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Deciphering the bacterial and fungal communities in clubroot-affected cabbage rhizosphere treated with *Bacillus Subtilis* XF-1

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ABSTRACT

Clubroot is an infection of cruciferous crops which results in considerable yield losses, caused by *Plasmodiophora brassicae* Woron. *Bacillus subtilis* XF-1 isolated from the rhizosphere of Chinese cabbages with severe clubroot in Guandu District of Kunming, Yunnan Province, China, has strong inhibitory effects on the resting spores of *P. brassicae*. However, its potential effects on the soil bacterial and fungal communities are still unknown. In this study, *B. subtilis* XF-1 was inoculated into cabbage rhizosphere and incidence of clubroot disease was surveyed, furthermore, an effect of this strain on soil microbial community in cabbage rhizosphere was investigated using Biolog™ MicroPlates and 454 pyrosequencing. *B. subtilis* XF-1 reduced the disease index (DI) by 17.14% and the control efficiency was 76.92%. The results of Biolog analysis and high-throughput pyrosequencing demonstrated that *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Firmicutes,* and *Gemmatimonadetes* were the dominant taxonomic phyla found among bacteria in ten samples and *Ascomycota*, *Basidiomycota* and early diverging fungal lineages were reported among fungi. The soil bacterial and fungal communities were reduced greatly at the beginning, but they were recovered gradually with the growth of plant. However, there was little difference between treatment and control at the mature stage. The present study demonstrated that the effect of *B. subtilis* XF-1 on soil fungal community in cabbages rhizosphere was just transient and that the high-throughput 454 pyrosequencing is a suitable method for the characterization of microbial communities of rhizosphere soil of cabbage.

1. Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is an important cruciferous vegetable all over the world which can be severely infected by *Plasmodiophora brassicae* Woron (Cook and Schwartz, 1930), resulting in one of most destructive disease known as clubroot (Voorrips, 1995; Dixon, 2009; Howard et al., 2010). The important methods to control this disease are crop rotation (Wallenhammar, 1996), liming (Murakami et al., 2002), agrochemicals (Naiki and Dixon, 1987; Tanaka et al., 1999; Kowata-Dresch and May-De Mio, 2012) (such as quintozene, chlorothalonil, flusulfamide), and resistant host strains. However, agrochemical controls in the form of fungicides and fluorinated organics created food quality problems (Wang and Jones, 1994; Key et al., 1997; Peñuela and Barceló, 1998; Sakkas et al., 2002). In addition, there are few known resistance genes for *P. brassicae* (Dixon and Robinson, 1986; Hirai, 2006), to be utilized by plant breeders. An alternative means to control diseases is the biological control and this approach has been studied for many years (Baker, 1987; Weller, 1988; Handelsman and Stabb, 1996).

The most serious plant disease clubroot has two phases of their life cycle. The infection of the root hairs are in the first phase and the second occurs in the cells of the root cortex. The resting spores of the *P. brassicae* produce zoospores with the germination of host plants. Further, this zoospore penetrate the root hairs and result in primary plasmodia, later, develops into zoosporangium develops. The secondary plasmodia emerge when zoosporangial zoospores fuse in pairs and started infecting the cortical tissues (Tommerup and Ingram, 1971).

Soil microbes play a significant role in the health and growth promotion of plants. The soil pH and root exudates composition results in soil microbial community changes at different stages of plant growth (Wang et al., 2017). Various biochemical factors in the soil not only affect the availability of nutrients for plant productivity but also

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influence growth and pathogen survival in the soil. Therefore, regulating soil fertility, promoting plant health, protecting plants from diseases and cycling of nutrients through soil microorganisms are important (Lauber et al., 2008; Faoro et al., 2010; Rousk et al., 2010). On the other hand, soil microbial community structure is also influence by other soil bio-chemical factors such as soil pH (Benizri et al., 2005; Zhang et al., 2016). Healthy soils with the balanced soil microbial community are beneficial for promoting plants growth and prevention of plant diseases (Benizri et al., 2005; Janvier et al., 2007). High abundances of beneficial microbes related to the high soil quality such as *Agromyces*, *Acremonium*, *Bacillus*, *Bradyrhizobium*, *Chaetomium*are, *Lysobacter*, *Micromonospora*, *Pseudonocardia*, *Mesorhizobium*, and *Microvirga*. The quality of this soil is indicated by nutrient and enzyme activities, better plant growth, higher soil pH, and lower disease incidence (Wang et al., 2017). However, in case of unhealthy soils, the soil microbial community structure is still unclear.

Bacillus subtilis XF-1, which has strong inhibitory effects on the pathogen of crucifer clubroot and many other pathogenic fungi, was isolated from the cabbage rhizosphere soil of clubroot-infected land (Guoru et al., 2009), produced a broad spectrum bioactive Fengycin-Type Cyclopeptides for clubroot disease control (Li et al., 2012; Li et al., 2013), and genome sequencing results revealed that this strain (CGMCC No. 2357) possesses a gene cluster that is involved in chitosanase synthesis results in suppressing the pathogen *P. brassicae* (Guo et al., 2013). *B. subtilis* XF-1 have great potential for the control of clubroot and other biocontrol applications. It is important to know the potential effects of XF-1 on agricultural soil bacterial and fungal communities to ensure the safety of introduction of this strain to soil microcosm (Van Elsas et al., 1998). However, there is hitherto no information on this issue.

Previous methods including DNA based techniques, such as Terminal Restriction Fragment Length Polymorphism (T-RFLP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Denaturing Gradient Gel Electrophoresis/Temperature Gradient Gel Electrophoresis (DGGE/TGGE) etc., and plate counting, phospholipid fatty acid (PLFA) analysis have been widely used to study soil bacterial and fungal communities (Nannipieri et al., 2003; Kirk et al., 2004). It has been suggested that common laboratory techniques could not culture at least 99% of soil microbes (Torsvik et al., 1990; Amann et al., 1995). This problem is started to solve through High-throughput sequencing (HTS) techniques. The quality, cost, and speed of this technology are rapidly improving. It is, therefore, whole communities of prokaryotes in many niches are widely studied (Di Bella et al., 2013). More comprehensive insight into microbial community diversity is achieved through these approaches without the bias associated with other technologies (Yang et al., 2016). HTS has revolutionized sequencing approaches by allowing to obtain a whole microbial genome in one run through technologies based on multi parallelized shotgun sequences (Caboche et al., 2014). Recently, it has been used to provide a deep understanding of the composition of the soil microbial communities (Roesch et al., 2007; Lauber et al., 2009; Uroz et al., 2010; Blaalid et al., 2012; Uroz et al., 2013).

In this study, we investigated the incidence of clubroot disease and determined the effects of biocontrol agent on the soil microbial communities of Chinese cabbages by measuring the soil physicochemical properties, plate counting, Biolog ECO-plates analyzing and 454 pyrosequencing. Our goal was to study the effect of *B. subtilis* XF-1 on rhizosphere microflora of Chinese cabbages and assess the risk of using this strain for biological control. We believe that dynamic monitoring of functional changes and structural shifts of soil microbiota associated with disease predisposition and progression may lead to a deeper understanding of the relationship between *P. brassicae* and rhizosphere soil microbes and the discovery of new tools for early prevention of the clubroot disease.

2. Materials and methods

2.1. Bacterial strain and inoculation preparation

B. subtilis XF-1 (CGMCC No. 2357) was obtained from Molecular Plant Pathology Laboratory, Yunnan Agricultural University, Kunming, China and inoculated on *Luria Bertani* (LB) agar plates to obtain separate colonies. The bacteria were grown in liquid LB broth at 30 °C at 170 rpm for 48 h. The bacterial density was measured at OD_{600} of the fermentation broth, with reference to a standard curve calibrated by plate enumeration.

2.2. Experimental site and soil sampling

The field of Aziying Township, Panlong District, Kunming Municipality, Yunnan Province, China (25°26′N, 102°52′E) was selected as the experimental site, where the average annual temperature is 13.8 °C and total rainfall is about 1000 mm per year. Ten plots with sandy loam soil were established in the experimental area, while each plot (10 m^2) contained thirty cabbage plants. All the experiments were performed through Randomized Block Design. Five plots were treated with *B. subtilis* XF-1, which had been diluted with sterile distilled water to 1×10^7 cfu/L. An aliquot of 0.5 L of the diluents was poured directly onto the root rhizosphere of every cabbage plant in each plot. The other five plots were treated with the same volume of sterile distilled water and referred as a control. Two treatments were performed at planting time, 7 and 14 days after sowing, respectively. The bioassay of the disease incidence (DI) and control efficiency was carried out 3 months after sowing (transplanting) when the cabbages were harvested. The disease incidence was conducted in the early stage of the clubroot gall. Based on the size of the gall of the root of the plant, three plants were randomly selected from each of the five plots for investigation. The grading standard of the disease was divided into five level as: 0 = No gall, 1 = main root swollen, its diameter less than two times the base of the stem, 3 = main root swollen, its diameter of 2 to 3 times the stem base, 5 = main root swollen, the diameter of the stem base of 3 to 4 times, 7 = main root swollen, its diameter is more than 4 times the base.

The incidence, disease index and control effect are calculated as follows:

Incidence rate (%) = number of diseased plants/total number of investigated plants × 100

Disease index = (Σ (number of diseased plants at each stage × relative value))/(total number of plants under investigation × highest incidence of disease) × 100

Control effect (%) = (control group disease index − treatment group disease index)/control group disease index × 100.

The soil was collected from cabbage rhizosphere of three randomly selected plants in each plot as mentioned at the cotyledon stage (Stage 1: B1), seedling stage (Stage 2: B2), rosette stage (Stage 3: B3), early heading stage (Stage 4: B4) and mature stage (Stage 5: B5). The C1-C5 was used as a control for each of these stages respectively. For each treatment, five soil samples were mixed in a plastic Ziploc bag which was carried to the experimental laboratory. Moreover, visible roots and rocks were removed from soil samples before they were manually sieved (2 mm) and homogenized for each plot. The samples were further analyzed for physicochemical properties, community-level physiological profiles and used for the extraction of DNA.

2.3. Soil physicochemical analysis and plate-counting technique

The pH, organic matter, electrical conductivity (EC), TN, available nitrogen (AN), phosphorus and potassium (mg kg^{-1}) of each sample

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