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Control of *Rhizoctonia solani* in a tobacco-float system using low rates of iprodione- and iprodione-resistant strains of *Gliocladium roseum*

Paola Tarantino*, Rosa Caiazzo, Angela Carella, Ernesto Lahoz

C.R.A. (Consiglio per la Ricerca e Sperimentazione in Agricoltura), Laboratory of Biology and Plant Pathology Scafati, Via P. Vitiello, 108; I-84018 Scafati (SA), Italy

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Abstract

Results obtained using low rates of the fungicide iprodione- and iprodione-resistant strains of *Gliocladium roseum* to control *Rhizoctonia solani* Kühn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) in a tobacco float system are reported. The objectives were three-fold: to obtain *G. roseum* (GNL) strains resistant to the fungicide iprodione; to verify that the mutants maintained the same characteristics of the wild type; and to control *R. solani* in a tobacco float system by the application of low doses of fungicide combined with a resistant biological control agent (BCA) strain. Two resistant strains were obtained and the IC_{50} values were 44.7 and 5000 $\mu g\,ml^{-1}$ for GNL wild type and mutants, respectively. The results showed the same level of disease control in plots with a high rate of iprodione and in plots with a low rate of fungicide, applied together with GNLr1 (96%) and GNLr2 (98%) iprodione-resistant strains of *G. roseum*. Less-disease control was obtained by a reduced dose of iprodione, when applied alone.

Keywords: *Rhizoctonia solani*; Biological control; *Gliocladium roseum*; Fungicide resistance

1. Introduction

Rhizoctonia solani Kühn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a widespread soil-borne pathogen of tobacco, in which it is known as the etiological agent of damping-off, sore shin and leaf target spot. The latter disease does not occur in all tobacco-growing areas of the world, since it is largely dependent on environmental conditions of high rainfall and relative humidity. However, damping off and sore shin are spread worldwide and crop losses up to 15% have been recorded (Nicoletti et al., 1999). Until now, isolates belonging to AGs one through five of *R. solani* have been reported as causal agents of tobacco diseases (Shew and Lucas, 1991; Nicoletti et al., 1999). In many areas, control is needed both in the seedbed (traditional or float system) and in the field. In recent years (Nicoletti and Lahoz, 1995; Caiazzo et al., 2005), *R. solani* has been reported as a pathogen of major interest in the tobacco seedbed and field, and the application of fungicides has been used to reduce losses. *Rhizoctonia*-disease epidemics are becoming a limiting factor for tobacco cultivations to such an extent that an increase in the use of chemical control can be foreseen. However, a reduction in the use of fungicides is also one of the research objectives for tobacco cultivation. The possible problem of fungicide resistance, coupled with pressure to reduce the overall use of pesticides in order to alleviate concerns about health and environmental issues, may result in less fungicide use in the future. Biological control, often used as part of integrated pest management, might help to reduce fungicide application. *Gliocladium roseum* (GNL) is one of the most powerful biological control agents (BCA), as shown in control of *Botrytis cinerea* in strawberry, raspberry, tomato (Sutton et al., 1997) and *R. solani* on tobacco (Lahoz et al., 2002) and bean (Tarantino et al., 2006). *G. roseum* is common in different environments (tropical, desert, temperate). It is associated with organisms such as fungi, nematodes and plants and has a remarkable ability to create associations with roots, leaves, fruits and

*Corresponding author. Tel.: +39 081 8563629; fax: +39 081 8506206.
E-mail address: paola.tarantino@entecra.it (P. Tarantino).

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seeds of many plants. This extraordinary ecological versatility renders *G. roseum* a good candidate for use in agriculture (Sutton et al., 1997). Many mechanisms have been reported for *G. roseum* antagonism (Sutton et al., 1997; Lahoz et al., 2004). The use of BCA combined with other control methods like solarization (Sivan and Chet, 1993) or fungicides has been proposed in the past (Elad et al., 1993; Chet et al., 1982). Another possibility to control soil-borne diseases, while reducing chemical applications, might be the use of lower fungicide application rates, but in this case, there are some concerns about the onset of pathogen resistance. In order to reduce fungicide use and to avoid the rapid appearance of resistance, the application of low rates of fungicides combined with a BCA should be practiced to achieve both the goals. The BCA strain used must be resistant to the fungicide, so that different mechanisms can act against the target pathogen. The UV method to obtain resistant strains of antagonists has been used successfully in the past (Papavizas et al., 1982; Papavizas and Lewis, 1983; Bensaci and Neumann, 1989). In addition to fungicide resistance, treatment with radiation has been reported to induce other effects in the biotype, such as altered growth, sporulation and biological control capabilities (Ahmad and Baker, 1988; Zhou and Reeleder, 1990; Kay and Stewart, 1994).

In this work, we chose iprodione as a model system due to its high ability to induce resistance or insensitivity and because grower pressure to register this fungicide for use on tobacco is increasing.

The objectives of the present work were: (i) to obtain iprodione-resistant strains of *G. roseum* effective against *R. solani*; (ii) to investigate if mutant strains maintained the same growth and biochemical characteristics (both related to antagonistic ability) as the wild type; (iii) to control *R. solani* on tobacco in a float system by the application of low doses of fungicide combined with the fungicide resistant strain of BCA.

2. Material and methods

2.1. *R. solani* and *G. roseum* isolates

The isolation of antagonistic *G. roseum* strains was made using Petri dishes containing potato dextrose agar (PDA) (Oxoid CM139) precolonized by *R. solani* mycelium (Mulligan and Deacon, 1992); *R. solani* (RT 27 belonging to AG 2–1 Nt) used in the present study was isolated from diseased burley tobacco plants grown in Southern Italy (Benevento province), and preserved in the mycological collection of the Tobacco Research Institute; it was chosen on the basis of its high level of virulence.

2.2. Mutation induction

Before obtaining mutant strains, a trial on the sensitivity of *G. roseum* to iprodione was carried out using the method of amended agar. In all in vitro experiments, malt extract agar (MEA) (Oxoid CM 59) was used as a solid medium, amended with iprodione, common name ROVRAL FL flowable 25% a.i. ($270\,g\,l^{-1}$), BASF Agro Spa, Italy. Sensitivity tests of fungal strains to the fungicide were carried out for each of seven fungicide concentrations (0.001, 0.01, 0.1, 1, 10, 100 and $1000\,\mu g\,ml^{-1}$) by inoculating three Petri dishes per concentration with a 5 mm mycelial plug, taken from 10-d old colonies.

Radial growth of GNL colonies was measured 4–10 d after incubation and expressed as a percentage of the growth in control plates without fungicide. The data were plotted in graphs placing the concentration in logarithmic scale on the abscissa and the radial growth as percentage of the control in linear scale on the ordinate. Relative toxicity was expressed as IC_{50}, the concentration causing 50% reduction in radial growth. Mutants were obtained after treatment of a conidial suspension by UV irradiation with a germicidal lamp (General Electric 15 W G15T8) for 0, 15, 30 and 60 min (Van Tuyl, 1977). Surviving colonies were transferred to media amended with iprodione (2000 and $5000\,\mu g\,ml^{-1}$) to confirm insensitivity of the mutant strains. Two mutant strains (GNLr1 and GNLr2) were obtained and tested, together with the wild type, following the above mentioned method, using five iprodione concentrations (10, 100, 1000, 2000, $5000\,\mu g\,ml^{-1}$) to be sure that they were really mutants and not escapers and to determine new IC_{50} values.

2.3. Evaluation of biological characteristics

The growth of mycelium of wild type and resistant strains was measured after 4 d at 27 °C on four Petri dishes per isolate containing MEA.

The ability to produce conidia was measured on $20\,mm^2$ of mycelium collected from five colonies of each isolate grown on MEA for 1 wk. Plugs were suspended in 5 ml of distilled water, homogenized and filtered through two layers of cheese cloth and the numbers of conidia were counted using a hemocytometer.

2.4. Detection and quantification of *N*-acetyl β-D-glucosaminidase, β-D-*N*-*N*′-diacetyl-chitobiosidase and endochitinase activities

Determinations of *N*-acetyl β-D-glucosaminidase and β-D-*N*-*N*′-diacetyl-chitobiosidase were made following the protocol reported by Tronsmo and Harman (1993). All isolates were grown in Richard's medium amended with 5 g colloidal chitin or alternatively 5 g of *R. solani* mycelium as carbon source. Before use, mycelium was homogenized, centrifuged and the pellets were re-suspended in sterile water and sterilized at 121 °C for 20 min. For activity determination, the release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-*N*-*N*′-diacetyl-chitobiose (Sigma Aldrich, Milan, Italy) were assayed, respectively. A test sample (30 µl) was added