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Induction of systemic resistance, root colonisation and biocontrol activities of the rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones

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Abstract Quorum sensing regulation, mediated by N-acyl homoserine lactone signals, produced by strain *Serratia plymuthica* HRO-C48 isolated from the rhizosphere of oilseed rape, was found to be responsible for this strain's ability to produce the broad spectrum antibiotic pyrrolnitrin. In this study, we have shown that some other biocontrol-related traits of strain HRO-C48, such as protection of cucumbers against *Pythium apahnidermatum* damping-off disease, induced systemic resistance to *Botrytis cinerea* grey mold in bean and tomato plants, and that colonisation of the rhizosphere also depends on AHL signalling. The results prove that quorum sensing regulation may be generally involved in interactions between plant-associated bacteria, fungal pathogens and host plants.

Keywords Antifungal activity · *Botrytis cinerea* · *Pythium aphanidermatum* · Quorum sensing

Abbreviations

AHLs	N-acyl-homoserine lactones
ISR	induced systemic resistance
PGPR	plant growth-promoting rhizobacteria
QS	quorum sensing
VOCs	volatile organic compounds
BCA	biocontrol agent

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Introduction

Quorum sensing (QS) is employed by numerous bacteria to regulate gene expression in response to cell density using small diffusible signal molecules, *N*-acyl homoserine lactones (AHLs). AHL-mediated QS controls diverse phenotypic traits in plant-associated Gram-negative bacteria. These signals play a central role in ecological interactions of organisms in microbial populations by affecting communication among bacterial populations as well as between bacterial populations and their eukaryotic hosts (Pierson et al. 1998; Loh et al. 2002). AHL signals are synthesised by members of the LuxI family of proteins. At low

population densities, cells produce a basal level of AHL. As cell density increases, AHLs accumulate in the growth medium. On reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor, a LuxR protein, which in turn either activates or represses target gene expression. The *luxI/R* family of genes, encoding synthesis of LuxI synthases and LuxR receptor proteins, are common among Gram-negative bacteria (Waters and Bassler 2005).

Several lines of data demonstrate that the production of secondary metabolites and exoenzymes, which are required for biocontrol activitives such as phenazine and pyrrolnitrin biosynthesis and exoprotease production in many beneficial rhizobacteria, are regulated by QS signalling (Pierson et al. 1998; Chin-A-Woeng et al. 2003; Liu et al. 2007). Evidence has been presented that AHLs are produced by the bacterial consortium that naturally colonises the roots of plants and probably coordinates the functions of the different populations within the rhizosphere community (Steidle et al. 2002).

In the genus *Serratia*, different AHL production profiles and target genes have been described, and many phenotypes are AHL-dependent, such as production of nuclease, lipase, protease and chitinase, swarming motility, biofilm formation, the lipB-secretion system, production of the red pigment prodigiosin, the β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid, and 2,3-butanediol fermentation (van Houdt et al. 2007). Several plant-associated strains of *Serratia plymuthica* were recently described as biocontrol agents (BCAs) of fungal pathogens (de Vleeschauwer and Höfte 2007). AHL signals produced by the root colonisers *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF are important for the ability of these strains to increase systemic resistance of tomato plants against the fungal leaf pathogen, *Alternaria alternata* (Schuhegger et al. 2006); In strain *S. plymuthica* HRO-C48 isolated from the rhizosphere of oilseed rape, AHL signals were shown to be responsible for the ability to produce the antibiotic pyrrolnitrin. The pattern of AHLs produced by this strain has been partially characterised, and the *splI/R* genes, which are the *luxI*/*R* homologues, were isolated and sequenced (Liu et al. 2007)

In this study, we report that besides pyrrolnitrin production, other important biocontrol-related traits of strain HRO-C48, including protection of cucumber against *Pythium aphanidermatum* damping-off disease, induction of systemic resistance to *Botrytis cinerea* in bean and tomato, and root colonisation of bean, are also regulated by AHL-mediated QS signalling. The data indicate that QS provides global regulation of diverse mechanisms that afford plant-beneficial properties of various Gram-negative inhabitants of the rhizosphere.

Materials and methods

Bacterial strains, pathogens, and plants

Strain *S. plymuthica* HRO-C48 was previously selected as a BCA of several phytopathogenic fungi (Kalbe et al. 1996; Kurze et al. 2001). A spontaneous mutant of strain HRO-C48 resistant to rifampicin and its derivatives, including the AHL-4 mutant with miniTn5 insertion into *splI*, and the complemented strain AHL-4/*splI* have been described previously (Liu et al. 2007). These strains were grown in liquid Luria-Bertani broth (LB) supplemented with corresponding antibiotics (rifampicin, 40 mg ml^{-1}; kanamycin, 50 mg ml^{-1}; tetracycline, 20 mg ml^{-1}) for 24 h at 30°C; the pellets were then collected by centrifugation at 10,000 rpm for 10 min and resuspended in tap water. The concentration of the bacterial suspension was adjusted to approximately 10^9 colony-forming units (CFU) ml^{-1} using a hemocytometer. The phytopathogenic isolates *P. aphanidermatum* and *B. cinerea* were from our laboratory collection, and potato dextrose agar (PDA; Difco) was used to cultivate the fungi at 25°C.

The conidia of *B. cinerea* were removed from 1 week-old PDA cultures and suspended in sterile distilled water (SDW). The conidial suspension contained 10^6spores ml^{-1} and was prepared in a water solution of 0.005% (*v*/*v*) Tween 80, 0.01 mol l^{-1} of glucose, and 6.7 mmol l^{-1} of KH_2PO_4 (Meziane et al. 2005). The suspensions were filtered through four layers of sterile cheesecloth in order to remove adhering mycelia. The conidial concentration desired was adjusted prior to use with the aid of a hemocytometer.

Cucumber (*Cucumis sativus* cv. Shennongchun # 3), bean (*Phaeseolus vulgaris* cv. Doujiawang), and tomato (*Lycopersicon esculentum* cv. Maofen802) seeds were surface-sterilised with 1% NaOCl, washed three times with SDW, and incubated at 28°C and

high relative humidity (RH) for 2-3 days. After germination, seeds were soaked in a bacterial suspension of different biocontrol strains or tap water as the control for 1 h and then sown in compost soil in plastic pots (10 cm×10 cm×12 cm); 20 ml of bacterial suspension (ca. 10^9 CFU ml^{-1}) was immediately poured on top of the pot soil. The seedlings were grown under greenhouse conditions at 25°C with a 16 h photoperiod and high RH for further greenhouse experiments.

In vitro antifungal activity

A plate assay for antagonism against the phytopathogenic *P. aphanidermatum* and *B. cinerea in vitro* was carried out. Strain HRO-C48 and its derivatives were grown in LB at 30°C with shaking at 150 rpm. Five mm diameter agar disks from an actively growing fungal culture were seeded at the centre of PDA plates (60 mm diam), and two 3 μl drops of overnight culture of strain HRO-C48 or its derivatives were spotted in a line 3 cm away from the centre of the plate; SDW served as a control. After incubating at 25°C for 3-4 days, the diameter of the inhibition zone was measured.

Biocontrol of cucumber damping-off caused by *P. aphanidermatum*

Before sowing, surface-sterilised and germinated seeds of cucumber were soaked in a suspension (ca. 10^9 CFU ml^{-1}) of wild-type *S. plymuthica* HRO-C48, *SplI*⁻ mutant AHL-4, complemented strain AHL-4/*splI*, or tap water as a control for 1 h, respectively. The treated seeds were sown in plastic pots (10 cm× 10 cm×12 cm) containing five plants in each pot with six pots per treatment; this was followed by drenching the soil with 20 ml of suspension or tap water (as a control). The cucumber seedlings with three leaves were again drenched with 20 ml of bacterial suspension or tap water and challenge-inoculated with *P. aphanidermatum* by applying three fresh mycelial disks (5 mm diam) to the soil in the vicinity of the roots 3 days later. Four days after inoculation, the percentage of cucumber seedlings developing symptoms of damping-off was determined, and the index of disease reduction (IDR) of each treatment was calculated (Ovadis et al. 2004).

Induced systemic resistance to *B. cinerea* on bean and tomato

To determine the induced systemic resistance to *B. cinerea*, germinated bean and tomato seeds were treated with either the suspension of HRO-C48 and its derivatives for 1 h or tap water as a control, followed by sowing the seeds in potting soil and drenching them with 20 ml of bacterial suspension or tap water in the greenhouse. Two week-old bean, and 5 week-old tomato plants were once again treated with bacteria, this time by pouring 20 ml of a bacterial suspension or tap water (as a control) on top of the soil in pots containing five plants per pot, six pots per treatment. Three days later, detached primary leaves of bean or tertiary leaves of tomato plants were challenge-inoculated with *B. cinerea* by pipetting 5 droplets (10 μl) of conidial suspension onto each leaf at a concentration of 10^6 CFU ml^{-1}. The detached leaves were covered with a membrane and kept at 100% RH (Meziane et al. 2005). After incubating at 23°C for 5 days, disease development was evaluated by recording the average lesion diameter.

Population dynamics of HRO-C48 and its derivatives in the bean rhizosphere

To determine the bacterial rhizosphere population dynamics, we randomly selected six bean seedlings, which were seed-treated and root-drenched with a bacterial suspension of HRO-C48 and its derivatives (ca. 10^9 CFU ml^{-1}) or tap water as previously described. The samples were taken at 1, 2, 3, and 4 weeks after planting. Populations of the bacteria were isolated from the rhizosphere according to Siddiqui and Shaukat (2005) with minor modifications. Briefly, root samples with adhering soil were collected and were shaken in a 250 ml sterilised flask containing 30 ml SDW at 150 rpm for 1 h. Ten-fold serial dilutions of the suspension were prepared, and 100 μl aliquots from the appropriate dilutions were plated onto LA supplemented with corresponding antibiotics (40 mg ml^{-1} rifampicin for HRO-C48; 50 mg ml^{-1} kanamycin for AHL-4 and 20 mg ml^{-1} tetracycline for AHL-4/*splI*). After incubation at 30°C for 48 h, the number of CFUs g^{-1} fresh weight of root with adhering soil was determined. No growth was observed after plating suspensions from controls on LA supplemented with rifampicin.

To assess colonisation of the interior of the plant, true leaves, stems, and root sections were surface-sterilised with 1% NaOCl and washed three times with SDW. Surface-sterilised plant tissues were homogenised with a sterilised mortar and pestle in 5.0 ml of 0.01 M $MgSO_4$. Suspensions were dilution-plated onto LA supplemented with corresponding antibiotics (Tran et al. 2007).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Fisher's least significant difference (LSD) and Duncan's multiple-range test to compare treatment mean values. Each trial was repeated at least twice with at least three replicates for each treatment (including the control). Population densities of the tested strains were $\log_{10}$-transformed before statistical analysis.

Results

Antifungal activities by strain HRO-C48 *in vitro* are positively regulated by QS

In a dual culture with strain HRO-C48 and the phytopathogenic *P. aphanidermatum* and *B. cinerea* on PDA plates, direct suppression of mycerial growth of both pathogens was observed. Moreover, the AHL-4 (*splI*::miniTn5) mutant significantly decreased the diameter of inhibition zones against both pathogens (P=0.01), while the inhibition zones formed by the complemented strain AHL-4/*splI* were almost the same as those formed by the parental HRO-C48 (Table 1). This indicates that complementation by the wild-type *splI* gene restored the mutant phenotype to the level of the original strain. *In vitro* bioassays of *P. aphanidermatum* and *B. cinerea* suppression supported and extended the previous observations (Liu et al. 2007) that the antifungal activities by strain HRO-C48 are partially AHL-dependent.

The *splI*-minus mutant AHL-4 is deficient in suppression of cucumber damping-off by *P. aphanidermatum* in the greenhouse

In a dual culture with *S. plymuthica* HRO-C48 and the phytopathogenic *P. aphanidermatum* on PDA plates, direct suppression of mycelial growth of the pathogen was observed, and the strain was shown to protect cucumber from damping-off caused by the oomycete *P. aphanidermatum* in the greenhouse (Ma et al. 2007). Therefore, we used this model to investigate if AHL signals produced by this strain are involved in its biocontrol activity against *P. aphanidermatum* under greenhouse conditions. The *splI*$^-$ mutant AHL-4 was impaired in the biocontrol of cucumber damping-off in comparison to the wild-type. The disease incidences in plants treated with HRO-C48 and AHL-4 were 44.4%±1.9% and 66.7% ±3.3%, respectively, with a significant difference of P= 0.01. However, the disease incidence for the complemented strain AHL-4/*splI* was 46.7%±3.3%; there was therefore no significant difference to the wild-type (Fig. 1). Compared with a 93.3%±3.3% incidence of disease control, the IDR of the wild-type, the mutant and the complementary strain was 52.38%, 28.57%, and 50%, respectively. These results indicate that AHL signalling plays a role in the biocontrol ability of *S. plymuthica* in the cucumber—*P. aphanidermatum* host-pathogen system.

Induced systemic resistance in both bean and tomato depends on AHL signalling

True leaves of 2 week-old bean and 5 week-old tomato plants raised from seeds treated with HRO-

Table 1 Antifungal activities by *Serratia plymuthica* HRO-C48 and its derivatives *in vitro*

Phenotypes	Strains		
	HRO-C48	AHL-4 (*splI*$^-$)	AHL-4/ *splI*
Suppression of *P. aphanidermatum* (mm)*	16.5±0.7a**	12.2±0.7 b	16.0±0.6a
Suppression of *B. cinerea* (mm)*	20.3±0.5a	16.7±0.4b	20.5±0.5a

* Diameter of inhibition zone on PDA plates in dual culture

**Different letters in the same line indicate significant differences at P=0.01 by one-way ANOVA test

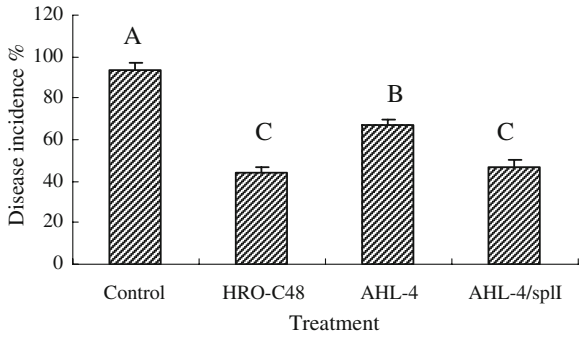

Fig. 1 Effects of *Serratia plymuthica* HRO-C48 and its derivatives on cucumber damping-off caused by *Pythium aphanidermatum* under greenhouse conditions. Different letters indicate significant differences at $P=0.01$. Control–treatment with water

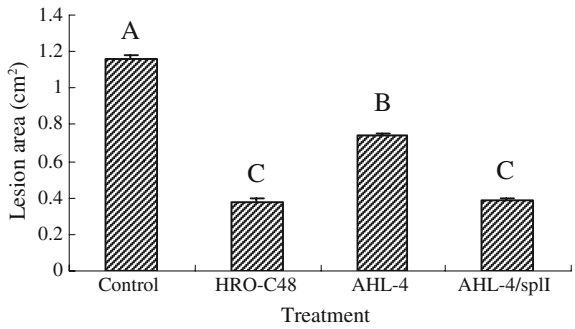

Fig. 3 ISR to *Botrytis cinerea* in tomato by HRO-C48 and its derivatives. Different letters indicate significant differences at $P=0.01$. Control–treatment with water

C48 and its derivatives were challenge-inoculated with a conidial suspension of *B. cinerea.* The bacteria and the challenging pathogen remained spatially separated to exclude direct interactions. Lesion areas of leaves were assessed 5 days after inoculation. The results showed that bean and tomato treated with the wild type HRO-C48 had significantly reduced lesion areas at $P=0.05$ compared with other treatments (Fig. 2) in bean, and $P=0.01$ in tomato (Fig. 3), respectively, whereas the mutant AHL-4 reduced lesion areas to values that were intermediate between the control and the wild-type treatments. Almost no difference between the complemented strain AHL-4/*splI* and the wild-type was observed. These data reveal that AHL signalling is required to induce systemic resistance to grey mould caused by *B. cinerea* in bean and tomato.

Disrupting *splI* in strain HRO-C48 affects bean root colonisation

We used bean plants as a model to investigate the role of AHL signalling in the colonisation of the rhizosphere by comparing the population dynamics of the wild-type HRO-C48, the mutant AHL-4, and the complemented strain AHL-4/*splI*. All three strains colonised the rhizosphere (Fig. 4), but none of them could be recovered from the interior tissue of roots, stems, or leaves after they were surface-sterilised and homogenised in 0.01 M $MgSO_4$, followed by plating serial dilutions. This indicates that strain HRO-C48 was not maintained in bean as an endophyte. From 1 to 4 weeks after planting, the wild-type HRO-C48 established a significantly higher population density in bean rhizospheres than the mutant AHL-4

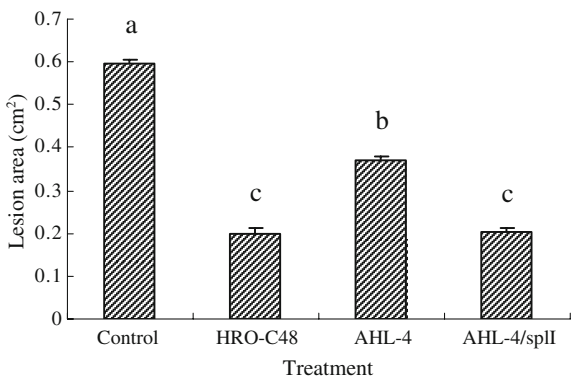

Fig. 2 ISR to *Botrytis cinerea* in bean by HRO-C48 and its derivatives. Different letters indicate significant differences at $P=0.05$. Control–treatment with water

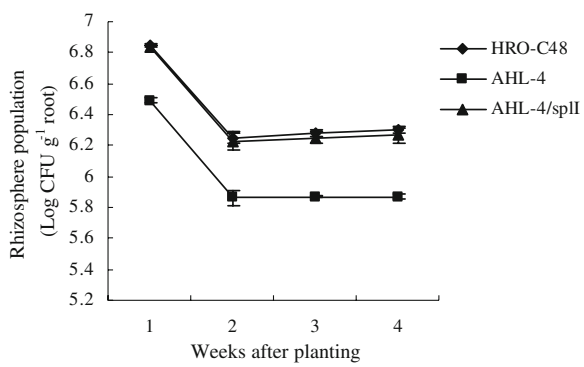

Fig. 4 Rhizosphere colonisation by HRO-C48 and its derivatives in bean Ca. 10^9 CFU ml^{-1} was applied at inoculation time for all strains

(P=0.05), while no difference was observed between the wild-type and complemented strain AHL-4/*splI* (Fig. 4). The results demonstrate that disruption of AHL signalling in HRO-C48 significantly reduced its colonisation ability in the bean rhizosphere.

Discussion

AHL-mediated QS systems have been identified in a broad range of plant-associated bacteria and regulate traits that are involved in symbiotic, pathogenic, and surface-associated relationships between microbial populations and their plant hosts (Pierson et al. 1998; Loh et al. 2002; Persello-Carteaux et al. 2003; Schuhegger et al. 2006). Bacterial infection of plants often depends on the exchange of QS signals between nearby bacterial cells. Plants, in turn, can perceive and often specifically respond to AHL molecules; examples include altering root exudation from pea seedlings, which interfere with QS regulation in the bacteria; significant change in the accumulation of proteins in *Medicago truncatula*, or the expression of plant defence genes in tomato (Bauer et al. 2004; Schuhegger et al. 2006; van Loon 2007). In plant-microbe interactions, the bacterial traits activated by AHLs can be beneficial or harmful to hosts; thus, identification of regulated function is of great interest (Fekete et al. 2007).

Serratia plymuthica is a ubiquitous bacterium that has been preferentially recovered from rhizospheres all over the world, both as a free-living and endophytic organism, and the development of bio-control products based on the genus *Serratia* is now gaining momentum (de Vleeschauwer and Höfte 2007). In this study, *S. plymuthica* HRO-C48 inhibited mycelial growth of *P. aphanidermatum* in dual cultures on PDA plates, which reveals the direct antagonism involved in the biocontrol of cucumber damping-off by HRO-C48. It is still not clear, however, which compounds produced by HRO-C48 are responsible for this effect. Prn is not the major factor involved in the control of the *Pythium* species (Sarniguet et al. 1995; Heungens and Parke 2000). Kai et al. (2007) demonstrated that small volatile organic compounds (VOCs) emitted from strain HRO-C48 negatively influence the mycelial growth of the soil-borne phytopathogenic fungus *Rhizoctonia solani*. Whether or not these VOCs play a role in suppression of *P. aphanidermatum* remain to be investigated.

Several *S. plymuthica* strains are endophytes (Benhamou et al. 2000; Berg 2000; Berg et al. 2005). The endophytic *S. plymuthica* R1GC4 reduced root rot in cucumber caused by *P. aphanidermatum* (McCullagh et al. 1996). Benhamou et al. (2000) demonstrated that strain R1GC4 stimulated defence reactions in cucumber seedlings inoculated with *P. ultimum*; cucumber plants treated with R1GC4 reacted more rapidly and more efficiently to *Pythium* attack through the formation of physical and chemical barriers at sites of potential fungal entry, meaning the mechanism of induced resistance in cucumber seedlings was activated. We were unable to recover strain HRO-C48 from the interior of surface-sterilised tissue in beans, suggesting that it was not an endophytic strain. In addition, we suggest that the mechanism of induced resistance could also be involved in the biocontrol activity of strain HRO-C48 against *P. aphanidermatum* under greenhouse conditions such as that observed in *S. plymuthica* strain R1GC4.

We have previously observed that production of chitinases by HRO-C48 is positively regulated by the QS system (Liu et al. 2004). Chitinases produced by this strain (Frankowski et al. 2001) are obviously not involved in the suppression of this fungus-like oomycete, which is unique in that its cell wall contains β-(1,3)-(1,6)-D-glucan and cellulose instead of chitin as the major structural components (Chet and Chernin 2002). It is possible, however, that oligomers produced by chitinases to lyse the cell walls of other fungi or arthropods in soil elicit induced resistance to cucumber damping-off (Navazio et al. 2007); the biocontrol activity of HRO-C48 against *P. aphanidermatum* might therefore be down-regulated by QS due to a reduction of chitinolytic activity.

We have characterised the *splI/R* genes in strain HRO-C48, which are responsible for synthesising the dominant signal molecule OHHL. The background level of the AHL signal in AHL-4 (Liu et al. 2007) could be the result of the presence of the second SpsRI QS system operating in strain HRO-C48, which we have recently cloned (GenBank accession No. EU730586). The signal encoded by this system and its role, and the relationship between these two QS systems in strain activities are now under investigation.

In the present study, we continued our previous observation that AHL signalling is important for the expression of biocontrol-related traits of strain HRO-C48, and have shown that the AHL-deficient mutant of this strain was less effective, not only in the production of the antibiotic pyrrolnitrin (Liu et al. 2007), but also in the suppression of cucumber damping-off caused by *P. aphanidermatum*. It was also less effective in its ability to induce systemic resistance against *B. cinerea* in tomato and bean and to colonise roots in bean, as compared to the wild-type under greenhouse conditions. Together, these data showed that AHL signalling plays a key role in global regulation of the interactions between the rhizobacterium HRO-C48, pathogens, and host plants.

Induce systemic resistance (ISR) elicited by plant growth-promoting rhizobacteria (PGPR) overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal resistance that depends on the signalling compounds jasmonic acid (JA) and salicylic acid (SA), respectively, and pathogens are differentially sensitive to the resistances activated by each of these signalling pathways (van Loon 2007). Schuhegger et al. (2006) first reported that AHL signals produced by *S. liquefaciens* MG1 and *Pseudomonas putida* IsoF in the rhizosphere increase systemic resistance of tomato plants against the fungal leaf pathogen, *Alternaria alternata,* and systemically induce SA—and ethylene (ET)-dependent defence genes. The rhizobacterium HRO-C48 and the challenging pathogen remained spatially separated; although suppression of *B. cinerea* by HRO-C48 on PDA plates was observed, direct antagonism can be ruled out. Our investigations further confirmed the role of AHLs in ISR against leaf pathogenic fungi other than *A. alternata* in tomato, but whether the signalling pathway involved in ISR mediated by HRO-C48 is dependent on SA or JA/ET is still unknown. It was recently reported (de Vleeschauwer and Höfte 2007) that systemic resistance induced by *S. plymuthica* IC1270 in rice is most likely due to its capacity to modulate the plant's oxidative machinery, accompanied by the generation of reactive oxygen species (ROS). However, the biochemical and cytological mechanisms involved in the ISR by strain HRO-C48 remain to be explored.

Colonisation of plant roots by rhizobacteria plays an essential role in biological control and growth promotion. The role of antibiotic production for successful colonisation has been demonstrated (Persello-Carteaux et al. 2003; Yan et al. 2003). AHLs might influence root colonisation by C48 via regulation of antibiotic production, because these compounds are critical in the competition with other microbes when substrate availability decreases (Landa et al. 2002).

Taken together, the multiple traits related to biological control in *S. plymuthica* HRO-C48 are partially dependent on AHL signalling. QS regulates the beneficial interactions between HRO-C48, phyto-pathogens, and host plants. Further studies of this regulation in the future may open up new ways to improve the biocontrol ability of rhizobacteria via manipulating the QS pathway.

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