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Full Length Article

Role of Pyrroloquinoline Quinone in Biocontrol Together with Induced Systemic Resistance: A Novel Resource Trialed for Rice Disease Control

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Abstract

Plants show strong levels of resistance to an extensive range of pathogens on account of root colonization through plant growth-promoting rhizobacteria (PGPR), namely, induced systemic resistance (ISR). Little is known about bacterial determinants and plant signaling pathways that underpin ISR in cereal crops associated with ISR in dicotyledonous plants. The present study evaluates the potential of *Pseudomonas* spp. QAU-92 using site directed mutagenesis of the pqqC gene to elicit ISR in rice (*Oryza sativa* L.) against the fungal pathogen *Cochliobolus miyabeanus.* The comparison between the wild-type strain and the mutant strain for biochemical attributes, *in vitro* and *in vivo* antagonistic activity, carbon source utilization assay and *in vivo* analyses on rice (cv. C-039) revealed the statistically significant role of Pyrroloquinoline Quinone (PQQ) in plant growth promotion. RT-qPCR analysis revealed that the plant recognition of QAU-92 results in the activation of ethylene (ET) and jasmonic acid (JA) pathways and also shows clear differences in resistance against *C. miyabeanus* disease compared with the pqq*C* mutants (QAU92-2). The expression of TF 89 (*EBP89*), a susceptible gene, as well as the pathogenesis-related protein 1a (*PR1a*) were much higher in the infected control and *pqqC* mutant plant than in wild type inoculated plants. Hence, this study is the first of the kind that has investigated the expressional analysis of PQQ against antifungal activity, phosphate solubilization and the induced systemic resistance of QAU-92 against C. *miyabeanus* in rice. Additionally, PQQ genes may act as a key regulator of *PR1a*/ET cross-talk and its interference with the fungal manipulation of plants. © 2021 Friends Science Publishers

Keywords: Induced systemic resistance; Fungal pathogens; Pyrroloquinoline Quinone; expressional analysis; *Oryza sativa*; plant growth-promoting rhizobacteria

Introduction

Generally, plants have elaborate levels of inducible resistance that triggers pathogen infection and builds an initial line of defense/resistance (Adamowicz *et al.* 1991). Plant hormones such as ethylene (ET), abscisic acid, salicylic acid (SA) and jasmonic acid (JA) play pivotal role in these inducible responses, which are controlled by a network of interrelated signal transduction pathways (Adie *et al.* 2007; Robert-Seilaniantz *et al.* 2007; Asselbergh *et al.* 2008). Much of the recently reported evidences support the view that these signaling pathways are not independent and are influenced by a multifaceted network of antagonistic and synergistic interactions (Koornneef and Pieterse 2008). Such advanced interactions between defense pathways are assumed to give plants the regulatory potential to adapt their resistance response to complement an encounter with the attacker.

An investigation into the signal transduction pathways in activated plants suggests that some of the analogous pathways triggered by *Bacillus* spp. are similar to those activated by *Pseudomonas* spp. In some instances, during the activity of signal transduction pathways, the defense gene PRI accumulates in plants using ISR induced by *Bacillus* spp. (Kumar *et al.* 2012). Besides the effective resistance against an immunent pathogen attack, plants also achieve the development of resistance at the site of infection. The occurrence of this, which is called induced resistance, may be activated by a range of abiotic and biotic stimuli (Bostock 2005). Research in recent years has shown that the improved defensive ability of plants does not need to be triggered directly but may be due to the quicker and stronger expression of the basal defense response upon encountering a pathogen attack (Huot *et al.* 2014).

In the category of crops, rice (*Oryza sativa* L.) is an essential component of diet for more than three billion individuals in the tropical and sub-tropical Asia (Khush 2004) and is only rivaled by maize and wheat in importance. Yet, more than 70 diseases that inflict fungi, nematodes,

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viruses and/or bacteria on rice, which hamper its production, have been well documented. Of these, brown spot disease (*Cochliobolus miyabeanus*), sheath blight (*Rhizoctonia solani*) and rice blast (*Magnaporthe oryzae*) are the significant fungal constraints. Recently, brown spot disease in rice, which is triggered by *C. miyabeanus,* is a major disease in rain-fed ecosystems that badly affects yield and significantly diminishes rice quality (Paz *et al*. 2006). In recent years, sheath blight and rice blast diseases have been controlled by multiple *PGPR* strains but there are little reports on brown spot. These include *Pseudomonas fluorescens* PF1 and FP7 (Nandakumar *et al*. 2001), *P. fluorescens* PfALR2 (Rabindran and Vidhyasekaran 1996), and *Bacillus subtilis* MBI 600 (Kumar *et al*. 2012), are effective against sheath blight; while the *Pseudomonas* strains, 7NSK2 and WCS374r, are effective against rice blast (Vleesschauwer *et al*. 2006). The root colonization of *PGPR* strains leads to form-induced resistance (discussed in terms of ISR) (Loon *et al*. 1998).

Only limited studies have reported the hormonal control or ISR-based analyses to control brown spot disease in rice. One of these was on *Magnaporthe grisea* KI-409 (a rice pathogen), the mutation of pqqA and pqqB genes in *E. intermedium* wiped out their bio-control capability. In addition to their capacity to improve the systemic resistance to infections activated by fungal pathogens, they advocated the involvement of PQQ in antifungal activity, as well as the embellishment of the systemic resistance and mineral phosphate solubilization of *E. intermedium* (Han *et al*. 2008). The PQQ biosynthesis pathway has been found to be comprised of six most conserved PQQ genes (pqqABCDEF) in most of the bacterial strains and pqqC (cofactor-less PQQ synthase) catalyzes the final step in PQQ biosynthesis pathway (Magnusson *et al.* 2004). According to review of the literature, very little attention has been paid on the expressional analysis of PQQ against pathogenic fungi in plants. Moreover, the bio-control potential of *Pseudomonas* spp. QAU-92 and its PQQ mutant (QAU92-2) in triggering ISR against brown spot disease in rice is yet to be tested. Therefore, the present study aimed to investigate the expression analysis of PQQ against antifungal activity; induced systemic resistance, phosphate solubilization of *Pseudomonas* spp. QAU-92 against *C. miyabeanus;* and finally, the extent of resistance against a *C. miyabeanus* attack on rice.

Materials and Methods

Microorganism and culturing conditions

The strain QAU-92 was isolated from wheat cultivar rhizospheres and cultured in *TY* medium at 28°C while the *E. coli* cultured in LB at 37°C was used for transformation and *S. cerevisiae* on *PDA* medium at 30°C (Table 1). All cultures were preserved at -80°C in LB medium supplemented with 40% glycerol.

Identification of isolate and amplification of PQQ operon

The genomic DNA extraction of bacteria was done by the CTAB method as described by Naveed *et al*. (2014a). The identification of the isolate was done with 16S rRNA through PCR recipe according to Naveed *et al*. (2014b). PCR amplification of PQQ genes was done with freshly designed oligonucleotides using sequence information of *Pseudomonas* spp. QAU-92 (Table 1). PCR was carried out according to the procedure described by Naveed *et al*. (2015). The amplified PCR products were sequenced commercially from LGC genomics (Germany) and compared with already published sequences in NCBI GenBank databases. The sequences generated were submitted to NCBI Genbank and enlisted at end of the text.

In vivo cloning and site-specific mutagenesis and Phosphate solubilization

To induce mutation in the pqq operon, the pqq biosynthesis "pqqC" was targeted for site directed mutation using PCR based knock out method (Naveed *et al*. 2015) as described for QAU-92. The deletion plasmid of *Pseudomonas* spp. QAU-92 was constructed in the same way (Table 1) and PCR confirmed the deletion of PQQ gene. The wild type strain was used as a negative control and *E. coli* strain with the plasmid was used as positive control. The absence of pqqC loci in deletion mutants were further confirmed by *in vitro* phenotypic analyses such as phosphate solubilization and secondary metabolite production and *in vivo* carbon source utilization, catabolism of enzymes and plant growth promotion. An experiment was conducted to assess the phosphate solubilization capacity in wild and mutant strains, i.e. by halo zone formation on *Pikovskaya* agar medium and pH measurement in broth medium as described by Naveed *et al*. (2014b).

PQQ mutant characterization

PQQ mutants were further characterized for utilization of carbon and nitrogen sources and their role in regulation of enzymatic activity for oxidation-reduction and fermentation processes by API-20E kit (Bio Merieux, U.S.A.), embedded with 20 biochemical tests based on enzyme and pathway regulation to assess differences among wild and mutant strains. PQQ mutants were tested to identify the enzymes for which pqq act as a cofactor was based on carbon source utilization assay. We used 1% of each eight carbon sources (glucose, acetate, Na-citrate, Na-succinate, manitol, glycerol, ethanol and methanol) with M-9 nutritional media (Naveed *et al*. 2015). The LB culture of all strains at 10^6 CFU was placed overnight into M9 media after taking OD at 620 nm then in 96 well plates in 12 replicates. After 24 h the differences in OD of wild and mutant type strains were observed.

PQQ role in plant growth promotion

The seeds of *Phaseolus vulgaris* 'Prelude' (Belgium) which is a model plant and easy to grow were used for *in vivo* plant experiments and Rice *cv*. C-039 (Japonica), having advantage to grow easily. After culturing the bacteria on KB medium, the seed coat was removed. The surface was sterilized twice with 2% NaOCl for 10 min (gently shaken to have better contact between seeds and NaOCl). Place the seeds on moist filter paper and keep them in an incubator at 28°C for 5 days to allow germination and sterilized the saline solution, cylinders and potting soil (2 times). The bacterial suspensions were (5×10^7 CFU/g soil) mixed with sterile potting soil (Structural; Snebbout, Kaprijke, Belgium) and distributed soil into white boxes (12 seedlings/700 g soil/box). Soil was mixed with non-sterile distilled water to make it wet before bacterial application. After this the seeds were sown. $FeSO_4$ and $(NH_4)_2SO_4$ (2:1 g/L) were used as nutrients to fertilize (250 mL solution/box) the plants.

The data on growth parameters (shoot length, shoot weight, leave area index, dry weight, total number of leaves and plant root) was documented on ten plants from three replicates by software package S.P.S.S. 15.0. The nonparametric data was analyzed using Kruskal-Wallis and Mann-Whitney comparisons ($\alpha = 0.05$).

Biocontrol activity of *Pseudomonas* strains against *Rhizoctonia* root rot

The bio-control activity *Pseudomonas* spp. QAU-92 and its derived pqqC mutant (*Pseudomonas* spp. QAU92-2) strains were tested *in vitro* for antifungal activity against *Rhizoctina solani* and *Pythium* spp. It was then re-confirmed *in vivo* with bean plants. The *P. vulgaris* 'Prelude' (Het Vlaams Zaadhuis, Belgium) was used to check the efficiency of *Pseudomonas* strain to suppress the *Rhizoctonia* root rot. Inoculum of *R. solani* (AG 2-2) was developed on water-soaked wheat seeds, which were then autoclaved two times on 2 successive days. The disease symptoms were recorded according to Nerey *et al*. (2010). All experiments were carried at 25°C with 16h photoperiod and repeated with four replications per treatment having ten bean plants per replication along with infected and healthy controls.

Root colonization by *Pseudomonas* spp. QAU92 and its PqqC mutant QAU92-2

The actual rating of disease severity depends upon *Pseudomonas* spp. QAU-92 and mutant colonization with bean roots which were determined as mentioned by D'Aes *et al*. 2011). S.P.S.S. 15.0 software package was used for statistical analysis of data. Neither the data of root colonization experiments nor the ordinal data of the disease severity met the conditions of homogeneity and normality of variances. Therefore, the nonparametric Kruskal-Wallis and Mann-Whitney analyses ($\alpha = 0.05$) were executed.

PQQ and Induced systemic resistance (ISR) in rice

Two experiments *i.e*., rice plants (*in vivo*) and with rice cell suspension cultures (*in vitro*) were performed to assess the role of wild and *pqqC* mutant strains in suppressing the rice brown spot disease through ISR.

The analysis of cell lines treated with *Pseudomonas* supernatant by *q*PCR

The wild type *Pseudomonas* spp. QAU-92 strain and its *pqqC* mutant *Pseudomonas* spp. QAU92-2 strains culture was scraped off from LB medium plates and then put into the sterile 10 mL of demineralized water. The suspended bacterial colonies were centrifuged at 10,000 *g* for 10 min. 1 mL of supernatant was passed through a filter of 0.22 μm and then added to 3 mL of 5-day-old rice cell. RNA extraction analysis was done from the cells collected at 1, 3 and 6 h post inoculation (hpi). The LB broth was used as control. Following treatment with wild type *Pseudomonas* and *pqqC* mutant strains, expression of JA marker genes *JAMYB* and *JiOsPR10* were checked along with the ET-related gene *EBP89* and Actin (*Os03g071810*) used as an internal reference (Vleesschauwer *et al*. 2010) to normalize the gene expression levels.

Induced resistance bio-assays and pathogen inoculations

Induced resistance bio-assays for *Pseudomonas* spp. QAU-92 and its *pqqC* mutant *Pseudomonas* spp. QAU92-2 were performed as described by Vleesschauwer *et al*. (2006) and Chandler *et al*. (2015) with some alteration. The *C. miyabeanus* strains Cm988 (brown spot) used for infection trials were grown on *PDA* at 28°C for sporulation. Seven days-old mycelia were spread on the medium under blue light for three days to prompt sporulation. Conidia were harvested upon sporulation and to make a final density of 1×10^4 conidia mL^{-1} re-suspended in 0.5% gelatin (Sigma-Aldrich). For inoculation, 6.5-leaf stage of five-week-old seedlings was glazed over with conidial suspension (1 mL per plant) by an artist airbrush. Straightway, plants were shifted to a precipitation chamber (30°C, with humidity > 92%) to assist fungal penetration and moved to greenhouse (28°C ± 4°C) after 18h for development of disease. Leaf samples of infected, mock and control rice plants were collected after fungal inoculation at four-time points (*i.e*., 12 h, 24 h, 36 h and 48 h after inoculation) in two biological repeats. For two bacterial inocula *Pseudomonas* spp. QAU-92 and its *pqqC* mutant; a total of 80 samples of *Pseudomonas* spp. QAU92-2 were collected; 40 for each of the two biological repeats, RNA extraction and RT-PCR expression analyses.

RNA extraction and cDNA synthesis

After harvesting, rice leaf tissues were submerged in liquid

nitrogen quickly to avoid possible RNA degradation. TRI reagent (Sigma) was used for total RNA extraction from frozen tissue and samples were quantified at A260/280 ratio (values should be < 1.6). After that it was re-suspended in 40 μL of DEPC-treated RNase-free Milli Q and incubated for 10 min at 65°C. The extracted RNA was treated with DNase (Turbo DNase, Applied Biosystems). The final concentration of extracted RNA was measured using Nanodrop ND-1000 Spectrophotometer. First cDNA strand was prepared by GoScript Reverse Transcription System (Promega, U.S.A.) from 1 μg of RNA.

Gene expression and quantitative real time PCR (*q*RT-PCR) analysis

Quantitative RT-PCR (*q*RT-PCR) amplifications and gene expressional analysis were done as mentioned by Chandler *et al*. (2015). The plant RNA from all samples was standardized with internal control of actin (*Os03g0718100*) or eukaryotic translation elongation factor 1A (*eEF1a* - *Os03g0178000*). The samples from control cell cultures; designated as a calibrator, pathogenesis-related (PR) class 1 (*PR1a*) and Ethylene-responsive TF89 susceptible gene (*EBP89*) were used for *pqq* gene expression analysis. Data was compiled by mean and standard error of three replicates from each representative experiment. Primer sequences listed in Table 5 were used for expressional analysis.

Statistical analysis

Analysis of variance (ANOVA) tests were used to analyze data at a confidence level of 95%, together with Kruskal-Wallis Test using Statistix 8.1 software (Tallahassee, USA) based on the method described by Steel *et al*. (1997).

Results

Identification based on the 16S rRNA and rpoB sequences revealed that QAU-92 is a *Pseudomonas* spp.; however, it has low bootstrap support that is, 35 and 43%, respectively (Fig. 1a and b). Furthermore, the *Pseudomonas* spp. QAU-92 showed a capability to solubilize phosphate, which further demonstrated its impact on plant growth. On top of this, it produced the lipopeptides that boosted strain efficiency against plant diseases (Table 1).

Amplification of the PQQ operon and characterization of the pqqC mutant

The PCR amplification and sequence homology of the PQQ operon (pqqAB, pqqBCD, pqqE and pqqF) demonstrated more than 97% sequence similarity with the PQQ sequences of *Pseudomonas* already available in the NCBI GenBank Accession number CP003190. The pqqBCD was chosen to develop mutant strains that were identified through the detection of 1 kb segments. Therefore, QAU-92 produced

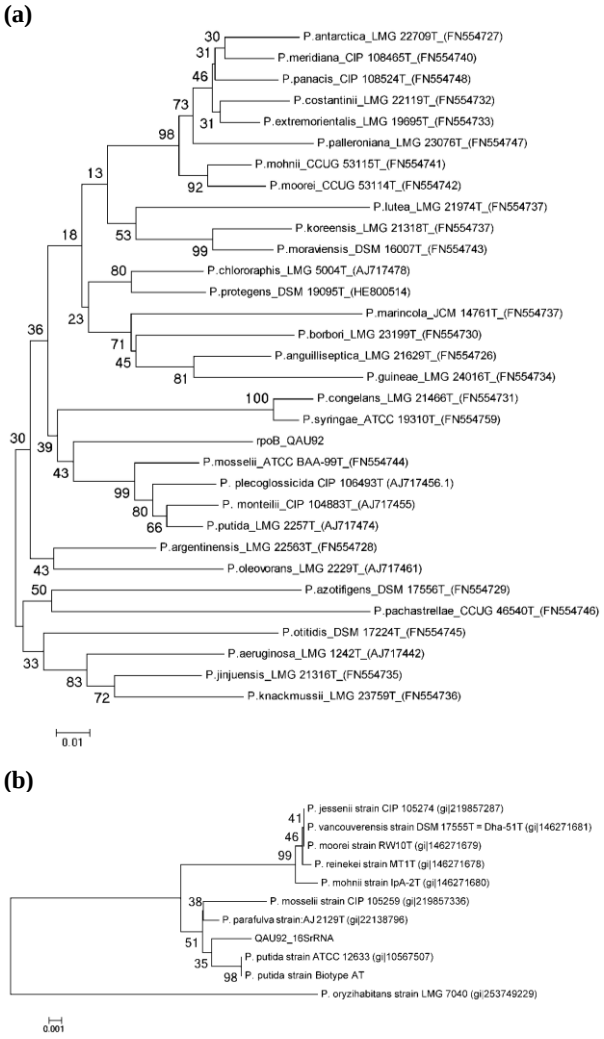

Fig. 1: Neighbor-joining phylogenetic tree showing (**a**) 16S rRNA and (**b**) rpoB gene sequence affinity of QAU-92 with *Pseudomonas* group. The 35% and 43% bootstrap value respectively provided statistical support base on 1000 interactions

eight mutants for the pqqC locus, and only the best characterized QAU92-2 was used here. The wild type and pqqC mutants were further confirmed and characterized for mutation and strain phenotype (Supp Fig. 1a, b and c).

The QAU-92 wild strain, tested for its capability to solubilize inorganic phosphate, demonstrated the utilization of ethanol as a carbon source. However, the pqqC mutant QAU92-2 was deficient in such activity, even in media enriched with ethanol (Table 2). Hence, the QAU-92 was an alcohol dehydrogenase (ADH) carrying system efficient in phosphate solubilization. The wild strain QAU-92 fermented various carbohydrates: L-omithin, glucose, sorbitol, L-rhamnose, D-melibiose, amygdaline and L-arabinose; while the mutant strain QAU92-2 lost this capacity (Table 2), suggesting the imminent role of PQQ in fermentation and redox reactions.

Table 1: Strains, plasmid vectors and primers used in this study

Strains/plasmid/ Oligonucleotides	Characteristics and sequences (5′→3′)	Reference/source
Pseudomonas spp. QAU-92	Biocontrol+, PGPR+, CLP+, pltC+, wild type (Pakistan)	This study
Pseudomonas spp.QAU92-2	Biocontrol−, PGPR−, CLP−, pqqC mutant (QAU92-2)	This study
E. coli WM3064	Donor strain for conjugation; carries the pir gene, which is necessary for plasmids with an oriR6K origin of replication	Dietrich *et al*. (2006)
S. cerevisiae InvSc	Yeast strain for in vivo recombination (ura3-52/ura3-52 mutation)	Invitrogen
Rhizoctonia solani AG 2-2 CuHav-Rs18	Causal agent of root rot on bean (intermediately aggressive) (Cuba)	Nerey *et al*. (2010)
Plasmid		
pMQ-30	Gene replacement vector for Pseudomonas spp.; sacB, URA3, GmR	Shanks *et al*. (2006)
Oligonucleotides		
pqqC-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTTCAAGATGCTCAGCCACTG	Naveed *et al*. (2015)
pqqC-Up-R	CAGTTCATAGGCCATGCTCAATGGGGATGTTCACCTGGTA	
pqqC-Down-F	TACCAGGTGAACATCCCCATTGAGCATGGCCTATGAACTG	Naveed *et al*. (2015)
pqqC-Down-R	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATCGATCTTGTCGATGTTGTGC	
pqqBCD-F	TTCAAGATGCTCAGCCACTG	Naveed *et al*. (2015)
pqqBCD-R	CGATCTTGTCGATGTTGTGC	
PqqAB-F	TGTGGACCAAACCTGCATACACTG	Naveed *et al*. (2015)
PqqAB-R	GATGCTCATGCCATCGAA	
PqqE-F	GATCGTCCTCGCCTGAGTT	Naveed *et al*. (2015)
PqqE-R	GATGACACGGGAGTTTCGAT	
PqqF-F	CCAACTTACCCTCGCCAAT	Naveed *et al*. (2015)
PqqF-R	CAGCGTTGGCCAAACATAG	
rpoB-F	CAGTTCATGGACCAGAACAACCCGCT	Naveed *et al*. (2015)
rpoB-R	CCCATCAACGCACGGTTGGCGTC	

PGPR: plant growth promoting rhizobacteria; PHZ+: Phenazine producer; CLP+: lipopeptides production

Table 2: Biochemical characterization by API-20E kit, root colonization and *in vitro* antagonistic activity of *Pseudomonas* spp. QAU-92 and their derivatives pqqC mutant (QAU92-2) against *R. solani* AG 2-2

Tests	QAU-92	QAU92-2 pqqC mutant
Source (Rhizosphere)	wheat	wheat
Strains identified by 16S rRNA and *rpo*B gene	*Pseudomonas* spp.	*Pseudomonas* spp.
Strain group	*Pseudomonas*	*Pseudomonas*
Drop collapse	drop collapse activity	no drop collapse activity
Phosphate solubilization[a]	3.7 ± 0.07	0.5 ± 0.03
Utilization of Glucose[b]	0.4 ± 0.02a	0.2 ± 0.03b
Utilization of ethanol	1.2 ± 0.04b	0.3 ± 0.01a
GDH[c]	2250 bp	2250 bp
PQQ[c]	4.9 kb	–
Production of acid[d]	4.14	5.92
Root colonization[e]	6.89 ± 0.24a	5.61 ± 0.35c
Antagonistic[f]	2	0
Disease severity (DS)	1.4 ± 1.2	3.2 ± 1.2
[g]Biochemical characterization (by API-20E kit)		
SOR, RHA, SAC, MEL, AMY and ARY	+	–
ADH and CIT	+	+
LDC, H2S, URE, TDA, IND, VP, GEL and INO	–	–

[a]Tri calcium phosphate ($Ca_3(PO_4)_2$ solubilization efficiency calculated according to Edi-Premoto *et al*. (1996) method on Pikovskaya medium plat with S.D of 3 replicates; [b]carbon source utilization assay data represent as averages ± standard deviations of three replicates per treatment with different letters indicate statistically significant difference in carbon source utilization; [c]PCR amplification of GDH, glucose dehydrogenase; PQQ, PyrroloQuinoline Quinone; [d]production of acid was checked with ethanol and glucose enriched Pikovskaya medium by wild and mutant strains (measured by drop in pH) but here data of GDH mutants were shown on ethanol and glucose. [e]The root colonization data represent as averages ± standard deviations of three replicates per treatment. Different letters indicate statistically significant difference between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests ($\alpha = 0.05$). [f]In vitro antagonistic activity against *R. solani* AG 2-2 tested on TY-agar (0, no inhibition of mycelial growth; 1, mycelial growth reached the edge of the bacterial colonies; 2, a clear inhibition zone could be observed). [g]Biochemical characterization by API 20E kit including following [d]OPNG (Ortho Nitrophenyl-βD-glactopyranoside), ADH (Arginine Dihydrolase), LDC (Lysine Decarboxylase), ODC (Omithin Decarboxylase), CIT (Citrate utilization), H_2S (H_2S production), URE (Urease), TDA (Tryptophane Desaminase), IND (Indole production), VP (Acetoin production), GEL (Gelatinase), GLU (Glucose), MAN (Manitol), INO (Inositol), SOR (Sorbitol), RHA (Rhamnose), SAC (Sacharose), MEL (D-melibiose), AMY (Amygdaline) and ARY (Arabinose)

Plant growth promotion activities in bean and rice

The Kruskal-Wallis statistical data clearly demonstrated the behavior of the wild type (*Pseudomonas* spp. QAU-92) with the pqqC mutant (*Pseudomonas* spp. QAU92-2) strains, and the control inoculated plants based on height as well as the fresh weight of rice and bean. It was further confirmed through statistical analysis. The statistical analysis compared the performance of the wild type and mutants as they were assembled based on parameters such as plant height and fresh weight. In both cases, the P value = 0.00 < 0.05=α, rejected our null hypothesis (Table 4) and there was no difference between the performance of the wild type and mutants. Therefore, based on the test scores there existed enough evidences to conclude that there was difference among the three methods. Furthermore, the groups were

Table 3: Percent growth inhibition of phytophathogens by antagonistic *Pseudomonas* strains

Strains	*Rhizoctonia solani*		*Fusarium solani*		*Pythium* spp.	
	Mycelium growth(mm)	Growth inhibition (%)	Mycelium growth(mm)	Growth inhibition (%)	Mycelium growth(mm)	Growth inhibition (%)
QAU-92	32.5c	59.3e	40a	50g	37b	53.7f
LSD, 0.5%	0.876	0.864	0.867	0.865	0.842	0.867

Least significant difference (LSD ≤ 0.05) was used separately to evaluate the response of each character. Different letters indicate statistically significant differences between growth inhibitions of fungi

Table 4: Statistical analysis of wild type and pqqC mutant strains in plant growth promotion

Wild and pqqC mutated Strains	Statistical parameters[a,b]	Inoculation with Bean plants				Inoculation with rice	
		Plant height	Root length	Fresh weight (S×R)	Leaf area (L×W)	Plant Height*	Fresh weight* (S×R)
QAU-92 and QAU92-2	Chi-Square	14.296	14.296	14.318	14.296	25.812	21.862
	Df	1	1	1	1	2	2
	Asymp. Sig.	0.000	0.000	0.000	0.000	0.000	0.000

Asymp. Sig (Asymptotic significance) = P-value, Df (Degree of freedom), a. Kruskal Wallis Test, b. Grouping Variable: CLASSES, L (Length), W (Width), S (Shoot), R (Root) and * control treatment along with wild and mutant strains.

Table 5: Gene-specific primers for quantitative real-time PCR (qPCR)

Pathway	Gene	Annotation	Locus number	Forward (5'-3')	Reverse (3'-5')
Housekeeping gene	*Actin*	Rice actin 1	Os03g0718100	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC
	eEF1a	Eukaryotic elongation factor 1A	Os03g0178000	GGCTGTTGGCGTCATCAAGA	CCGTGCACAAAACTACCATT
Ethylene (ET)	*EBP89*	Ethylene responsive TF 89	Os03g0182800	TGACGATCTTGCTGAACTGAA	CAATCCCACAAACTTTACACA
Jasmonic acid (JA)	*JAMYB*	JA-inducible Myb TF	Os11g0684000	TGGCGAAACGATGGAGATGG	CCTCGCCGTGATCAGAGATG
	JiOsPR10	JA-inducible PR10 protein	Os03g0300400	CGGACGCTTACAACTAAATCG	AAACAAAACCATTCTCCGACAG
	OsPR1a	pathogenesis-related protein (PR) class 1	Os07g03710	GTCGGAGAAGCAGTGGTACG	GGCGAGTAGTTGCAGGTGAT

tested and the median scores were equal. However, p = 0.00 < 0.05=α provided reasons to reject the null hypothesis. The statistical analyses clearly showed and corresponded to the plant growth promotions.

Assessing the bio-control capacity of *Pseudomonas* spp. QAU-92

***In vitro* antagonistic activity:** The *Pseudomonas* spp. QAU-92 strain significantly inhibited the growth of *R. solani* (59.3f) with 0.864 of least significant difference (LSD ≤ 0.05). Furthermore, it showed an almost equal inhibition capability against *Pythium* spp. and *Fusarium solani* with 0.867 and 0.865 of least significant difference values, respectively. The statistical analysis further confirmed the bio-control potential of the *Pseudomonas* spp. QAU-92 strain. The results indicated it is a potential candidate for disease control in plants (Table 3).

***In vivo* antagonistic activity:** The *in vivo* bio-control activity of *Pseudomonas* spp. QAU-92 against *Rhizoctonia* root rot revealed a substantial decrease in disease severity triggered by *R. solani* on bean plants. In contrast, the *pqqC* mutant showed only a reduced potential in disease control. In another experiment, *Pseudomonas* spp. QAU-92 protected against a moderately aggressive isolate of *R. solani* AG 2-2 and caused a reduction in disease severity (DS): from ≈4.0 ± 0.9 to 1.4 ± 1.2 (p = 0.000). Treatments with mutant strains showed less disease control activity; from ≈4.0 ± 0.9 to 3.2 ± 1.2 (p = 0.000). The QAU92-2 mutant (deficient in PQQ production) demonstrated a complete loss in bio-control capacity and the DS for this strain was very near to that of the control (Table 2). This datum was also valid for a second trial, which yielded similar results. The count of the wild type (*Pseudomonas* spp. QAU-92) and the *pqqC* mutant strain (*Pseudomonas* spp. QAU92-2) showed bacterial population differences on the bean roots (Table 2). The concentrations of the wild and mutant bacterial strains were observed to be variable between repetitions and treatments of time. However, the concentration factor did not affect the disease-suppressive ability and revealed that the root colonization was appropriately higher for optimal biological control. For the most part, the mutant QAU92-2 lacking *pqq* had the lowest bacterial root concentration and root colonization.

Induced systemic resistance in rice

The JA/ET signaling pathways: Following the treatment with *Pseudomonas* spp. QAU-92 and the *Pseudomonas* spp. QAU92-2 mutant supernatant, the hormone signaling pathways were observed. In addition to this, the expression levels of the JA marker genes; *JiOsPR10* and *JAMYB* were recorded. 4- and 2-fold increase in the expression of genes respectively, was observed in infected plants. A 5- and 5-fold increase was recorded in the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) inoculated plants compared with the wild type (*Pseudomonas* spp. QAU92), which had a higher expression of approximately 13- and 9-fold at 3 hpi, respectively (Fig. 2a and b). Under similar conditions, the ET-related gene *EBP89* showed an a6-fold expression by the infected control plants, a 9-fold expression by the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) inoculated plants, and an upregulation of wild type (*Pseudomonas* spp. QAU-92), expressed about 20-fold at 6 hpi (Fig. 2c).

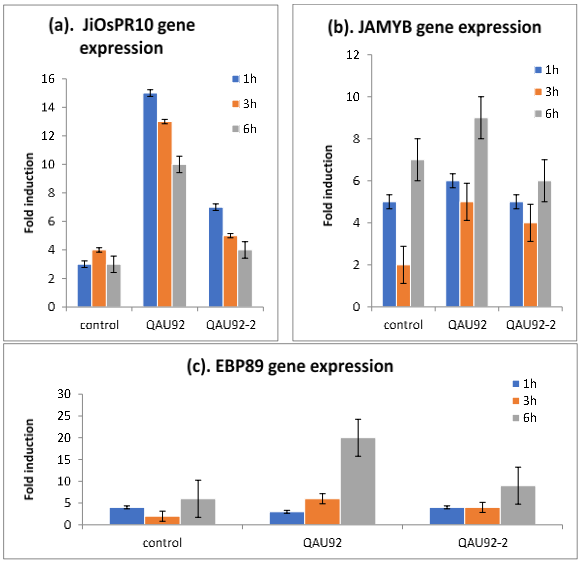

Fig. 2: Expression of hormone marker genes in rice cell cultures treated with supernatant of *Pseudomonas* spp. QAU-92 and its pqqC mutant (*Pseudomonas* spp. QAU92-2). At different time points after inoculation (1, 3 and 6 h), cell cultures were harvested and subjected to quantitative RT-PCR analysis for the following transcripts: (**a**) JiOsPR10, (**b**) JAMYB and (**c**) EBP89. Actin (Os03g071810) was used as an internal reference to normalize the gene expression levels and calculated relative to the expression in mock-treated control cells at 1, 3 and 6 h. Data presented are means and standard error of three replicates from a representative experiment

Together, these results suggested that *pqq* genes produced activation of JA and ET pathways, while the *pqq* deletion mutant was unable to activate the hormone to the level of the wild type *Pseudomonas* spp. QAU-92 strain.

Similarly, the quantitative reverse transcription analysis showed an accumulation of JA transcripts upon treatment with the *Pseudomonas* spp. QAU-92 supernatant (Fig. 2a and b) but not as much as with the *pqqC* mutant *Pseudomonas* spp. QAU92-2 supernatant. The application of the QAU-92 supernatant caused a strong and fast accumulation of *JiOsPR10*, with mRNA levels topping 1 hpi, and as 3-fold was found in mock-inoculated controls, 7-fold in the *pqqC* mutant (QAU92-2) and 15-fold in the upregulated with wild type (QAU92).

The use of the QAU-92 supernatant also induced a strong 11-fold upregulation of *EBP89* (Fig. 2c) gene expression in comparison to the control and *pqqC* mutant strain, while much weaker changes were observed in response to *EBP89* at 1 and 3 hpi than with the mock control. These changes indicated that the PQQ produced in the LB broth-culture by the strain QAU-92 triggered the JA/ET signaling pathways.

ISR against *Cochliobolus miyabeanus* in rice

The PQQ-based induced resistance in rice (*Oryza sativa* subsp. *indica cv* CO39) was assessed against the *C. miyabeanus* strain, Cm988, using the wild type (*Pseudomonas* spp. QAU-92) and its *pqqC* mutant (*Pseudomonas* spp. QAU92-2). The analysis showed clear differences in resistance against *C. miyabeanus* disease induced by wild type strains compared with the *pqqC* mutants. Gene expression in the mock control, control and bacteria-treated samples were articulated as a ratio to actin or *eEF1a* expression through measured efficiency for each gene by RT-PCR. Based on the expression of reference genes (*actin* and *eEF1a*), the CT (cycle threshold) value of *Eef1a* fluctuated more than did the actin gene and therefore actin was chosen as the reference gene.

Expression analysis

Ethylene-responsive TF 89 (*EBP89*), a susceptible gene used as a reference, was upregulated more in infected control plants than in the mock treatment, suggesting the infection-prone nature of plants. It was further observed that the *EBP89* manifested a 21- and 18-fold higher expression in infected control plants than in mock control plants after 36 and 48 h post-inoculation (hpi), respectively (Fig. 3b). For both the wild type strains and pqq mutant strains, it showed less susceptibility to pathogens than did the control plants, which demonstrated resistance to the pathogen. All mutants showed greater susceptibility to pathogens than did wild type strains. The results of disease susceptibility in two biological repeats were the same, but the expression was much higher in the second biological repeat (Fig. 3a and b).

The *EBP89* expression responded strongly to pathogen infection in the infected control plants and *pqqC* mutants than it did in both wild types. This resulted in the expression of approximately 10-fold by the infected control plants and 5-fold by the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) plants compared to the wild type (*Pseudomonas* spp. QAU-92). The expression of the wild type (*Pseudomonas* spp. QAU-92) was only 0.26-fold at 36 hpi and 19-fold induction by the infected control plants. Furthermore, 13-fold induction by the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) was compared to the wild type (*Pseudomonas* spp. QAU-92), which induced only 6-fold at 48 hpi in the second biological repeat (Fig. 3a). In conclusion, the suppression of Cm988-induced EBP89 expression consequently from the wild type (*Pseudomonas* spp. QAU-92) plants was higher than the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) in two biological repeats. The results revealed that the pqq-induced resistance against *C. miyabeanus* in the wild type inoculated plants was higher than in the pqq deleted mutant plants.

Pathogenesis-related protein (*PR1a*) expression analysis

The expression of PR proteins is generally pathogen- and host-specific. The expression of the PR1a gene was much higher in the infected control and *pqqC* mutant plant than in

the wild type inoculated plants, resulting in the wild-type (*Pseudomonas* spp. QAU-92) plants exhibiting a higher induced systemic resistance than the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) in two biological repeats at all observation points (12 h, 24 h, 36 h and 48 h hpi) (Fig. 3b). Approximately, a 47-fold induction was observed by infected control plants and a 39-fold induction by *pqqC* mutant (*Pseudomonas* spp. QAU92-2) inoculated plants. In contrast, the wild type (*Pseudomonas* spp. QAU92) induced about a 15-fold at 36 hpi, a 46-fold induction by infected control plants and a 37-fold induction by the *pqqC* mutant (*Pseudomonas* spp. QAU92-2). Also, the wild type (*Pseudomonas* spp. QAU-92) showed a 17-fold induction at 48 hpi in the second biological repeat (Fig. 3b). Hence, PQQ-induced resistance against *C. miyabeanus* in the wild type inoculated plants was recorded higher compared with the *pqq* deleted mutant plants. The *OsPR1a* gene selected here clearly upregulated in a compatible *C. miyabeanus* fungus interaction, indicating that the *pqq* gene has an effect in suppressing the disease.

Discussion

The identification of the microbes under study remains a fundamental yet tricky task and is usually done through amplifying conserved loci. The molecular phylogeny constructed, therefore, extends our knowledge with regard to organismic relationship and provides the basis for accurate identification (Singh *et al*. 2007). One of the most widely used loci has been the 16S rRNA gene sequences, and the rationale behind this is that the bacterial strains with a similarity of less than 97% can be stated as novel-although after complete characterization. In a similar study, the QAU-68 and QAU-63 strains showed 95 and 97% 16S rRNA sequence similarity, and may be regarded as novel by further characterization (Naveed *et al*. 2014b). In the present study, the 16S rRNA sequence homology for some strains was found low compared with the already submitted sequences at NCBI. The QAU-92 isolates showed 89% homology with *Pseudomonas putida*, a level much lower than the threshold, which therefore pointed to its possible novel status. The housekeeping gene/MLSA analysis revealed that the QAU-92 strain also showed low-level homology and a poor bootstrap value (43%) with loci used in MLSA (rpoB). The results therefore endorsed its novel nature. However, it is important to mention that its novel status requires complete taxonomic characterization (Lim *et al*. 2006), which is in progress.

Although PQQ has been reported in several bacterial genera, many of the species are living in an anaerobic environment and do not use glucose as a carbon source. These bacteria do not have a PQQ cofactor alone but have it in the form of PQQ-dependent GDH. The role of PQQ as cofactor is so important that the GDH enzyme remains inactive. The majority of *Pseudomonas* species (such as *P. fluorescens*) are strictly aerobic in nature and act as a glucose oxidizer (Choi *et al*. 2008). Such bacterial strains produce PQQ-dependent GDH and thus PQQ remains active in such cases. The API-20E system has been found appropriate for the identification of Gram-negative rods and enteric bacteria. The API-20E system for the biochemical characterization of the *pqqC* mutant and wild type strains (QAU92) was used to check the effectiveness of the pqqC gene. Their role was deciphered through comparing the wild type and *pqqC* mutants in the biochemical utilization of carbon, nitrogen sources and enzymatic action, which demonstrated that PQQ has an effect on processes like fermentation and oxidation-reduction (Table 2). These analyses highlighted the clear difference in phenotype of QAU92 wild and mutant strain.

To an extent, PQQ's role is connected to the uptake of phosphate via plants as a cofactor for rhizobacteria dehydrogenases. The PQQ assists in making the soil and the surrounding environment acidic (Rodriguez *et al*. 2004) and consequently more phosphate becomes available to plants. After the *pqqC* deletion, the mutant strains (*Pseudomonas spp.* QAU92-2) lost their capability to solubilize phosphate *in vitro* and also their ability to acidify the medium. The present study confirmed that the phosphate solubilization activity and plant growth promotion is stopped after mutation in the *pqq* gene when compared with the wild type strain phenotype. Previously, it was reported that PQQ enhanced pollen germination *in vitro* in certain plant species such as, *Tulipa, Lilium, Camellia, lettuce, bean* and *tomato* (Naveed *et al*. 2015), but the mechanism remains unclear. The present study provided evidence that PQQ is a plant growth-promoting factor, which was demonstrated through comparison of such activity in *Pseudomonas* wild type strains and the loss of such activity in the *pqqC* mutants (both *in vitro* with lettuce as well as *in vivo* in bean and rice). A significant difference ($P < 0.05$) was noted for plant height, shoot length, dry weight, root weight and total number of leaves in the wild type strains with the *pqqC* mutants, and has shown that the plant growth promotion is mediated by PQQ. It is anticipated that this will enrich our present understanding of the plant growth promotion mechanism (Naveed *et al*. 2016). The PQQ synthesized from *P. fluorescens* B16 has been reported as a growth promoter in tomato, cucumber, *Arabidopsis* and hot pepper (Choi *et al*. 2008).

The *Pseudomonas* spp. QAU-92 produced bio-surfactant (lipopeptides) also demonstrated biological control of *R. solani* root rot. Previously, Perneel *et al*. (2007) reported that *P. fluorescens* CMR12a produced phenazines and bio-surfactants in cocoyam root rot, suggesting strong antagonistic activity against *R. solani*. The reduced antifungal capacity and plant growth promotion might be due to low acid production by the *pqqC* mutants compared with the wild type strains and further suggested the possible control of PQQ over such a process.

The pBKminiTn7- gfp2 tagging system revealed successful root colonization in lettuce by the *Pseudomonas* QAU-92, which colonized the root hair zone of the plant.

This clearly indicated the expression of this gene in lettuce roots. This system has been found useful in environmental studies, disease control, and in addressing the gene expression and population dynamics in a plant's rhizosphere (Koch *et al*. 2001). The previously reported failures in plant growth promotion studies under field conditions have often been associated with poor root colonization (Bloemberg and Lugtenberg 2001). We found in the present study that the plant growth promotion by *pqqC* mutants (QAU92-2) maintained the capability to colonize roots, which emphasized that many other factors might be involved in plant growth promotion beyond root colonization. This recognized a new *PGPR* factor PQQ from *Pseudomonas* spp. QAU-92, which also produced lipopeptides. The root colonization may be influenced by the production of the lipopeptides of both strains because the rhizosphere competence of the bio-surfactant was increased (D'Aes *et al*. 2011).

Many recent studies have accredited the importance of lipopeptides for bacterial root colonization and motility, which are often crucial aspects of biocontrol agents for the soil borne pathogens (Andersen *et al*. 2003; Tran *et al*. 2007). Very few studies are available on plant signaling pathways and bacterial factors underlying ISR in key cereal crops like rice, although the data on dicots is relatively high. The present study focused on PQQ genes of the QAU-92 strain producing a cyclic lipopeptides (CLP) type bio-surfactant, and showed antagonistic activity against fungus and their role in increasing ISR in the model monocot rice. We observed that the application of *Pseudomonas* QAU-92 protected the foliar tissues of rice against brown spot diseases compared with the pqqC mutant strain QAU92-2, which showed a clear symptom of the disease on the leaves. We also identified pqq in *Pseudomonas* with ISR eliciting-activity (Fig. 2) and how it triggers the activation of SA and JA pathways but represses ET signaling, showing the presence of multiple ISR resistance pathways in rice (Fig. 2–3).

The first line of plant defense becomes activated upon pathogen recognition, which results in a basal level of resistance (Pieterse *et al*. 2009). When *PGPR* colonizes the host roots, it leads to ISR (Loon *et al*. 1998). The expression of the pathogenesis-related (*PR*) protein is generally pathogenic and host-specific. In rice, it has shown infection with *C. miyabeanus*, inducing the transient expression of *PR1a*. An approximately 47-fold induction by infected control plants, and a 39-fold induction by *pqqC* mutant (*Pseudomonas* spp. QAU92-2) inoculated plants as the wild type (*Pseudomonas* spp. QAU-92) induced approximately 15-fold at 36 hpi (Fig. 3b). Therefore, the PQQ induced resistance against *C. miyabeanus* in the wild type inoculated plants was recorded as higher compared with the *pqq* deleted mutant plants. The *OsPR1a* gene selected here clearly upregulated in a compatible *C. miyabeanus* fungus interaction, indicating that the *pqq* gene has an effect in suppressing the disease.

Studies in rice and *Arabidopsis* have revealed that rhizobacterial-mediated ISR functions have components of ET and JA response pathways and are independent on SA (Verhagen *et al*. 2004). The expression of the JA marker genes *JiOsPR10* and *JAMYB* responded strongly to the QAU-92 treatment compared with the *pqqC* mutant (QAU92-2). At the same time point and for the same treatment, the ET-related gene *EBP89* showed upregulation over the mock-treated controls and *pqqC* mutant (Fig. 3a). These results also suggested that *pqq* genes of QAU-92 in LB broth produced activation of mainly JA and ET pathways, while the *pqq* deletion mutant (QAU92-2) was unable to activate the hormone up to the level of wild type *P. fluorescens* QAU-92. A clear difference between the wild type (*Pseudomonas* spp. QAU92) and the *pqqC* mutant (*Pseudomonas* spp. QAU92-2) strains in the expression of an ET-related gene *EBP89* and the JA marker genes *JiOsPR10PR1a* gene and *JAMYB* showed the fundamental role of PQQ in induced systemic resistance against *C. miyabeanus* in rice and against *R. solani* root rot in bean.

Conclusion

This is the first study that has investigated the expressional analysis of PQQ from *Pseudomonas* spp. QAU-92 against antifungal activity, phosphate solubilization and the induced systemic resistance against *C. miyabeanus* in rice. Furthermore, evaluated the extent of resistance against a *C. miyabeanus* attack on rice

GenBank sequence submissions

The EMBL GenBank accession numbers for the 16S rRNA gene sequence of QAU-92 strain is KM251450 and rpoB gene sequences is KM251446. The PQQ operon of QAU-92 are pqqA (KM251432), pqqB (KM251433), pqqC (KM251434), pqqD (KM251435) and pqqE (KM251436) and glucose dehydrogenase (gdh) encoding gene sequence is KM251439.

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Author Contributions

MN performed core research work in this article which includes role of Pyrroloquinoline Quinone (PQQ) in biocontrol and induced systemic resistance in rice disease control and expressional analysis of PQQ against phosphate solubilization, antifungal activity. ASM supervised this work, provided facilitates for bench work and generously

made available the chemicals, materials, and equipment this research work. MAS improved the quality of manuscript and did the proof reading.

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