Prospects and challenges for practical application of rhizobacteria-mediated induced systemic resistance

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Abstract: Selected strains of plant growth-promoting rhizobacteria are able to induce a systemic resistance (ISR) in plants, which is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). The generally non-specific character of induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens may be present. ISR has been shown to be effective in radish and cucumber under field conditions. However, when induced plants are infected, disease development or severity are reduced but not prevented. Resulting economic losses to farmers make induced resistance alone insufficiently attractive for commercial application in modern intensive agriculture. For practical applications, durable strategies may be devised in which the growth-stimulating properties of resistance-inducing rhizobacteria are combined with other bacterial mechanisms of disease suppression through mixtures of rhizobacterial strains or by combinations with biocontrol fungi, low doses of chemical crop protectants, chemical SAR inducers or partial resistance. In *Arabidopsis* ISR and SAR are effective against a different, though largely overlapping spectrum of pathogens, depending on the signaling pathways involved in basal resistance. Combination of ISR and SAR can increase protection against pathogens that are resisted through both pathways, as well as extend protection to a broader spectrum of pathogens than ISR or SAR alone.

Key words: *Arabidopsis*, biological disease control, induced systemic resistance, plant growth-promoting rhizobacteria, *Pseudomonas*, radish, systemic acquired resistance

Introduction

Developing plant roots become colonized rapidly by soil-inhabiting fungi and bacteria that can be either detrimental – as pathogens – or beneficial – as mutualists or symbionts – to plant growth and survival (Schippers et al., 1987). Various rhizobacterial strains fall into the latter category and possess growth-promoting and disease-suppressive properties (Kloepper, 1996; Glick et al., 1999). Of these plant growth-promoting rhizobacteria (PGPR) several strains have been shown to be able to induce a systemic resistance in plants which is phenotypically similar to pathogen-induced systemic acquired resistance (SAR) (Van Loon et al., 1998). This rhizobacteria-mediated induced systemic resistance (ISR) is just one of the mechanisms by which PGPR improve plant emergence and development. Selected rhizobacterial strains can promote plant growth by activities that depend on increasing nutrient supply and/or involve growth-stimulating hormonal effects. Increased growth can also result from the suppression of diseases as a result of competition of the rhizobacteria with soil-borne pathogens for nutrients, notably iron, or through rhizobacterially-produced antibiotics or lytic enzymes (Handelsman and Stabb, 1996). ISR extends the disease-suppressive properties of root-colonizing bacteria to the phyllosphere and can reduce the extent or severity of diseases caused by foliar pathogens. Because induced resistance has been shown to be active against plant pathogenic fungi, bacteria, viruses and insects, rhizobacteria-mediated ISR can be an effective means to combat a wide range of plant diseases.

The disease-suppressive agents are harmless bacteria that are naturally present in soils and able to colonize roots of many plant species. Moreover, they ideally fit with the concept of sustainable agriculture, as they multiply in the root environment and continuously deliver triggers that induce resistance to root cells. However, so far practical applications of biocontrol bacteria for plant disease control have made little progress (Mathre et al., 1999). The biocontrol bacterium *Pseudomonas aureofaciens* AB 254 provided effective control of Pythium seed rot in sweet corn, as well as in several cucurbitaceous crops, but development as a commercial product was not pursued because of the high cost of the freeze-dried formulation of the organism. The doses required for commercial application of *Pseudomonas fluorescens* and *Bacillus* spp. strains for biocontrol of take-all are hampering further development of these strains. Similarly, formulating *Phialophora* sp. for biocontrol of wheat take-all is currently too expensive for commercial purposes.

Field studies with resistance-inducing rhizobacteria have been described for radish (Leeman et al., 1995a) and cucumber (Wei et al., 1996). Cucumber seeds were treated with different rhizobacterial strains and in some experiments a soil drench with bacteria was given at transplanting. Most treatments significantly reduced the severity of angular leaf spot upon challenge inoculation with the pathogen *Pseudomonas syringae* pv. *lachrymans*, and also resulted in significant protection from naturally occurring anthracnose caused by *Colletotrichum orbiculare*. Yield (cumulative fruit weight) was significantly increased by two of three PGPR strains in a first trial, two of four strains in a second and three of four strains in a third trial. Radish seeds film-coated with the rhizobacterial strain *P. fluorescens* WCS374 were drilled into greenhouse soil naturally infested with the wilt fungus *Fusarium oxysporum* f.sp. *raphani*. During four consecutive years in six out of 11 crops WCS374 significantly suppressed Fusarium wilt. The relative reduction of disease ranged from 19 to 68%, with an average of 43%, whereas the relative increase in yield ranged from 20 to 100%, with an average of 45%. In these experiments, disease suppression was likely to result from ISR, because in a bioassay with the same greenhouse soil WCS374 suppressed disease but a mutant lacking the resistance-inducing O-antigenic side chain of the outer membrane lipopolysaccharide did not (Leeman et al., 1995b).

Although these results demonstrate that ISR-eliciting rhizobacteria can reduce crop losses, the efficacy and reliability of this type of biocontrol are insufficiently attractive for commercial application in modern intensive agriculture. Indeed, induced resistance constitutes an enhanced defensive capacity of plants to a range of pathogens but it does not prevent disease from occurring, whereas protective chemicals are usually very effective as well as cheap. Therefore, biocontrol by resistance-inducing rhizobacteria can be attractive only in situations where chemical treatments are ineffective or undesirable and, otherwise, alternatives are not available. Such conditions pertain to small markets involving specific diseases in minor crops, for which the development of selective crop protectants is too costly. A similar situation essentially holds also for the application of SAR. However, compared to SAR, treatment with biocontrol bacteria offers advantages in that those organisms may also promote plant growth and counteract pathogens by more than a single mechanism. It is generally taken that the enhanced defensive capacity of SAR, marked by the accumulation of pathogenesis-related proteins (PRs), poses a cost to the plant, as exemplified by the small stature of various mutants in which the inducible defense mechanisms are expressed constitutively (Glazebrook, 1999). In contrast, rhizobacterially-mediated ISR is not associated with an accumulation of PRs. ISR-expressing plants appear only to be sensitized to respond to subsequent infection by activating defenses more quickly and to an increased level (Van Wees et al., 1999). Hence, the metabolic costs associated with the state of ISR seem negligible and both growth promotion and ISR may be apparent at the same time. When applied to suppress

soil-borne pathogens, ISR-eliciting rhizobacteria may also antagonize the pathogen by competition for nutrients, antibiosis or lytic activity. However, root colonization can become a limiting factor when strains express highly effective antagonistic activities. Both for effective competition for e.g. iron through secretion of siderophores, and for induction of systemic resistance a minimum population density of 10^5 colony-forming units of bacteria per gram root appears to be required (Raaijmakers et al., 1995). Therefore, competitive colonization of plant roots in the rhizosphere, which has been shown to involve multiple factors (Lugtenberg et al., 2000), is an important trait for biocontrol.

Strategies to improve rhizobacteria-mediated disease suppression

Screening of bacterial strains for induction of systemic resistance is more expensive, time-consuming, and labor-intensive than screening for antagonistic mechanisms, such as antibiotic production. Moreover, to achieve commercially acceptable levels of crop protection by ISR-eliciting rhizobacteria, both the efficacy and the reliability of disease suppression need to be improved. This may be achieved by judicious choices of bacterial strains targeted to specific diseases. In naturally occurring Fusarium wilt-suppressive soils a concerted action of several disease-suppressive microorganisms and mechanisms is considered to be responsible for the highly consistent reduction in disease seen (Lemanceau and Alabouvette, 1991). Thus, mixtures of different rhizobacterial strains expressing complementary mechanisms of disease suppression are likely to be more effective in biocontrol of soil-borne diseases than single strains. For instance, WCS374 suppresses Fusarium wilt of radish through ISR, whereas the biocontrol strain *Pseudomonas putida* WCS358 antagonizes the pathogen through siderophore-mediated competition for iron (Raaijmakers et al., 1995). However, combining WCS374 and WCS358 was not more effective in suppressing disease than application of either of the single strains alone (Raaijmakers, 1994). This result could be ascribed to non-compatibility between these two strains, because WCS358 reduced the population density of WCS374 up to 30-fold as a result of its iron-sequestering activity. When WCS358 was combined with *P. putida* strain RE8, another strain capable of inducing resistance in radish, the mixture did result in enhanced disease suppression (De Boer, 2000). Rhizosphere population densities of both WCS358 and RE8 were similar whether inoculated singly or in combination, indicating that these strains did not antagonize each other in the rhizosphere. In some bioassays one of the strains failed to suppress disease, but plants were still protected through the activity of the other strain. Combining RE8 with *P. fluorescens* strain RS111a – whose disease-suppressive activities have not been characterized yet – likewise resulted in significantly better disease suppression as compared to the single strains (De Boer et al., 1999).

Non-pathogenic fungi may protect plants by mechanisms similar to those employed by rhizobacteria, and combinations of biocontrol bacteria and fungi are another option. Non-pathogenic *F. oxysporum* Fo47 combined with WCS358 efficiently suppressed Fusarium wilt of carnation. This suppression was significantly higher than that obtained by treatment with either antagonistic microorganism alone and appeared to depend on competition for carbon by the biocontrol fungus together with competition for iron by the bacterium (Lemanceau et al., 1992, 1993). However, by using a split-root system, Duijff et al. (1998) demonstrated that Fo47 can induce resistance in tomato, leaving the possibility open that the effect in carnation also depended, in part, on induced resistance. Also in the suppression of Fusarium wilt in radish by WCS374 under commercial greenhouse conditions, non-pathogenic fungi naturally present in the soil may have contributed to biocontrol. Indeed, in pot experiments, WCS374-elicited ISR led to a rather modest reduction of Fusarium wilt symptoms, whereas the

effective non-pathogenic *F. oxysporum* strain FoC8 reduced disease from over 80 to 30%. However, the combination of WCS374 and FoC8 was even more effective, lowering the percentage of diseased plants to about 15. When both microorganisms were applied together at low doses at which neither suppressed disease on its own, the combination did reduce disease substantially (Leeman et al., 1996). Thus, combinations of disease-suppressing bacteria, or of bacteria and fungi, can act synergistically to reduce crop losses caused by soil-borne fungal pathogens.

Reducing the dose of a biocontrol bacterium required for disease suppression would lower the cost and increase acceptance of the treatment. To enhance the disease-suppressive effect of rhizobacteria-mediated ISR against both soil- and air-borne pathogenic fungi, application of inducing bacteria might be combined with low doses of fungicides. Specific data on such combinations are lacking, but additive effects have been obtained on yield of mildewed barley by inducing resistance with a culture filtrate of the biocontrol bacterium *Bacillus subtilis*, combined with fungicide treatment (Kehlenbeck et al., 1994). Similarly, combined treatment of rosemary cuttings with an isolate of the biocontrol fungus *Laetisaria arvalis*, that was selected for tolerance to the experimental fungicide CGA 173506, and a foliar spray of this fungicide at half the recommended rate reduced aerial blight caused by *Rhizoctonia solani* AG-4 more than treatment with either the fungus or the fungicide alone (Conway et al., 1997).

Strategies for exploiting rhizobacteria-mediated ISR and SAR

Pathogen-induced SAR depends on the signaling molecule salicylic acid (SA), whereas, at least in *Arabidopsis*, rhizobacteria-mediated ISR requires jasmonate (JA) and ethylene signaling (Pieterse et al., 1998). Cross-talk between the SA-dependent pathway and wound-induced JA-dependent signaling has been shown to result in inhibition of JA-mediated defense responses (Doares et al., 1995; Van Wees et al., 1999). However, simultaneous activation of SAR by an avirulent strain of the pathogen *Pseudomonas syringae* pv. *tomato* (avrPst) and ISR by the rhizobacterial strain *P. fluorescens* WCS417 resulted in an additive effect on the level of induced resistance against virulent *P. syringae* pv. *tomato* (Pst) (Van Wees et al., 2000). In *Arabidopsis* genotypes that are incapable of expressing either SAR or ISR, the additive effect was not evident. Conversely, treatment of ISR-expressing plants grown in soil containing WCS417 with the SAR-inducing chemical SA led to a higher reduction of disease symptoms and a greater inhibition of pathogen proliferation than treatment with either inducer alone. Growing mutant *cpr1* plants, that constitutively express SAR, in soil containing WCS417, also led to a significantly higher level of protection against Pst. Thus, simultaneous activation of both the SAR and the ISR pathway results in an enhanced level of systemically induced resistance, whether induction occurs by biological or chemical means (Van Wees et al., 2000). Although this additive effect has been demonstrated so far only in *Arabidopsis*, it holds promise for combining rhizobacteria-mediated ISR with application of chemical SAR inducers, such as BION.

Some rhizobacterial strains with biocontrol properties have been shown to be themselves capable of producing SA under iron-limiting conditions, to activate the SAR pathway, and to induce systemic resistance against selected pathogens (Maurhofer et al., 1998; De Meyer et al., 1999a,b). Some of those strains might activate both the ISR and the SAR pathway through different bacterial determinants. However, experiments with cucumber, radish, tobacco and tomato and different biocontrol strains have raised considerable doubts as to whether the bacteria actually produce SA on plant roots, because in most cases SA does not appear to be the primary determinant of the systemically induced resistance observed (Press et al., 1997;

Van Loon et al., 1998). Both the use of mutant and transgenic plants impaired in the expression of either ISR or SAR, and application of chemicals that selectively induce ISR or SAR will be needed to further investigate in how far ISR and SAR act additively in plant species other than *Arabidopsis*.

The mechanisms by which biocontrol bacteria elicit ISR are not known, although several bacterial determinants have been implicated (Van Loon et al., 1998). Recently, SA-biosynthetic gene clusters were isolated from *Pseudomonas aeruginosa* CHA0 (Serino et al., 1995) and *P. fluorescens* WCS374 (Mercado-Blanco et al., 2001), opening up possibilities for constitutive synthesis of SAR-inducing SA by genetically modified biocontrol bacteria in the rhizosphere. Further optimization can be envisaged by genetically engineering other resistance-inducing and disease-suppressive traits into bacterial strains with excellent root-colonizing abilities.

Induced resistance as an enhancement of basal resistance

Of ten different *Arabidopsis* accessions tested, two (RLD and Ws-0) were found to lack the ability to express ISR. Both RLD and Ws-0 are affected in the same locus and the non-responsive phenotype is associated with both an enhanced susceptibility to infection with Pst and a reduced sensitivity to ethylene (Ton et al., 2000, 2001). These observations point to a link between basal resistance and induced resistance. Such a link was apparent also in WCS417-induced resistance against Fusarium wilt in carnation, which was more strongly and consistently observed in the moderately resistant cultivar Pallas than in the susceptible cultivar Lena (Van Peer et al., 1991). Resistance against *F.oxysporum* f.sp. *dianthi* in carnation is polygenic and a certain level of partial resistance seemed required for ISR to be manifested. However, no such relationship between level of partial resistance against Fusarium wilt and inducibility of ISR by WCS374 was apparent in radish when disease pressure was moderate (Leeman et al., 1995c). The data indicate that a very high disease incidence precludes significant disease reduction by ISR-eliciting rhizobacteria. Thus, an apparent lack of inducibility of highly susceptible cultivars might be due not to an inherent incapability of expressing ISR, but to the overwhelming of basal defenses by the pathogen. Such considerations might explain data that of three cucumber cultivars susceptible to anthracnose, caused by *Colletotrichum orbiculare*, all could be induced by *P. putida* 89B-27, but only two by *Serratia marcescens* 90-166 (Liu et al., 1995). Such a biocontrol strain – cultivar interaction was also described for two bean cultivars. The biocontrol strain *P. aeruginosa* 7NSK2 was unable to induce resistance to anthracnose in cultivar Boterkoning, which is completely sensitive to the pathogen *Colletotrichum lindemutianum*, but did in the partially resistant cultivar Prelude. However, WCS417 was able to induce resistance against anthracnose in both these cultivars (Höfte et al., 2000). In the absence of mutants that have lost any resistance to a pathogen, it is difficult to determine in how far a level of partial resistance is still present in susceptible cultivars. However, partial resistance may be associated with the constitutive accumulation of specific defense-related gene products, which are boosted more strongly upon induction of ISR than in fully susceptible relatives (Tuzun, 2001).

There is ample evidence that the generally non-specific character of induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens may be present. However, due to negative cross-talk between SA-dependent SAR and the JA-dependent response to injury, plants tend to become more vulnerable to insect herbivory when induced to express SAR to pathogens. Inhibition of JA-mediated signalling by SA may also interfere

with the attraction by wounded plants of the natural enemies of herbivores (Bostock, 1999; Felton et al., 1999; Paul et al., 2000). Rhizobacteria-mediated ISR is dependent neither on SA, nor on increases in JA level or ethylene production (Pieterse et al., 2000), and an effect of the expression of ISR on insect herbivory has been documented (Zehnder et al., 1997). However, whereas in *Arabidopsis* ISR was found to be effective against pathogenic oomycetes, fungi and bacteria, it was not effective against turnip crinckle virus, whereas SAR was (J. Ton, unpublished results). Induction of SAR and ISR was equally effective against *F. oxysporum* f.sp. *raphani*, Pst, and *Xanthomonas campestris* pv. *armoraciae*. Activation of ISR resulted in a significant level of protection against *Alternaria brassicicola*, whereas SAR was ineffective against this pathogen. Conversely, activation of SAR resulted in a high level of protection against *Peronospora parasitica*, whereas the level of protection conferred by ISR was relatively weak (Ton, 2001). Thus, SAR and ISR are effective against a different, be it largely overlapping, spectrum of pathogens.

Remarkably, the pathogens that are resisted by SAR are more virulent on transgenic *Arabidopsis* plants that are impaired in the accumulation of SA (NahG plants), but not on mutants with reduced sensitivity to JA or ethylene. Conversely, ISR is effective against pathogens that are more virulent on JA- or ethylene-insensitive mutants, but not on NahG plants. Thus, the type(s) of induced resistance to which individual pathogens are sensitive is coupled to the type(s) of defense signaling compounds that confer basal resistance, and induced resistance appears to constitute an enhancement of basal resistance mechanisms (Ton, 2001). These observations cannot only explain the differences in effectiveness of ISR and SAR against specific pathogens, but also support the concept that combination of ISR and SAR can both increase protection against pathogens that are resisted through both pathways, and extend protection to a broader spectrum than by either ISR or SAR alone. Moreover, ISR and SAR can reduce pathogen pressure and, thereby, prolong the efficacy of monogenic resistances that tend to be overcome by new races of the pathogen.

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