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Selecting bacterial strains for use in the biocontrol of diseases caused by *Phytophthora capsici* and *Alternaria alternata* in sweet pepper plants

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Abstract

More than 500 isolates of bacteria were obtained from the aerial part and rhizosphere of sweet pepper (*Capsicum annuum* L.) plants harvested from different places in the Region of Murcia (Spain). The isolates were purified and assayed *in vitro* against *Phytophthora capsici* and *Alternaria alternata*. Sixty isolates (12 %) produced an inhibition zone against at least one of the pathogens, while ten had a strongly inhibitory effect on both pathogens assayed. Microscopic observation of interactions zone showed cell vacuolisation, hyphae lysis and spilling of cytoplasm content of the pathogens in the culture media. These ten isolates were then chosen for biocontrol of *Phytophthora* root rot and *Alternaria* leaf spots of pepper plants *in vivo*. Four of them denominated HS93, LS234, LS523 and LS674 reduced *P. capsici* root rot by 80, 51, 49 and 54 %, respectively, and *A. alternata* leaf spots by 54, 74, 62 and 53 %. HS93 belongs to the genus *Bacillus* and probably the species *subtilis*, while LS234, LS523 and LS674 belong to the genus *Bacillus* and probably the species *licheniformis*. Dry mass of plants treated with these bacteria was significantly higher than that of non-treated and inoculated plants.

Additional key words: antagonism, plant growth-promoting rhizobacteria, *Capsicum annuum*.

Introduction

Root rot of pepper (*Capsicum annuum*) caused by *Phytophthora capsici* is a serious and economically important disease. To control this and other diseases, farmers have used preventive treatments with chemicals such as preplant treatment of soil with methyl bromide, metham sodium or curative treatments with metalaxyl and fosetyl aluminium. These chemicals can produce good disease suppression, but their secondary effects are not desirable. The use of antagonistic microorganisms to control plant pathogens is a strategy to control disease and reduce the risks related with the use of chemical fumigation. Among the microorganisms of potential use for the control of plant pathogens are the bacteria of the genus *Pseudomonas* (Burr *et al.* 1978, Howell and Stipanovic 1979, Weller and Cook 1983, Elad and Baker 1985, O'Sullivan and O'Gara 1992) and the *Bacillus* ssp (Broadbent *et al.* 1971, Merriman *et al.* 1974, Utkhede 1984, Kim *et al.* 1997, Sindhu *et al.* 2002). All these microorganisms have been isolated from the soil surrounding plants and from plant surfaces.

One of the methods used to introduce these microorganisms in the soil involves coating the seeds with antagonists, a method which can be economically competitive (Cook 1992, Timothy 1992). According to the first author, the presence of the antagonist on the seed during the first stage of germination facilitates contact between the antagonist and the emerging organs, establishing itself on the root before competitors arrive, thus excluding pathogens. This competitive advantage can be increased by sowing the treated seed in a sterile substrate in such a way that the seed germinates in the presence of the antagonist alone and is not exposed to competitors.

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Abbreviations: cfu - colony forming units; I - inoculated; NBY - nutritive both yeast; NI - non-inoculated; NT - non-treated; PDA - potato dextrose agar; PW - peptone water; T - treated; WA - water-agar.
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The object of this work was to isolate and identify antagonistic bacterial strains collected from pepper plants and their rhizosphere and, after treating the seeds and the aerial parts of plants with the isolated bacterial strains, to analyse their effect on *Phytophthora capsici* root rot and *Alternaria alternata* leaf spots.

Materials and methods

Microorganisms: *Phytophthora capsici* isolate 17 (Candela *et al.* 1995) and *Alternaria alternata,* isolated in our laboratory from diseased pepper plant, were cultivated in potato dextrose agar (PDA) (*Difco*, Detroit, USA) medium at 25 °C and maintained at 4 °C. The isolated bacteria were conserved in nutritive broth yeast (NBY) medium containing 0.8 g of nutritive broth (*Oxoid*, Hampshire, England), 15 cm^3 of glycerine, 0.2 g of yeast extract (*Difco*, Detroit, USA), 0.5 g of glucose and 100 cm^3 of water (pH 6.8) at -20 °C.

Plants: Pepper (*Capsicum annuum* L., cv. Yolo Wonder) seeds were disinfected with 2 % sodium hypochlorite for 20 min, rinsed with sterile distilled water and kept in sterile flasks until being used.

Isolation of antagonistic bacterial strains: The antagonistic bacterial strains were isolated from pepper plants taken from various agricultural zones of the Region of Murcia, SE Spain. Roots were shaken to remove excess soil and rinsed with 0.1 % (m/v) sterile peptone water (PW). The rinsing solutions was then serially diluted and plated on NBY-agar. The rinsed roots were cut in 1 cm lengths and then placed in NBY-agar Petri plates (*Difco*, Detroit, USA). Plates were incubated at 25 °C during 24 to 48 h.

Screening of bacterial strains with an antifungal activity: All the isolated and purified bacterial strains were confronted with the pathogens: *P. capsici* and *A. alternata* in plates containing different culture medium: PDA, V8c agar, Czapek agar (*Difco*, Detroit, USA), or water-agar (WA) at 2 % (m/v). The plates were incubated at 25 °C in darkness. From the third day, the plates were observed by optical microscope. The isolates with an inhibitory effect against the pathogens were conserved for subsequent evaluation of their biocontrol capacity on detached leaves and *in vivo*.

Preparation of pathogen inocula and bacterial suspensions: The *A. alternata* inoculum was prepared by flooding 7-d-old culture with 9 cm^3 of 0.1 % (m/v) PW and scarping gently with a sterilised glass rod. The suspension obtained was adjusted to 4×10^6 conidia cm^{-3} using a haemacytometer. The *P. capsici* inoculum was prepared according to the method described by Smith *et al.* (1990).

The bacterial strains suspensions were prepared from 48-h-old cultures. Plates containing the bacteria were flooded with 9 cm^3 of 3 % (m/v) sucrose solution or 0.1 % (m/v) PW and the colonies were gently scarped with a sterilised glass rod. The suspensions obtained were collected into sterilised glass tubes, and adjusted to the required concentrations by measurement of absorbance at 660 nm using a spectrophotometer (*Unicam UV2*, Cambridge UK).

Test of antibiosis on pepper detached leaves: Young pepper detached leaves were surface disinfected with hypochlorite sodium at 5 % during 1 - 2 min, washed twice with sterile distilled water, and the excess was removed. Finally the leaves were sprayed with a 0.1 % peptone suspension containing 8×10^6 colony forming units (cfu) cm^{-3} of bacterial strain until wet, and maintained at laboratory temperature. After 24 h, the leaves were inoculated with *A. alternata* at 4×10^6 spores cm^{-3} and then maintained in the same conditions. Leaves non-treated and inoculated (NTI) with *A. alternata*, leaves treated and not inoculated (TNI) and leaves treated with sterile peptone-water (NTNI) were used as three different controls. Five plates (2 - 3 leaves per plate) per treatment were used and the experiments were repeated three times.

Treatment of the pepper seeds with the isolated bacterial strains: The seeds were soaked in a 3 % saccharose suspension containing 10^8 - 10^9 cfu cm^{-3} of bacterial strains for 10 min. The treated seeds were sown in plug trays with sterile substrate (peat and sand (3:1, v/v) and grown in a *Fison*® (USA) culture chamber at 25 °C and 75 - 85 % relative humidity (RH) with a 16-h photoperiod, until the appearance of five true leaves, at which stage the plants were used.

Preparation of the substrate used for the plants infection: The pepper plants were transferred to pots filled with a mix of 100 g of above substrate and 12 g of vermiculite where the fungus *P. capsici* had grown (initial population 3.1×10^4 propagules g^{-1} of substrate) (Papavizas *et al.* 1981).

Evaluation of the effect of bacteria on *P. capsici* root rot and *A. alternata* leaf spot in controlled conditions: When the target disease was leaf spot caused by *A. alternata*, the aerial part of 50-d-old pepper plants were sprayed with the suspension of selected bacterial strains ($7 - 8 \times 10^6$ cell cm^{-3}) until all aerial part of plant running. The plants were then placed in a greenhouse at

15 - 25 °C and 85 - 95 % R.H. 24 h later, the leaves were inoculated in eight different points with drops (0.01 cm^3 per drop) of the *A. alternata* suspension (4.6 × 10^6 conidia cm^{-3}) and the plants were covered with transparent plastic bags. Controls were the same used in detached leaves. Five plants were used in each treatment and the treatments were repeated three times. After 4 d, the results were recorded using the same scale as described above.

When *P. capsici* was the target pathogen, the control plants used were *1*) grown from treated seeds planted in sterile substrate, *2*) grown from untreated seeds planted in substrate inoculated with *P. capsici*, and *3*) grown from untreated seeds planted in sterile substrate. The pots for all the treatments were placed in a greenhouse and each experiment was repeated three times. At the end of the experiments the plants were dried at 80 °C and their mass was recorded.

Identification of antagonistic bacteria: Identification of bacteria was carried out according to Sneath (1986) by observation of the bacterial colony's growth in the Petri plate, microscopic examination, Gram reaction, spore staining, mobility and by biochemical tests involving oxygen requirements, catalase and oxidase reactions, nitrate reduction, gelatine hydrolysis and indole production. The *API* system *20E* (*API*, Vercieu, France) were also used.

Statistical analysis: Effects of treatments on plant mass, and disease severity were analysed by one way *ANOVA* using *Statgraphics* plus software for Windows, *V.2.1* (*Statistical Graphics Corp.*, Maryland, USA) and the means were compared using Least Significant Difference (LSD) test at $P = 0.05$.

Results

Selection of antagonistic bacterial strains: From the fifty isolates obtained from the roots and aerial parts of pepper plants, only ten bacteria (denominated HS11, HS45, HS93, HS126, LS234, LS523, LS674, LS741, LS756 and LS766) showed an inhibition zone of vegetative growth of both pathogens *in vitro*. All of them were obtained from the root of the pepper plants and their rhizosphere. The inhibition intensity observed *in vitro* varied with culture media used, antagonistic isolate, pathogen and temperature of incubation. Microscopic examination of pathogen hyphae in the inhibition zone showed cell vacuolisation and hypertrophy. Except in the WA medium where the interactions were very weak, HS93 and LS674 inhibited the vegetative growth of the pathogens assayed. LS234 and LS523 inhibited the pathogens in V8c and Czapek but not in PDA. In the presence of these bacteria, *P. capsici* hyphae were morphologically deformed and the contents disorganized (Fig. 1*A,B*), while *A. alternata* hyphae showed spherical hypertrophic structures, that did not resemble chlamydospores, and whose contents after lysis were spilled into the culture medium (Fig. 1*C,D*).

Effect of detached leaf treatments on *A. alternata* leaf spots: Severity of leaf spot caused by *A. alternata* was highly inhibited on leaves treated by the selected bacteria. Inhibition varied significantly among the bacterial strains and between trials. The bacteria HS11, HS45, HS93, HS126, LS234, LS523, LS674, LS741, LS756 and LS766 reduced significantly leaf spots. High reduction was observed when leaves were treated with HS93, LS234, LS523 and LS674. The leaves non-treated and inoculated showed various degree of susceptibility to *A. alternata* (Table 1).

Table 1. Effect of bacterial leaf treatment on spot leaf caused by *A. alternata*. Results were recorded after 4 d of incubation by mean of the following scale: 0 = diameter of spot lower than 1 mm, 1 = 1 - 2 mm, 2 = 2 - 3 mm, 3 = 3 - 4 mm, 4 = 4 - 5 mm, 5 > 5 mm. Disease severity was calculated as the sum of the ratings for all the inoculated points and the effect of treatment was expressed as percentage reduction, R [%] = 100 - [(STI × 100)/SNTI] where STI is the mean of the ratings per leaf in treated and inoculated leaves and SNTI the mean of the ratings per leaf in non-treated and inoculated leaves. In all trials, some leaves non treated had become necrotic over the entire surface at the end of the sixth day. Values are the mean of ratings per leaf from 30 to 40 leaves. Means followed by the same letter are not significantly different according to LSD test at $P = 0.05$. NTI - leaves not treated.

Treatments	Disease severity [sum of ratins $leaf^{-1}$]
HS11	13.5 b
HS45	14.0 b
HS93	4.3 a
HS126	14.4 b
LS234	5.6 a
LS523	4.6 a
LS674	6.8 a
LS741	15.1 b
LS756	14.1 b
LS766	15.7 b
NTI	36.0 c

Effect of selected bacteria on *P. capsici* and *A. alternata* in controlled conditions: Among the isolates tested *in vivo* to control the pathogens, HS93, LS234, LS523, and LS674 significantly ($P = 0.05$) reduced *P. capsici* root rot (reduction by 80, 51, 49 and

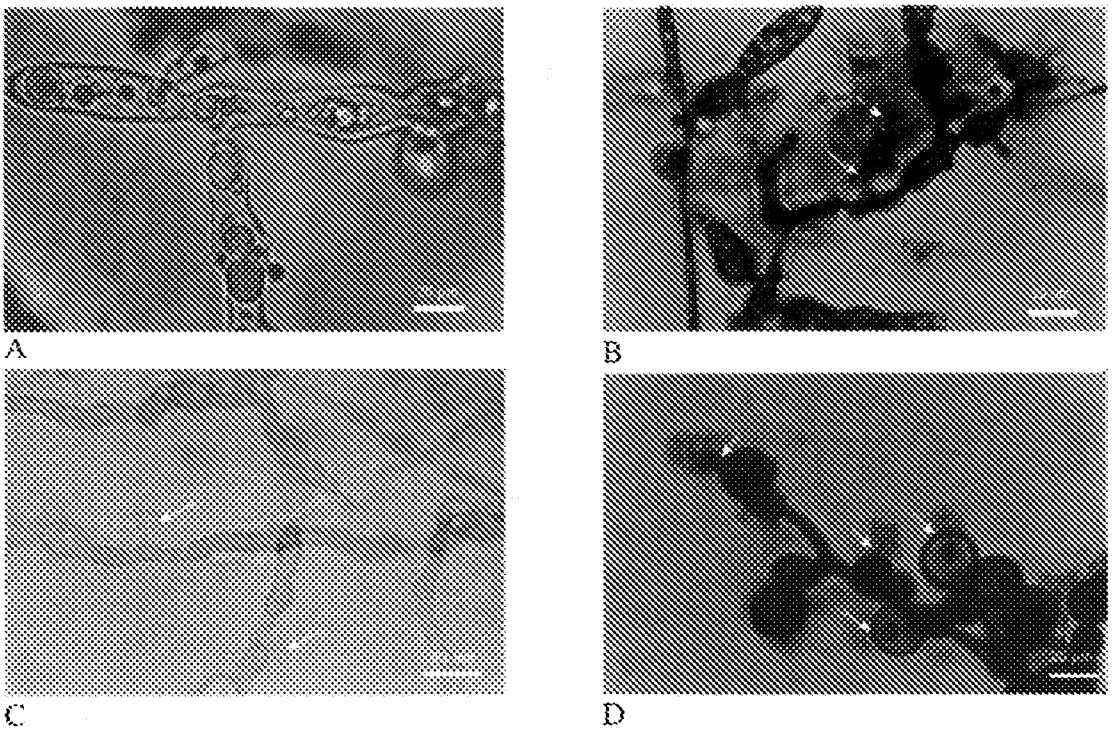

Fig. 1. Photomicrographs showing morphological changes in fungal hyphae in presence of HS93 on PDA medium. *A, B - P. capsici* hyphae vacuolization and wall lysis. *C, D - A. alternata* hyphae deformation, lysis and spilling of their content.

Table 2. Effect of bacterial seed and aerial parts treatment on disease severity and dry mass of pepper plants inoculated by *P. capsici* and *A. alternata* in controlled conditions. Treatments of the leaves: TNI - treated and non inoculated, NTI - non-treated and inoculated, NTNI - non-treated and non-inoculated. The severity of *P. capsici* root rot was evaluated at the end of the second month by applying the following scale: 0 = < 1 % of root rotted, 1 = 1 - 25 %, 2 = 26 - 50 %, 3 = 51 - 75 %, 4 = 76 - 90 %, 5 = >91 % or died plant. Values are the means of the disease severity recorded in three experiments with five replicates each. Means followed by the same letter are not significantly different according to Fisher's LSD test at $P = 0.05$.

Treatments	Plants inoculated with *P. capsici*		Plants inoculated with *A. alternata*		TNI
	root rot severity	plant dry mass [g]	spot leaf severity	plant dry mass [g]	plant dry mass [g]
HS11	4.0 ef	2.6 bc	37 a	2.7 a	4.2 a
HS45	4.5 f	2.4 ab	35 a	3.3 bc	4.0 a
HS93	0.7 a	4.7 h	18 c	4.0 de	4.2 a
HS126	4.0 ef	2.6 bc	36 a	2.6 a	4.1 a
LS234	1.9 b	3.5 f	10 d	4.2 e	4.2 a
LS523	1.9 b	2.9 de	15 cd	4.0 de	4.2 a
LS674	1.7 b	2.8 cd	19 c	3.8 d	4.1 a
LS741	4.5 f	3.0 g	34 a	3.0 b	4.0a
LS756	3.5 d	3.7 e	30 ab	2.9 ab	4.0 a
LS766	4.0 ef	2.2 a	38 a	2.5 a	4.1 a
NTI	3.8 d	2.6 bc	40 a	3.0 b	
NTNI					4.0 a

54 % compared with NTI plants) and *Alternaria* leaf spots (reduction by 54, 74, 62 and 53 %, respectively, compared with NTI plants) (Table 2).

Compared with the control, the plant dry mass was significantly (P = 0.05) improved in presence of the isolates tested, increasing by 21 to 46 %. However, the presence of both pathogen and isolate did not always produce this favourable effect. For example, while the combination HS93 + *P. capsici* stimulated an increase in plant mass, this was not the case with HS93 + *A. alternata.*

Identification of antagonistic isolates: Only the bacterial isolates with antagonistic effect on diseases

were identified. HS93 formed medium-sized whitish colonies that were smooth and opaque. In the same media, LS234, LS523 and LS674 formed medium-sized colonies in solid medium. These colonies were creamy white with undulating borders, a convex profile and a rough surface. Cells of all bacterial isolates were Gram positive and formed oval, regularly shaped spores that were sub terminal in LS674 but central in HS93. The API 20E tests showed that HS93 belongs to the genus *Bacillus* and probably the species *subtilis,* while LS234, LS523 and LS674 belong to the same genus and probably the species *licheniformis*.

Discussion

HS93, LS234, LS523 and LS674 reduced *P. capsici* root rot and *A. alternata* leaf spot *in vivo*. Plant biomass was significantly increased when seed were treated with the mentioned bacteria. These bacteria were isolated from repeatedly washed pepper root and its rhizosphere which suggest that these latter sites are probably natural niches for these bacteria. *In vitro* inhibition test for screening antagonists are not always adequate for recovering biocontrol agents. Analysis of results demonstrate that the inhibition of the vegetative growth of the pathogens observed *in vitro* was not necessarily a reliable indicator of their *in vivo* performance. Two of the strains (LS741 and LS756) showed an *in vitro* antagonistic effect against *P. capsici* and *A. alternata* although their introduction with the seeds in the greenhouse experiment did not reduce the pathogen produced diseases. On the other hand, *in vitro* antibiosis of HS93 was correlated with biocontrol of the two pathogens *in vivo*. Antibiosis activity observed in agar media might be affected by the pathogen, plant and surrounding medium. One of the possible reasons for the reduced effectiveness of a microorganism *in vivo* is its response to different stimuli in the medium into which it is introduced (Weller 1988). The antifungal properties exhibited by antagonists *in vitro* might be altered when they are introduced into the substrate where the plant is grown (Cook 1992).

The greatest reduction in the severity of the diseases was brought about by HS93, this effect being seemingly related with the inhibition in agar medium of the responsible fungi.

The biocontrol of the diseases observed might not only be the result of antibiosis but also other mechanisms such as parasitism, competition and induced resistance (Elad and Chet 1987, Sid-Ahmed *et al.* 1999). The actual mechanism responsible was not investigated in this study.

The increase in plant mass observed in the presence of antagonistic isolates was probably due to the production of growth factors by the strains used. According to Broadbent *et al.* (1971), Kerr (1972), and Lifshitz *et al.* (1987), increased plant biomass is the result of hormone production by plant growth-promoting rhizobacteria. However, such stimulation was not observed in the presence of all the pathogens studied. For example, the combination HS93 + *P. capsici* produced such stimulation but the combination of the same isolate with *A. alternata* did not have such an effect. This lack of stimulation may have been due to the fact that the diseases were only partially reduced.

It may be concluded, then, that bacterial strains like HS93 with its broad antifungal spectrum against two different pathogens could be useful for control of the pathogens of pepper used and possibly others in commercial greenhouses.

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