of HT-1 strain, LX-7 strain and its mixture was 0 mg/L.

The presence of transparent circles on PDA medium containing potassium indicated that the strain had the ability to absorb potassium. The results showed that the HT-1 strain could not form transparent circles on the culture medium containing potassium, which indicated that it had no ability to absorb potassium, while LX-7 could form transparent circles on the culture medium containing potassium, which proved that it had the ability to absorb potassium. The quantitative determination results showed that the highest
potassium absorbable content of the LX-7 strain was 2.05 mg/L, followed by LX-7:HT-1 = 2:1 (1.57 mg/L) (Table 1, Figure 3).

The above data indicated that the mixture of two different *Trichoderma* strains (*T. atroviride* LX-7 and *T. citrinoviride* HT-1) could be used as multifunctional biological stimulants to provide sufficient nutrients for plant growth and development.

![Figure 3. Potassium absorbable ability of LX-7 strain.](image)

3.2. Effects of HT-1 and LX-7 Strains on Seeds Germination and Seedlings Growth of Pakchoi

The treatments of single strains and mixed strains can affect germination of pakchoi seeds. Among them, the highest GP (94.67%), GI (127.91), GE (62.67%) and VI (565.45) were observed after the treatment of LX-7:HT-1 = 1:1, which was significantly higher than in the single strain treatments and control, respectively (Table 2).

| Treatment | GP (%)  | GI      | GE (%)  | VI       |
|-----------|---------|---------|---------|----------|
| LX-7      | 91.00 ± 1.00 b | 127.91 ± 3.01 c | 62.67 ± 1.15 c | 565.45 ± 23.00 d |
| HT        | 91.67 ± 1.15 b | 132.33 ± 2.51 b | 66.67 ± 2.08 b | 608.10 ± 10.80 c |
| LX-7:HT-1 = 1:1 | 94.67 ± 1.15 a | 139.36 ± 1.01 a | 71.33 ± 1.53 a | 705.43 ± 6.62 a |
| LX-7:HT-1 = 1:2 | 92.67 ± 1.53 ab | 135.51 ± 1.79 ab | 68.33 ± 1.53 b | 654.41 ± 7.76 b |
| LX-7:HT-1 = 2:1 | 92.33 ± 1.15 ab | 135.44 ± 2.19 ab | 68.00 ± 1.00 b | 631.43 ± 19.26 bc |
| CK        | 88.67 ± 1.53 c | 112.54 ± 3.08 d | 57.33 ± 1.53 d | 424.14 ± 12.95 e |

Note: Different letters in the table indicate the differences are significant at *p* < 0.05.

The treatments of single strains and mixed strains could effectively improve the growth of pakchoi seedlings. Among them, the highest plumule length (2.90 cm, Figure 4a), radicle length (5.06 cm, Figure 4b), fresh weight (0.39 g/10 seedlings, Figure 4c) and dry weight (0.035 g/10 seedlings, Figure 4d) of pakchoi seedlings were obtained when HT-1 and LX-7 strains were mixed in 1:1 ratio, which was significantly higher than in the single strain treatments and control, respectively.
Figure 4. Effects of HT-1 and LX-7 strains on plumule length (a), radicle length (b), fresh weight (c) and dry weight (d) of Brassica chinensis L. seedlings. Values are mean ± SD (n = 30 seedlings). Different letters above the bars indicate the differences are significant at $p < 0.05$.

3.3. Effect of HT-1 and LX-7 Strains on Pakchoi Growth in Greenhouse

In our experiment, after treatment of single strains and mixed strains, the growth of pakchoi plants showed a notable improvement trend (Figure 5). The highest shoot length (6.82 cm, Figure 5a), root length (7.01 cm, Figure 5b) and maximum leaf length × width (86.07 cm², Figure 5c) were obtained by the treatment in which LX-7 and HT-1 strains were mixed in 1:2 ratio, which was significantly higher than in the single strain treatments and control, respectively.

The biomass of pakchoi plants was increased by the treatment of single strains and mixed strains (Figure 5). The treatment of LX-7 and HT-1 strains, which were mixed in 1:2 ratio, led to the highest fresh weight (13.16 g/plant, Figure 5d) and dry weight (0.92 g/plant, Figure 5e), which was significantly higher than in the single strain treatments and control, respectively.

3.4. Effect of HT-1 and LX-7 Strains on Pakchoi Quality in Greenhouse

The quality of pakchoi plants could be improved by the treatments of single strains and mixed strains. The contents of vitamin C (Vc), soluble sugar and soluble protein of pakchoi plants showed notable improvement, and nitrate content decreased significantly (Figure 6).
Figure 5. Effects of HT-1 and LX-7 strains on root length (a), shoot length (b), maximum leaf length × width (c), fresh weight (d) and dry weight (e) of *Brassica chinensis* L. plants. Values are mean ± SD (n = 15 plants). Different letters above the bars indicate the differences are significant at $p < 0.05$.

The highest contents of Vc (103.78 mg/100 g, Figure 6a), soluble sugar (5.14 mg/g, Figure 6b) and soluble protein (14.30 mg/g, Figure 6c) of pakchoi were obtained in the treatment of LX-7 and HT-1 strains mixed in 1:2 ratio, which was significantly higher than in the single strain treatments and control, respectively. The different treatments could decrease the nitrate content of pakchoi plants; the lowest nitrate content of pakchoi was obtained under the treatment of LX-7 and HT-1 strains mixed in 1:2 ratio, which reduced it by 294.06 mg/kg compared with the control (Figure 6d).

The chlorophyll (a + b) content of pakchoi plants was significantly increased by different treatments (Figure 7). The highest chlorophyll (a + b) content (604.84 µg/g) of a pakchoi leaf was observed under the treatment of LX-7 and HT-1 strains mixed in 1:2 ratio, which was significantly higher than in the single strain treatments and control, respectively.
Figure 6. Effects of HT-1 and LX-7 strains on vitamin C (a), soluble sugar (b), soluble protein (c) and nitrate content (d) in *Brassica chinensis* L. plants under greenhouse. Values are mean ± SD (n = 15 plants). Different letters above the bars indicate the differences are significant at *p* < 0.05.

Figure 7. Effect of HT-1 and LX-7 strains on chlorophyll (a + b) content in *Brassica chinensis* L. plants in greenhouse. Values are mean ± SD (n = 15 plants). Different letters above the bars indicate the differences are significant at *p* < 0.05.

4. Discussion

*Trichoderma* fungi can have positive effects on the growth of several plants, which has been demonstrated by many reports [15]. The secretion of plant growth hormone or its analogs is one of the mechanisms by which *Trichoderma* promotes plant growth and yield [34]. IAA can promote root and shoot development [34,35]. In this study, the IAA content of HT-1 and LX-7 strains was 10.06 and 9.29 mg/L, respectively, which was lower than that reported by Eslahi et al. [15], who found that *T. harzianum* T13 and T15 can produce IAA concentrations of 61.29 and 54.67 μg/mL. This result may be caused by the difference in IAA production capacity of the strains. We also found that the combined
strains produced more IAA than single strains, which indicated that the two strains were not excluded by each other. Another plant growth-promoting mechanism of *Trichoderma* is that it can produce siderophore, which is a micromolecular iron-chelating ligand that promotes plant growth by improving the Fe III solubilization [36]. In our tests, HT-1 and LX-7 strains showed that the siderophore production was 39.41% and 31.22%, and that compared with single strains, the siderophore production of combined strains was better. This indicated that the strain combination had a synergistic effect on the ability of siderophore production. In addition, some *Trichoderma* strains can promote element solubility (such as phosphorus and potassium) by changing soil fertility [37]. In this study, the LX-7 and HT-1 strains had no ability of phosphate solubilization. We inferred that there are differences among *Trichoderma* strains, and there are *Trichoderma* strains in nature without the ability of phosphate solubilization [38]. The mixed strains still had no phosphate solubilization ability. Only LX-7 had the capacity to absorb potassium, while the HT-1 strain did not. The potassium absorbable contents of mixed strains were lower than that of the single LX-7 strain because the HT-1 and LX-7 strains were mixed in 1:1, 1:2 and 2:1 ratios, which reduced the inoculation density of the LX-7 strain. In addition, *Trichoderma* reduced soil pH, which was related to the phosphate and potassium solubilization process, as it can produce acids that dissolve phosphate, potassium and other microelements [39].

*Trichoderma* has been used as a biostimulant in many reports [40], and several factors can influence its growth-promoting ability. For example, the same species may or may not have the ability to promote plant growth, or crops and/or varieties for which the species is suitable [41]. Similarly, the combined use of *Trichoderma* strains and other fungi has been reported in several studies stating, for example, that the combined use of AM fungi and *Trichoderma* can promote plant growth [42]. However, contrasting results have emerged, which reported that combined strains can reduce the stem and root dry weight of plants [43,44]. De Jaeger et al. [45] reported that *T. harzianum* destroyed the external and internal hyphae continuity of *Glomus intraradices*, which resulted in reducing P acquisition of mycorrhizal plants. Therefore, the compatibility of strains should be determined firstly to ensure the effective application of inoculants. In this study, we chose two *Trichoderma* strains, and we compared the combined use of two *Trichoderma* strains and single *Trichoderma* strains to promote the difference of pakchoi growth and quality. The results showed that seed germination and seedlings growth were significantly increased under the treatment of mixed *Trichoderma* strains, which, compared with the single strains and CK, respectively, yielded similar results to those of Esitken et al. [17] and Colla et al. [21], who reported that combination use of *Bacillus OSU-142* and *Pseudomonas BA-8*, *Glomus intraradices* and *Trichoderma atroviride* can improve the yield, growth and nutrition content of plants. There are many reasons that may explain these results: the microbiome may provide important nutrients and basic substances for the growth of another microbiome, create favorable conditions for the continued growth of another microbiome, or even depend on other microorganisms to survive, and some can promote the growth and reproduction of other microbiomes [17]. Therefore, the mixed strains had better growth-promoting effects on plants than single strains did. In this study, we considered that the combined strains could produce better growth-promoting effects than the single strains because the combined strains produced more substances for plant growth. However, the molecular mechanism of the mixed strains promoting the growth of pakchoi needs to be further studied.

While fertilizers and pesticides increased crop yields, they have also had negative effects. With the extensive use of biopromoters or biofertilizers, environmental pollution can be reduced and food safety ensured.

5. Conclusions

The above data proved that combined use of two *Trichoderma* strains had greater benefits for the growth and quality of pakchoi, compared with single strains, making this formulation attractive for future field applications.
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