Chapter 5

The enhanced disease susceptibility of ethylene-insensitive tobacco is not counteracted by inducing resistance or bacterial antagonists

Bart P.J. Geraats, Peter A.H.M. Bakker, Huub J.M. Linthorst, Jan Hoekstra, and L.C. van Loon

1 Institute of Molecular Plant Sciences, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

Abstract

Transgenic, ethylene-insensitive tobacco (Tetr) plants are more susceptible to several necrotrophic pathogens than non-transformed plants. To investigate if this enhanced susceptibility can be reduced, Tetr plants were treated with agents that induce resistance, or with antagonistic rhizobacteria. Treatments with β-aminobutyric acid (BABA), benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), methyl jasmonate (MeJA), or salicylic acid (SA), did not reduce spontaneous wilting of Tetr plants growing in non-autoclaved soil. Neither did they reduce disease development when Tetr seedlings growing in autoclaved soil were inoculated with Pythium, even though the chemicals each induced expression of at least one of the defense-related genes PR-1g, PR-5a, or PR-5c. However, the induction of PRs in Tetr plants by the chemicals was quantitatively less than in non-transformed seedlings, indicating that ethylene is required to stimulate PR gene expression. In transgenic Tetr lines overexpressing PR-1g, PR-5c, or both, no significant reduction in disease development was apparent. Treatments with Bacillus cereus UW85, Pseudomonas aeruginosa 7NSK2, Pseudomonas fluorescens WCS417r or Q8r-196, Pseudomonas putida WCS358r, or antibiotic-producing derivatives of WCS358r, did not reduce symptoms caused by Pythium, even though all isolates were capable of inhibiting growth of the pathogen in vitro. These results indicate that ethylene insensitivity impairs enhancement of resistance to Pythium and that ethylene signaling plays a central role in disease resistance of tobacco against necrotrophic pathogens.

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Introduction

Transgenic, ethylene-insensitive tobacco plants (Tetr) have lost non-host resistance to *Pythium sylvaticum* (Knoester et al. 1998) and are hyper-susceptible to various necrotrophic pathogens (Chapters 2, 3). In contrast to non-transformed plants, Tetr plants that were grown in non-autoclaved soil developed symptoms of wilting and stem rot due to infection by soil-borne microorganisms. Inoculation studies revealed that Tetr plants were less resistant to *Botrytis cinerea*, *Cercospora nicotianae*, *Erwinia carotovora*, *Fusarium oxysporum*, *Fusarium solani*, *Pythium* spp., and *Thielaviopsis basicola*, but resistance to several biotrophic pathogens, i.e. *Oidium neolycopersici* and *Peronospora tabacina*, was not reduced (Chapters 2, 3). This indicates that resistance to some pathogens is impaired in ethylene-insensitive Tetr tobacco, but defense against other pathogens is still fully functional. In *Arabidopsis*, resistance to biotrophic and viral pathogens has been demonstrated to depend on salicylic acid (SA) signaling (Reuber et al. 1998; Thomma et al. 1998; Kachroo et al. 2000), whereas resistance to several necrotrophic pathogens requires jasmonic acid (JA) and ethylene signaling (Thomma et al. 1999; Norman-Setterblad et al. 2000). Moreover, treatment of *Arabidopsis* with either SA, methyl jasmonate (MeJA), or ethylene, increased resistance to selected pathogens. One might envisage, therefore, that Tetr tobacco plants can be protected against soil-borne pathogens by inducing resistance through the activation of defense pathways that do not depend on ethylene.

Systemic acquired resistance (SAR) is a SA-dependent mechanism that is triggered upon infection by necrotizing pathogens and enhances the plants defensive capacity against a broad spectrum of pathogens (Van Loon 1997). The induction of SAR is associated with the accumulation of pathogenesis-related (PR) proteins (Van Loon 1975). Exogenous treatment of tobacco with SA induced the expression of PR genes (Ward et al. 1991) as well as resistance to tobacco mosaic virus (TMV) (White 1979), the fungus *B. cinerea* (Chivasa et al. 1997), the bacterium *E. carotovora* (Palva et al. 1994), and oomycete *Pythium* spp. (Chen et al. 1996). In addition, treatments with the SA-analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) or with β-aminobutyric acid (BABA), which induces accumulation of SA, also triggered PR expression and SAR to TMV in tobacco (Siegrist et al 2000). In contrast, culture filtrates of *E. carotovora* induced resistance to this pathogenic bacterium in a SA-independent manner (Vidal et al. 1998). Apparently, in tobacco resistance to *E. carotovora* can be induced through SA-dependent, as well as SA-independent defense signaling pathways. Also several non-pathogenic, root-colonizing bacteria can induce disease resistance through either SA-dependent or -independent pathways. In tobacco,
the rhizobacterium *Pseudomonas aeruginosa* strain 7NSK2 has been demonstrated to induce resistance to TMV in a SA-dependent fashion (De Meyer et al. 1999), whereas induced resistance against *Pseudomonas syringae pv. tabaci* by *Serratia marcescens* operated through a SA-independent pathway (Press et al. 1997). In *Arabidopsis*, root colonization by *Pseudomonas fluorescens* strain WCS417r or *Pseudomonas putida* strain WCS358r induced resistance to *P. syringae pv. tomato* independent of SA accumulation, but dependent on JA/ethylene signaling (Van Wees et al. 1997; Pieterse et al. 1998).

Antagonistic rhizobacteria can also protect plants against pathogens by competing for iron through the production of siderophores or by producing antibiotics (Bakker et al. 1991; Agrios 1997). 7NSK2 protected tomato against *Pythium* damping-off, whereas its non-siderophore producing mutant KMPCH had lost most of its biocontrol activity (Buysens et al. 1996). Likewise, WCS417r and WCS358r have been demonstrated to antagonize *F. oxysporum* by competition for iron in the rhizosphere of carnation (Duijff et al. 1993, 1994). Other rhizobacteria antagonize root pathogens through the production of antibiotics. *Bacillus cereus* UW85 produces at least two antibiotics, i.e. zwittermicin A and antibiotic B (Silo-Suh et al. 1994), and reduced damping-off of tobacco seedlings caused by *Pythium aphanidermatum*, *Pythium torulosum* and *Phytophthora parasitica* (Chen et al. 1996). *P. fluorescens* Q8r1-96 produces the antibiotic 2,4-diacetylphloroglucinol (DAPG), a compound that was shown to reduce *Pythium* diseases in several plant species, and antagonized the root pathogen *Gaeumannomyces graminis* in wheat (Raaijmakers and Weller 2001).

Phenazine-1-carboxylic acid (PCA) production by *P. aeruginosa* strain PNA1 contributed to the suppression of *Pythium* damping-off in bean (Anjaiah et al. 1998).

In this study it was investigated if the increased disease susceptibility of Tetr tobacco plants can be counteracted either by applying chemicals that induce resistance, or by growing the plants in soil containing root-colonizing bacteria that were demonstrated to possess antagonistic and resistance-inducing properties.

**Results**

**Chemical treatments**

To examine whether chemical inducers of resistance can rescue ethylene-insensitive tobacco from spontaneous disease development, Tetr plants were grown in non-autoclaved soil and sprayed every two weeks with the chemicals BABA, BTH, MeJA, or SA. Since Tetr plants are insensitive to ethylene, the ethylene-precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was
included as a negative control. In time, individual plants spontaneously developed wilting symptoms, leading to a gradual increase in the percentage of diseased plants from day 41 onwards. Disease development was similar in all treatments: neither BABA, MeJA, or SA (Fig. 1A), nor BTH (Fig. 1B) significantly reduced the percentage of Tetr plants developing symptoms of wilting compared to control plants that were sprayed with water, ACC (Fig. 1A), or wettable powder (ingredient of BTH formulation; Fig. 1B). Although MeJA and SA showed a slight, and BTH a strong, tendency to reduce the percentage of diseased plants, results were not consistent and showed high variation. This high variation was due to the unpredictable outcome of the natural infection and spontaneous disease development. When applied as a soil drench every two weeks, SA and BTH similarly tended to reduce the percentage of spontaneously diseased Tetr plants, although effects were again non-significant (data not shown). Under the same conditions, both chemicals were effective in inducing SAR against TMV, reducing lesion sizes by 15–50% (data not shown). Thus, the chemicals effectively induced SAR to TMV, but did not protect Tetr plants from spontaneously wilting.

Wilted Tetr plants that were grown in non-autoclaved soil might have been infected by root pathogens already before the chemical treatments had
effectively induced resistance. To control infection and reduce variation in disease development, plants were grown in autoclaved soil and inoculated with *Pythium* isolate Nt59d (Chapter 2) three days after chemical treatment. Eleven-day-old seedlings were sprayed with water, ACC, wettable powder, BABA, BTH, MeJA, or SA. To verify that the chemical treatments had been effective, expression of genes encoding acidic and basic PR-proteins was studied in both non-transformed and Tetr seedlings. Water-treated control seedlings of non-transformed tobacco had low background expression of basic PR-1g and acidic PR-5a, and a significant expression of basic PR-5c (Fig. 2). In the ethylene-insensitive Tetr seedlings no expression was observed, indicating that these basal levels are controlled by ethylene. ACC treatment induced PR-1g expression in non-transformed seedlings as compared to the water treatment, but not in Tetr seedlings. PR-5c expression might have been slightly induced in non-transformed tobacco as well, but this was hard to differentiate due to the high background level. MeJA treatment slightly induced PR-5c expression in Tetr seedlings. BABA induced expression of PR-1g, PR-5a, and PR-5c in non-transformed and in Tetr seedlings. However, expression in Tetr seedlings was considerably lower (Fig. 2), indicating that ethylene signaling stimulates induction of these genes. Treatment with BTH induced the expression of PR-5a and PR-5c in non-transformed and in Tetr seedlings, as compared to the wettable powder control treatment, but again expression levels were lower in Tetr. Similar to BTH, SA induced PR-5a and PR-5c expression. However, upon treatment with SA expression levels were almost as high in Tetr seedlings as in non-transformed seedlings. Apparently, induction of PR genes by BABA, BTH, or SA is differentially dependent on ethylene perception.

When non-transformed tobacco seedlings were inoculated with *Pythium*, some plants initially developed disease symptoms, but almost all of them fully recovered within 18 days (data not shown; cf. Chapter 2). For this reason, it was not possible to study induced resistance against this pathogen in non-

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**Figure 2.** PR-1g, PR-5a, and PR-5c expression in hypocotyls of two-weeks-old non-transformed (n) or Tetr (T) tobacco seedlings. Three days before RNA extraction, seedlings were sprayed with water, 3 mM SA, 0.5 mM MeJA, 3 mM ACC, 30 ppm BABA, 180 ppm wettable powder (w.p.), or 60 ppm BTH.
transformed seedlings. In contrast, of the Tetr seedlings an average of 20% already showed symptoms of disease within one day after inoculation (Fig. 3). The percentage of diseased seedlings increased linearly during the first week after inoculation and stabilized thereafter at approximately 85% diseased plants. As expected, ACC had no effect on disease development (Fig. 3A). Neither did treatment with MeJA (Fig. 3B), BABA (Fig. 3C), BTH (Fig. 3D), or SA (Fig. 3E), reduce disease in the Tetr seedlings to a significant extent. Similar to the observations in naturally infected full-grown Tetr plants, SA tended to reduce disease development, but this was not significant (Fig. 3E).

Figure 3. Disease development after inoculation of two-weeks-old Tetr tobacco seedlings with *Pythium* sp. Nt59d. Three days prior to inoculation seedlings were sprayed with A: water or 3 mM ACC, B: water or 0.5 mM MeJA, C: water or 30 ppm BABA, D: 180 ppm wettable powder or 60 ppm BTH, or E: water or 3 mM SA.
Overexpression of PR-1g and PR-5c

Basic PR-1 and PR-5 proteins have been reported to possess anti-oomycete activity \textit{in vitro} (Vigers et al. 1992; Niderman et al. 1995). Therefore, it was investigated if overexpression of genes encoding these PR-proteins enhances resistance to \textit{Pythium} in Tetr plants. Three-weeks-old Tetr seedlings constitutively overexpressing PR-1g (lines EPRO1-16 and EPRO1-31), PR-5c (EPRO5-7 and EPRO5-12), or both PR-1g and PR-5c (EPRO15-17), were inoculated with \textit{Pythium} and transplanted into autoclaved soil. Overexpression of the PR genes at the time of inoculation was confirmed by Northern blotting (Fig. 4). In all transgenic lines plants developed wilting symptoms within two

\textbf{Figure 4. PR-1g and PR-5c expression in hypocotyls of three-weeks-old Tetr tobacco seedlings transformed with PR-1g (EPRO1-16 and EPRO1-31), PR-5c (EPRO5-7 and EPRO5-12), or PR-1g and PR-5c (EPRO15-17). Line 22-11 was transformed with the empty vector (pMOG22).}

\textbf{Figure 5. Disease development after inoculation of three-weeks-old Tetr tobacco seedlings overexpressing PR-1g (EPRO1-16 and EPRO1-31), PR-5c (EPRO5-7 and EPRO5-12), or PR-1g and PR-5c (EPRO15-17), with Pythium isolate Nt59d. The control line (22-11) was transformed with the empty vector (pMOG22). A and B represent two independent experiments.}

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days (Fig. 5). In two independent experiments (Fig. 5A, B), the disease development curves for the seedlings overexpressing PR-1g, PR-5c, or both, did not differ significantly from the curve of the control seedlings (line 22-11), as tested with repeated measures. Nevertheless, in the first experiment (Fig. 5A), disease development of EPRO1-16 plants stabilized already by day 7 at approximately 36% of diseased plants, whereas the percentages of diseased plants in the other lines kept increasing. By 18 days the percentage of diseased EPRO1-16 plants (32%) was significantly lower than that of the control plants (82%), as tested with t-test and Bonferroni correction ($P=0.02$). In the second

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$^a$ DAPG: 2,4-diacetylphloroglucinol.

$^b$ PCA: Phenazine-1-carboxylic acid.

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### TABLE 1. Rhizobacterial strains used in this study with their relevant characteristics.

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$^a$ DAPG: 2,4-diacetylphloroglucinol.

$^b$ PCA: Phenazine-1-carboxylic acid.
experiment (Fig. 5B), disease developed much faster and no reduction was observed in any of the overexpressing lines. Therefore, overexpression of PR-1g and PR-5c appears to be insufficient to significantly reduce disease development of Tetr tobacco after inoculation with *Pythium*.

**Rhizobacterial treatments**

In view of the ineffectiveness of the chemical inducers, it seems unlikely that Tetr plants can be protected against natural infections by inducing resistance. To reduce disease caused by *Pythium* spp., root-colonizing bacteria that directly antagonize soil-borne pathogens were tested. A selection of siderophore- and / or antibiotic-producing bacteria (Table 1) was tested for *in vitro* antagonism against *Pythium* isolate Nt59d. The antibiotic-producing bacteria *P. fluorescens* strain Q8r1-96, *P. putida* WCS358r::phl, and WCS358r::phz, inhibited mycelial growth of *Pythium* on all media tested, whereas *B. cereus* UW85 did so only on King’s B (KB) agar medium, but not on potato dextrose agar (PDA) medium (Table 2). Bacteria that are able to compete for iron by producing siderophores, i.e. *P. fluorescens* WCS417r, *P. putida* WCS358r, and *P. aeruginosa* 7NSK2, all inhibited *Pythium* growth on KB medium under iron-limiting conditions, but not when an excess of iron was added to the medium. In addition, the non-siderophore producing mutant of 7NSK2, KMPCH, did not inhibit *Pythium* growth on iron-limited KB agar, confirming the involvement

**TABLE 2. In vitro growth inhibition of Pythium isolate Nt59d and in planta colonization of Tetr tobacco roots by *Bacillus cereus* and fluorescent *Pseudomonas* strains with resistance-inducing and / or antagonistic properties.**

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<th>in vitro inhibition</th>
<th>in planta root colonization</th>
<th>log (cfu / g) ± S.E.</th>
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<tr>
<td></td>
<td>PDA</td>
<td>KB</td>
<td>KB + FeCl₃</td>
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<tr>
<td><em>B. cereus</em> UW85</td>
<td>-</td>
<td>+ / -</td>
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a Assays on *in vitro* inhibition of *Pythium* were performed on potato dextrose agar (PDA) medium or King’s B (KB) agar medium with or without 500 µM FeCl₃. -: no inhibition of mycelial growth; +: reduced mycelial growth around bacterial colony; ++: no mycelial growth around bacterial colony.

b Root colonization of Tetr plants was determined two weeks after root dip inoculation with *Pythium*.
of competition for iron. Interestingly, both 7NSK2 and its mutant KMPCH inhibited growth of *Pythium* on PDA medium (Table 2), suggesting secretion of an antibiotic by both strains on this medium. These rhizobacteria were tested *in planta* for their capacity to suppress disease of Tetr tobacco upon inoculation with *Pythium* isolate Nt59d. To maximize the potential antagonistic activities, plants were bacterized at the seed stage, the seedling stage, and prior to pathogen inoculation. In control plants that were not inoculated with *Pythium*, none of the bacterial treatments had any significant effect on plant growth. Whereas in previous experiments inoculations with *Pythium* were performed by dipping the roots of two-weeks-old seedlings into a suspension of the pathogen, the rhizobacteria-treated plants

**Figure 6.** Disease development after inoculation of four-weeks-old, bacterized Tetr tobacco plants with *Pythium* sp. Nt59d. Before transplanting into *Pythium*-infested soil, plant roots were treated with MgSO₄ or bacterial strains UW85, Q8r1-96, or WCS417r (A), WCS358r, WCS358r::phz, or WCS358r::phl (B), or 7NSK2, or KMPCH (C).
were inoculated when they were four weeks old by transplanting them into soil infested with *Pythium*. Under the same conditions, non-transformed plants did not develop any symptoms of disease, confirming earlier results (Chapter 2). In Tetr plants, disease symptoms were apparent within 4 days after transplantation, and disease severity increased to varying levels thereafter. None of the bacterial treatments reduced the percentage of diseased plants to any significant extent, as tested with repeated measures (Fig. 6). The lack of disease reduction by the bacteria was probably not due to poor root colonization, since all strains had maintained population densities of approximately $10^5$ cfu / g root or higher at 14 days after inoculation (Table 2).

**Discussion**

Ethylene-insensitive Tetr tobacco plants that are grown in non-autoclaved potting soil spontaneously develop symptoms of disease due to natural infection by soil-borne microorganisms, notably *Pythium* spp. (Knoester et al. 1998; Chapters 2, 3). Spray treatments with chemicals that are known to induce resistance did not prevent spontaneous wilting of Tetr plants, although BTH, MeJA, and SA tended to delay disease development. Since the experimental set-up used generally resulted in high variations in disease severity and pathogen infection was not controllable, a standardized bioassay was developed, in which seedlings were grown in autoclaved soil and inoculated with *Pythium* three days after chemical treatment. Also under these conditions disease development was not reduced significantly by any of the chemicals used, although SA again tended to lower disease of the Tetr seedlings.

It is unlikely that the concentrations of the chemicals used were too low to be effective. Application of much lower concentrations induced resistance in *Arabidopsis* (Van Wees et al. 1999; Zimmerli et al. 2001). Moreover, higher concentrations were toxic to both non-transformed and Tetr plants (data not shown). All chemical treatments induced *PR* gene expression in the non-transformed seedlings, and to a lesser extent in the Tetr seedlings. In *Arabidopsis* concentrations of MeJA and SA that were too low to induce *PR* gene expression, were sufficient to induce full resistance (Van Wees et al. 1999). Therefore it is likely that in the tobacco seedlings defense-related signal transduction pathways had been activated at the time of inoculation.

When seedlings were sprayed with ACC, basic *PR-1g* was induced in non-transformed, but not in Tetr tobacco, confirming the ethylene insensitivity of Tetr18 (Knoester et al. 1998). MeJA induced some expression of basic *PR-5c* in Tetr seedlings. Although it was hard to distinguish in how far *PR-5c* expression was induced in non-transformed seedlings, MeJA has been reported
to induce PR-5c expression in full-grown tobacco (Niki et al. 1998). The fact that resistance to *Pythium* was not increased in Tetr seedlings by treatment with MeJA may be explained by a requirement for ethylene signaling. Alternatively, *Pythium* may not be sensitive to the defense mechanisms that are induced by MeJA. In *Arabidopsis* the MeJA-induced resistance to *B. cinerea* was still functional in ethylene-insensitive mutant ein2-1 (Thomma et al. 1999), but MeJA-induced resistance to *P. syringae* in the ethylene-insensitive *etr1-1* mutant was abolished (Pieterse et al. 1998).

Similar to MeJA, BABA induced the expression of PR-5c in Tetr seedlings, but no resistance to *Pythium*. As described previously (Siegrist et al. 2000), BABA induced acidic PR gene expression as well in the tobacco seedlings, demonstrating that SA-dependent defense must have been effectively activated. Treatment of the seedlings with SA or BTH induced acidic PR gene expression, although to different extents (Fig. 2). The BABA- and BTH-induced expression of PR genes was lower in Tetr seedlings than in non-transformed seedlings. Likewise, in *Arabidopsis* reduced PR-1 expression was observed in the ethylene-insensitive *etr1-1* mutant (Lawton et al. 1995), indicating that ethylene signaling is required for full expression of acidic PR genes.

Soil application of SA or BTH reduced lesion expansion upon TMV infection of leaves of full-grown Tetr plants (data not shown), confirming that SA-dependent defense to TMV is not impaired in Tetr tobacco (Knoester et al. 2001). Although SA did not induce resistance to *Pythium* in Tetr tobacco, in non-transgenic tobacco SA has been reported to induce resistance to two *Pythium* spp. (Chen et al. 1996). Apparently, SA-dependent resistance to *Pythium* is abolished in Tetr plants, whereas resistance to TMV is still functional, indicating that ethylene signaling is required for the expression of some, but not all SAR-related responses.

Some PR-1 and PR-5 proteins have been reported to possess anti-oomycete activity (cf. Van Loon 1997). Tobacco PR-1g (Niderman et al. 1995) and PR-5c (Woloshuk et al. 1991; Vigers et al. 1992) inhibited growth of the oomycete *Phytophthora infestans*. To investigate if overexpression of the genes encoding these proteins contributes to *Pythium* resistance in ethylene-insensitive tobacco, Tetr seedlings that constitutively express basic PR-1g, basic PR-5c, or both PR-1g and PR-5c were tested. The two PR-5c overexpressors were as susceptible as control Tetr seedlings that were transformed with the empty vector. This indicates that overexpression of PR-5c does not contribute to resistance against *Pythium*. In line with this result, overexpression of PR-5c in tobacco cv. Wisconsin-38 did not increase resistance to the oomycete *Phytophthora parasitica* (Liu et al. 1994). In contrast, overexpression of acidic PR-1a was shown to increase resistance to *P. parasitica* and *Peronospora tabacina*.
in tobacco (Alexander et al. 1993), although the effect was fairly small. Niderman et al. (1995) reported that the basic PR-1g protein was far more effective in inhibiting \textit{in vitro} growth of \textit{Phytophthora infestans} than the acidic PR-1a. Overexpression of PR-1g in Tetr seedlings reduced disease severity in one of the two transgenic lines (EPRO1-16), but only when disease developed relatively slowly (Fig. 5). In radish rhizobacteria-mediated induced systemic resistance to \textit{F. oxysporum} was effective at moderate, but not at high disease incidence (Leeman et al. 1995). Apparently, the induced resistance is overwhelmed when disease pressure is high. Because disease of naturally infected Tetr plants growing in non-autoclaved soil generally develops more slowly than upon inoculation, overexpression of \textit{PR} genes might still have some protecting effect under the former conditions.

Because the induction of resistance appeared to be insufficient to protect Tetr tobacco, rhizobacterial strains that possess antagonistic properties against soil-borne pathogens were tested for suppression of \textit{Pythium}. To check the effectiveness of root colonization by the bacteria, their population levels on bacterized roots were examined. Population densities of \(10^5\) cfu / g root were demonstrated to be sufficient for pseudomonads to control Fusarium wilt in radish and take-all in wheat (Raaijmakers et al. 1995; Raaijmakers and Weller 1998). All strains used had colonized the Tetr tobacco roots to at least this level 14 days after transplanting the seedlings in \textit{Pythium}-infested soil, suggesting that the requirement for effective biocontrol was met. The bacterial isolates 7NSK2, WCS417r, and WCS358r, which have been demonstrated to compete for iron with soil-borne pathogens in different pathosystems (Duijff et al. 1993, 1994; Raaijmakers et al. 1995; Buyens et al. 1996), did not reduce disease severity, although they effectively inhibited growth of \textit{Pythium in vitro} under iron-limited conditions. Possibly, the iron concentration in the soil used was not limiting. Similarly, the antibiotic-producing bacteria \textit{B. cereus} UW85, \textit{P. fluorescens} Q8r1-96, and WCS358r derivatives WCS358r::phl and WCS358r::phz, inhibited \textit{Pythium growth in vitro}, but did not reduce disease severity in Tetr plants. Perhaps, the antibiotic concentrations in the rhizosphere did not reach levels that are required to suppress the pathogen.

In summary, none of the tested chemicals or biocontrol agents reduced \textit{Pythium} disease in Tetr tobacco, even though some bacteria were capable of expressing different antagonistic mechanisms (Table 1). As \textit{Pythium} is a major pathogen infecting Tetr plants that are grown in non-autoclaved soil (Knoester et al. 1998; Chapters 2, 3), counteracting the spontaneous disease development of these plants appears to be very difficult. Some of the agents might induce resistance dependent on ethylene signaling or induce mechanisms that require ethylene for the expression of resistance. Other agents might be active in Tetr tobacco, but the mechanisms could be ineffective against soil-borne pathogens.
In order to develop treatments or biocontrol agents that counteract enhanced disease susceptibility of ethylene-insensitive plants, more insight in which mechanisms are impeded is indispensable.

**Materials and methods**

**Plant material**

$T_2$ seeds of transgenic, ethylene-insensitive Tetr18-5 tobacco (Knoester et al. 1998), homozygous for the *Arabidopsis etr1-1* transgene, and seeds of corresponding non-transformed tobacco (*Nicotiana tabacum* cv. Samsun NN) were sown on either autoclaved ($2 \times 20$ min with a $24$ h interval) or non-autoclaved potting soil. The seedlings were grown for two weeks at RH 100% in a temperature-regulated greenhouse, at $24\, ^\circ\text{C}$ during the day and $21\, ^\circ\text{C}$ at night, and a photoperiod of $16\, \text{h}$.

All PR-overexpressing lines were developed in the Tetr18-5 genomic background. The cDNAs corresponding to tobacco *PR-1g* and *PR-5c* (AP24) were obtained by RT-PCR using upstream and downstream primers containing KpnI and HindIII sites. After digestion with KpnI and HindIII, the resulting PCR fragments were cloned between the cauliflower mosaic virus 35S promoter/alfalfa mosaic virus RNA4 leader (35S) and potato inhibitor terminator (Term) of pMOG843. The identity of the cDNAs was confirmed by sequencing. The 35S-PR-cDNA-Term cassettes from the pMOG843 constructs were obtained after digestion with EcoRI and XbaI, and subsequently cloned in the T-region of the pMOG22 transformation vector (hygromycin-resistance). The resulting transformation plasmids were named pMOGPRb1 and pMOGPRb5. A double construct containing both the *PR-1g* and the *PR-5c* genes was obtained by ligation of an XbaI fragment with the 35S-basic PR-5c-cDNA-Term cassette to XbaI-digested pMOGPRb1. This resulted in transformation plasmid pMOGPRb15. All transformation plasmids were electroporated into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium*-mediated leaf disc transformation was performed as described previously (Linthorst et al. 1989). Primary transformants were allowed to self-pollinate. The PR-overexpression lines were selected on the basis of PR gene expression.

$T_1$ seeds of Tetr18-5 overexpressing *PR-1g* (designated EPRO1-16 and EPRO1-31), *PR-5c* (EPRO5-7 and EPRO5-12), both *PR-1g* and *PR-5c* (EPRO15-17), or no PR genes (22-11; empty vector), were sterilized by immersion in 1% NaClO for 20 min, washed three times with sterile water, and sown on MS agar medium, containing 1% (w/v) glucose and 20 µg / ml hygromycin. The seedlings were grown with an 8-h light / 24 °C and 16-h
dark / 21 °C cycle at RH 70% for three weeks. Hygromycin-resistant seedlings were selected for the bio-assays, in which they were inoculated and transplanted into autoclaved soil.

**Chemical treatments, natural infection, and inoculation with *Pythium***

To obtain natural infections, Tetr plants were grown in non-autoclaved soil. Two-weeks-old seedlings were planted out at about 380 plants / m² in non-autoclaved soil and their leaves were sprayed evenly with either water (control), 3 mM ACC, 30 ppm BABA, 60 ppm BTH (applied as 240 ppm Bion [Syngenta]; 75% wettable powder + 25% BTH), 180 ppm wettable powder (control for Bion formula), 0.5 mM MeJA, or 3 mM SA. After another two weeks, 16 individual plants were transplanted into 0.6 l pots and sprayed according to schedule.

For controlled infection, plants were grown in autoclaved soil. When applicable, chemical treatments were performed by spraying 11-days-old seedlings as described above. Two-weeks-old chemically treated seedlings or three-weeks-old seedlings overexpressing PR genes were inoculated by dipping the roots for 1 s into a suspension containing 15 g / l mycelium of *Pythium* sp. isolate Nt59d, as described previously (Chapter 2). Seedlings were transplanted into autoclaved soil at four plants per pot and seven pots per treatment.

**Quantification of disease symptoms and statistical analysis**

At specific time points, disease severity was assessed based on the percentage of plants developing disease symptoms, as described previously (Chapter 2). Data were statistically analyzed using the repeated measures option of SPSS for Windows, release 10.0. All bioassays were repeated at least once with similar results.

**PR gene expression: RNA isolation and Northern blotting**

Total RNA from 20 seedling shoots per treatment was extracted, blotted onto Hybond-N⁺ membrane (Amersham, The Netherlands), and hybridized as described previously (Chapter 2). Hybridization was performed at 62 °C with random-primed-α-³²P-labeled (Ready-To-Go labeling kit, Amersham Pharmacia Biotech, The Netherlands) probes of tobacco *PR-1g* cDNA (Hooft van Huijssduijnen et al. 1986), *PR-5α* cDNA (Cornelissen et al. 1986), *PR-5c* cDNA (Stintzi et al. 1991), or 5.8S rDNA (RNA-loading control; Chapter 2). After hybridization, the membrane was rinsed once with 0.3 M NaCl, 0.037 M Na-citrate, 0.5% SDS, washed three times for 15 min in the same solution.

Enhanced disease susceptibility cannot be counteracted.
at hybridization temperature, and exposed to a Molecular Imager® FX screen (Bio Rad).

*In vitro antagonism of Pythium*

Four different bacterial isolates per Petri dish (diameter 9 cm) were inoculated with a sterile needle at regular distances onto potato dextrose agar (PDA; Difco), King’s B (KB) agar, and KB agar supplemented with 500 µM FeCl₃. After incubation at 28 °C for 3 days, a PDA plug containing freshly-grown mycelium of *Pythium* isolate Nt59d was put in the middle of the agar plate. After incubation at 24 °C for 4 days, the mycelial growth of *Pythium* surrounding the bacterial colonies was determined visually. All bacteria were tested in triplicate, and the experiments were repeated at least once with similar results.

*Rhizobacterial treatments and inoculation with Pythium*

The rhizobacterial strains used and their relevant characteristics are listed in Table 1. All *Pseudomonas* spp. were grown on KB agar and *Bacillus cereus* UW85 was grown on half-strength tryptic soy agar (TSA; Difco) at 28 °C for 48 hours. Bacteria were washed off the agar plates with 10 ml 10 mM MgSO₄, and the numbers of bacteria in the suspensions were determined spectrophotometrically at 660 nm.

Tetr tobacco seeds were sown in autoclaved soil mixed with 50 ml bacterial suspension (2 × 10⁹ bacteria / ml) per kg, and transplanted in similarly bacterized soil when two weeks old. After another two weeks, 20 plants per treatment were uprooted and bacterized again by dipping the roots for 1 s in a suspension containing 10⁹ bacteria / ml. Subsequently, plants were inoculated with *Pythium* isolate Nt59d by transplanting them individually into pots containing autoclaved soil mixed with the pathogen (100 mg mycelium / kg).

*Determination of root colonization*

Two weeks after inoculation with *Pythium*, roots from seedlings treated with rhizobacteria as described above, were examined for rhizobacterial colonization. For each treatment, five replicate root samples from pools of four plants were analyzed. One gram of roots was vortexed in a glass tube containing 5 ml 10 mM MgSO₄ and 0.5 g of glass beads (0.56–0.80 mm) for 30 s. Dilutions of the resulting suspensions were plated on agar medium. The samples from plants treated with rifampicin-resistant *Pseudomonas* strains WCS358r, WCS358r::phz, WCS358r::phl, WCS417r, or Q8r1-96, were plated on KB agar + rifampicin (150 mg / l), and incubated at 28 °C for 48 h. Strains 7NSK2 and KMPCH were enumerated on KB + ampicillin (40 mg / l) + chloramphenicol (13 mg / l), after incubation at 37 °C for 14 h. Numbers of
B. cereus UW85 were determined on half-strength TSA + polymyxin β-sulfate (12.5 mg / l), after incubation at 28 °C for 48 h. For comparison, suspensions obtained from non-bacterized roots (10 mM MgSO₄) were plated on all media. None of the applied rhizobacterial strains was detected in the control root samples.

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