European Journal of Soil Biology 61 (2014) 58–67

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Integration of biofumigation with antagonistic microorganism can control Phytophthora blight of pepper plants by regulating soil bacterial community structure

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ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form
20 November 2013
Accepted 9 December 2013
Available online 13 January 2014
Handling editor: Bryan Griffiths

Keywords:
Biofumigation
Combining biofumigation with BS211
Disease incidence of pepper
Soil bacterial community

ABSTRACT

Soil biofumigation with brassica plant residues has been shown to suppress soilborne plant pathogens. However, few studies reported the impact of biofumigation, especially combining biofumigation with antagonistic microbes, on disease incidence of Phytophthora blight of pepper and soil bacterial community structure. Biofumigation (BF) and combining biofumigation with antagonistic *Bacillus amyloliquefaciens* strain BS211 (BF + BS211) were tested to control the pepper disease caused by *Phytophthora capsici* at 0, 15 and 20 days after biofumigation (DAB) under controlled conditions. BF + BS211 treatment showed the lowest disease incidence among these treatments. Real-time PCR and denaturating gradient gel electrophoresis (DGGE) were used to investigate the microbial effects, and the results indicated that the BF and BF + BS211 treatments affected certain microbial populations and increased soil bacterial diversity, which might play significant roles in the suppression of Phytophthora blight of pepper. There was a negative correlation between soil bacterial diversity and disease incidence. Cloning of the bacterial community showed that the bacterial community structures were altered by BF and BF + BS211 treatments. These findings suggested that disease control could be improved by this integrated approach.
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1. Introduction

Pepper has been grown as a monoculture in greenhouses in Huaian, Jiangsu province (China) for many years. To control the main pathogens of the crop (*Phytophthora* spp) and to avoid crop decline effects associated with repeated monoculture, soils used to be disinfested every year with methyl bromide until it will be banned in 2015 [1]. Environmental concern over conventional agricultural fertilization and disease control measures has led to more interest in finding environmentally friendly alternatives. Biofumigation, performed by the incorporation of rapeseed meal into the soil, has been proposed as an alternative method [2,3]. Biofumigation is a term that refers to the suppression of soil-borne pathogens and decomposing organic material, including agricultural by-products or manure [2,4–7]. The volatile chemicals released during the process own the capacity of reducing fungal, bacterial and nematode pathogens [8]. However, Omirou et al. [4] pointed out that the suppression of soilborne pathogens induced by biofumigation with broccoli plant residues reflected the response of the microbial community to soil enrichment with fresh and decomposable organic matter rather than a direct toxicity effect by the release of isothiocyanates from hydrolyzed plant-derived glucosinolate. Cohen et al. [3] proposed that increasing *Streptomyces* spp. populations in response to *Brassica napus* seed meal are associated with the observed control of *Rhizoctonia* root rot, with nitric oxide (NO) production by *Streptomyces* spp. having a role on induction of plant systemic resistance. So far, most studies concerning biofumigation have focused on its efficacy against soilborne pathogens [1,6,9], while any effects on the structure and function of the microbial community has been overlooked. More recently, disease control was even related to functional mechanisms other than biofumigation, but occurring as a consequence of seed meal incorporation, involving stimulation of resident streptomycetes or actinomycetes [10].

Biocontrol is also proposed as a promising method for the management of soilborne diseases [11–13]. Because directly deploying the biocontrol agent into the soil may lead to poor activity, integrated approaches which enhance the activity of the biocontrol agents by the addition of organic amendments are more attractive [14]. Many reports suggested that significant biocontrol of Fusarium wilt of banana could be obtained by the application of a mixture of neem cake and *Pseudomonas fluorescens* [15]. *Bacillus* spp. and agricultural compost were also used together to suppress

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1164-5563/$ – see front matter © 2014 Published by Elsevier Masson SAS.
http://dx.doi.org/10.1016/j.ejsobi.2013.12.004

soilborne disease [15–17]. Currently, it is believed that a combination of antagonistic microbes with organic material may be more efficient in inhibiting disease than using single antagonistic microbe [15,18]. Seed meal incorporation into the soil also involves an enrichment in carbon source that may alter or even stimulate the resident or introduced microflora, both beneficial and pathogenic [19]. Here we isolated one *Bacillus amyloliquefaciens* strain, BS211, which could significantly inhibit the growth of *Phytophthora capsici* from soils in which pepper was continuously cropped. The present study was, therefore, carried out to assess the capability of the combination of rapeseed meal and antagonistic microorganisms to control root rot of pepper.

The objective of this study was to assess the capability of the combination of rapeseed meal and antagonistic microorganisms to control root rot of pepper; explore the feasibility of a combined incorporation of *B. amyloliquefaciens* BS211 with rapeseed meal, which could increase the disease control efficacy through an integrated biological approach.

2. Materials and methods

2.1. P. capsici, B. amyloliquefaciens BS211 and rapeseed meal preparation

Pepper plants were collected from fields suffering from Phytophthora blight in Huaian, Jiangsu province, China. Soils was gently shaken from the roots, and the remaining soil plus roots was considered the rhizosphere soil. A pathogen of Phytophthora blight was isolated from rhizosphere soil of a wilted plant using potato dextrose agar. The isolated strain was initially recognized by colony morphology characteristic of *P. capsici*. The strain was also checked for the presence or absence of *P. capsici*-specific fragments with PCR amplification using CAPFW (5′TTTAGTTGGGGGTCTTGTACC3′) and CAPRV1 (5′CCTCCACAACCAGCAACA3′) primers [20] and was finally confirmed as the responsible pathogen of Phytophthora blight of pepper.

For preparing zoospores of *P. capsici*, one of its isolates was grown on V8 agar (10 ml V8 juice (Campbell, USA), 90 ml distilled water, 0.02 g $CaCO_3$, and 2 g agar) at 25 °C for 3 days. Eight agar blocks (4 × 4 mm) of the actively growing culture of *P. capsici* were transferred into an Erlenmeyer flask containing 20 ml of 10% V8 broth. After 3 days, the broth was replaced with sterile water. The flask was chilled at 4 °C for 30 min; subsequently, the culture was filtered through cheesecloth to remove the agar blocks and attached mycelia. The resulting zoospore suspension was quantified using a hemocytometer.

The antagonistic strain, *B. amyloliquefaciens* BS211, was isolated from the root of healthy pepper in a field severely affected by root rot in Huaian, Jiangsu province, China. The sequence of 16S rRNA gene of BS211 has been deposited in the GenBank under accession number KF040978.1. The strain was stored at −80 °C in 15% glycerol and routinely cultured on LB medium at 30 °C. BS211 was tested for antagonism against *P. capsici* on potato dextrose agar (PDA) by the dual culture technique as described elsewhere [15]. A mycelia plug from actively growing *P. capsici* on PDA was taken with a cork borer of 1-cm in diameter and put in the center of the agar medium in a 90-mm Petri plate. Four spots were made on the edges of the plate with an actively growing suspension of the bacterial isolate 24 h after the fungal inoculation. The plates were incubated at 28 °C, and the inhibition of fungal growth was noted after 5–7 days.

The rapeseed meal used in this study possessed a total allyl glucosinolate content of 85.71 μmol g^{-1} and 5.04% of total N. Rapeseed meal was generated by grinding dried seed-containing rapeseed pods in a waring blender.

2.2. Soils

The soil for the pot experiment was collected from a field in Huaian, Jiangsu province, where the field had been plant with red pepper since 1980. The soils had the following properties: pH 7.20, organic carbon, 19.21 g kg^{-1}, total nitrogen, 1.87 g kg^{-1}, available P 172.31 mg kg^{-1}, and available K 31.56 mg kg^{-1}. The soil was pre-inoculated with the *P. capsici* spore suspension to obtain a concentration of 10^3 g^{-1} soil.

At 1 day after infesting soil with *P. capsici*, the rapeseed meal was incorporated into soil at a rate of 4 g kg^{-1} dry soil and the appropriate quantity of water was added to adjust soil moisture content to 50% of the water holding capacity. Moisture content was maintained constant at these levels by regular addition of water when needed. Amended soil was immediately transferred to 5-L pot and covered with double layer plastic film to minimize fumigant emissions. Pots were transferred in a growth room and incubated at 25 ± 2 °C for 20 days. The plastic cover was maintained for 20 days, and after its removal, the soil was gently mixed to ensure the release of any residues of isothiocyanate.

Antagonistic bacteria BS211 was grown separately in nutrient broth in 5-L fermentation tanks. The bacterial culture was suspended with 0.01 M phosphate buffer and the bacterial density was adjusted to 10^9 cfu ml^{-1} using a spectrophotometer. The suspension of BS211 was inoculated into soil immediately after biofumigation as to obtain a final concentration of 10^7 cfu g^{-1}.

2.3. Inhibition of BS211 growth by isothiocyanates from rapeseed meal

BS211 was grown on Luria–Bertani (LB) and aliquots of 0.1 ml were spread onto 90 mm Petri dishes on LB. Rapeseed meal was confined in the remaining sector to avoid direct contact with BS211. The dose of 1 g of rapeseed meal was chosen on the basis of our previous experiments. To avoid losing volatile compounds, the dishes were sealed with parafilm immediately after wetting the rapeseed meal with 1 ml of distilled water, and the dishes without rapeseed meal were used as Controls. Dishes were incubated at 28 °C for 3 days, and the results were the mean of three replicates.

2.4. Pot experiment design

Pepper seeds (*Capsicum annuum* L.) were surface-sterilized with H_2O_2 (5%) for 30 min, washed with sterile water three times, placed in Petri dishes containing wet filter paper for germination at 30 °C for 48 h. The germinated seeds were moved into nursery plates each containing healthy matrix. By 6-leaf age (~25 days growth), the seedlings were transplanted to pots. Each pot containing 5 kg of infested soil was transplanted with one seedling. The plants were grown in a greenhouse and the temperature ranged from 23 °C to 30 °C. Twenty DAB (days after biofumigation), the pepper plants were harvested.

Three treatments were applied in pot experiment, and the treatments was as follows: (1) CK (no fumigation), (2) BF (biofumigation with rapeseed meal), (3) BF + BS211 (The pot soil were supplemented with *B. amyloliquefaciens* BS211 after biofumigation). Three blocks were randomly laid out to replicate the three treatments and each treatment with 20 replicates (pots) was randomly arranged within each block. This pot experiment was carried out from September to November in 2012. Soil samples at 0 DAB and rhizosphere soil samples at 15 and 20 DAB were collected and stored at −20 °C until analyzed.

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