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Research Article

Development of an Effective Nonchemical Method against *Plasmodiophora brassicae* on Chinese Cabbage

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Clubroot disease, caused by *Plasmodiophora brassicae*, is a serious soil-borne disease of crucifer worldwide, and it can significantly reduce yield and quality. Although some agrochemicals have been used to manage clubroot and can provide effective control, increasing use of chemical inputs causes several negative effects. In this study, using Chinese cabbage (*Brassica rapa* L. subsp. *chinensis*) as the test crop, we developed an effective nonchemical method that would protect the roots against *P. brassicae* infection by using a combination heat treatment and a cocktail of biocontrol agents. The data showed that this method could cause 91.7% inhibition of *P. brassicae* infection. The average height of plants (13.5 cm) using this method was about twice higher than that in control group (6.7 cm), and the average plant weight (3.19 g) was about three times increased compared to that in control set (1.23 g).

1. Introduction

Clubroot disease, caused by the pathogen *Plasmodiophora brassicae*, is one of the most serious problems in crucifer cultivation worldwide [1]. Clubroot disease occurs when the pathogen invades the plant's root system. Infected roots develop galls or "clubs" and cannot take up water and nutrients, so plants become stunted and wilt, with subsequent losses of quality and yield [2]. Because the pathogen is wholly confined to the soil and its resting spores are able to survive for a long period of time in the soil, agricultural practices such as liming and crop rotation are insufficient to keep crops healthy [3, 4]. Although some agrochemicals have been used to manage clubroot and can provide effective control, increasing use of chemical inputs causes several negative effects, that is, development of pathogen resistance to the applied agents and their nontarget environmental impacts [5].

Besides agrochemicals treatment, soil also can be sterilized with nonchemical methods. Soil steam sterilization is a farming technique that sterilizes soil with steam in open fields or greenhouses [6, 7]. Pathogens, including bacteria, fungi, and viruses, can be effectively killed by heating the soil to levels that cause protein coagulation or enzyme inactivation. However, after heating, soil microbiota (living soil organisms) is commonly affected to varying degrees. The exact impact of heating on soil microbiota is complex and for the most part still poorly understood.

Biological control is another way of reducing the use of chemicals in agriculture [8, 9]. There has been a large body of literature describing potential uses of plant associated bacteria as agents stimulating plant growth and managing soil and plant health [10–12]. Soil microorganisms with beneficial activity on plant growth and health represent an attractive alternative to conventional agriculture. In recent years, several microbial inoculants have been formulated, produced, marketed, and applied successfully by an increasing number of growers [13, 14]. It was observed that Bacillus subtilis could suppress clubroot on canola under controlled conditions but its performance was inconsistent under field conditions [15]. Also *Gliocladium catenulatum* can reduce clubroot severity [16], but the mechanism interfering by *P. brassicae,*

Gliocladium catenulatum or other potential biocontrol agents is not clear.

Based on nonchemical control of clubroot, this study focused on the effects of three different treatments on *P. brassicae*, leading to inhibition of the development of clubroot on Chinese cabbage. The aim of this study was to develop an effective nonchemical method that would protect the roots against *P. brassicae* infection by binding heat-sterilized soil and effective microorganisms' biocontrol.

2. Materials and Methods

2.1. Preparation of Effective Soil Microbial Agent. The effective soil microbial agent (ESMA) used in this study was a home-made combination of *Bacillus megaterium* [15], *Clostridium tyrobutyricum* [17], and *Saccharomyces cerevisiae* [16, 18] based on previous studies and reports. The various culture media and cultivation conditions of *B. megaterium* (ATCC 14581) [19], *C. tyrobutyricum* (ATCC 25755) [20], and *S. cerevisiae* (ATCC 16664) [21] were followed protocols in the previous reports. After fermentation, the three microorganisms were mixed in the ratio of 5 : 5 : 1 with the total amount of 10^8 CFU/mL.

2.2. Artificially Infested Soil. Uncontaminated soil was collected from nearby field, air-dried, and passed through 2 mm sieve for using it in the greenhouse experiment. Air-dried clubroot galls (induced by *P. brassicae*) collected from Chinese cabbage were homogenized by hammer milling and passed through 1 mm sieve. The artificially infested soil was prepared via mixing the clubroot gall powder with the dried soil as the weight ratio of 1 : 200.

2.3. Experimental Design and Procedure. A greenhouse pot experiment was conducted using Chinese cabbage (*Brassica rapa* L. subsp. *chinensis*) as the test crop. The infested soil was divided into four sets following different treatments to test the effect against *P. brassicae*. Set 1 was used as a control experiment, which only included soil and crushed clubroot galls. The infested soil in Set 2 was heated at 200°C for 2 min. In Set 3, ESMA was mixed with the infested soil following the ratio of 1 : 5 (volume : weight) (e.g., 1 mL ESMA mixed with 5 g infested soil). To find the effective method to inhibit clubroot and to recover populations of soil microorganisms, the infested soil used in Set 4 was firstly heated at 200°C for 2 min, and after being cooled to room temperature it was mixed with ESMA with a ratio of 1 : 5 (volume : weight). Each treated soil sample was divided into four plastic pots. Ten seeds were sown in each pot. Two days after seedling emergence, plants were thinned to six plants per pot. Every treatment included 4 pots, so a total of 24 plants were grown in each set. All plants were grown in a greenhouse with a temperature of 22–25°C, a photoperiod of 16L : 8D, and 60% of relative humidity.

2.4. Data Collection. At 6 weeks after seedling emergence, plants were harvested and data were collected. Whole plants were removed from the soil, and the roots were cut off, washed, and scored for disease severity. The height and fresh weight of plant (without root) were measured to assess the effect of clubbing on plant growth. Clubroot severity was assessed on a scale of 0–4 [22], where 0 (healthy) = no clubbing; 1 (slight) = very small galls, or diam. <4 mm; 2 (moderate) = galls <10 × 5 mm, or 7.5 mm diam.; 3 (severe) = galls <20 × 10 mm, or 15 mm diam.; 4 (very severe) = galls >20 × 10 mm, or 15 mm diam. The disease severity data were used to calculate an index of disease (DI) using the formula of Horiuchi and Hori [23] as modified by Strelkov et al. [24] as follows:

$$\mathrm{DI}\,(\%) = \frac{(\text{sum of all numerical grades})}{(\text{total number of plants} * \text{maximum grade})} * 100. \quad (1)$$

2.5. Microbiological Examination. The colony-forming units (CFU) of culturable bacteria, including actinomycetes, and fungi in soil were determined following methods described by Stotzky et al. [25]. Three replicate soil samples were collected from each pot. Briefly, 1 gram soil was suspended in 10 mL sterile tap water and 10-fold serially diluted. The CFU of bacteria was determined by spreading 100 all of diluted samples on agar plates and incubating at 30°C for 3 days. Bacterial and actinomycetes isolates were characterized based on cultural characteristics, staining reactions, and biochemical reactions. The CFU of fungi was estimated on Rose Bengal-streptomycin agar, of which 100 μL of 10-fold serially diluted soil diluted samples was spread. After incubation at 25°C for 5 days, the CFU of fungi was counted.

2.6. Statistical Analyses. All data are presented as mean ± SEM. Difference of plant height and fresh weight and microbiological CFU between treatments were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Ridit analysis [26] was used to examine the effects against clubroot incidence among the four different treatments. Ridit analysis calculates one aggregate score that is the probability of a higher/lower score in the distribution under investigation relative to a common reference distribution. In this study, the null hypothesis is a priori Ridit of 0.5, which implies a 50/50 distribution. Statistically significant differences were identified when P values <0.05.

3. Results and Discussion

3.1. Effects on Clubroot Disease Incidence and Severity. Differences in clubroot disease incidence and severity were found between the different treatments (Table 1). The Ridit scores and 95% confidence interval limits were also shown in Table 1. There was no overlap between Set 1 and Set 2, Set 3, and Set 4 on 95% CI. In Set 1, all of the 24 plants had clubroot, meaning the disease incidence was 100%. What is more, about half of them had a very severe infection, and the disease index was 80.2%. Cabbage clubroot disease incidence was decreased for plants grown in heat-sterilized soil with or without the application of ESMA. The decreased disease

Table 1: Clubroot disease incidence and severity in four sets.

Treatment	Number of plants*					Ridit score**	95% CI	Disease incidence (%)	Disease index (%)
	0	1	2	3	4				
Set 1	0	1	4	8	11	0.805 (a)	0.716~0.895	100	80.2
Set 2	19	3	1	1	0	0.323 (b)	0.234~0.413	20.8	8.3
Set 3	7	1	6	6	4	0.598 (c)	0.508~0.687	70.8	48.9
Set 4	22	2	0	0	0	0.274 (b)	0.184~0.363	8.3	2.1

*Clubroot severity, where 0 (healthy) = no clubbing; 1 (slight) = very small galls, or diam. <4 mm; 2 (moderate) = galls <10 × 5 mm, or 7.5 mm diam. 3 (severe) = galls <20 × 10 mm, or 15 mm diam. 4 (very severe) = galls >20 × 10 mm, or 15 mm diam.
**Ridit scores within columns followed by different letters are significantly different ($P < 0.05$).

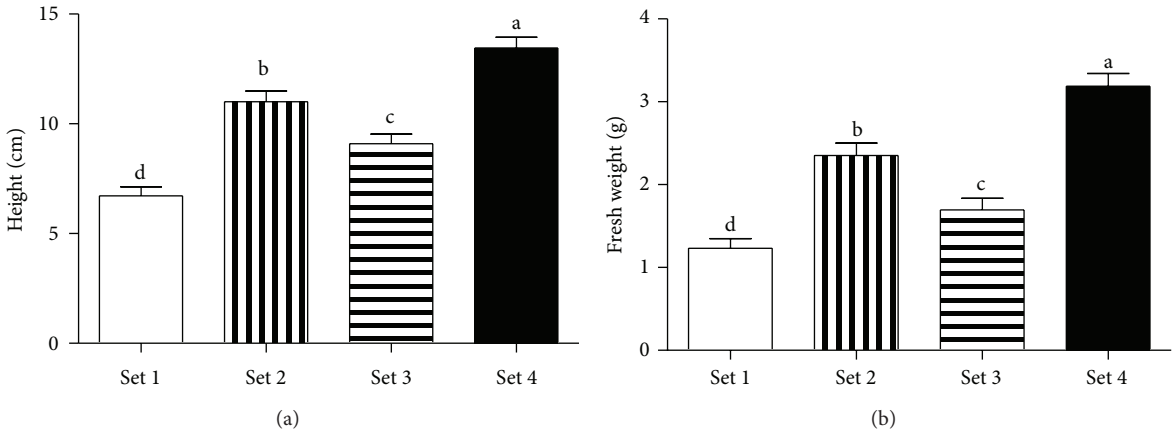

Figure 1: Plant height and fresh weight. (a) Plant height and (b) plant fresh weight. Means with the different letter are significantly different from each other (Tukey-Kramer test, $P < 0.05$).

incidence was observed after the soil was treated at high temperature as expected. In this study, treated with high temperature, *P. brassiace* was mostly killed. The soil treated with high temperature could inhibit infection of *P. brassiace*, causing 79.2% and 91.7% inhibition without ESMA and with ESMA, respectively. In further study, we will try to use higher temperature (more than 200°C) to treat soil or heat soil for longer time to reach 100% inhibition of infection of *P. brassiace*.

Interesting to is that even in the soil only mixed with ESMA (Set 3), clubroot disease incidence also decreased in this study. Although there was no report that *B. megaterium* or *C. tyrobutyricum* could inhibit infection of *P. brassiace*, the current result showed that *B. megaterium* or *C. tyrobutyricum* maybe looked at as potential biocontrol agents. Effective microorganism, referred to as EM, was first suggested by Higa [27]. In fact, EM is a combination of beneficial microorganisms, which can improve soil quality, enhance crop production and protein, conserve natural resources, and ultimately create a more sustainable agriculture and environment [28, 29]. The effective soil microbial agent (ESMA) used in this study might be looked at as a simplified effective microorganism, because the plants grown in the soil treated with ESMA had less clubroot infection, and the data of plant height and fresh weight (shown in 3.2 part) also proved that the plants grew better with ESMA treatment.

Although two plants grown in Set 4 developed clubroot, those two galls were very small (diam. less than 4 mm), and the clubroot severity (index of disease) was 2.1%. The following results about plant height and fresh weight showed that this kind of slight infection only had a very small effect on plant growth and yield. Compared to Set 1 (without any treatment), the clubroot severities in Set 2 and Set 3, which only had one treatment done, were 8.3% and 48.9%, respectively. Based on these data, we could know that the combination of heat-sterilized soil and ESMA may be an effective way to control clubroot.

3.2. Plant Height and Fresh Weight. The plant height and fresh weight responses of Chinese cabbage to heated-soil, ESMA, and heated-soil/ESMA mixture factors were shown in Figure 1. Compared to the control group (Set 1), plants grown in the infested soil with three different treatments were taller and greater. The order of average plant height was Set 4 > Set 2 > Set 3 > Set 1 (Figure 1(a)). The highest plant height was observed in Set 4, where the soil was treated with high temperature and then mixed with ESMA. The mean plant height was 13.5 cm in Set 4, and it was twice higher than that in Set 1 (6.7 cm).

In association with increased plant height, the average of fresh weight of aboveground plant in Set 4 was greater than that in the other three sets (Figure 1(b)). The average value

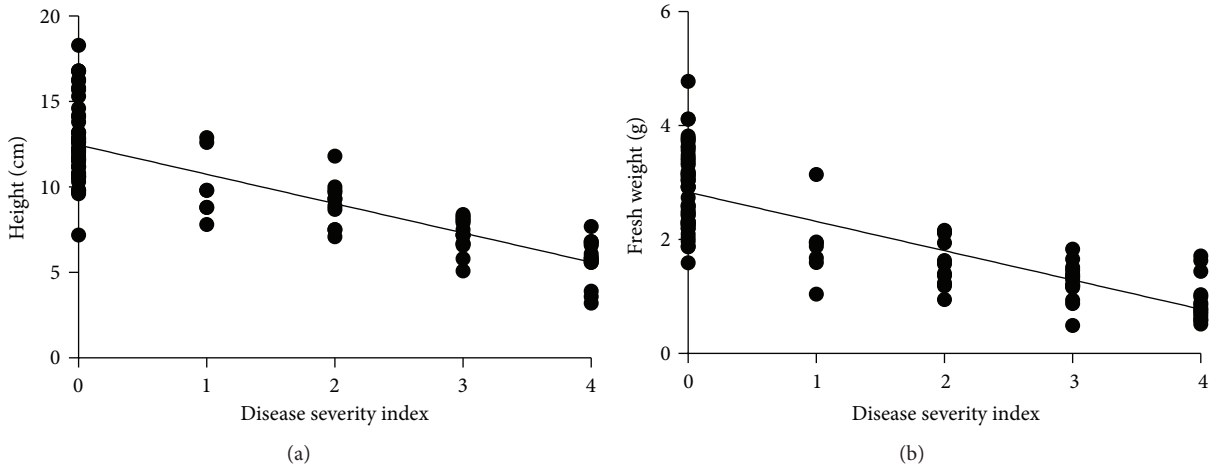

Figure 2: Correlation between clubroot disease incidence and plant height (a) and fresh weight (b).

of plant weight in Set 4 was 3.19 g, and it was about three times than that in Set 1 (1.23 g). The order of the mean fresh weight was also Set 4 > Set 2 > Set 3 > Set 1. In other studies, the data showed that plant growth and yield were better in heat-sterilized than in nonsterilized soil [30], or with EM application than without EM application [31, 32].

As expected, the plant's growth is affected by clubroot. With the disease more severe, the height of the plant was shorter (Figure 2(a)). DI and plant height were negatively correlated, $r = -0.819$ ($P < 0.001$). And DI and weight were also negatively correlated, $r = -0.810$ ($P < 0.001$) (Figure 2(b)). It has been reported that the major effect of clubroot on the growth of young cabbage plants was a change in the distribution of dry matter [33]. As galls developed, the growth of the shoots and fibrous roots decreased, reducing total plant development [34]. In this study, we only measured fresh weight, but it also clearly showed the major negative effect of clubroot on plant growth.

3.3. Influences of Soil Microflora. This study analyzed the influences of different treatment measures on soil microflora. The results showed that there were no statistically significant differences in the CFU of culturable bacteria (including actinomycetes) and fungi among four different treatments. Although the difference did not reach significance, there was a decreased trend on the total number of actinomycetes and fungi in Set 2, where the soil was only treated with high temperature. Compared with the control (Set 1), the amounts of actinomycetes and fungi in Set 2 were decreased by 21.2% and 22.1%, respectively. The decreased microflora in Set 2 may be affected by heat. To test the reduction of microflora, the soil samples might be collected at 1 or 2 weeks after heat treatment. After heat treatment, living soil organisms were killed. In Set 4, the effective soil microbial agent (ESMA) was added to set up the new microbial ecology quickly. In this study, after 6-week recovery, we could observe the number of soil biota that also recovered. However, we do not know the exact difference of microbial species in the soil from 4 sets. To answer this question, it would be better to do a metagenomic analysis on soil microflora using ultradeep genome sequencing, which have to be used in human gut flora study [35, 36].

4. Conclusion

The present study concludes that the treatment with heat-sterilized soil and the application of a combination of *Bacillus megaterium*, *Clostridium tyrobutyricum*, and *Saccharomyces cerevisiae* have the potential to be used as an effective nonchemical method against *Plasmodiophora brassicae* on Chinese cabbage. Also this method could improve the growth and yield of Chinese cabbage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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