Crop Protection 56 (2014) 87–90

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Short communication

Control of tobacco mosaic virus by *Pseudomonas fluorescens* CZ powder in greenhouses and the field

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ARTICLE INFO

Article history:
Received 30 August 2013
Received in revised form
7 November 2013
Accepted 9 November 2013

Keywords:
Pseudomonas fluorescens
Tobacco mosaic virus
Disinfector
Nicotiana tabacum

ABSTRACT

Pseudomonas fluorescens strain CZ has been reported to inhibit tobacco mosaic virus (TMV) by producing an antibiotic protein. In this study the effects of CZ powder on TMV infection were investigated on tobacco grown in greenhouses and in the field. CZ stocks were cultured in a mixture medium (5 g wheat bran, 1 g soybean flour, 1 g corn flour, 1 g peanut flour), and then mixed with silica white at a ratio of 6 ml:1 g to make CZ powder. The effective cell concentration in a 100-fold dilution of CZ powder was 49.8×10^{10} cfu ml^{-1}. In greenhouse experiments where *Nicotiana tabacum* cv. Samsun NN plants were challenged by mechanical inoculation with a mixture of the 100-fold diluted CZ powder and TMV, the *in vitro* suppression of the TMV infection was 88.3% compared to the controls in which a mixture of water and TMV was used as inoculum. Similarly when *N. tabacum* cv. NC89 plants were inoculated by cutting the leaves with virus-contaminated scissors, dipping the scissors in a 100-fold dilution of CZ powder before cutting showed a disinfection effect of 96.3% compared with water dipping controls. Drenching and spraying a 100-fold dilution of CZ promoted growth and inhibited virus infection by 59.2% on *N. tabacum* cv. NC89 plants in the greenhouse. In field trials, 100-fold dilution of CZ suppressed TMV infection by 58.2% and 47.6% in 2010 and 2011, respectively, which was similar to the effect of Ningnanmycin (antibiotic purified from *Streptomyces noursei* var. xichangensis), a registered antiviral agent in tobacco. All these results indicated that CZ has a potential to be used as a hand tool disinfector and an antiviral agent against TMV.

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1. Introduction

Pseudomonas fluorescens Migula is an important plant growth-promoting rhizobacterium (PGPR), which can promote plant growth, increase yield and inhibit pathogen infection. Kloepper and Schroth (1978) reported that certain root-colonizing bacteria could promote growth, reduce soil pathogen infection and increase yield of radish plants. Raupach et al. (1996) reported that two PGPRs, which had been previously reported to be able to induce resistance against fungal and bacterial diseases in cucumber were able to induce TMV resistance in both cucumber and tomato. A number of studies on tomato demonstrated that PGPR could trigger 'induced systemic resistance' (ISR) against various viruses, including TMV, cucumber mosaic virus (CMV) and tobacco leaf curl virus (TLCV) (Murphy et al., 2000, 2003; Zehnder et al., 2000; Vasanthi et al., 2010; Wang et al., 2011; Dashti et al., 2012). It has also been shown that when applied as bio-fertilizer through seed coating and soil soaking in agricultural production, PGPR could significantly promote plant growth and inhibit infection by bean common mosaic virus (BCMV), sunflower necrosis virus (SNV) and bhendi yellow vein mosaic virus (BYVMV) (Shankar et al., 2009; Srinivasan et al., 2009; Patil et al., 2011).

P. fluorescens is an effective PGPR biocontrol agent with the characteristics of wide distribution, availability of a large number of strains with various properties, simple nutritional requirements, rapid propagation and strong competitiveness. *P. fluorescens* has several important uses. It could efficiently colonize plant roots, form protective bacteria films and induce disease resistance. Maurhofer et al. (1994) showed that 6 weeks after application of *P. fluorescens* CHA0 in the soil growing *Nicotiana glutinosa* L. and a *Nicotiana tabacum* cultivar, resistance against tobacco necrosis virus (TNV) was induced through producing pyoverdine and increasing salicylic acid content in leaves. Maurhofer et al. (1998) reported that expression of salicylic acid biosynthetic genes in *P. fluorescens* P3 improved ISR against TNV in tobacco.

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0261-2194/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cropro.2013.11.020

In earlier studies, we found that *P. fluorescens* strain CZ significantly suppressed TMV *in vitro* by producing an antibiotic protein and that this PGPR could provide efficient protection against TMV infection by forming protective bacteria films and triggering ISR when sprayed on tobacco leaves (Wu et al., 2008, 2009; Shen et al., 2012). The objective of this study was to evaluate the effectiveness of this bacterial antagonist CZ powder in controlling TMV when applied in greenhouses and in the field.

2. Material and methods

2.1. Preparation of CZ powder and TMV suppression

P. fluorescens strain CZ was isolated from tobacco field soils at Qingdao Experimental Farm and stored at 4 °C. The stocked bacteria culture was incubated in nutrient broth medium (10 g peptone, 3 g beef grease, 5 g NaCl, 1 g glucose) or in a mixture medium in a microbial fermentor at 28 °C for 72 h to prepare CZ sub-cultures. *P. fluorescens* CZ cultures in mixture medium were mixed with silica white at a ratio of 6 ml:1 g and spray dried. Silicone surfactant (ethoxy modified trisiloxane) 2‰ (v/v) was added before use.

The number of colony forming units (cfu) of CZ culture in nutrient broth medium, mixture medium and a 100-fold dilution of CZ were determined by means of dilution plate count using an automatic colony counter. The *in vitro* suppression of TMV was determined by rub-inoculating *N. tabacum* cv. Samsun NN with a mixture containing an 80-fold dilution of TMV (1 g TMV-infected tobacco leaf tissue ground in 80 ml PBS) and the same amount of CZ. The suppression effects of CZ powder at 10, 25, 50, 100 and 150-fold dilutions were also determined. Each treatment was performed with three replications, each consisting of six plants. The first three leaves were inoculated and the number of necrotic spots was examined. Water was applied as control.

2.2. Disinfection of TMV on scissors

Scissors contaminated with TMV (by dipping in TMV suspension for 1 min) were dipped in 100-fold dilution CZ, nutrient broth medium or water for 30 s and were used to cut the first three leaves of *N. tabacum* cv. NC89 plants (cut off half of every leaf). Scissors dipped in TMV suspension only were used as controls. Each treatment consisted of twenty-five plants and was repeated three times. Disease severity was recorded 15 days after inoculation.

2.3. Growth promotion and TMV disease prevention on N. tabacum cv. NC89

N. tabacum cv. NC89 plants were grown in plastic pots till the three leaf stage and were treated as follows: 1) the soil in each pot was drenched three times at 6-day intervals with 10 ml of 100-fold CZ dilution, nutrient broth medium or water. Fresh weight, plant height and area of the biggest leaf (leaf width × leaf length × 0.63) were measured 15 days after first drenching. Each treatment to six plants was repeated three times; 2) plants were sprayed three times at 6-day intervals with 10 ml of 100-fold CZ dilution, nutrient broth medium or water. The plants were inoculated with 160-fold TMV suspension 6 h after the last spray. All plants were examined for disease severity 15 days after inoculation. Each treatment consisted of twenty-five plants, and all experiments were repeated three times.

2.4. Field experiments

Field experiments were performed in 2010 and 2011 at Qingdao Experimental Farm. *N. tabacum* cv. NC89 plants were sprayed with 100-fold dilution CZ three times at 7-day intervals after transplanting. Three replicates were arranged per treatment in a randomized block design, each consisting of forty plants in single-row plots. 200-fold Ningnanmycin dilution or water was applied to plants as controls. The tobacco field was drip-irrigated. All plants were examined for disease severity before flowering. TMV symptoms were rated according to the standard of GB/T23222-2008 (Ren et al., 2008).

2.5. Statistics

All data were calculated using the following formulas and analyzed with Duncan's new multiple range test using the DPS data processing system.

$$\text{Disease severity} = \sum(\text{disease grade} \times \text{number of plants in each grade}) \times 100/(\text{total number of plants} \times \text{highest disease grade})$$

$$\text{Disease ratio} = (\text{infected plants}/\text{total plants}) \times 100\%$$

$$\text{Inhibitory effect}\% = [(\text{local necrosis number or disease index of control} - \text{local necrosis number or disease index of treatment})/\text{local necrosis number or disease index of control}] \times 100\%$$

3. Results

3.1. Suppression of TMV by CZ powder in vitro

The effective cell concentration of CZ cultured in mixture medium was 90.9×10^{10} cfu ml^{-1}, showed an 89.3% *in vitro* suppression of TMV, similar to the treatment of CZ cultured in nutrient broth medium. This result suggested that mixture medium was able to meet the nutritional requirements of CZ, and could replace nutrient broth medium to reduce cost. CZ powder diluted by 100 times to 49.8×10^{10} cfu ml^{-1} showed an *in vitro* suppression of 87.8% against TMV on *N. tabacum* cv. Samsun NN compared with the water control (Table 1). The mean numbers of local necrosis lesions increased, so the *in vitro* suppression effects decreased gradually with the increasing dilution of the CZ powder. Compared with the water control, the *in vitro* suppression effects by the CZ powder diluted 100-fold, were all higher than 88.3%, significantly higher ($P < 0.01$) than the 40.4% suppression by the 150-fold diluted CZ powder (Table 2). The EC90 of the CZ powder was a 97.27-fold dilution (approximately 100-fold dilution), which could be used as a standard concentration in future applications.

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