ISOLATION AND EVALUATION OF BACTERIA AND FUNGI AS BIOLOGICAL CONTROL AGENTS AGAINST *RHIZOCTONIA SOLANI*

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SUMMARY

Rhizoctonia solani is one of the most important limiting factors for potato production and storage in Belgium and worldwide. Its management is still strongly dependent on chemical treatments. The aim of this work was to evaluate the possibility of exploiting bacteria and fungi in order to control this pathogen. Among a collection of 220 bacterial strains isolated from different organs of healthy potato plants and rhizospheric soils, 25 isolates were selected using screening methods based on *in vitro* dual culture assays. The mycelial growth inhibition rate of the pathogen was ranged from 59.4 to 95.0%. Also seven fungal strains isolated from the rhizospheric soil and potato roots showed a highly mycelial growth inhibition of *R. solani*. The mycelial growth inhibition rate obtained with these fungi was included between 60.0 and 99.4%. From this preliminary study, the further investigations will be planned to determine the bacterial isolates systematic, species of fungal strains by using molecular tools and to assess their efficacy against *R. solani* in greenhouse trials.

Key words: Bacterial isolates, Fungal isolates, Potato plant, *Rhizoctonia solani*, Selection.

INTRODUCTION

Rhizoctonia solani Khün (teleomorph, *Thanatephorus cucumeris*) is one of the most important soilborne pathogens which develops in cultured soils, causing serious diseases worldwide, and which has a wide host range (Woodhall *et al*., 2007). Stem canker and tuber blemishes are two major diseases associated with *R. solani* in potato (*Solanum tuberosum* L.). These diseases can cause both quantitative and qualitative damages to the potato crop. The predominance of the anastomosis group AG-3 in causing potato disease has been reported by several investigations (Bains and Bisht, 1995; Balali *et al*., 1995; Virgen-Calleros *et al*., 2000; Campion *et al*., 2003).

Infection of potato plants by *R. solani* may be initiated by soilborne (Hide *et al*., 1973; Banville, 1978; Platt, 1989) or tuber-borne inoculum (Carling *et al*., 1989), and the use of infected potato seed tubers or planting in contaminated soil can cause reduction in the marketable tuber yield (Platt *et al*., 1993). Planting disease-free seed tubers, or treating tubers with fungicides or antagonists, are helpful. However, fungicide treatments may not provide complete control due to the occurrence of soilborne inoculum (Tsror and Peretz, 2005).

Different methods have been used to control *R. solani*. The most used ones are cultural practices, solarization, chemical and biological control. Considering the cost of chemical pesticides and the hazard involved, biological control of plant diseases is now increasingly capturing the imagination of plant microbiologists. This last method has been successfully developed during the last years. It's based on the reduction of inoculum or pathogenic activity due to the natural presence of one or more organisms, through the management of the environment, the host or antagonists (Baker and Cook, 1978). So, several microorganisms, including the obli-

gate mycoparasite fungus V*erticillium biguttatum*, have been reported as effective biocontrol agents against *R. solani* in potato (Van Den Boogert and Jager, 1984; Jager *et al*, 1991; Wicks *et al*., 1996). Applying *V. biguttatum* in combination with *Gliocladium roseum* has repeatedly proved successful in reducing *R. solani* on stolons and stems. In field experiments, this ultimately led to a reduced formation of sclerotia on new tubers, particularly in natural sandy and clay loam soils (Jager and Velvis, 1985). To date, the filamentous fungi included in the genus *Trichoderma* remain economically important efficient biocontrol agents that are commercially produced to prevent development of several pathogenic fungi (Papavizas, 1985; Samuels, 1996). These species of *Trichoderma* can produce extracellular enzymes (Haran *et al*., 1996) and/or antifungal antibiotics (Ghisalberti and Rowland, 1993), or they may also be competitors to fungal pathogens (Simon and Sivathamparan, 1989), promote plant growth (Inbar *et al*., 1994), and induce resistance in plants (De Meyer *et al*., 1998; Grondona *et al*., 1997).

Beneficial bacteria have been widely investigated for use in agriculture. During last 20 years, several works reported the importance of bacterial isolates to suppress soilbrone pathogens in different crops. Many reports or reviews in this area have already appeared (e.g., Weller, 1988; Handelsman and Stabb, 1996) and show that *Bacillus* and *Pseudomonas* are the most investigated. In the case of *Rhizoctonia* diseases, there is an increasing interest in developing biological approaches based on finding bacteria to suppress *Rhizoctonia* diseases (Asaka and Shoda, 1996; Siddiqui and Shaukat, 2002; Szczech and Shoda, 2004; Szczech and Shoda, 2006). Recently, it has been demostrated that bacterial endophytes may have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Adhikari *et al*., 2001; Sturz *et al*., 2000; Tjamos *et al*., 2004). It has been suggested that these bacteria might interact more closely with the host plant and therefore could be efficient biological control agents in sustainable crop production. In litterature, a few studies were interested in the potential of endophyte and non-endophyte bacteria associated with potato plants to suppress soilborne pathogens of tubers (Berg *et al*., 2005). In this context, the main aim of this study was to screen and to evaluate the potential of endophyte and non-endophye bacterial and fungal isolates associated with different potato plant organs (leaves, stems, roots and tubers) as well as with the surrounding soil to control *R. solani* in *in vitro* dual culture assays.

MATERIAL AND METHODS

Fungal strain

Rhizoctonia solani strain MUCL30286, anastomosis AG-3 used in this study was obtained from MUCL/Louvain la Neuve, Belgium. This strain was stored at -80% in 25% glycerol.

Isolation of bacterial isolates from potato plant organs (leaves, stems and roots)

The isolation of endophyte bacteria from leaves, roots and stems of healthy potato plants was carried out based on methods described by Berg *et al*. (2005). The different plant organs were delicately separated and cut into small fragments of 2 to 3 cm long and then disinfected in 1.5% sodium hypochlorite (NaOCl) for 3 min followed by three washes in sterile distilled water. The plant material was placed on

nutrient agar (NA) as a sterility check (4 fragments per Petri dish). After incubation of 5 days, only samples for which no bacterial growth occurred in the sterility check were used for endophyte bacteria isolation. All samples of each plant organ were homogenized with mortar and pestle, serially diluted with sterile 0.85% NaCl and plated onto R2A medium (Merck). Plates were incubated for 5 days at 20°C. For each plant organ, the bacterial colonies with visually different morphologies were transferred to NA medium, purified and then stored at -70°C in nutrient broth containing 15% glycerol. Unlike endophyte bacteria, non-endophyte bacteria were isolated from disinfected organ fragments from which microorganisms has developed.

Isolation of bacterial isolates from tubers and surrounding soil (Rhizosphere)

Tubers obtained from healthy potato plants were cleaned with sterile distilled water, disinfected as described above during 20 min and then washed twice in sterile distilled water. After drying for one hour, tubers were cut into small slices and mixed in isotonic solution (0.85% NaCl). After serial dilutions, 100 µl were plated onto R2A medium. Petri dishes were then kept at 20°C in a growth chamber. After 2 to 3 days of incubation, visible colonies were differentiated according to their size and color and subcultred and purified individually on NA medium. All isolates were considered as endophytes.
Samples (3g) of soil surrounding tubers of healthy potato plants were homogenized and sieved. One hundred ml of isotonic solution (0.85% NaCl) was added and then homogenized. After serial dilutions, 100 µl were spread over on R2A medium (3 Petri dishes per dilution). Petri dishes were then sealed and kept at 20°C. After an incubation of 2 to 3 days, colonies that differ in size and color were transplanted individually on NA medium and then purified. All isolates were considered as non-endophytes.

Isolation of fungal strains

The same techniques and methods described above were used to isolate fungal species from different organs of healthy potato plants.

Screening of bacteria and fungi for antagonism against *R. solani*

Bacterial isolates were screened for their ability to produce antifungal substances against *R. solani* by *in vitro* dual culture assays on Potato dextrose agar (PDA) (Landa *et al*. 1997). Each bacterial isolate was spotted at 4 equidistant points along the perimeter of the plate (3 plates per isolates). After 48 h of incubation at 28°C in the dark, a 5-mm plug from the leading edge of a 7-day-old culture of *R. solani* on PDA was placed in the center of the plate. Plates without bacteria were used as control. Plates were incubated at 28°C for 5 days, after which the length of hyphal growth toward the bacteria (Ri) and that on a control plate (Rc) were measured. The relative growth inhibition was expressed as [(Ri-Rc)/Rc x 100]. The isolates that caused significant inhibition of the pathogen growth were also examined for gram staining, endospore formation, and fluorescent pigment on PAF and hypersensitive reaction on White Burley tobacco leaves. Regarding the fungal isolates, the same dual culture assays were applied to assess their antagonistic activity against

Rhizoctonia, except that the PDA plates were inoculated with a plug from leading edge of 7-day-old culture of the fungal isolate and the pathogen at the same time.

Statistical analysis

Data expressed as inhibition rate (%) of mycelial growth of *R. solani* were analyzed using SAS software (SAS Institute, version 8.2, Cary, NC, USA) and the treatment mean values were compared by Duncan's multiple range test at $P<0.05$.

RESULTS

Importance of bacterial isolates within potato plant organs and in the surrounding soil

In leaves and roots, we have isolated as much endophyte as non-endophyte bacterial isolates (Table 1). In stems, however, we have isolated much more non-endophytes compared to endophytes. If we consider tubers and the surrounding soil, we have isolated less endophytes (88) than non-endophytes (132).

Importance of antagonistic bacteria among endophyte and non-endophyte bacterial populations

All bacterial isolates were tested for their ability to produce antifungal substances against *R. solani* (Figure 1A). Whatever the sample considered, the highest number of antagonistic bacterial isolates was observed in non-endophyte bacteria which represented 16.04% of the total bacterial population (220 isolates) compared to endophytes representing 4.09% only (Table 2). Table 3 represents 25 bacterial isolates showing an inhibition rate higher than 60%. Among them, 3 isolates (RE032 from roots, TE02 and TE012) are endophytes whereas the 22 others are non-endophytes.

Table 1. Number of endophyte and non-endophyte bacterial isolates in potato plant organs and in the surrounding soil

Potato plant organs	Endophytes	Non-endophytes
Leaves	19	19
Stems	16	25
Roots	39	28
Tubers	14	0
Soil	0	60
Total	88	132

Table 2. Antagonistic bacterial isolates (expressed as % of total population of endophyte or non-endophyte bacteria) in potato plant organs and in the surrounding soil

Antagonistic bacterial isolates		
Potato plant organs	**Endophytes (%)**	**Non-endophytes (%)**
Leaves	5.26	13.15
Stems	2.43	21.95
Roots	5.97	7.46
Tubers	14.28	-
Soil	-	26.66
Total	4.09	16.04

Screening for fungal isolates showing an antagonistic activity against *R. solani*

Among 14 fungal isolates isolated from potato roots and rhizosphere (tubers and surrounding soil), only 9 showed an antagonistic activity against *R. Solani* (Table 4). The inhibition rate ranged from 60% to 99% in *in vitro* dual culture assays. Statistical analysis revealed a significant difference between fungal isolates. The Duncan's multiple range test distinguished six homogenous groups and the highest hyphal growth of *R. solani* was obtained with isolate FRHN01 followed by FRHN08, FRHN07 and FRHN04.

DISCUSSION

R. solani causes stem and stolon canker and black scurf on potato tubers, reducing plant health, yield quality and quantity. The management of *Rhizoctonia* disease requires an integrated approach and knowledge of each stage of the disease because no single tactic is totally effective (Banville *et al*., 1996; Elbakkali and Martin, 2005). During the ten last years, several workers underlined the ability of bacterial isolates to inhibit soilborne pathogens and almost of them belong to the *Pseudomonas* and *Bacillus* genus. However, the implication of endophyte bacteria in biological control of soil pathogens has received less attention as compared to epiphyte bacteria.

Table 3. Bacterial isolates showing high antagonistic activity (more than 60%) against *R. solani* in *in vitro* dual culture assays

Bacterial isolate	Origin	Inhibition rate (%)
LN05^{X}	Leaves	76.82^{FGY}±2.12
LN07	Leaves	75.52^{FIGH}±3.39
LN08	Leaves	74.87^{FIGH}±7.50
LN010	Leaves	84.56^{CD}±1.98
LN016	Leaves	76.18^{FGH}±2.36
SN019	Stem	62.02^{K}±3.22
SN01L	Stem	75.62^{FIGH}±1.86
SN010L	Stem	62.50^{K}±2.02
SNRL01	Stem	59.37^{K}±2.18
SN010	Stem	83.75^{DE}±0.00
SN09L	Stem	68.75^{J}±0.00
RN01	Roots	79.40^{EF}±3.56

Bacterial isolate	Origin	Inhibition rate (%)
RN02	Roots	76.85^{FG}±4.90
RN016	Roots	74.24^{GIH}±0.22
RN018	Roots	60.06^{K}±3.58
RE032	Roots	71.66^{IJH}±3.11
TE02	Tuber	79.37^{EF}±0.00
TE012	Tuber	87.5^{CD}±0.00
RH000	Rhizosphere	86.5^{CD}±1.78
RH111	Rhizosphere	77.50^{FG} ±1.05
RH222	Rhizosphere	88.75^{BC} ±1.29
RH333	Rhizosphere	85.62^{CD}±0.88
RH444	Rhizosphere	79.37^{EF}±0.75
RH555	Rhizosphere	92.5^{AB}±0.00
RH666	Rhizosphere	95^{A}±0.00
Control[z]		0.00^{J}

[x]L: leaves, R: roots, S: stem, T: tuber, RH: Rhizosphere, N: non-endophytes and E: endophytes.
[y]Treatments having the same letters are not significantly different (P <0.05).
[z]Treatment inoculated only with *R. solani*.

Identification and selection of effective antagonistic organisms is the first and foremost step in biological control (Kamalakannan *et al*., 2004). In this work, we have screened and evaluated for the first time endophyte and non-endophyte bacterial isolates from healthy potato plants and rhizosphere for their antagonistic activity against *R. solani* in Belgium. Among a total of 220 bacterial isolates, only 25 showed a highly significant inhibition rate against *R. solani* in *in vitro* dual culture assays superior to 60%. However, only three endophyte bacterial isolates seemed to be effective against *Rhizoctonia* in *in vitro* conditions. These antagonistic bacterial isolates are isolated from roots and tubers. The highest number of isolates with antifungal activity against *R. solani* was obtained from the rhizosphere. These results are in contradiction with those found by Berg *et al*. (2005) who reported that the highest number of effective isolates against *R. solani* in Germany was observed on phyllosphere followed by the endorhiza, rhizosphere and endosphere. The fact that our endophyte and non-endophyte isolates were obtained on R2A medium after samples disinfection may explain the small number of bacterial isolates in our study. In most cases, effective bacteria as biocontrol agents of fungal diseases belong to the genera *Bacillus*, *Pseudomonas* and *Streptomyces* (Handelsman and Stabb, 1996). The use of bacteria to exert an appropriate biological control of *R. solani* and other soilborne pathogens relies on their ability to efficiently colonize roots; otherwise, their biocontrol character would not have any sense.

Table 4. Fungi selected for their antagonistic activity against *R. solani* in *in vitro* dual culture assays.

Fungi	Origin	Inhibition rate (%)
FRHN 01	Rhizosphere	99.36^{Ay} ±1.26
FRHN 02	Rhizosphere	72.35^{D} ± 1.54
FRHN 03	Rhizosphere	71.71^{D} ± 2.13
FRHN 04	Rhizosphere	83.15^{C} ±1.98
FRHN 05	Rhizosphere	66.73^{E} ± 1.13
FRHN 07	Rhizosphere	88.14^{B} ±0.87
FRHN 08	Rhizosphere	86.89^{B} ±1.95
FRE 09	Roots	60.00^{F} ± 2.50
Control^{x}		0.00^{G}

^{x}Treatment inoculated only with *R. solani*.
^{y}Teatments having the same letters are not significantly different (P<0.05)

As with bacteria, we also tested the ability of fungal isolates isolated from healthy potato roots to inhibit the mycelial growth of *R. solani* in *in vitro* dual culture assays (Figure 2B). Among 14 fungal isolates, only nine showed an antagonistic activity higher than 60% and four isolates gave an inhibition rate superior to that reported here for bacterial isolates. In the literature, *Trichoderma* and *V. biguttatum* remain the most cited antagonistic fungi against *R. solani* (Jager *et al*., 1991; Samuels, 1996).

According to results obtained in this preliminary study, further works are planned to characterize the selected antagonistic bacterial and fungal isolates, to test their ability to produce volatile or diffusible antibiotics and to evaluate their ability to inhibit *R. solani* in potato greenhouse trials.

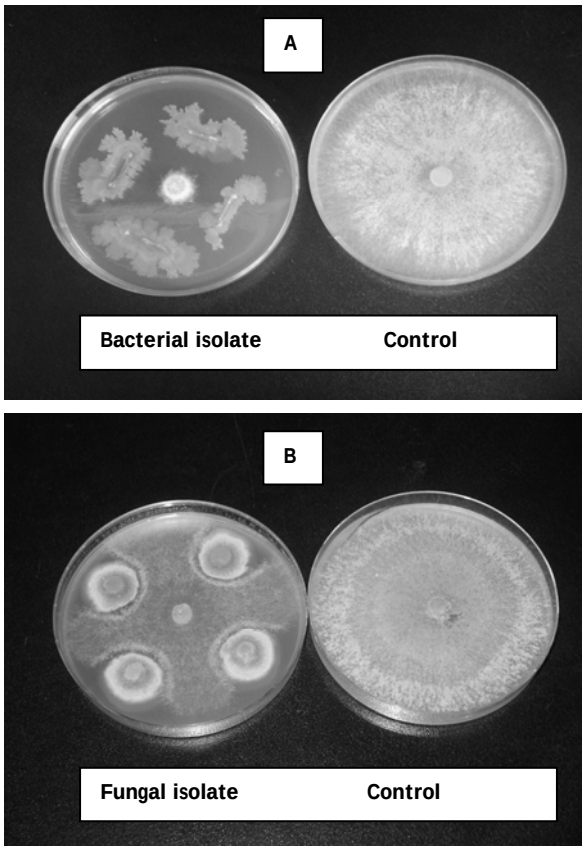

Figure 1. Two representative examples of *in vitro* dual culture assays: the first one (A) is for a bacterial isolate and the second one (B) is for a fungal isolate

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