Biological Control 92 (2016) 45–54

Contents lists available at ScienceDirect

Biological Control

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

Cowpea resistance induced against *Fusarium oxysporum* f. sp. *tracheiphilum* by crustaceous chitosan and by biomass and chitosan obtained from *Cunninghamella elegans*

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HIGHLIGHTS

- Chitosan inhibited the growth of *Fusarium oxysporum* f. sp. *tracheiphilum*.
- Chitosan caused morphological change in *Fusarium oxysporum*.
- Chitosan and bioprotector induced natural plant defenses in cowpea.
- Chitosan and bioprotector increased the peroxidase and catalase in cowpea.

ARTICLE INFO

Article history:
Received 2 June 2015
Revised 10 September 2015
Accepted 22 September 2015
Available online 25 September 2015

Keywords:
Biopolymer
Antioxidative enzymes
Plant resistance
Reactive oxygen species

ABSTRACT

Pathogenic microorganisms increase the production of reactive oxygen species (ROS), and the response can cause oxidative damage and even death in a plant. Plant resistance inducers activate antioxidant enzymes such as catalase (CAT) and peroxidase (POX). The aims of this study were to evaluate the antimicrobial activity and the potential to induce the CAT and POX activities by the biopolymer chitosan in cowpea plants inoculated with the phytopathogen *Fusarium oxysporum* f. sp. *tracheiphilum*. The antimicrobial activity of crustaceous chitosan (CrCh) was tested in a laboratory assay and analyzed by optic and scanning electron microscopy. In a greenhouse experiment, the effectiveness of CrCh, fungal chitosan obtained from *Cunninghamella elegans* (C.eCh), and *C. elegans* biomass (C.eCh source) plus NPK biofertilizer, named bioprotector (*C. elegans* + NPKB), were evaluated in relation to the disease severity and the CAT and POX activities in cowpea plants infected with *F. oxysporum* f. sp. *tracheiphilum*. The higher rates of CrCh (4.0–6.0 mg mL^{-1}) showed the total fungicide ability, and the other concentrations (0.5–3.0 mg mL^{-1}) promoted a greater fungistatic effect and morphological changes on the growth of *F. oxysporum* f. sp. *tracheiphilum* during the antimicrobial test in vitro. Chitosan effectively protected cowpea plants against this pathogen. The higher chitosan concentrations (4.0 and 6.0 mg mL^{-1}) were directly related to CAT and POX activity, which controlled the ROS equilibrium for plant resistance and resulted in a significant reduction of the disease severity in cowpea. These findings are important for the establishment of a sustainable agriculture and to avoid the use of pesticides.

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a drought-tolerant and warm-weather crop of great socioeconomic importance in semi-arid tropical countries, especially in Brazil, because it represents a popular dietary source of protein, carbohydrates, iron, potassium and phosphorus. Cowpea legumes can be used for animal feed and

Abbreviations: CAT, catalase; C.eCh, fungal chitosan obtained from *Cunninghamella elegans*; CrCh, crustaceous chitosan; HMDS, hexamethyldisilazane; NPKB, NPK biofertilizer; OMB, organic matter biofertilizer; PKB, PK biofertilizer; POX, peroxidase; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.biocontrol.2015.09.006
1049-9644/

for the recovery of soil fertility as green manure; additionally, it has a considerable tolerance to drought and heat (Gogile et al., 2013; Rodrigues et al., 2006).

Phytopathogenic fungi are economically important agricultural microorganisms, because they induce decay on a large number of agricultural crops during the growing and postharvest (Rabea and Steurbaut, 2010; Badawy and Rabea, 2011). *Fusarium oxysporum* f. sp. *tracheiphilum* (Smith) Snyder and Hansen is a pathogenic fungus responsible for one of the most frequent diseases in cowpea, known as *Fusarium* wilt. This pathogen inhabits the soil, can grow and survive for long periods in the form of chlamydospores, and the main symptoms of the disease in cowpea are a reduction in plant growth, chlorosis, wilting and premature leaf fall, which almost always result in the death of infected plants (Rodrigues et al., 2006).

Currently there has been a public concern with the presence of pesticide residues in food and the environment due to the use of synthetic fungicides in agriculture. Chitosan has been reported as a safer alternative to control phytopathogenic fungi during both pre- and postharvest processes (Rabea and Steurbaut, 2010; Badawy and Rabea, 2011). The biopolymer chitosan has antimicrobial activity against pathogenic fungi and the ability to induce plant defense mechanisms (Romanazzi et al., 2013). Chitosan is a biodegradable, biocompatible, a cationic and linear polymer that is essentially composed of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues (Berger et al., 2014).

Chitosan has been proven to inhibit several plant pathogens fungal *in vitro* and in host plants. This polymer demonstrates ability to induce marked morphological changes, structural alterations and molecular disorganization of the fungal cells. On the other hand, this biopolymer displays indirect activity when induce defense mechanisms in plants, stimulates the production of reactive oxygen species, inhibits the action of proteinases, alters the metabolism of phytoalexins, promotes lignification, induces the formation of phenolic compounds, stimulates the accumulation of pathogenesis related (PR) proteins such as chitinase, β-1,3-glucanase (Ali et al., 2014; Li et al., 2015), and activates peroxidase (Ortega-Ortiz et al., 2007), superoxide dismutase and catalase enzymes (Zeng and Luo, 2012). The PR proteins assist in the reduction of plant diseases, such as β-1,3-glucanase act as a mechanical barrier for blocking the invasion of fungi into the plant tissue and also protects the tissue against phytotoxic substances produced by the fungus. The accumulation of PR proteins is also related to the hydrolysis of β-1,4-glucan, the major fungal cell wall components (Ali et al., 2014).

The intense production of reactive oxygen species (ROS), referred to as 'the oxidative burst', is one of the responses of the plant during a pathogenic infection. ROS play a key role in plants as signal transduction molecules involved in mediating responses to a pathogen (Miller et al., 2010). ROS can also act as direct antimicrobial agents, inhibiting the development of the pathogen; ROS can produce hypersensitivity responses and cause cell death and systemic acquired resistance in the host (Pascholati et al., 2008). On the other hand, the increase in ROS production has the potential to cause oxidative damage to cells during host–pathogen interaction. The enzymes catalase (CAT) and peroxidase (POX) are among the antioxidants responsible for carrying out the detoxification of ROS in the host plant (Miller et al., 2010). POX is also involved in the process of cell wall lignification. This enzyme catalyze the biosynthesis of metabolites such as phytoalexins and phenols to form lignin which strengthen the defense system in plants against pathogen infection (Li et al., 2015).

The influence of chitosan to increase CAT and POX has been evaluated in tomato plants (Ortega-Ortiz et al., 2007), and to increase POX activity alone in tobacco (Falcón-Rodríguez et al., 2009), in tomato (Liu et al., 2007), in orange fruits (Rappussi et al., 2009) and in cowpea (Rodrigues et al., 2006).

The aim of this study is to evaluate the antifungal activity of chitosan (*in vitro*) and the effectiveness of crustaceous (CrCh), fungal chitosan obtained from *Cunninghamella elegans* (C.eCh) and a bioprotector fertilizer (*C. elegans*-NPKB) in the resistance against *F. oxysporum* f. sp. *tracheiphilum* on cowpea.

2. Materials and methods

2.1. Materials

2.1.1. Fungal and bacterial isolates

F. oxysporum f. sp. *tracheiphilum*, phytopathogenic fungi of cowpea plants, was purchased from the Culture Collection of pathogenic fungi at the University Federal Rural of Pernambuco, Recife, Brazil. The strain was maintained in Petri dishes containing Potato Dextrose Agar (PDA), pH 5.6, at 4 °C until be used in laboratory assay and in the greenhouse experiments. To increase the phytopathogenic activity, the pathogenic fungi were inoculated and re-isolated from cowpea plants prior to use in the greenhouse experiments. This re-isolated pathogen was grown in Petri dishes with PDA medium for 7 days at 25 °C.

The *F. oxysporum* f. sp. *tracheiphilum* cultures were transferred to Erlenmeyer flasks (1000 mL) containing 400 mL of PD medium (pH 5.6) and incubated for 7 days at 25 °C. After this incubation period, the culture was filtered and the final concentration of the macroconidial suspension was standardized to 10^7 macroconidia mL^{-1}.

The fungal chitosan (C.eCh) was obtained from *C. elegans* (UCP/WFCC 0542). This strain was isolated from mangrove sediment situated in the district of Rio Formoso, Pernambuco, Brazil, and purchased from the Culture Collection of the Catholic University of Pernambuco, registered in the World Federation for Culture Collection (WFCC). *C. elegans* was maintained at 4 °C on PDA slants and transferred to a new medium every 4 months. The cell wall of this Mucorales fungi is a potential source of chitosan (Berger et al., 2014).

The diazotrophic bacteria *Bradyrhizobium* strain (BR 3267) was used as a nitrogen source for cowpea plants in greenhouse experiment, recommended and purchased by the Nucleus of Nitrogen Fixation of the University Federal Rural of Pernambuco – UFRPE.

2.1.2. Production of the biofertilizer (NPKB)

The mixed biofertilizer (NPKB) was used in the greenhouse experiments as source of N, P and K for cowpea plants. The NPKB was produced from PK rock biofertilizers mixed with organic matter (OM – earthworm compound) used in a PK:OM proportion equivalent to 1:3 (v/v) following Berger et al. (2013) and Stamford et al. (2007). An analysis of the P and K rock biofertilizers in accordance with two methodologies, namely (A) Mehlich 1 and (B) extraction with citric acid, yielded the following results: (P-biofertilizer)-pH = 3.8, available P (A) = 60 (g kg^{-1}) and (B) = 48 (g kg^{-1}); and K rock biofertilizer-pH = 3.3, available K (A) = 10 (g kg^{-1}) and (B) = 5 (g kg^{-1}).

The OM was enriched in N by inoculation with free living diazotrophic bacteria (NFB 1001) selected in the Nucleus of Nitrogen Fixation at the University Federal Rural of Pernambuco (UFRPE). The selected diazotrophic bacteria were cultured in LG liquid media (50 mL) in 125 mL Erlenmeyer flasks, 180 rpm for 96 h at ±28 °C and applied in 100 mL per tray (6 L) according to Lima et al. (2010). Analysis of the OM produced the following results: pH 7.85; organic carbon 120.7 g kg^{-1}; total N 8.6 g kg^{-1}; total sulfur 2.9 g kg^{-1}; and total P 11.2 g kg^{-1}. The NPKB analysis was as follows: pH 6.2; organic carbon 100.1 g kg^{-1}; total N 16.8 g kg^{-1}; total sulfur 3.2 g kg^{-1}; available P 13.4 g kg^{-1}; and available K 15.4 g kg^{-1}.