Journal of Animal & Plant Sciences, 2008. Vol. 1, Issue (1): 9 - 20.
Publication date: 7 November 2008; http://www.biosciences.elewa.org/JAPS; **ISSN 2071 - 7024**

Comparison of physiological changes induced by three rhizobacteria that lead to systemic resistance against *Fusarium* wilt in tomato

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Key words
Biocontrol, *Fusarium oxysporum*, Induced systemic resistance, phenols, PR-proteins

SUMMARY
The rhizobacteria isolates *Pseudomonas fluorescens* T58, *Pseudomonas putida* 53 and *Bacillus sphaericus* B43, selected after greenhouse tests for biological control of *Fusarium* wilt in tomato, were all able to induce systemic resistance (ISR) against *Fusarium oxysporum* f.sp. *lycopersici*. The effects of bacteria on accumulation of phenolic compounds, pathogenesis-related proteins and structural changes in cells showed that each bacteria affected plants differently. Phenol accumulation patterns were not affected by bacteria treatments, but a phenolic compound that was accumulated after *Fusarium*-infection was not detected in plants treated with *P. fluorescens* T58 or *B. sphaericus* B43. Peroxidase activity in the stem was stimulated by all three bacteria. The increase due to *B. sphaericus* B43 occurred transiently three days after bacteria treatment, while peroxidase activities in plants treated with *P. fluorescens* T58 or *P. putida* 53 increased six days after bacteria treatment. Chitinase or ß-1, 3-glucanase are not associated with ISR against *F. oxysporum*. Cell wall modifications were observed after all bacteria treatments and subsequent *Fusarium*-infection. *P. fluorescens* T58 induced deposition of callose on cell walls, while isolates *P. putida* 53 and *B. sphaericus* B43 induced extrusion of occluding material into the lumina of xylem vessels and thereby inhibited *Fusarium*.

1 INTRODUCTION

Systemic induction of resistance after infection through a necrotising pathogen was initially described as an unspecific resistance to a pathogen that is not related to the inducer and was termed "systemic acquired resistance" (SAR; Ross, 1961). Similar reactions have been shown for fungal, bacterial and viral pathogens after infection of plants with weakly aggressive, avirulent or incompatible strains of the disease-causing organism (Kuc, 2001). Non- pathogenic rhizobacteria also induce systemic resistance against virus, bacteria, fungi, insects, or nematodes in different host plants (van Peer *et al*., 1991; Maurhofer *et al*., 1994; Hasky-Günther *et al*., 1998; van Loon *et al*., 1998).

Rhizobacteria mediated induced systemic resistance (ISR) as well as SAR is associated with host metabolic changes, culminating in a number of physical and biochemical responses that limit pathogen penetration and development in host tissues (Buchenauer, 1998). Upon resistance induction, plants respond by

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accumulating soluble pathogenesis-related (PR) proteins (Tuzun *et al*., 1989). Key PR-proteins include the enzymes chitinase, ß-1,3-glucanase, and peroxidase (van Loon & van Strien, 1999). Chitinases and ß-1,3-glucanases have attracted considerable interest because of their proven inhibitory activity against fungi *in vitro* (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988). These lytic enzymes degrade fungal wall components and inhibit growth. Peroxidases are involved in phenol oxidation, lignin biosynthesis, and in the cross-linking of phenols, lignin and suberin (Bradley *et al*., 1992; Scott *et al*., 1995; Vidhyasekaran *et al*., 2001) and are used as an indicator of defence related reactions in plants under infection (Heerschap & Bakker 1999). SAR and rhizobacteria-induced systemic resistance differ in the involvement of salicylic acid and induction of PR-proteins (Pieterse *et al*., 1996; Pieterse & van Loon, 1999).

Limitation of pathogen penetration can occur directly by the action of antimicrobial substances such as phytoalexins and phenolic compounds (Benhamou *et al*., 1996a). In tomato the main phytoalexin is the alkaloid tomatine (Fontaine *et al*., 1948), but rishitin and several other compounds also occur (Tjamos & Smith, 1974). Phenolic compounds have been associated with resistance to *Fusarium* and *Verticillium* in carnation (van Peer *et al*., 1991; Baayen *et al*., 1996) and tomato (Elgersma & Liem, 1989). Phenolic compounds are infiltrated at sites of potential penetration where they can be directly toxic to pathogens and also contribute to the elaboration of physical barriers restricting pathogen spread (Benhamou *et al*., 1996a).

Plants also respond to vascular pathogens by rapidly mobilising perivascular barriers to halt fungal ingress (Bishop & Cooper 1984; M'Piga *et al*., 1997). Resistant tomato plants have been observed to block *Fusarium* and *Verticillium* infection by depositing callose, tylose occlusion and lignification (Bishop & Cooper 1984; Beckman *et al*., 1989). Fortified plant cell walls are more resistant to degradation and penetration by pathogens, and can also reduce the diffusion of nutrients from the cells to pathogens and the diffusion of toxins from the pathogen to the plant cells (Kuc, 2001).

In a previous study (Hauschild *et al*., 2000), seven bacteria isolates were evaluated for biocontrol of tomato *Fusarium* wilt in the greenhouse and *Pseudomonas fluorescens* T58, *P. putida* 53, and *Bacillus sphaericus* B43 were selected. The main mechanism of action of the effective isolates, was determined to be induction of systemic resistance (Mwangi, 2003). *Bacillus sphaericus* B43, in addition, was able to induce systemic resistance against the cyst nematode *Globodera pallida* in potato (Hasky-Günther *et al*., 1998). The objective of the study reported in this paper was to characterise changes in plant physiology related to systemic resistance (ISR) induced against *Fusarium* in tomato by the effective rhizobacteria. The aspects investigated were: (1) changes in phenolic compounds, (2) changes in lignin content in roots, (3) changes in the activity of PR-proteins, and (4) histological changes in cells that may affect colonisation of plants by *Fusarium*.

2 MATERIALS AND METHODS

2.1 Plant material: Seedlings of tomato cv. Rheinlands Ruhm were sown in a low-nutrient seedling substrate (Klasmann-Deilmann, Groß-Hesepe, Germany) and maintained constantly moist in the greenhouse at 23 $\pm$ 5°C. Seedlings were transplanted when they had two fully differentiated leaves into an organic growth substrate or a sand:soil mixture (1:1) as indicated below.

2.2 Bacteria isolates: Names and origin of the bacteria isolates evaluated are shown in Table 1. Cultures were grown in 100 ml Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) in 300 ml flasks for four days at 23 $\pm$ 2°C while shaking at 100 rpm in the dark. Bacterial cells were centrifuged at 5000 x *g* for 20 min at 6°C and re-suspended in 0.1 M $MgSO_4$. Bacterial suspensions were adjusted to OD_{560} = 2, corresponding to approximately 5 x 10^7 -

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2 x 10^8 cfu ml^{-1}, depending on the bacteria strain. Bacteria were infiltrated into the rhizosphere by drenching the soil surface with 5 ml of the cell suspension. The isolates *P. fluorescens* T58, *P. putida* 53 and *B. sphaericus* B43 were included in all experiments whereas three additional isolates were included for comparison in the phenols evaluation experiment.

Table 1. Bacteria isolates evaluated for biocontrol effect against *Fusarium oxysporum* f.sp *lycopersici*.

Isolate	Source	Effective against	Reference
Bacillus megaterium 4	Tomato	*F. oxysporum* f.sp. *lycopersici*	Terhardt, 1998
Bacillus sphaericus B43	Potato	*Globodera pallida*	Racke and Sikora, 1992
Bacillus thuringiensis 2	Tomato	*Meloidogyne incognita*	Terhardt, 1998
Paenibacillus macerans 60	Tomato	*F. oxysporum* f.sp. *lycopersici*	Terhardt, 1998
Pseudomonas fluorescens T58	Sugar beet	*Heterodera schachtii*	Oostendorp and Sikora, 1989
Pseudomonas putida 53	Tomato	*F. oxysporum* f.sp. *lycopersici*	Terhardt, 1998

2.3 *Fusarium* isolate: The isolate used was *Fusarium oxysporum* f.sp. *lycopersici* race 1 from the German Collection of Micro-organisms and Cell Cultures (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Göttingen, No. 62059). A spore suspension with a concentration of 10^6 conidia ml^{-1} was prepared by the method of De Cal *et al.* (1997) using TSB as the culture medium. *Fusarium* spores were infiltrated into the rhizosphere by drenching the soil surface with 5 ml of the spore suspension adjusted to attain an average of 2 x 10^3 conidia g^{-1} soil.

2.4 Phenolic compounds: Seedlings were transplanted into Klasmann growth substrate in 0.5 l pots and kept in the greenhouse at 23 ± 5°C. The six bacteria isolates shown in Table 1 were applied 3 weeks after transplanting and *Fusarium* was added one week later. The total phenol content was determined by the Price & Butler method as modified by Waterman & Mole (1994). Qualitative changes in phenols were evaluated by Thin Layer Chromatography (van Peer *et al.*, 1991; Wagner & Bladt, 1997). Plant stems were sampled 2 and 7 days after inoculation with *Fusarium*. Shoots were cut off at the soil level and after detaching the leaves, the stems were washed in running tap water, then frozen immediately in liquid nitrogen, and stored at -20°C until extraction. To extract phenolic compounds, 5 g of material were chopped while frozen in 20 ml acetone and homogenised for 1 min at 19,000 rpm using an Ultra Turrax T25 basic macerator. The homogenised material was centrifuged at 5000 x *g* at 20°C for 20 min to separate the plant debris from the filtrate. The filtrate was evaporated to constant volume under vacuum at 45°C using a Rotavapor RE 111 (Büchi, Switzerland). The remaining aqueous suspension was further extracted with 5 ml toluene. The toluene fraction was evaporated until dry under vacuum at 65°C and the extracts re-dissolved in 1 ml toluene. 50 µl volume of the extracts was separated on Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) using toluene:ethylacetate (93:7) as the solvent. Plates were examined under UV light at 254 and 366 nm. Bands corresponding to phenolic compounds could be detected under 254 nm as dark UV absorbing spots. The R_f value of detected compounds was calculated as R_f = Distance moved by compound / Distance moved by mobile phase.

2.5 Lignin content in the roots: Seedlings were transplanted into sand:soil mixture in 0.5 l pots and kept in the greenhouse at 23 ± 5°C. *P. fluorescens* T58, *P. putida* 53 and *B. sphaericus* B43 were applied two weeks after transplanting. *Fusarium* was added one week after bacteria application. One week later, lignin in the roots was quantified using the acetylbromide photometric method described by Johnson *et al.* (1961). Zero to 10 mg synthetic lignin (Aldrich, Irvine, UK) treated exactly as the plant material was used to generate a standard curve for comparison.

2.6 Pathogenesis related proteins: Seedlings were transplanted into a sand:soil mixture in 0.25 l pots and kept in a growth chamber at 23 ± 2°C and a 12/12 h light/dark cycle. *P. fluorescens* T58, *P. putida* 53 and *B. sphaericus* B43 were applied three weeks after transplanting. *Fusarium* was added 3 days thereafter. At 0, 3, 6 and 10 days after bacteria treatment three plants per treatment were sampled for protein extraction. After carefully washing off the soil, the 5th leaf (counting from below) was detached first, then the stem was separated from the

roots. The plant material was frozen immediately in liquid nitrogen and stored at –20°C until protein extraction. Proteins were extracted and their activity quantified as described by Reitz *et al.* (2001).

2.7 Histological changes in cells: Plants were transplanted into Klasmann organic substrate in 0.5 litre pots and kept in the greenhouse at 23 ± 5°C. *P. fluorescens* T58, *P. putida* 53 and *B. sphaericus* B43 were applied one week after transplanting and *Fusarium* was added one week thereafter. Four plants were sampled from each treatment at 7, 10, 14 and 21 days after *Fusarium* inoculation. Hand sections were made both longitudinally and in transverse from the part of the stem 1 cm above the soil line. The KOH-aniline blue fluorescence technique described by Hood & Shew (1996) was used to investigate the extent of colonisation and histological changes in plant tissues.

2.8 Data analysis: All experiments were repeated at least once and treatments were arranged in a completely randomised design. Data were analysed by One Way ANOVA using Statgraphics Plus 3.1 software (Manugistics Inc., Rockville, USA) and means separated using Fisher's LSD procedure at $P \leq 0.05$.

3 RESULTS

Treatment of tomato plants with the resistance-inducing rhizobacteria *P. fluorescens* T58, *P. putida* 53, and *B. sphaericus* B43 led to changes in phenol composition, enzyme activities and structural modifications in vascular elements of treated tomato plants.

3.1 Quantitative changes in phenols: Plants infected with *Fusarium* alone had increased total phenol content, but not always statistically significant, when compared to the uninfected control plants (Figure 1). Plants treated with bacteria did not have significant changes in amounts of phenols when compared to the untreated control plants, even after adding *Fusarium*.

3.2 Qualitative changes in phenols: Extracts from plants infected with *Fusarium* alone without prior bacteria treatment contained a phenolic compound migrating with an *Rf* value of 0.65, which was not detected in the extracts from untreated, non-infested plants (Figure 2a & 2b). A comparison with extracts from *Fusarium* grown in TSB culture did not yield a similar band. The compound with an *Rf* value of 0.65 was also found in the extracts of plants treated with *P. putida* 53, *P. macerans* 60, *B. megaterium* 4 and *B. thuringiensis* 2 before *Fusarium* infection (Figure 2a). The 'new' phenol was not detected when plants were treated with *P. fluorescens* T58 and *B. sphaericus* B43 before *Fusarium* infection. Treatment with bacteria alone (without *Fusarium* infection) did not lead to production of the 'new' phenolic compound (Figure 2b).

3.3 Lignin content in the roots: Lignin contents were not significantly altered between untreated plants infected or not with *Fusarium*. Plants treated with *P. fluorescens* T58 and *B. sphaericus* B43 had insignificantly higher amounts of lignin when compared to the untreated control plants (data not shown), even after adding *Fusarium* to the treated plants.

3.4 Peroxidase activity: In roots, peroxidase activity was almost not detectable and did not change over time regardless of treatment. In the leaves there was a transient increase in peroxidase activity in all bacteria treatments which was detectable at three days after treatment. This increase was followed by a decrease in activity below activities of untreated plants at six and ten days after treatment, except in plants treated with *P. fluorescens* T58 where activity remained at the same level as in the untreated plants (data not shown).

In the stem, plants treated with *B. sphaericus* B43 had a short-lived increase in peroxidase activity three days after treatment with or without *Fusarium*. After six days, activity in the stems of plants infected with *Fusarium* alone was non-significantly higher than in untreated plants. At the same time, plants treated with *P. fluorescens* T58 or *P. putida* 53 plants had significantly higher peroxidase activity than the untreated, non-infested plants (Figure 3). *Fusarium* infection in *P. putida* 53-treated plants led to insignificant increase in peroxidase activities while for *P. fluorescens* T58 peroxidase activities were significantly reduced after *Fusarium* infection.

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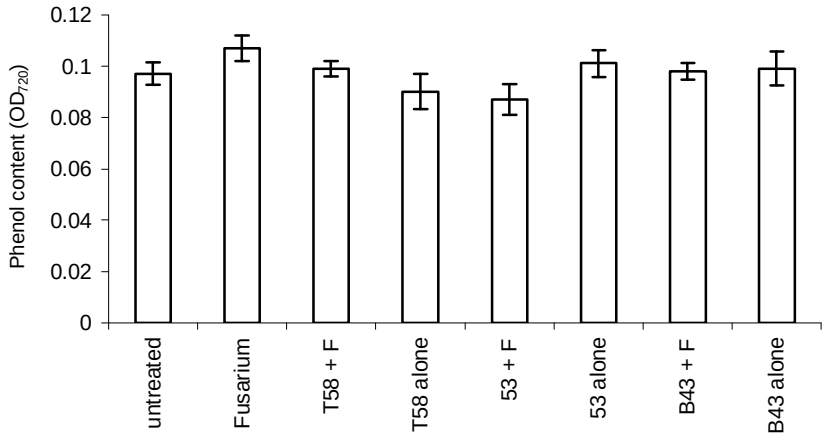

Figure 1: Relative content of total phenols in extracts from tomato plants treated with *Pseudomonas fluorescens* T58, *Pseudomonas putida* 53 or *Bacillus sphaericus* B43 alone or in combination with *Fusarium oxysporum* f.sp. *lycopersici*. +F means with *F. oxysporum*. Bars show standard deviation. Data are the average of three experiments (each n = 4).

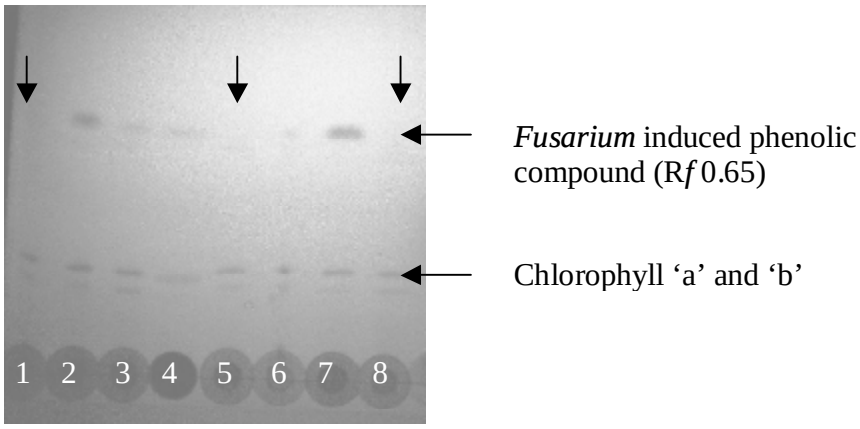

Figure 2a: Thin Layer Chromatography Separation of phenolic compounds from extracts of plants treated with rhizobacteria and subsequently infected with *Fusarium oxysporum*. Extracts were from plants with following treatments: **(1)** *Pseudomonas fluorescens* T58, **(2)** *Pseudomonas putida* 53, **(3)** *Paenibacillus macerans* 60, **(4)** *Bacillus megaterium* 4, **(5)** *Bacillus sphaericus* B43, **(6)** *Bacillus thuringiensis* 2, **(7)** no bacteria treatment with *F. oxysporum* alone **(8)** no bacteria treatment and no *F. oxysporum* infection. The *Fusarium*-induced phenolic compound is missing on lanes 1, 5 and 8 (arrows).

3.5 Chitinase and ß-1, 3 -Glucanase activity: Following bacteria treatment, chitinase activity changed significantly only in the leaves. Infecting plants with *Fusarium* alone did not cause a significant change in chitinase activity when compared to the untreated control plants (Figure 4). An increase in chitinase activity in the plants treated with *P. putida* 53 was observed six days after treatment but this increase was lower in the presence of *Fusarium*. *P. fluorescens* T58 had no measurable effect on chitinase while *B. sphaericus* B43 caused a non-significant change in chitinase activity (data not shown). No significant changes were observed in ß-1, 3-Glucanase activity over time regardless of treatment or plant organ examined (data not shown).

3.6 Histological changes in cells

***Fusarium* alone**: Plants treated only with *Fusarium* started reacting to infection visibly after 10 days. The plant tissue sections showed dense non-fluorescent matter on the periphery and dispersing loosely towards the lumen of xylem vessels (Figure

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6a & 6b). Vessel collapse (Figure 5b) and vascular browning which was evident by 14 days intensified 21 days after *Fusarium* application.

P. fluorescens* T58 + *Fusarium: In the plants treated with *P. fluorescens* T58 deposits on some cell walls were observed 10 days after adding *Fusarium* (Figure 5c & 5d). The densely deposited material had a smooth texture and appeared to push and to indent the walls. Plants treated with *P. fluorescens* T58 and *Fusarium* had far much less mycelia and material extruded into xylem lumina after 21 days. At this time only a few xylem vessels had collapsed when compared to the plants treated only with *Fusarium*.

P. putida* 53 + *Fusarium: Loosely dispersed material started appearing in the lumen of xylem vessels by 14 days after adding *Fusarium* to plants treated with *P. putida* 53 (Figure 6a & 6b). Although occlusion had intensified by 21 days, none of the vessels were as yet completely blocked at this time. Frequent vessel collapse was observed where infection was intense with no occluding material present.

B. sphaericus* B43 + *Fusarium: Occluding material started filling up the lumen of some xylem vessels 14 days after *Fusarium* infection of the plants treated with *B. sphaericus* B43 (Figure 6a & 6b). *Fusarium* colonisation was observed in some parenchyma cells, with adjacent cell walls appearing thickened and more fluorescent (Figure 6d). A few xylem vessels were already completely occluded by 21 days (Figure 6c), and slight vessel collapse and vascular browning could be detected.

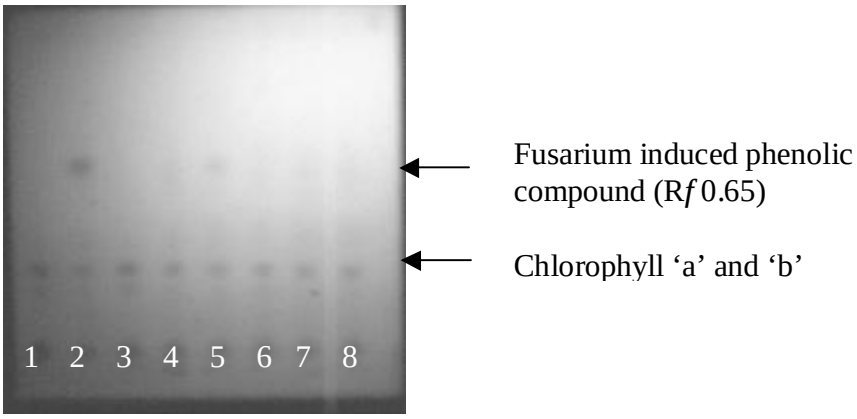

Figure 2b: Thin Layer Chromatography Separation of phenolic compounds from extracts of plants treated with rhizobacteria alone and in combination with *Fusarium oxysporum*. Extracts were from plants with following treatments: **(1)** untreated plants **(2)** *Fusarium oxysporum* alone **(3)** *Pseudomonas fluorescens* T58 + *Fusarium*, **(4)** *P. fluorescens* T58 alone, **(5)** *Pseudomonas putida* 53 + *Fusarium*, **(6)** *P. putida* 53 alone, **(7)** *Bacillus sphaericus* B43 + *Fusarium*, **(8)** *B. sphaericus* B43 alone. The 'new' phenolic compound with an R*f* is seen in lanes 2 and 5.

4 DISCUSSION

The bacteria strains *P. fluorescens* T58, *P. putida* 53, and *B. sphaericus* B43 were previously shown to induce systemic resistance against *Fusarium oxysporum* f.sp. *lycopersici* (Mwangi, 2003). The systemic effects induced by these bacteria on phenol accumulation, PR-proteins and structural changes associated with resistance against *Fusarium* were further investigated. Phenolic compounds that are relevant in disease resistance should be selectively toxic to the pathogen and produced promptly and in high amounts following pathogen infection (van Peer *et al*., 1991; Waterman & Mole, 1994). Systemic induction of phenolic compounds by plant health promoting rhizobacteria was reported by van Peer *et al*. (1991) and has been further discussed by Benhamou *et al* (1996a, b). However, the measurement of total phenols is unreliable as an indicator of disease resistance as phenols with antimicrobial activity often represent only a small proportion of phenols in plant tissues (Waterman & Mole, 1994). In this study, therefore, qualitative changes during resistance induction were analysed using Thin Layer Chromatography.

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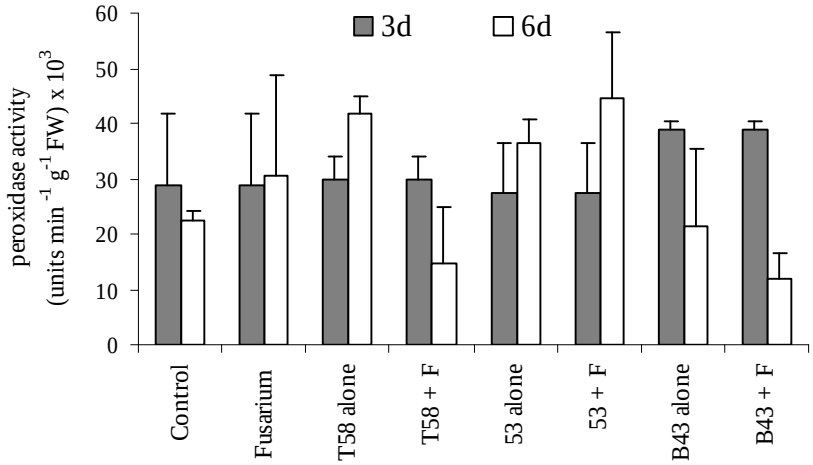

Figure 3: Peroxidase activity in the stem of tomato 3 and 6 days after treatment with bacteria alone and in combination with *Fusarium oxysporum* f.sp. *lycopersici*. Treatments were *Pseudomonas fluorescens* (T58), *Pseudomonas putida* (53), *Bacillus sphaericus* (B43) and *Fusarium oxysporum* (+F) Control were untreated uninfected plants. FW is fresh weight. Bars show standard deviation, (n = 3).

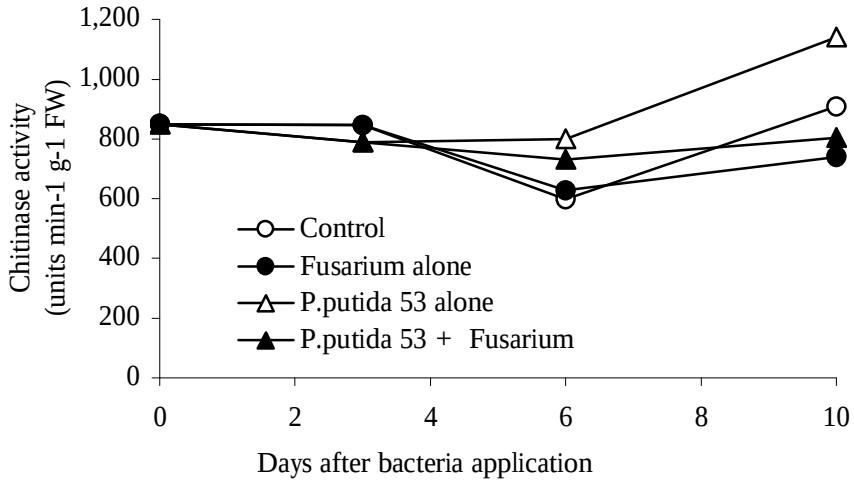

Figure 4: Chitinase activity in the leaves of tomato plants treated with *Pseudomonas putida* 53 alone or in combination with *Fusarium oxysporum* f.sp. *lycopersici*. FW is fresh weight, (n = 3).

A phenolic compound was detected where *Fusarium* had managed to infect and colonise plant tissues in the absence of ISR. This phenolic compound accumulated in plants infected with *F. oxysporum*, indicating that the plant recognises the presence of the pathogen and responds by changes in phenol metabolism. Plant treatment with rhizobacteria that are inefficient in reduction of *Fusarium* infection, e.g. *Bacillus megaterium* 4, *B. thuringiensis* 2, or *Paenibacillus macerans* 60 had no effect on accumulation of this compound. On the other hand, this compound was not detected where systemic resistance induced by *P. fluorescens*T58 or *B. sphaericus* B43 led to reduced infection. No compound similar to the one detected from plant extracts could be detected from *Fusarium* culture filtrates, but we cannot determine whether *Fusarium* is able to synthesise this compound *in planta*. On the other hand, it is possible that the 'new' compound is a phytoalexin produced for plant defence, but it is accumulated too slowly to inhibit fungal colonisation efficiently. Susceptible cultivars, such as the one used in this study are known to have late accumulation of phenolic compounds (Tjamos & Smith, 1974; MacCance & Drysdale, 1975; Elgersma & Liem, 1989).

The 'new' compound can be described as a marker for successful infection rather than a defence component. Suppression of this substance by *P. fluorescens* T58 and *B. sphaericus* B43 coincides with symptom suppression. Two hypotheses are possible: plants treated with *P. fluorescens* T58 or *B. sphaericus* B43 induce degradation (or suppress accumulation) of the compound and therefore, disease does not develop. Alternatively, plants treated with *P. fluorescens* T58 or *B. sphaericus* B43 suppress disease development by other means than phenol metabolism, as PR-proteins or the deposits observed in the histological examinations and therefore the new component does not accumulate. Such restriction of pathogen spread is well known (Hillocks, 1986) and could lead to a suppression of phytoalexin synthesis. The result obtained with *P. putida* 53 indicates that this resistance-inducing rhizobacterium induces a different response from *P. fluorescens* T58 or *B. sphaericus* B43, but leading to a similar suppression of infection. The function of the 'new' phenolic compound as well as its origin and chemical nature remain to be elucidated.

Plants treated with *B. sphaericus* B43 showed increased peroxidase activity after 3 days in presence or absence of *Fusarium*, which presents a possible defence mechanism against the pathogen. This response occurs earlier than the one observed in plants treated with one of the two *Pseudomonas* isolates and therefore may be more effective. Peroxidase has a role as a quick response to *Fusarium* ingress within the vascular system in the stem (Scott *et al*., 1995; M'Piga *et al*., 1997; Heerschap & Bakker, 1999). While peroxidase activities slightly increased after *Fusarium* infection in *P. putida* 53-treated plants, activities reduced after infection in *P. fluorescens* T58-treated plants. However, colonisation and disease symptoms were similar in both treatments. To determine clearly if alterations in peroxidase activity are linked to changes in lignification the activity of specific isoenzymes has to be determined (Scott *et al*., 1995).

The constitution of lignin in infected tissues is different from healthy tissues. In wheat leaves (Ride, 1975) and horseradish roots (Asada & Matsumoto, 1972) the main lignin component changes from syringyl propane to guaiacyl after infection with fungi. In our study the activity of guaiacol-peroxidases was specifically determined and peroxidase activity can be used as a good indicator of the lignification process. Thus, it would be expected that plants treated with *P. fluorescens* T58 and *P. putida* 53, which had the highest peroxidase activities, would also have the highest lignification in the stem. Lignin content was determined in the roots since they are expected to form the first line of defence to soilborne pathogens. A slight increase in lignin content in the roots was observed in plants treated with *P. fluorescens* T58 and *B. sphaericus* B43, but it did not vary after addition of *Fusarium*. Peroxidase activities in roots do not seem to be limiting for lignin synthesis. Increased lignin contents may reflect strengthening of cell walls as a preformed barrier that can reduce *Fusarium* penetration and spread when bacteria-treated plants come into contact with the pathogen.

Chitinases and ß-1,3-glucanases have been widely associated with defence responses due to their ability to hydrolyse cell wall material exposed at the surface of hyphal tips (Bacon *et al*., 1996) and represent typical PR-proteins induced during SAR (van Loon & van Strien, 1999). However, these enzymes apparently do not play a role in resistance induction by the bacteria analysed in this study. ß-1,3-glucanase activity was not affected by bacteria treatments and chitinase activity only increased in

the leaves. This increase induced by *P. putida* 53 and slightly by *B. sphaericus* B43 on their own was lost after infection of these plants with *Fusarium*. Increased chitinase activity in leaves would be ineffective against *Fusarium* since the pathogen would not have reached the leaves in 6 days after inoculation.

The data obtained show that induced systemic resistance mediated by the rhizobacteria *P. fluorescens* T58, *P. putida* 53, and *B. sphaericus* B43 is independent from induction of chitinase and ß-1,3-glucanases activities. Comparable plant reactions were also observed for other bacteria inducing systemic resistance: *Pseudomonas aeruginosa* 7NSK2 induces resistance against *Botrytis cinerea* on bean without accumulation of PR-proteins (De Meyer und Höfte, 1997) and resistance induction against *F. oxysporum* and *Pseudomonas syringae* in *Arabidopsis thaliana* is independent from salicylic acid accumulation and expression of genes encoding PR-proteins (Pieterse *et al*., 1996; van Wees *et al*., 1999). Induction of systemic resistance against cyst nematodes on potato by *Rhizobium etli* G12 is also independent of PR-protein activities (Reitz *et al*., 2001). Similarly, Bokshi *et al*. (2003) treated potato plants with benzothiadiazole to induce resistance against foliar pathogens and did not observe increases in ß-1,3- glucanases activities in potato roots, but in shoots and leaves. In our study, the only enzymatic activity associated with PR-proteins that we found to be implicated in ISR triggered by *B. sphaericus* B43, and with a different timing also by *P. fluorescens* T58 and *P. putida* 53 is peroxidase.

Rapid material deposition at sites of pathogen entry may contribute to delay the infection process, allowing sufficient time for the plant to build up other defence reactions (M'Piga *et al*., 1997). Structural barriers and toxic substances are considered to be among the first line of defence before the synthesis of PR-proteins which may contribute to complete fungal cell breakdown (Benhamou *et al*., 1996b). In our study, non-treated plants infected with *Fusarium* showed collapsing vessels and tissue browning. Cell wall appositions were observed in plants treated with *B. sphaericus* B43 where they occured in response to *Fusarium* infection, and in plants treated with *P. fluorescens* T58 where they occurred without any visible *Fusarium* infection. The material in cell walls could be callose, because it fluoresced with Aniline blue stain (Hood & Shew, 1996). Since *Fusarium* alone did not induce deposition of callose, it can be concluded that treatment of plants with *B. sphaericus* B43 or *P. fluorescens* T58 sensitised plants to react this way. Callose enriched deposits induced by plant health promoting bacteria have been reported in tomato (M'Piga *et al*., 1997) and in pea and cucumber (Benhamou *et al*., 1996a, 2000).

Fusarium infection after bacteria treatment was associated with extrusion of occluding material into the lumen of xylem vessels, with differing intensity depending on the bacteria. Vascular plugs and vessel coatings inside xylem elements have been described as host responses against *Fusarium* in tomato, and early tylose formation is essential for rapid localisation of infections (Bishop & Cooper 1984). When infection occurs through the roots *Fusarium* may take several days to penetrate and start spreading within plants. Therefore, the response observed about 10 days after *Fusarium* application may still be sufficiently early for plant defence. Apparently extrusion of occluding material into the lumen of xylem vessels is a major mechanism of defence in plants treated with *B. sphaericus* B43. The presence of this isolate caused an increase in the amount of material extruded into the xylem vessels and by 21 days some xylem vessels were already completely occluded. At 21 days after *Fusarium* application the xylem vessels of plants treated with *P. putida* 53 were considerably occluded, but the number of xylem vessels totally or nearly collapsed was just slightly lower than in the plants treated with *Fusarium* alone. However, the intensity of wilting symptoms was lower on the plants treated with *P. putida* 53 than in untreated plants infected with *Fusarium*. Apparently, *P. putida* 53 must support a different mechanism to protect plants than the other two bacteria, which may be partly explained through the increased peroxidase activity discussed above.

The results of this study suggest that a combination of effects induced by bacteria play a role in ISR to *Fusarium* wilt in tomato. Modifications of cell walls and vascular plugging, together with the changes in peroxidase activities are apparently involved in rhizobacteria-induced systemic resistance to *Fusarium* in this study. Changes in phenol metabolism as observed for *P. fluorescens* T58 and *B. sphaericus* B43 are possibly as well involved, but their precise role remains to be determined. Other possible effects of bacteria, e.g. inactivation of *Fusarium* toxins need to be investigated.

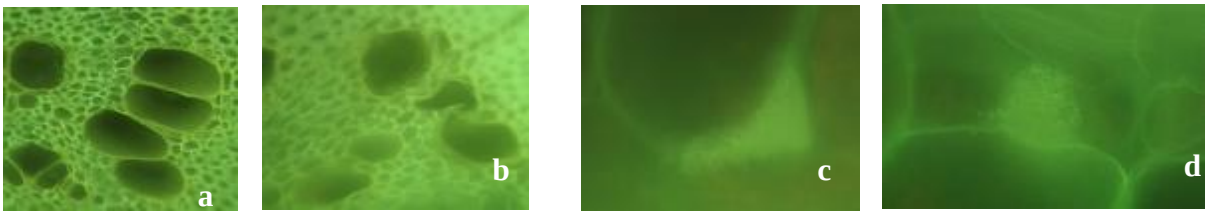

Figure 5: (a) Healthy xylem vessels with open lumina and no deposits. (b**)** Xylem vessel collapse due to *Fusarium oxysporum* infection. **(c & d)** Deposits on cell walls observed in plants treated with *Pseudomonas fluorescens* T58 and *Fusarium oxysporum*

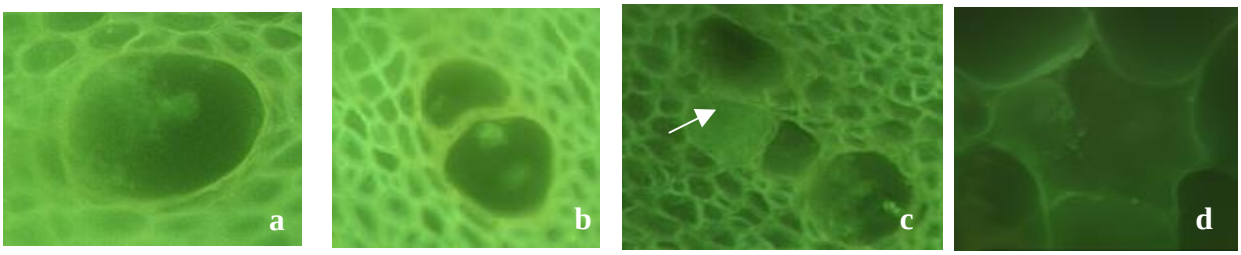

Figure 6: (a & b) Occluding material filling in xylem vessels of tomato plants treated with *Fusarium oxysporum* f.sp. *lycopersici* and *Bacillus sphaericus* B43 or *Pseudomonas putida* 53. **(c)** Complete occlusion of a xylem vessel in plants treated with *B. sphaericus* B43. **(d)** Thickened fluorescent wall after *F. oxysporum* infection of a cell in plants treated with *B. sphaericus* B43

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