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Short communication

Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants

A. Sid Ahmed, M. Ezziyyani, C. Pérez Sánchez and M.E. Candela*
*Department of Plant Biology, Faculty of Biology, University of Murcia, Campus de Espinardo, 30100, Espinardo, Murcia, Spain; *Author for correspondence (Fax: 96 83 63963; E-mail: mcandela@um.es)*

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Abstract

Two bacterial isolates and one strain of *Trichoderma harzianum* were tested alone and in combination with chitin for efficacy in control of root rot disease caused by *Phytophthora capsici* and *Rhizoctonia solani* in pepper plants under greenhouse conditions. These bacteria (*Bacillus subtilis* HS93 and *B. licheniformis* LS674) were isolated from repeatedly washed roots of pepper plants. In *in vitro* assays, HS93, LS674 and *T. harzianum* were antagonistic against *P. capsici* and *R. solani* and produced high levels of chitinase. Seed treatment and root drenching with bacterial suspensions of HS93 with 0.5% chitin was more effective against *Phytophthora* and *Rhizoctonia* root rot than addition of the organisms without chitin. LS674 and *T. harzianum* reduced *Rhizoctonia* but not *Phytophthora* root rot. In two greenhouse tests, seed treatment and root drenching with HS93 amended with chitin enhanced its biocontrol activity against *P. capsici* but not on *R. solani.* The effects of LS674 and *T. harzianum* against *R. solani* were significantly enhanced when they were used as suspensions with 0.5% chitin for root drenching, but this had no effect on *P. capsici*. In both greenhouse experiments, the use of 0.5% chitin alone for root drenching reduced *Rhizoctonia* root rot. Reduction of root rot disease was accompanied by increased yield. These results show that the antagonistic activity of HS93, LS674 and *T. harzianum* may be stimulated by chitin resulting in significant improvements in their effectiveness against pathogens.

The biological control activity of several antagonistic microorganisms has been correlated with chitinase production (Zhang et al., 2000; Zhang and Yuen, 2000). For this reason, chitin was used to activate the chitinolytic microorganisms able to affect the growth of chitinous fungi (De Boer et al., 1999). Chitin amendment of soil may have effects in the rhizosphere, such as the stimulation of growth of chitinolytic microorganism (De Boer et al., 1999; Manjula and Podile, 2001), their increased biocontrol activity and elicitation of plant defense proteins (Roby et al., 1987). All these effects may culminate in enhancing plant protection.

The objective was to isolate and select antagonistic bacteria from pepper plants, analyze chitinase activity of selected bacteria and a strain of *Trichoderma harzianum* and, to evaluate the effect of these organisms, alone and in combination with chitin, on *Phytophthora capsici* and *Rhizoctonia solani* causal agents of root rot of pepper. Pepper plants collected from the agricultural field were used for isolation of antagonistic microorganisms. Roots were washed under running sterile-distilled water and the rinsed soil was serially diluted and plated on nutrient agar (NA) medium (Difco). The dishes were incubated in darkness for 36 h at 25 °C. The washed roots were excised into 1-cm long sections, washed twice in sterile distilled water and placed aseptically on plates with NA medium and incubated at 25 °C in darkness for 36 h. All the bacterial isolates and one strain of *T. harzianum* (Sid Ahmed et al., 1999) were tested *in vitro* against *P. capsici* and *R. solani*. All antagonistic

microorganisms were subjected to chitinase analysis by (Roberts and Selitrennikoff, 1988).

Of all the bacterial strains isolated, ninety bacterial isolates produced a zone of inhibition around at least one of the pathogens, while ten inhibited both pathogens. Microscopic examination of the morphological changes in the hyphae of the inhibition zone showed cell vacuolization and hypertrophy of *P. capsici* and *Rhizoctonia*.

The actual mechanisms of pathogen inhibition were not investigated in the present study. However, in other studies, the mechanisms proposed for *Bacillus subtilis* and other biocontrol agents (Raupach and Kloepper, 1998) of plant diseases have included competition for space and nutrients, the production of antifungal metabolites and the induction of systemic resistance in plants. The inhibition of vegetative growth, the alteration of cell organization and the lysis of the hyphae observed in agar media may be caused by the diffusion of antifungal metabolites. Analysis of chitinase activity revealed that only HS93, LS234, LS523, LS674 and *T. harzianum* are chitinase active (Table 1). However, the *in vitro* antagonistic effect of these strains bore no relation to their *in vivo* effect.

To evaluate the effect of selected isolates evaluated for chitinase activity on *Phytophthora* or *Rhizoctonia* root rot *in vivo*, plants with five true leaves, grown from seeds treated (dipped for 10 min in bacterial suspensions containing 10^7–10^9 cells ml^{-1}) with the selected strains, were transplanted to plastic pots containing a sterile mixture of peat and sand (3 : 1, v/v) infested with inocula of *P. capsici*, (Sid Ahmed et al., 1999) or *R. solani* prepared in Petri dishes containing a steril mixture of peat and 1% wheat bran. The roots of these plants were drenched with 10 ml of bacterial suspension alone or containing 0.5% of chitin (Sigma). The pots of each treatment in each experiment were transferred to a growth chamber with a 16 h photoperiod at 25 °C, arranged in a randomized complete block design and watered with tap water every three days. All pots received Planta Vite (2 ml l^{-1}) nutrient solution (Reckitt and Colman. S.A., Bilbao, Spain) every two weeks. Each treatment had five replicate pots. The treatments were repeated three times.

Among the selected isolates evaluated for chitinase activity only HS93, LS674 and *T. harzianum* were effective in inhibiting disease (Table 1). Seed treatments and root drenching with strains HS93, LS674 and *T. harzianum* without chitin reduced *Phytophthora* root rot by 55%, 50% and 44%, respectively, and *Rhizoctonia* root rot by 44%, 55% and 38%. These strains, when combined with 0.5% chitin, reduced *Phytophthora* root rot by 70%, 64% and 52%, respectively, and *Rhizoctonia* root rot by 50%, 62% and 56% (Table 1).

Table 1. Effect of bacterial seed treatments and root drenching on disease severity of pepper plants caused by the two pathogens *in vivo*

Treatments	Chitinase activity[a] (U/100 ml)	Disease severity[b]	
		P. capsici	*R. solani*
Pathogen alone		4.5 e	4.5 c
Pathogen alone + chitin		4.25 e	4.0 bc
HS93	3.9 gh	2.0 b	2.5 ab
HS93 + chitin	11.9 d	1.25 a	2.0 a
LS234	4.0 g	3.6 d	3.6 bc
LS234 + chitin	8.0 f	4.8 ef	3.6 bc
LS523	2.4 h	3.65 d	3.65 bc
LS523 + chitin	9.7 e	3.4 d	3.7 b
LS674	4.6 g	2.25 b	2.0 a
LS674 + chitin	18.0 c	1.5 a	1.5 a
T. harzianum	27.6 b	2.5 bc	2.75 ab
T. harzianum + chitin	49.5 a	2.0 b	1.75 a

[a]Chitinase activity was evaluated by colorimetric determination of *p*-nitrophenol released from *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose. Values representing activity are the means from five independent replicates. Means followed by the same letter are not statistically different ($P = 0.05$) according to LSD test. The culture media used for chitinase production was PDB with or without chitin.

[b]Severity of *Phytophthora* and *Rhizoctonia* root rot was evaluated using a scale 0 (no symptoms) to 5 (100% of root rotted or dead plant). Values representing disease severity are the means per plant and treatment from three experiments (data from all three experiments were pooled, because the experiment × treatment interaction was not significant). Means in each column followed by the same letter are not significantly different at $P = 0.05$ according to LSD test.

Bacterial strains HS93 and LS674 were identified according to Bergey's Manual of Determinative Bacteriology (Sneath, 1986). In agar medium, HS93 formed medium-sized whitish colonies that were smooth and opaque. In the same media, LS674 formed medium-sized colonies. These colonies were creamy white with undulating borders, a convex profile and a rough surface. Cells of both strains were Gram positive and formed oval, regularly shaped spores that were subterminal in LS674 but central in HS93. The API 20E tests showed that the two strains belong to the genus *Bacillus*. Analysis by the Spanish Type Culture

Collection indicated that the LS674 strain was probably *B. licheniformis* and HS93 a strain of *B. subtilis*.

To evaluate the effect of these strains on *Phytophthora* root rot in greenhouses, plants with five true leaves (which had been grown from seeds treated with the bacterial strains) were transplanted to the greenhouse soil artificially infected with inoculum (Sid Ahmed et al., 1999). When *Rhizoctonia* root rot was the target disease, plants with five true leaves grown from treated or nontreated seeds were transplanted to the greenhouse soil naturally infected by the pathogen.

Twenty days after transplanting, half of the plants growing from treated or nontreated seed were root drenched with 10 ml of the suspension of the corresponding bacteria or *T. harzianum* containing 0.5% of chitin. For each pathogen biocontrol experiment, treatments consisted of plants spaced at 40 cm intervals in rows 100 cm apart. All treatments were arranged in a randomized complete block design with 55–64 plants per treatment and each treatment was replicated three times. Drip irrigation was used ($4\,l\,h^{-1}$) and fertilizer was injected every three weeks from a fertilizer tank into the irrigation system.

Of the isolates that were inhibitory *in vitro* and *in vivo*, only HS93, LS674 and *T. harzianum* suppressed diseases in greenhouse assays. HS93 significantly reduced *Phytophthora* root rot but failed to control *Rhizoctonia* root rot. The other isolates, LS674 and *T. harzianum,* reduced disease severity caused by the latter pathogen but failed to control *Phytophthora* root rot.

In the first year, without chitin, *B. subtilis* HS93 significantly ($P = 0.05$) reduced *Phytophthora* root rot by 22% compared with the control but failed to control *Rhizoctonia* root rot (Table 2). Strains *B. licheniformis* LS674 and *T. harzianum* significantly ($P = 0.05$) reduced the severity of *Rhizoctonia* root rot by 43% and 39%, respectively, but fail to control *Phytophthora* root rot (Table 2). The addition of 0.5% chitin to the microbial suspension increased the effect of HS93 on *Phytophthora* root rot, which was reduced by 41% compared with plants inoculated with chitin alone but not on *Rhizoctonia.* Likewise, the effect of LS674 and *T. harzianum* on *Rhizoctonia* root rot was increased (reduced by 69% and 61%, respectively) when chitin

Table 2. Effect of treatments with *Bacillus* spp. and *T. harzianum* strains on diseases and yield of pepper plants grown in the greenhouses

Year	Treatment	*P. capsici* root rot		*Rhizoctonia* root rot	
		Severity[a]	Yield[b] (kg)	Severity[a]	Yield[b] (kg)
2000	Not treated	5.0 c	ND	2.3 e	ND
	HS93	3.9 b		1.6 de	
	LS674	4.8 c		1.3 bcd	
	T. harzianum	4.7 c		1.4 cd	
	Not treated + chitin[c]	5.0 c		1.3 bcd	
	HS93 + chitin	2.95 a		0.7 abc	
	LS674 + chitin	5.00 c		0.4 a	
	T. harzianum + chitin	4.8 c		0.5 ab	
2001	Not treated	4.8 c	0.19 c	1.95 e	0.72 d
	HS93	3.95 b	0.90 b	1.7 de	0.75 d
	LS674	4.65 c	0.24 c	1.15 cd	0.90 c
	T. harzianum	4.50 c	0.31 c	1.1 bcd	1.01 b
	Not treated + chitin	4.75 c	0.21 c	1.25 cd	0.85 c
	HS93 + chitin	2.50 a	1.20 a	0.6 abc	0.92 c
	LS674 + chitin	4.6 c	0.12 c	0.45 ab	1.02 b
	T. harzianum + chitin	4.4 c	0.34 c	0.4 a	1.13 a

[a]The values representing disease severity are the means of 80–120 plants (data from three replicate treatments were pooled and analyzed). Disease severity was estimated on a scale of 0 (healthy plant) to 5 (dead plant), as indicated in the text. The means followed by the same letter in each column are not significantly different according to LSD test at $P = 0.05$.

[b]Yields are the means per plant and treatment from 50 to 70 plants from the middle of each row treatment.

[c]Chitin was added at a rate of 0.5% to the microbial suspensions used in root drenching.

was added although no significant effect was observed on *P. capsici* (Table 2).

In the second year, treatment with HS93 alone significantly reduced (17%) *Phytophthora* root rot compared with nontreated plants. LS674 and *T. harzianum* reduced *Rhizoctonia* by 41% and 43%, respectively, compared with the nontreated plants. When the above treatments included 0.5% of chitin the effect of HS93 on *Phytophthora* root rot was enhanced (47% reduction compared with plant control inoculated with chitin alone) but not its effect on *Rhizoctonia* (Table 2). Addition of chitin increased the effect of LS674 and *T. harzianum* on *R. solani* which was reduced by 64% and 68%, respectively, compared with plants inoculated with chitin alone. In the two years of the experiment, the addition of chitin alone increased plant protection against *R. solani* (reduced by 43%, in the first year and 35% in the second year, as compared with plants not amended with chitin). In both years, plant mortality caused by *Phytophthora* and *Rhizoctonia* root rot was significantly slower in plants treated with HS93, LS674 and *T. harzianum* compared with nontreated plants (data not shown).

The yield obtained from the nontreated plants inoculated with *P. capsici* was very low because most of the plants died (Table 2). Except in the case of HS93-treated plants, the yield of the plants treated with the isolates assayed was not significantly different from that obtained from nontreated plants. The addition of chitin to the HS93 suspension increased the yield over that obtained from plants treated with HS93 alone (Table 2). The yield obtained from plants treated with LS674 and *T. harzianum* in *R. solani* infested soil was significantly ($P = 0.05$) greater than that obtained from the nontreated plants (Table 2). Addition of 0.5% of chitin increased the yield obtained from plants treated with *T. harzianum*, HS93 and LS674 compared with the yield obtained without chitin.

The ability of these strains to inhibit the pathogens is noteworthy since the suppression obtained is a result of a single seed and root drenching. The addition of chitin increased the effect of HS93 on *P. capsici* and the effect of LS674 and *T. harzianum* on *R. solani*. These results suggest that chitin stimulated the inhibitory activity of HS93, LS674 and *T. harzianum*. Amendment of soil with chitin might increase plant protection by favoring the fast growth of chitinolytic microorganisms and therefore stimulate the production of related metabolites which may help antagonistic activity. Root drenching with chitin alone reduced *Rhizoctonia* disease severity by 43%. This result suggests that chitin might stimulate indigenous chitinolytic microbiota and/or the plants which also help in plant protection. A similar result was observed by De Boer et al. (1999) when dune soil was amended with chitin.

T. harzianum and bacteria produced a high chitinase activity in culture media amended with chitin. This substrate, in addition to its possible effect on stimulating microbial growth and inhibition activity, may stimulate the defense capacity of the plants. Products of chitin hydrolysis elicited plant defense proteins such as chitinase (Roby et al., 1987). These defense proteins were also elicited when *T. harzianum* (Sid Ahmed et al., 2000; Yedidia et al., 1999) and *Bacillus* spp. (Sid Ahmed et al., unpublished data) were used as seed and root treatment of pepper and cucumber plants. Chitinase may be involved in cell wall degradation of some fungi that contain chitin. However, the cell wall of *P. capsici* does not contain chitin, which suggests that the enhanced effect observed when HS93 was used in combination with chitin is possibly the result of other mechanisms. Other studies are needed to elucidate the mechanism responsible for the biocontrol observed in this study.

In the second year, the failure of the treatment with LS674 and *T. harzianum* in controlling *Phytophthora* root rot was accompanied by non-significant increases in fruit yields. Simultaneously, the reduction of *Phytophthora* root rot by treatment with HS93 was related to significant increases in yields. This behavior suggests that the increases in fruit yield may be a result of a reduction in disease severity.

The results obtained indicate that the addition of chitin together with strains like HS93, LS674 or *T. harzianum* may stimulate their antagonistic effect against the pathogens studied and help pepper plant protection.

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