SUPPRESSION OF INCIDENCE OF *RHIZOCTONIA SOLANI* IN RICE BY SIDEROPHORE PRODUCING RHIZOBACTERIAL STRAINS BASED ON COMPETITION FOR IRON

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Abstract

Rice is a major crop in much of the developing world, where disease management using agrochemicals is not economically practical, nor environmentally safe. The identification of biocontrol agents therefore presents a useful alternative. Here, we screened bacterial strains isolated

from the rhizosphere of rice plants, and identified a number of these that exhibit antagonistic activity towards the fungal pathogen, *Rhizoctonia solani*, the causative agent of rice sheath blight disease. Correlation analysis with different metabolites produced by these bacteria revealed that antagonism was strongly correlated with the quantity of siderophores produced by individual strains, and was increased under iron-limiting conditions. Selected high-siderophore-producing strains were found to promote the growth of rice plants, possibly via the solubilisation of soil phosphates, nitrogen fixation and the production of phytohormones. These same PGPR also conferred resistance against sheath blight disease, which resulted in significant yield increases in infected plants. A consortium of the selected strains was especially effective in both growth promotion and disease suppression, and generally performed better than treatment with the fungicide, benlate. Molecular analysis indicated that the PGPR strains tested enhance plant defence gene expression, and may therefore activate induced systemic resistance in rice. Our work has identified a series of rhizobacterial strains able to promote plant growth and provide effective resistance against sheath blight disease in rice and which therefore have potential for application as biocontrol agents in agriculture.

Keywords: Sheath blight, siderophore, competition, iron, biocontrol

Introduction

Rice is the most widely cultivated food crop worldwide, whose production is constrained by diseases of fungal, viral and bacterial origin. Among the fungal diseases of rice, sheath blight caused by *Rhizoctonia solani* kühn is one of the most destructive diseases causing losses of around 5% to 50% in severe cases (Saikia *et al.,* 2006). Crop loss assessment studies have revealed that this disease reduces grain yield to varying levels depending upon the stage of crop, degree of cultivar susceptibility and to a great extent, the conduciveness of the environment in which it occurs. The severity and significance of damage caused by this disease has necessitated the development of strategies to control and manage the disease, so as to reduce the crop loss and to avert epidemics (Slaton *et al.,* 2003).

Seed treatment, soil application and foliar sprays with systemic fungicides and antibiotics have given effective control of sheath blight (Kazempour, 2004). However, there are no economically viable and environmentally safe control measures presently available to manage the disease. Moreover, no fully resistant rice varieties are known, with partial resistance/tolerance to sheath blight identified in some lines being attributed to multiple genes (Che *et al.,* 2003). In this context, sheath blight management through biological control agents that are easy to handle, cost

effective and environmentally-friendly is gaining worldwide attention of researchers as an alternative strategy. In the past few years, plant growth-promoting rhizobacteria (*PGPR*) have drawn considerable interest due to their plant growth-promoting and biocontrol activities. PGPR are able to promote plant growth through the mobilisation of nutrients and the biosynthesis or modification of phytohormones (Zahir *et al.,* 2004). Their ability to control plant disease may either be as result of direct antagonism of the growth of pathogenic microorganisms via the production of metabolites such as antibiotics, siderophores (iron-chelating compounds), HCN and hydrolytic enzymes, or an indirect affect of inducing host plant resistance mechanisms. This latter effect is generally known as induced systemic resistance (ISR; Van Loon *et al.,* 1998).

In natural environmental conditions combat between soil microbes and pathogenic fungi is mainly based on competition for iron (Naureen *et al.*, 2005). Root associated plant growth promoting bacteria secrete siderophores under iron limiting conditions which have a high affinity towards iron and thus can deprive their fungal competitors of iron (Naureen *et al.*, 2009). Based on this fact we hypothesized that rhizobacterial strains producing high quantities of siderophore will provide efficient biological control against fungal pathogen *R. solani* which dwells rice rhizosphere in form of sclerotia and germinates when finds suitable environmental conditions and optimum iron supply. We also proposed that these bacteria may play a role in inducing expression of defence genes in rice thus playing a role in combat against the pathogen. The present study reports evaluation of PGPR strains based on quantitative estimation of siderophore production; suppression of sheath blight disease; enhancement of plant growth and induction of defence genes in rice.

Materials and methods

Isolation and collection of bacterial and fungal isolates

Bacterial isolates and growth conditions

A total of 63 bacterial strains were used in this study. Twenty two of these bacterial strains, previously isolated from rhizosphere and rhizoplane of maize and rice plants, were obtained from the BIRCEN culture collection, Plant microbiology division, NIBGE, Faisalabad, Pakistan. All other strains were isolated from the rhizosphere of the rice variety *Super Basmati*, cultivated in saline soils near Shorkot, Pakistan, some of which were previously identified by 16S rDNA sequencing (Naureen *et al.*, 2009).

Isolation of rhizoctonia solani

The rice plants showing symptoms of sheath blight were collected from the fields of the nuclear institute for agriculture and biology, faisalabad,

pakistan. Infected parts of rice stem and leaves were cut into small pieces of 3-4 mm and surface sterilized with 5% sodium hypochlorite. The pieces were placed on petri dishes containing potato-dextrose-agar (PDA, Difco) and incubated at 28ºC for 72 hrs. The emerging colonies of *R. solani* cultures were purified by subsequent sub culturing and identified as belonging to anastomosis group AG1-1a based on morphology (Sneh *et al.,* 1991). Sclerotia of uniform size were harvested from the pure cultures of selected *R. solani* isolates cultivated on pda medium and preserved at 4ºC (Bashar *et al*., 2010).

Pathogenicity of *R. solani*

Pathogenicity tests for isolated fungal strains were performed in green house facility of national institute for biotechnology and genetic engineering (NIBGE). Sclerotia (1 mm diameter) of a 1-week-old culture of *R. solani* isolates were placed (two-three per tiller) on the sheaths of thirty days old rice seedlings. The inoculated parts of the plants were covered with absorbent cotton and tied with parafilm. Sheaths were maintained at high humidity by regularly moistening the cotton with sterile water. *R. solani* isolates which produced typical symptoms of sheath blight were selected and re-isolated from the infected plants.

In a separate experiment sclerotia (1 mm diameter) from 1-week-old culture of *R. solani* isolates were placed at the rate of 1g/kg soil in autoclaved clay loam soil. Batches of soil were infested separately with each isolate. Infested soil was dispensed in 15-cm-diam. Clay pots and these were planted with 10 seedlings per pot (thirty days old rice seedlings of *Super Basmati* variety). Pots were randomly distributed on a greenhouse bench under a temperature 28±3°c. Benches were covered with a plastic sheet and high humidity was maintained by regular watering the plants and their sheaths. Symptoms of sheath blight were recorded after 7 days and *R. solani* isolates which produced typical symptoms of sheath blight were selected and re-isolated from the infected plants.

Characterization of bacterial strains

All of the bacterial strains were screened for the production of secondary metabolites such as siderophores, extracellular hydrolytic enzymes, diffusible and volatile antibiotics and HCN as reported previously (Naureen et al., 2009).

Siderophore production and quantification

Bacterial strains exhibiting siderophore production on universal chrome azurol ‘s’ (CAS) medium were selected for quantification of siderophores. CAS plates were spread with overnight grown cultures and

incubated at 28±2°c for 72 hours (Schwyn and Neilands, 1987; Sharma and Johri, 2002). Amount of siderophores produced by bacterial strains was quantified according to Meyer and Abdallah (1978). Quantification of siderophores was based on the extinction coefficient of culture supernatants at 400 nm and ph 7.0. In brief, the culture broth was inoculated with actively growing culture (12 h in King's 'B') and grown in standard succinate broth at 28 ± 2ºC for 48 -72 hrs under shaking (120 rpm). Aliquots of culture broth were withdrawn and centrifuged at 10,000 rpm (sigma 3K30) for 10 min. Absorbance of the supernatant was read at 400 nm on a beckman du 640 spectrophotometer and extinction coefficient was determined.

Bioantagonistic activity of bacterial strains

In vitro inhibition of R. solani in different media

To check the effect of availability and non-availability of iron on antagonistic activity of bacteria against *R. solani* several dual culture assays were performed using different media including PDA, standard succinate medium (iron free), Nutrient agar, LB agar and specially designed iron-free medium (IFM; 0.02 % $MgSO_4$, 1 gL^{-1} of K_2HPO_4 and (NH_4) H_2PO_4, 5 gL^{-1} of NaCl, 5% peptone and 1.5% bacteriological agar) supplemented with different iron concentrations.

We performed dual culture assay by four different methods and observed best results in case of disc circle method which is described as under.

A 10 mm disk of a pure culture of *R. solani* grown on potato dextrose agar (PDA) was placed at the centre of a petri dish containing different media as mentioned above. A circular inoculum, made with a 6 cm diameter petri dish dipped in a suspension of bacteria ($5x10^9$-cfu mL^{-1}), was placed surrounding the fungal culture. In control plates circular bacterial inoculum was replaced by (a) autoclaved distilled water and (b) 1000 ppm solution of fungicide benlate. Plates were cultured for 72 hrs at 28 ± 2ºC and inhibition of pathogen growth calculated as under.

% inhibition = {1- (mean fungal growth on test plate/ mean fungal growth on control plate)} x 100.

Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times.

Effects of bacteria on plant growth

Bacterial strains selected as a result of antagonistic assays were then checked for their any positive or negative effects on plant growth and health. Seeds of rice variety *Super Basmati* were immersed in a suspension of the antagonistic bacteria ($5x10^9$ cfu ml^{-1}) for 5 minutes (Raupach and Kloepper, 1998). Sterile distilled water was used as a control. Once treated, seeds were

germinated in autoclaved soil in 20 cm pots. Seedlings were maintained for 60 days under glasshouse conditions at 25-28ºC with daily watering and addition of half of the recommended dose of nitrogen and phosphorus fertilizers once a month. Plant height, number of leaves, fresh weights were measured 30 days after planting. Numbers of tillers were recorded 60 days after planting. Experiments were repeated three times.

In vivo inhibition of rhizoctonia solani

Rice seeds (var. *Super Basmati*) were sterilised and germinated on moist filter paper in sterile petri dishes. Ten days after germination, the seedlings were transplanted into small trays containing autoclaved soil and grown for a further 20 days. Seedlings were then transferred to 40 cm pots containing autoclaved soil and grown for another 30 days in a mesh-house under natural conditions at an average temperature of 30ºC during the day and 25ºC at night. The experiment was carried out in a completely randomized block design with six replications for each treatment. Parallel experiments were performed in which plants were grown in autoclaved sand rather than soil. Plants were grown in same conditions, watered regularly with autoclaved water and were given ¼ strength Hoagland's solution for nutritional requirements. Each experiment was repeated three times.

Bacterial inoculation of rice seedlings

For inoculation of plants, bacterial cultures were grown in lb broth for 24-48 hrs at 28 ± 2ºC. Cell pellets were obtained by centrifugation at 6000 x *g* for 5 min, washed and resuspended in sterile water. Thirty days after transplantation of seedlings into pots, the soil adjacent to the roots was inoculated with 10 mL of bacterial suspension (10^8 - 10^9 cells/mL) and left for seven days under the same growth conditions. Plants growing in uninoculated soil served as controls while plants grown in soil treated with 1000 ppm of the fungicide benlate in distilled water were used to compare the efficiency of bacteria in controlling sheath blight.

Challenge inoculation

Thirty seven days after transplanting, sclerotia from a 1-week-old culture (1 mm diameter) of *R. solani* were placed (two per tiller) on the sheaths of the rice plants in order to provide maximum chance to the pathogen to cause disease. The inoculated parts of the plants were covered with absorbent cotton and tied with parafilm. Sheaths were maintained at high humidity by regularly moistening the cotton with sterile water. Moreover plants were kept in a relative humidity above 90 % for 3 days to allow the sclerotia to germinate. Symptom development was observed after 7

days and graded on a 0 to 5 point scale based on lesion size (Sriram et al. 1997). The disease index was calculated by using the formula:

Disease index = (total grade points/number of sheaths observed).

After initial disease scoring, plants were kept under normal greenhouse conditions for another period of 45 days before being scored again for disease index and determination of percentage tiller mortality. Total biomass, harvest index (harvest index = (grain yield/biomass) x 100), straw and grain yield (dry mass) were calculated at the time of harvesting.

Gene expression analysis

Leaf samples from rice plants grown in soil were collected after one week of challenge inoculation with *R. solani*. The samples were homogenized with liquid nitrogen and 1 g of powdered sample was used to extract total RNA using the SV40 total RNAisolation system (Promega). Extracted RNAwas checked for purity on 1.2% agarose gels prepared in 0.5 x tris borate EDTA buffer. For cdna synthesis, extracted RNA was treated with DNase in a mixture containing total RNA(75 ng), 1 x DNase buffer (2 µL), 0.5 units DNase I and DEPC-treated water. This mixture was incubated at 37ºC for 15 minutes and then at 65ºC for 10 minutes to inactivate the dnase. The reaction mixture was then transferred to ice and to this was added oligo dt primers (10 µL) and 5 x rtase buffer (10 µL). This was subsequently incubated at 70ºC for 5 minutes and then transferred to ice. Finally, to this mixture were added 10mm dNTP mix (5 µL), 0.1 m DTT (5 µL) and superscript ii reverse transcriptase (1 µL; invitrogen, uk) and then incubated at 37ºC for 1 hour and 92ºC for 2 minutes. PCR reaction mixtures contained in a 50 µL volume, 2 µL red taq dna polymerase (sigma, uk), 1 µL of 10 mm dntps, 5 µL of 10x pcr buffer, 2 µL cDNA and 1 µL each of 50 µM forward and reverse primers (table 1; operon technology) and underwent 35 rounds of temperature cycling (94°c for 2 min, 94°c for 30 seconds, and 55 for 30 seconds). This was followed by incubation at 72°c for 1 min. Products were detected using agarose gel electrophoresis.

Ephipytic and rhizospheric colonization of rice variety *Super Basmati* by bacteria

Leaf and root apex samples from rice plants grown in sand inoculated with different bacterial strains and consortium were taken 7, 25 and 45 days after inoculation to check for ephiphytic and rhizospheric colonization of bacteria. Leaf and root extracts were made and serial dilutions of 10^{-3}, 10^{-5} and 10^{-7} were prepared. For both types of samples, 0.1 ml of each dilution was spread on Luria Bertani (LB) agar and incubated at 28 ± 2ºC for 48-72 h or until colony development was observed. The isolated bacterial strains were checked for gram staining and homology with the inoculated bacterial

strains. These were also checked on cas medium to confirm siderophore production.

Statistical analyses

Plant growth and yield data were analyzed using duncan's multiple range tests. Disease indices were analysed using single factor ANOVA with tukey's multiple comparison post-test. Correlation between siderophore production and antagonism was assessed using pearson's correlation coefficient.

Results

A total of 63 bacterial strains isolated from the rhizosphere and rhizoplane of rice and maize plants (taken from the BIRCEN culture collection NIBGE) were screened for siderophore production using the cas assay and bioantagonism against *R. solani* by dual culture methods. From these, 20 bacterial isolates that showed siderophore production and were able to bioantagonise *R. solani* in preliminary experiments were selected and quantitative assays of siderophore production performed (table 2). We collected isolates of the sheath blight pathogen *R. solani* ag1-1a from infected rice plants and tested whether the selected siderophore-producing bacterial strains showed *in vitro* antagonistic activity against the fungus using a dual culture assay technique. Many of the bacterial strains were found to significantly inhibit fungal growth initially, but most of them lost their activity over a period of 21 days (table 2, fig 5). No physical contact was observed between any of the bacteria tested and *R. solani.* The bacteria *Aeromonas hydrophila* BPS10, *Bacillus cereus* Z2-7, and *Enterobacter sp*. B41 strain SPR7, which were found to produce high quantities of siderophores, remained antagonistic at a range of 45% to 100% even after 28 days of assay. The high siderophore producers SPR7, Z2-7 and BPS10, along with *Enterobacter sp*. BPS12 which produced a much lower quantity of siderophores and did not show antagonistic activity, were selected for further experiments.

To test the hypothesis that siderophores producing bacteria could contribute to antagonism based on competition for iron, dual culture assays were carried out on a range of media with different nutrient levels, including iron-free medium (ifm) supplemented with different iron concentrations. In general, bacterial isolates showed highest antagonistic activity on media with low iron concentration (ifm and ssm), and antagonistic activity decreased with increasing iron concentration in supplemented ifm (table 3).

Effect of bacterial strains on plant

To determine whether any plant growth promotion or deleterious effects were provided by the bacteria; seeds were immersed in broth cultures of selected isolates and then germinated in small pots containing autoclaved soil. We measured the performance of the inoculated seedlings after 30-60 days. Most of the individual strains significantly increased the mean fresh weights, number of tillers and shoot height of seedlings, and when applied in a consortium, the four strains tested produced an even greater positive effect on plant growth (table 4).

Disease suppression in plants

The selected bacterial strains and a consortium of all four were tested for suppression of rice sheath blight in 67-day-old rice plants of the variety *Super Basmati* grown in a net house facility in natural conditions during the rice growing season in pakistan. When bacterial cultures were applied to the soil, BPS12, Z2-7, SPR7 and BPS10 were observed to suppress the incidence of sheath blight disease to a range of 32% to 100% relative to controls seven days post-inoculation (fig. 1). Plants that were inoculated with a consortium of these four bacterial strains also showed high resistance to disease. Interestingly, plants grown in soil inoculated with fungicide benlate also showed high disease resistance indicating a possible role of benlate in inducing resistance in rice against sheath blight.

After initial scoring for disease, plants were kept for a further period of 45 days under normal growth conditions to assess the longer-term impact of PGPR on disease. Although disease developed further over the next 45 days (disease indices rose for all experimental groups), plants inoculated with the high siderophore-producing bacterial strain still showed much more resistance than the control ($p < 0.001$ by one-way anova), and plants treated with *bacillus cereus* Z2-7 or *aeromonas hydrophilla* BPS10, performed similarly to those treated with benlate (fig. 2). Moreover, plants treated with *Enterobacter sp*. B41 strain SPR7 or with the consortium of bacterial strains were almost completely protected against the disease. Similar results were observed for rice plants grown in autoclaved sand (figs. 1 & 2). In addition to the reduction in disease symptoms on treated plants, PGPR treatments also resulted in similar decreases in tiller mortality in both soil- and sand-grown plants (fig. 3)

To determine the impact on the observed suppression of sheath blight disease on productivity, yield data were also recorded from the infected plants. As shown in tables 5 and 6, the suppression of disease symptoms by *b. Cereus* Z2-7, *Enterobacter* b41 strain SPR7 and the consortium of bacteria coincided with increased plant biomass, grain and straw yield and harvest index. The application of the bacterial consortium had especially positive

effects on all yield variables measured. In general, plants inoculated with PGPR out-performed both control and fungicide (benlate)-treated plants.

Effects of PGPR on defence gene expression

To gain a more detailed understanding of the mechanism of disease suppression in sheath blight-infected plants grown in the presence of PGPR, we examined the expression of three defence-related genes from rice in control and PGPR-inoculated plants infected with sheath blight.RNAsamples were collected at the time of disease index estimations and the expression of genes encoding a β-1,3-glucanase, a chitinase and a peroxidase, along with an actin control, was assayed by rt-pcr. All three defence genes were up-regulated following challenge with *R. solani* in plants not treated with PGPR, confirming their pathogen-responsiveness (data not shown). Enhanced levels of mrnas for some of these genes could be detected in disease-inoculated plants grown in soils containing PGPR (fig. 4). Expression of chitinase iib was particularly high 7 days after pathogen challenge in plants treated with SPR7, BPS10 and the bacterial consortium, suggesting that these PGPR may have enhanced plant defences against the sheath blight pathogen. Expression of peroxidise pox22.3 on the other hand, appears to be increased by all bacterial treatments at seven days post-infection relative to control plants without PGPR. Interestingly, however, treatment with benlate fungicide also appears to promote a similar increase in pox22.3 mrna levels. Expression of the β-1,3-glucanase that we assayed in these experiments was not significantly affected by benlate or PGPR treatments.

Discussion

Biological control with rhizobacteria offers a simple and cost-effective strategy for managing soil-borne diseases. There are various modes of actions of biocontrol bacteria, such as antibiosis, competition for iron through production of siderophores (Duffy and Défago, 1999), parasitism that may involve production of extracellular enzymes, (for example, chitinases and proteases that can lyse pathogen cell walls), and induction of plant resistance mechanisms (Whipps, 2001).

Siderophore production is an important property of practical utility for some biocontrol agents, and plays an important role in plant growth promotion (Katiyar and Goel, 2004), phytopathogen antagonism and induction of systemic resistance (Leong, 1986; Sturz and Christie, 2003; Bais *et al.,* 2004). In the present study, we selected those rhizobacterial strains that produced higher quantities of siderophores and evaluated their efficiency in plant growth promotion and the management of sheath blight disease in the rice variety *Super Basmati* . Our results indicated a strong link between siderophore production and fungal antagonism *in vitro*. Statistical analysis

indicated a significant association between antagonistic activity and siderophore production by the bacteria (table 2). It should be noted, however, that this correlation appears to be driven by the three high siderophore-producing strains, since no correlation between siderophore quantity and antagonism was observed at any time point when these strains were removed from the analysis (fig 5; table 2). The high siderophore-producing bacterial strain *Enterobacter sp.* B41 strain SPR7 was especially effective against the sheath blight pathogen, *R. solani*. In addition to the production of a high quantity of siderophores, this bacterium was also found to produce volatile and diffusible antibiotics and HCN (Naureen *et al*., 2009), and this may be related to the strong antagonistic behaviour of this bacterium. Similarly, bacterial strains *Bacillus cereus* Z2-7 and *Aeromonas hydrophilla* BPS10 showed varying levels of antagonism against *R. solani* which might be due to the production of siderophores, chitinases and diffusible antibiotics by these strains (Naureen *et al*., 2009). This is consistent with the findings of other workers who have previously found the production of chitinases (Velazhahan *et al.,* 1999), β-1,3-glucanase, HCN and salicylic acid (Nagarajkumar *et al*., 2004) to be associated with antagonism of *R. solani* by *Pseudomonas fluorescens.* Despite this, in the diverse range of bacterial species that we have tested here, only siderophore production showed a statistically significant correlation with fungal antagonism.

Iron is of fundamental importance for microbial aerobic respiration. Microorganisms that are able to produce siderophores in low iron conditions have a competitive advantage over those that do not produce siderophores. Subsequent dual culture assays performed on a range of media with controlled iron concentrations provided further evidence for the importance of siderophores in bioantagonism with our strains. Inhibition of growth of *R. solani* was maximal in iron-limiting conditions, in which siderophore production would be expected to be highest. In rich media, when competition for iron is less important, antagonism was reduced. This is in contrast to the findings of Montealegre *et al.,* (2003) who observed no difference in bioantagonistic activity of different *bacillus* species with increase in iron concentration. Moreover, it is interesting to note that the low-siderophore-producing bacterial isolate *Enterobacter sp.* BPS12 (which had originally shown no bioantagonistic activity on pda media) was found to antagonise *R. solani* on media with no or low iron concentrations, again indicating competition for iron as a major component of antagonism.

Those strains exhibiting maximal bioantagonistic activity against *R. solani* also provided protection against sheath blight disease when inoculated onto the roots of rice plants. A number of groups have isolated PGPR capable of generating resistance against sheath blight disease (*e.g.* Gnanamanickam *et al.,* 1992; Velazhahan *et al.,* 1999; Nandakumar *et al.,*

2001a; Rangarajan *et al.,* 2003), but to our knowledge, the almost complete absence of disease afforded here by *Enterobacter sp.* B41 SPR7 has not previously been observed using PGPR as biocontrol agents. The other bacterial strains, *Bacillus cereus* Z2-7 and *Aeromonas hydrophilla* BPS10, also provided protection against sheath blight, but were less effective than SPR7. This may reflect either a reduced biocontrol capacity and/or a lower persistence in the rhizosphere.

Many factors contribute to the performance of bacterial inoculants and key amongst these is colonization. If the introduced strain does not survive and colonize, it will not be effective in promoting plant growth and biocontrol activity (Goddard *et al.,* 2001). In the present study, all of the bacterial strains were found to colonize well in the rhizosphere. *Enterobacter sp*. B41 strain SPR7 and *bacillus cereus* Z2-7 were found to persist longer than the other two strains, and this might be the reason for the better performance of SPR7 and Z2-7 in terms of biocontrol and plant health. Siderophore production may also be responsible for better colonization of these strains as the soil iron concentration during the experiment was as low as 5 ppm. This better colonization of bacterial strains SPR7 and Z2-7 was in turn associated with the increased disease suppression and improved plant growth by these strains.

Our preliminary gene expression assays indicate that at least part of the mechanism for disease resistance in plants inoculated with PGPR is induced systemic resistance. We observed enhanced expression of chitinase iib and the peroxidase pox22.3 in response to blight inoculation in plants previously inoculated with PGPR, especially in the case of strains SPR7, BPS10 and the consortium, which also promoted the highest levels of resistance. This enhanced gene expression may reflect the 'primed' state induced by isr. Enhanced expression of chitinases and peroxidases has also been suggested previously to be important in PGPR-induced isr against sheath blight (Nandakumar *et al.,* 2001b; Radjacommare *et al.,* 2004). Our results are somewhat complicated by the fact that treatment with the fungicide benlate also appears to increase pox22.3 expression to a similar extent to the PGPR compared with the control, which at first sight does not support the conclusion that the bacteria contribute directly to increased gene expression. However, there are other examples in the literature in which fungicides also promote plant defence responses as well as acting directly against the pathogenic organism (Herms *et al.,* 2002; Pasquer *et al.,* 2005; Andreu *et al.,* 2006). That disease resistance is a consequence of isr rather than a direct interaction between PGPR and pathogen is supported by two additional lines of evidence. First, no epiphytic colonisation by PGPR was detected, and second, resistance was observed in plants inoculated with *Enterobacter sp.* BPS12, which was unable to inhibit fungal growth *in vitro*.

Previous workers have identified siderophores as molecular determinants of isr in several other systems, including radish (Leeman *et al.,* 1996), tomato (Audenaert *et al.,* 2002) and *Arabidopsis* (Meziane *et al.,* 2005). Although we also found a good relationship between siderophore production and disease resistance, it is important to acknowledge that we did not test for any causal link between siderophore production and pathogen resistance.

As might be expected, the reduction of disease by PGPR treatments was accompanied by increases in plant growth and yield. However, growth measurements on un-infected plants indicated that the PGPR strains under test also directly promote the growth of rice plants. The consortium of bacteria had the greatest effect, with the analysis of individual strains indicating that SPR7, Z2-7 and BPS10 all contribute. Plant growth promotion may reflect the phyto-stimulatory properties of these bacteria, including IAA synthesis, P-solubilisation and nitrogen fixation. The optimum performance of the consortium probably represents an additive or synergistic interaction between the strains, since each possesses different combinations of these growth-promoting characteristics. Importantly, similar results were obtained whether plants were grown in soil or in sand (with supplementary nutrition), showing that the efficacy of these PGPR is maintained under different growth conditions, which may be important from an applied perspective.

In general, this study has not only led to the isolation and characterization of rice rhizosphere-associated bacteria, but has also helped in the selection of specific strains, with special reference to siderophore production, that can improve plant health and increase plant yield both directly by production of plant growth-promoting substances, and indirectly by enhancing plant resistance mechanisms against the sheath blight pathogen, *R. solani.* In terms of disease suppression, the efficacy of the consortium of selected strains was comparable to the fungicide benlate, which is normally used for sheath blight control, and was better than benlate in case of plant growth promotion and grain yield. It would be interesting in the future to engineer siderophore deficient mutants of *Enterobacter sp*. B41 strain SPR7 and *Bacillus cereus* Z2-7, for example, to test the importance of siderophore production on both direct fungal antagonism and the ability to generate disease resistance on rice plants.

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Table 1. Primers used for rt-pcr

Gene	Database annotation	Forward primer (5'- 3')	Reverse primer (5'- 3')
Actin	Os03g0718100 osrac1	TCCATCTTGGCATCTCTCAG	GTACCCGCATCAGGCATCTG
Chitinase	Os04g0493400 pr-3, chitinase iib; class iv chitinase	TCTACACACGGCAATCGTTC	GGCCACTGTTTGTTGCTCTT
Glucanase	Os07g0539900 beta-1,3-glucanase-like protein	CCAGACGGGACGGAGGTTTAC	AGATGCGTGTGCGACCAAGG
Peroxidase	Os07g0677200 peroxidase (pox22.3)	ACGACGCCCAACGCCTTC	CTTCCAGCAACGAACGCATCC

Table 2. Correlation of siderophore production with bioantagonistic activity of bacterial strains.

Bacterial strain	**Siderophore quantitation (µg/l)**	**Mean inhibition of growth of *R. solani* (%) on pda**				
		3 days	7 days	11 days	15 days	21 days
		R = 0.539 P = 0.0171	R = 0.668 P = .0018	R = 0.812 P<0.0001	R = 0.960 P<0.0001	R = 0.973 P<0.0001
Z.2.4	12.2± 0.1	10	10	0	0	0
Z2-7	52.9± 0.02	78	80	80	80	80
Z.2.10	5.3± 0.03	23	0	0	0	0
N4	6.36± 0.12	31	22	15	5	0
Sps1	7.2± 0.1	5	10	10	4	3
Sps4	14.76± 0.22	8	0	0	0	0
Spr4	10.02± 0.14	52	33	21	0	0
SPR7	60.67± 0.04	100	100	100	100	100
BPS10	41.8± 0.02	60	58	45	45	45
BPS12	3.9± 0.04	0	0	0	0	0
Bpr1	7.14± 1	48	24	22	14	0
Wbpr2	7.66± 0.23	34	0	0	5	2
Wbps6	4.28± 0.01	22	18	0	0	0
Z.3.2	6.6± 0.01	64	30	6	1	1
4.2.1.a	1.16± 0.03	80	67	33	5	0
F9	1.35± 0.01	6	33	0	0	1
5.1.a	7.98± 0.01	100	78	66	15	0
F11	7.3± 0.01	4	33	6	0	0
18 a-1	6.2± 1.0	2	0	0	4	2

R = pearson correlation coefficient

Table 3. Antagonistic activity (mean percentage inhibition) of selected bacterial isolates again *R. solani* on different media (with different iron concentrations).

Treatment	Ifm[a]	Ifm±5 ppm fe	Ifm±15 ppm fe	Ifm±25 ppm fe	Ssm[b]	Nb	Lb
Control	0± 0.00	0± 0.00	0± 0.00	0± 0.01	0± 0.00	0± 0.00	0± 0.01
Bacillus cereus Z2-7	95± 0.5	91± 0.51	87± 0.11	79± 0.42	93± 0.51	80± 0.12	80± 0.3
Enterobacter sp. B41 SPR7	100± 0.01	100± 0.1	98± 0.23	95± 0.1	100± 0.11	100± 0.03	100± 0.5
Aeromonas hydrophilla BPS10	89± 2.1	82± 1.41	74± 0.31	60± 0.51	87± 0.21	61± 0.14	60± 0.02
Enterobacter sp. BPS12	25± 1.51	18.5± 0.21	10± 0.1	0± 0.1	21± 0.32	0± 0.01	0± 0.43

[A]ifm iron free medium [B]ssm standard succinate medium ± is standard deviation. Means are calculated for three replicates of each treatment

Table 4. Effect of application of different bacterial strains on growth of rice seedlings of the variety *Super Basmati*

Treatment	Mean fresh weight (g)/ plant at 30 days.	Mean shoot length (cm)/ plant at 30 days	Mean no. Of tillers/ plant At 60 days
Control	6.63± 2.1bc	21.4± 0.5c	11± 2.0cde
Benlate	7.06 ± 1.1b	22.5± 0.51c	8± 1cdef
Bacillus cereus Z2-7	8.60± 0.1 ab	27.3± 0.43 b	20± 1.2ab
Enterobacter sp. B41 SPR7	8.90± 0.2 a	29.7± 0.33 a	20± 0.7ab
Aeromonas hydrophilla BPS10	8.75± 0.42 a	25.1± 0.5 bc	20± 2.0ab
Enterobacter sp. BPS12	8.07± 1.0 b	23.7± 0.47 bc	17± 3.2b
Consortium	9.01± 0.51 a	30.1± 0.26 a	23± 1.0a

Table 5. Effect of application of different bacterial strains on yield of rice variety *Super Basmati* grown in sand after being challenged with sheath blight fungus.

Treatment	Biomass (kg/pot)	Grain yield (kg/pot)	Straw yield (kg/pot)	Harvest index
Control	0.65±0.05 bc	0.23± 0.01d	0.41± 0.01cd	35.38± 0.03cd
Benlate	0.71± 0.11b	0.24± 0.02d	0.5± 0.13b	33.80± 0.1de
Bacillus cereus Z2-7	0.90 ± 0.01a	0.38± 0.04c	0.52± 0.2[a]	42.22 ± 0.02c
Enterobacter sp. B41 SPR7	0.92 ± 0.01a	0.49± 0.1b	0.43± 0.21c	53.26± 0.14a
Aeromonas hydrophilla BPS10	0.91± 0.03ab	0.39± 0.03c	0.54 ±0.14 a	42.82± 0.06c
Enterobacter sp. BPS12	0.81± 0.24b	0.36±0.12 c	0.46± 0.02c	44.44± 0.30c
Consortium	1.21± 0.11a	0.68± 0.01a	0.54± 0.24a	56.33± 0.2a

For Table 4 and 5 the letters a, b, c, d, e represents different statistical grouping of the data. The data with similar letters are statistically similar and *vice versa*. Letters in one column show variation within the column only ± is standard deviation, Means are calculated for three experiments. Each experiment had six replicates for each treatment and each replicate consisted of 3 plants.

Table 6. Effect of application of different bacterial strains on yield of rice variety *Super Basmati* grown in soil after being challenged with sheath blight fungus.

Treatment	Biomass (kg/pot)	Grainyield (kg/pot)	Strawyield (kg/pot)	Harvest index
Control	0.71± 0.04 bc	0.27± 0.03 d	0.45± 0.05 c	38.02± 0.51 cd
Benlate	0.79± 0.01 b	0.28± 0.021 d	0.51± 0.1 b	35.44± 0.21 de
Bacillus cereus Z2-7	0.9± 0.12 a	0.36± 0.04 c	0.55± 0.05 b	40± 0.34 c
Enterobacter sp. B41 SPR7	0.9± 0.03 a	0.45± 0.01 b	0.5± 0.02 b	50± 0.21 bc
Aeromonas hydrophilla BPS10	0.87± 0.21 ab	0.37± 0.1 c	0.5± 0.11 bc	42.52± 0.51 c
Enterobacter sp. BPS12	0.75± 0.03 b	0.33± 0.01 c	0.44± 0.01 c	44± 0.41 c
Consortium	0.96± 0.14 a	0.58± 0.01 a	0.37± 0.01 d	60.42± 0.43 a

The letters a, b, c, d, e represents different statistical grouping of the data. The data with similar letters are statistically similar and *vice versa*. Letters in one column show variation within the column only ± is standard deviation, Means are calculated for three experiments. Each experiment had six replicates for each treatment and each replicate consisted of 3 plants.

Table 7. Epiphytic and rhizospheric colonization of *Super Basmati* rice by different bacterial isolates

Treatment	Bacterial population (cfu ml^{-1})					
	7 days		21 days		45 days	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	0	0	0	0	0	0
Bacillus cereus Z2-7	0	$4.5x10^9$	0	$5.0x10^9$	0	$4.1x10^8$
Enterobacter sp. B41 SPR7	0	$4.6x10^9$	0	$5.0x10^9$	0	$4.0x10^8$
Aeromonas hydrophilla BPS10	0	$4.3x10^9$	0	$4.3x10^9$	0	$4.5x10^6$
Enterobacter sp. BPS12	0	$4.5x10^9$	0	$4.0x10^8$	0	$3.0x10^5$

Means are calculated for three experiments. Each experiment had six replicates for each treatment and each replicate consisted of 3 plants.

Figure 1

Effect of application of different bacterial strains on disease incidence in rice plants, seven days after inoculation with *R. solani* ((grey bars for plant grown in soil; black bars plant grown in sand).
letters above bars indicate statistically different groupings as determined by one-way ANOVA with Tukey's multiple comparison test.

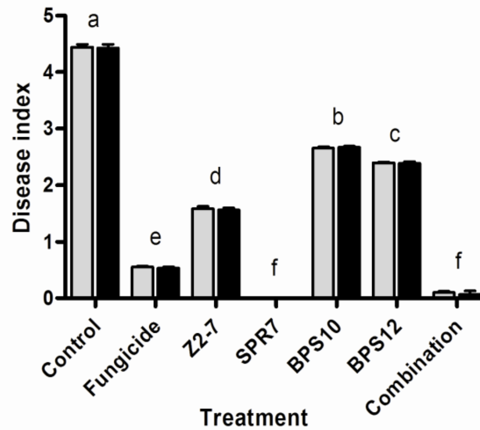


Figure 2

Effect of application of different bacterial strains on disease incidence in rice plants, 52 days after inoculation with *R. solani* (grey bars for plant grown in soil; black bars plant grown in sand).
Letters above bars indicate statistically different groupings as determined by one-way anova with tukey's multiple comparison test.

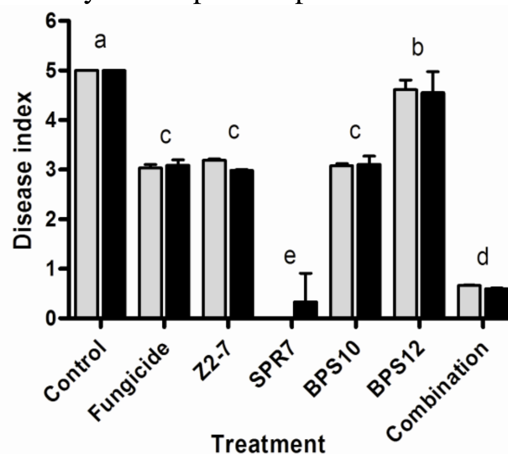


Figure 3.

Effect of application of different bacterial strains on tiller mortality in rice plants, 52 days after inoculation with *R. solani* (grey bars for plant grown in soil; black bars plant grown in sand).

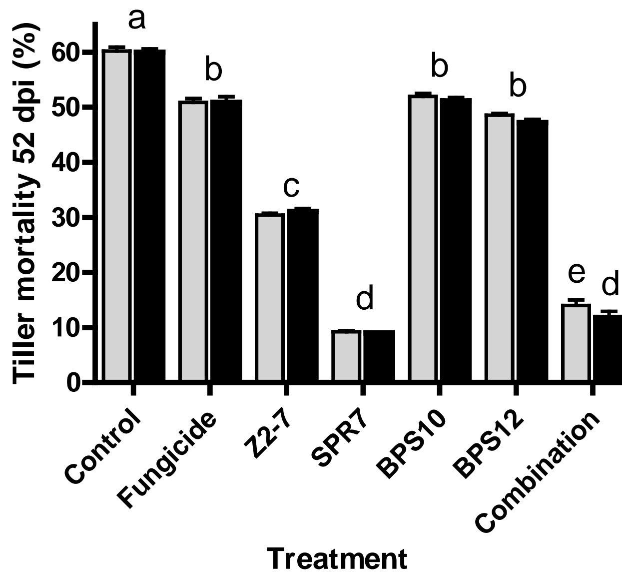
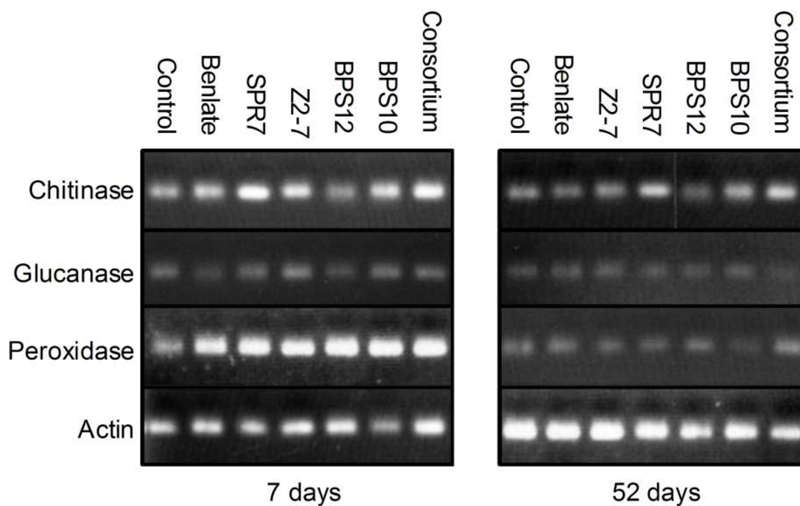


Figure 4.

Induced expression of different defence enzymes in rice plants inoculated with bacterial strains SPR7, Z2-7, BPS10, BPS12 and consortium of these after 7 and 52 days of inoculation



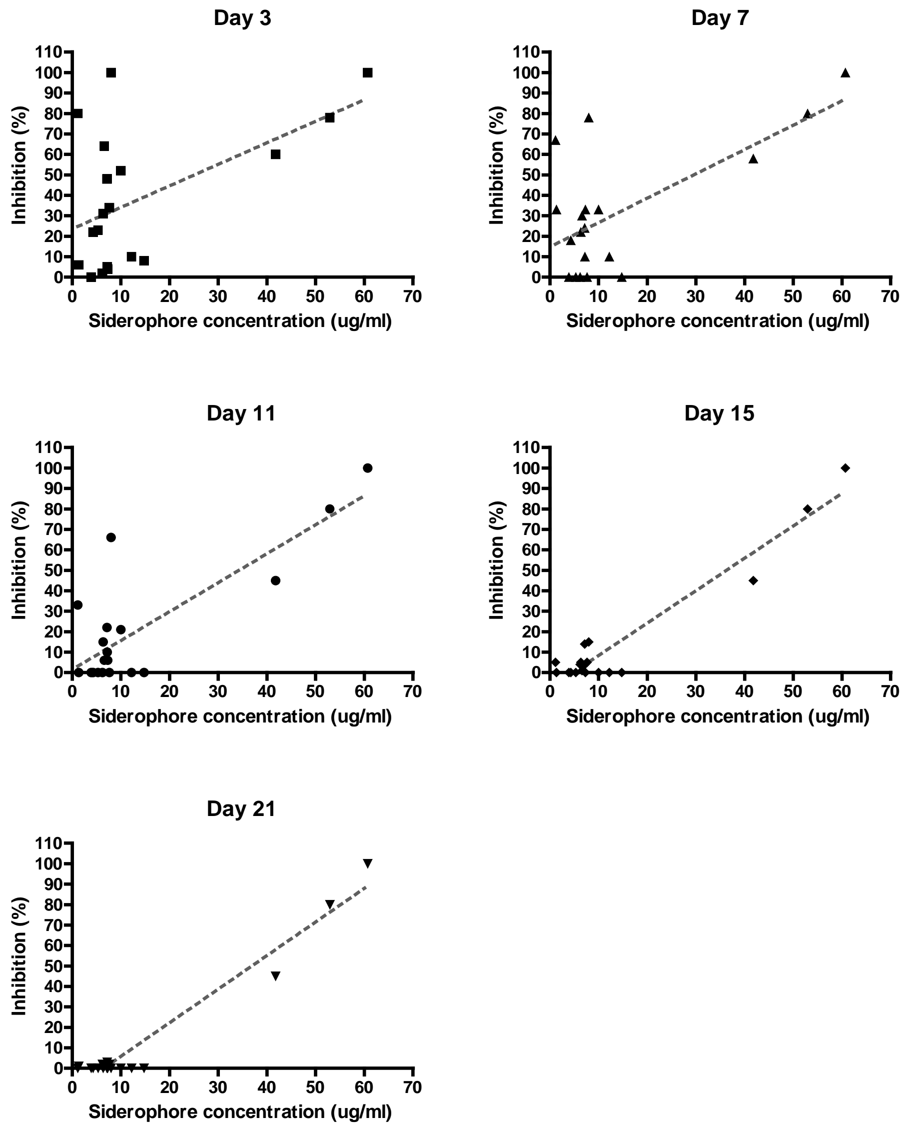


Figure 5.
Significance of correlation between quantity of siderophores produced and *in vitro* antagonism.