https://doi.org/10.56093/ijas.v90i3.101491

Indian Journal of Agricultural Sciences **90** (3): 593–6, March 2020/Article

Zinc enriched *Pseudomonas fluorescens* triggered defense response in rice against bacterial leaf blight

PRANAMIKA SHARMA, LOHIT CHANDRA BORA, PALASH DEV NATH, SUMITA ACHARJEE, POPY BORA* and JAGDALE MAHESH VASANTRAO

Assam agriculture university, Barbheta, Jorhat, Assam 785 013, India

Received: 26 June 2019; Accepted: 26 August 2019

ABSTRACT

Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* is a major disease in rice and *Pseudomonas fluorescens* is known as a successful bioagent against the pathogen. The present investigation was carried out to study the effect of micronutrients, viz. boron (B), iron (Fe), zinc (Zn), and molybdenum (Mo) on the efficacy of the bioagent and induction of defense mechanism in rice. The study revealed that *P. fluorescens* strain *Pf*As1 enriched with 50 ppm Zn could suppress the blight pathogen by 45.92% compared to control and other micronutrients under *in-vitro* condition. The results also demonstrated a reduction of 82.99% disease intensity in rice due to application of Zn enriched *Pf*As1. Further study was made to understand whether the Zn enriched *Pf*As1 (Zn *Pf*As1) induces defense response in rice. The levels of expression of defense related genes, viz. *OSPR1a*, *OSPR1b*, *OSPR10a*, *Xa1* and *Xa26* against *Xoo* was quantified by quantitative PCR (qPCR). During the interaction of rice with Zn *Pf*As1, a 20 fold increase in the expression of rice BLB resistance gene *Xa26* and pathogenesis related (PR) gene *OsPR1b,* was observed when compared with controls. Similarly, 14 and 10 fold increase in levels of expression of *OsPR1a and OsPR10a* genes was recorded, respectively. An 8 fold increase in the level of expression of *Xa1* gene was also recorded in the study. This is the first report on enhanced defense response in rice due to the antagonistic potential of Zn enriched *P. fluorescens* strain against BLB pathogen.

Key words: Biocontrol, Defense related genes, Micronutrients, Systemic resistance, *Xanthomonas oryzae* pv. *oryzae*

Bacterial leaf blight (BLB) is one of the most important and oldest known diseases of rice caused by *Xanthomonas oryzae* pv. *oryzae* which infects at the maximum tillering stage of the crop, resulting in 20–40% reduction in yields (Nino-Liu *et al.* 2006). The use of plant growth promoting rhizobacteria (PGPR) has been widely adapted in the field of agriculture as it offers an attractive way to improve crop growth and development, replacing or supplementing fertilizers and pesticides. In terms of environmental health, biocontrol agents are considered to be environmental friendly alternative to chemical protections. One of the dominant PGPR under the order Pseudomonds, *Pseudomonas fluorescens* acts as an active biocontrol agent of soil borne diseases on a wide range of crop plants (Bora and Bora 2008).

P. fluorescens isolated from rice rhizosphere is naturally suppressive to BLB, but level of control may depend on the status of soil micronutrient, predominant type of clay mineral, their effect on the pathogen or host or host pathogen interactions (Jeyalakshmi *et al.* 2010). However, plant defense responses are interconnected and both antagonism and synergy between phytohormones signaling network is known (Bostock 2005). The inconsistency in efficacy of the biocontrol agents has been a matter of concern and must be reduced in order to increase the effectiveness against diseases and to create a reliable alternative of chemical control. Zinc has been identified as a factor to improve the biocontrol potential of *P. fluorescens* against *Fusarium* crown and root rot of tomato. Similarly Iron (Fe) is important for siderophore production, a major mechanism of antagonism by *P. fluorescens* (Bora *et al.* 2016). The influence of minerals on suppression of soil borne pathogens has received little attention, and the potential for utilizing mineral amendments for optimizing biocontrol of crop diseases remains largely unexplored.

With this background in the present investigation, attempt was made to enhance the aggressiveness of bioagent *P. fluorescens* through micronutrient enrichment technique and also to study the expression of defense related genes in rice against BLB pathogen in response to application of micronutrient enriched bioagent.

MATERIALS AND METHODS

Sources of rice cultivar, bioagent, bacterial culture and micronutrients: Rice seeds (var. Mahsuri) were collected from ICAR farm AAU, Jorhat (2015–18). The bacterial

*Corresponding author e-mail: pbora.sonitpur10@gmail.com

bioagent *Pf*As1 was collected from Biopesticide laboratory, Department of Plant Pathology, AAU Jorhat and the culture of *X. oryzae* pv. *oryzae* causal agent of BLB was supplied by Regional Agriculture Research Station, Titabor. All the collected organisms were preserved in refrigerator at 4°C for subsequent experimentations.

In vitro evaluation of micronutrients for enhanced efficacy of PfAs1: Aggressive isolate *Pf*As1 was tested against *Xoo* plates enriched with micronutrients (Zn, Mo, B, Fe) at the concentration of 0, 5, 10, 50, and 100 ppm under *in vitro* condition. The assay plates were incubated at 28 ± 2°C for 48 h and percentage inhibitions of the pathogenic bacterial growth were calculated. Micronutrient unamended plates (*Xoo* inoculated *Pf*As1) were maintained as a control for comparison.

In planta *evaluation of various treatment combinations*: The best treatment combinations found *in vitro* were evaluated for their ability to suppress BLB disease in pot grown rice plants (var. Mahsuri). The treatment combinations compared were, *Xoo* uninoculated, *Xoo* inoculated, *Pf* AS1 alone, 50 ppm Zinc alone, *Pf* AS1 with 50 ppm Zinc. The treatments were applied as seed, root dip treatment and foliar spray on 20 days old rice seedlings. The pathogen was inoculated into rice plants adopting clip method. Inoculated leaves were wrapped in moist plastic bags to conserve moisture in net house at 25–27°C. Fourteen days after inoculation, disease intensity (%) was continuously recorded up to 60 days of inoculation. The best treatment obtained from this experiment in induction of host defense, was screened for analysis of pathogenesis-related gene expression along with uninoculated and inoculated control for comparative study.

Gene expression through qRT-PCR analyses: Six-week-old Mahsuri rice plants were inoculate for the virulence assay with bacterial suspensions of approximately 10^8 cfu/ml. Inoculated rice leaves from inoculated control and mixture of Zn*Pf*As1 were collected at 16 and 96 hrs post inoculation. The leaves of Zn*Pf*As1 and uninoculated control were also collected for comparative study with the inoculated ones. RNA was extracted from the collected leaves using Spectrum Plant Total RNA kit (Sigma) as per the manufacturer's instructions with liquid nitrogen.

The isolated RNA and synthesised cDNA was quantified and its purity was estimated with the help of Nanodrop 1000 (Thermo Scientific). The quality of isolated RNA and cDNA was accessed based on A_{260}/A_{280} and A_{280}/A_{260} ratios. Total RNA (10 µg) was used as template to obtain a cDNA yield of 200 ng/µl with the help of Prime Script RT reagent kit with gDNA erase (Takara) kit. Quantitative real time PCR was performed to get quantitative measurements of transcription of tested defense genes with gene specific primers on first strand cDNAs using SYBR premix Ex Taq (Takara) kit. The temperature regime followed were: 95°C for 10 min; 40 cycles of 95°C for 15s, 55°C for 30s, 62°C for 30 s; and 60°C for 1min.

Primers were synthesized for amplification of respective genes, 5'-CGTCTTCATCACCTGCAACTACTC-3' and 5'-CATGCATAAACACGTAGCATAGCA-3'were designed from the sequence of rice *PR1a*, 5'-AGCTGGCCATTGCTTTGG-3' and 5'-CGTT-GTGGAGCCTCACGTAGT-3' designed from rice *PR1b*, 5' -CGCCGCAAGTCATGTCCTA-3' and 5'-GCTTCGTCT-CCGTCGAGTGT-3' designed from rice *PR10a*, *Xa1* gene 5'-ACTGCCCTCTTGCACACGCCTTTGG- 3' and 5'-CCGGTACATCAGTATTGTCCATCGG-3', *Xa26* gene 5'-TGGTCAAATACCGGAAGGAG-3' and 5'-CAGTCCACCACATGGACAAG-3', with endogenous reference gene *OsP1E6* 5'-TTGCACCTAGGAGCGTGGAT-3'and5'-AACTGCAC-ACAACAGTTTGCTCTT-3'. Expression of each target *OsPR* gene was normalized to expression of the constitutively expressed, endogenous reference gene *OsP1E6* and to its expression in untreated, matched control tissues. Master mixture was prepared for 11 cDNA samples to avoid pipetting error. Master mixture of 7 µl was added to each reaction containing 3 µl cDNA, making the total reaction volume to 10 µl and then subjected to 96 well micro titre plate (Applied Biosystem). The components were then mixed well by spinning in a mini centrifuge. For expression levels of each defense gene, cDNAs synthesized from ten independent RNA samples were analyzed in triplicates.

RESULTS AND DISCUSSION

Effect of micronutrients on enhanced efficacy of P. fluorescens As1 against Xoo: The *in vitro* study revealed that micronutrient enrichment could show both positive and negative effects on aggressiveness of *Pf*As1 in their antagonistic properties. Except Zn and Fe at a concentration of 50 ppm, all other micronutrients at various concentrations significantly lowered the antagonistic properties of *Pf*As1 against *Xoo*. *Pf*As1 alone without any micronutrients enrichment showed 36.83% inhibition whereas 50 ppm Zn and Fe exhibited 45.92% and 37.84% growth inhibition respectively, which was significantly higher than positive control. In the case of Zn enriched plates, a gradual increase in antagonism was observed with increase in concentration, but above 50 ppm exhibited a steep decline in percent inhibition. Similar trend was also observed in Fe enriched plates. When 10 or 100 ppm of Mo was supplemented to *Pf*As1 very low levels (8.48% and 7.03%) of suppression of *Xoo* growth was recorded (Table 1).

Effect of micronutrient and PfAs1 on bacterial leaf blight of pot grown rice: In all the test pots, significantly lower disease intensity (% DI) has been observed when micronutrients and *Pf*As1were applied in combinations (Table 2). The highest disease reduction (82.99%) or lowest DI (17.00%) was recorded in treatment comprising Zn enriched *Pf*As1 (10^8 cfu/ml) and Zn applied as root dip and spray method at 2%. PfAs1 alone treated plants showed 62.58% disease reduction in pots. The highest disease intensity of rice blight (84.42%) was recorded in the *Xoo* inoculated rice plant without any treatment. Thus Zn enriched *Pf*As1 (Zn*Pf*AS1) was found to have superior biocontrol efficacy.

Table 1 Effect of micronutrients on enhanced aggressiveness shown *Pseudomonas fluorescens As1* against *Xoo in vitro*

	Treatment	Zone of inhibition (mm diam)	Inhibition (%)
T_1	Xoo + *Pf*As1	33.15	36.83(37.35) *
T_2	Xoo + *Pf*As1 +Zn (5 µg/ml)	18.00	20.20 (25.10)
T_3	Xoo + *Pf*As1+Zn (10 µg/ml)	30.66	34.06(35.67)
T_4	Xoo + *Pf*As1 +Zn (50 µg/ml)	41.33	45.92 (42.65)
T_5	Xoo + *Pf*As1+Zn (100 µg/ml)	16.66	18.51 (25.48)
T_6	Xoo + *Pf*As1+Mo (5 µg/ml)	9.48	10.53 (18.91)
T_7	Xoo + *Pf*As1+Mo (10 µg/ml)	7.64	8.48 (16.85)
T_8	Xoo + *Pf*As1 + Mo (50 µg/ml)	13.33	14.81 (22.63)
T_9	Xoo +*Pf*As1 + Mo (100 µg/ml)	6.33	7.03 (15.34)
T_{10}	Xoo + *Pf*As1 +B (5 µg/ml)	10.08	12.00 (20.27)
T_{11}	Xoo + *Pf*As1 + B (10 µg/ml)	9.33	10.36 (18.72)
T_{12}	Xoo + *Pf*As1 + B (50 µg/ml)	12.87	14.30 (22.22)
T_{13}	Xoo + *Pf*As1 + B (100 µg/ml)	8.68	9.64 (18.05)
T_{14}	Xoo + *Pf*As1 +Fe (5 µg/ml)	8.66	9.62 (18.05)
T_{15}	Xoo + *Pf*As1 + Fe (10 µg/ml)	26.66	29.62 (32.96)
T_{16}	Xoo + *Pf*As1 + Fe (50 µg/ml)	34.06	37.84 (37.94)
T_{17}	Xoo + *Pf*As1+ Fe (100 µg/ml)	11.00	12.22 (20.44)
T_{18}	Control	0.00	0.00 (0. 57)
	S.Ed.(±)		0.933
	$CD_{0.05}$		2.080

* Data in the parenthesis are angular transformed values; Data are mean of three replications.

P. fluorescens employs an array of mechanisms inhibit pathogens through various mechanisms, viz. antibiotics, siderophores, etc. The enhanced crop vigour along with induction of ISR by *P. fluorescens* results in low disease incidence (Bora *et al.* 2016). Application of *Pf*As1 to various crops improves the percentage of seed germination, enhance crop growth and yield. This may be due to the fact that the *P. fluorescens* isolates could exhibit more than two or three plant growth traits, stimulates phytohormone, which promote growth directly, indirectly, or synergistically and protects plants against pathogens (Kumar *et al.* 2012). Minorsky (2008) reported that *P. fluorescens* application on plants increased the height, flower number, fruit number and total fruit weight of tomato plants. This was also established by Rani *et al.* (2012) where they reported that PGPR inoculation in pigeon pea significantly improve seedling emergence, shoot and root length, dry matter production, nodule number and mass than uninoculated control. Deng *et al.* (2012) reported that one of the rice CCCH-type zinc finger proteins, C3H12 partially enhanced resistance to *Xoo*, accompanied by the accumulation of Jasmonic acid (JA) and induced expression of JA signalling genes in rice.

Expression of defense genes in rice due to application of Zn enriched PfAs1: Expression of five important defense related genes, viz. *OsPR1a*, *OsPR1b*, *OsPR10a*, *Xa1* and *Xa26* in rice was studied to understand the underlying molecular mechanism of resistance to BLB upon application of Zn*Pf*As1.

Table 2 Effects of different treatments on bacterial blight intensity (%) in pot grown rice

Treatment		Disease intensity (%)	Disease reduction (%)
T_0	Negative control (*Xoo* uninoculated)	0.00 (4.05)*	--
T_1	Positive control (*Xoo* inoculated)	84.42 (66.74)	--
T_2	*P. fluorescens* AS1 alone	37.42 (37.70)	62.58
T_3	Zn (50 ppm) alone	67.86 (55.43)	32.14
T_4	*P. fluorescens* AS1 + Zn (50 ppm)	17.00 (24.35)	82.99
SEd±		0.304	--
CD 5%		0.532	--

* Data in the parenthesis are angular transformed values; Data are mean of five replications.

The qPCR data revealed a slight up-regulation of defense genes, *OsPR1a* and *Xa26* when compared with the untreated plants. However, no expression of *OsPR1b, OsPR10a* and *Xa1* was observed due to application of Zn*Pf*As1. We have also analyzed the changes in expression profile in the leaves treated Zn*Pf*As1 followed by inoculation of *Xoo* in leaves after 7 days. We found that the expression of defense gene in Zn*Pf*As1 treated leaves were highest when compared to the *Xoo* inoculated leaves and untreated controls. The relative expression of *Xa1* was observed after 16 h of inoculation of *Xoo* in rice leaves treated with Zn*Pf*As1. The gene *Xa1* up-regulated after 16 h, however, after 96 h more than 3 fold increase in the gene expression was observed when compared with uninoculated and *Xoo* inoculated controls at 16 h and 96 h. Similarly, expression of *Xa26* was 4 fold higher than after 96 h of *Xoo* inoculation in rice leaves treated with Zn*Pf*As1. No expression of *Xa26* gene was found in uninoculated control and *Xoo* inoculated control at 16 h and 96 h, respectively.

The qPCR assays of the defense gene *OsPR1a* revealed expression of *OsPR1a* in *Xoo* inoculated control after 96 h of inoculation. Interestingly, the expression of *OsPR1a* was eight-fold higher at 16 h of *Xoo* inoculation rice leaves treated with Zn*Pf*As1 when compared with the *Xoo* inoculated samples. Moreover, the expression was two-fold higher after 96 h compared to the expression at 16 h. The expression pattern of the PR gene *OsPR1b* was different from *OsPR1a.* However, after 96 h the expression of *OsPR1b* was four fold up-regulated as compared to *Xoo* inoculation in rice leaves and controls. Expression of *OsPR10a* was observed after 16 h of inoculation *Xoo* in the *Pf*As1 + Zn (50 ppm) treated samples. Similarly, *OsPR10a* up-regulated after 96 h of *Xoo* inoculation in Zn*Pf*As1 treated samples.

The above findings confirmed that the up- regulation of *OsPR1a*, *OsPR1b* and *OsPR10a* genes were observed only after exposure to the pathogen *Xoo*. Expression of defense genes up-regulated in samples where Zn *Pf*As1 were applied 7 days prior to inoculation of *Xoo*. We also found that the application of *Xoo* did not induce up-regulation of these defense genes in susceptible rice plants. The expression of *Xa1, OsPR1a* and *OsPR10a* was highly up-regulated after 16 h of inoculation in samples treated with Zn *Pf*As1.

In the present experiment we studied the expression of 5 important defense related genes, viz. *OsPR1a*, *OsPR1b*, *OsPR10a*, *Xa1* and *Xa26* in uninoculated rice (control), *Pf*As1 + Zn (50 ppm) treated rice plants, *Xoo* inoculated rice plants and *Pf*As1 + Zn (50 ppm) treated rice plants challenged by *Xoo* inoculation. We found up-regulation of *Xa1* and *Xa26* in samples where *Pf*As1 was applied. Interestingly result showed that no up-regulation of *PR* genes, viz. *OsPR1a*, *OsPR1b* and *OsPR10a* was found in rice due to application of *Pf*As1. Up-regulation of these defense genes were detected in samples where *Pf*As1 +Zn (50 ppm) was applied 7 days prior to inoculation of *Xoo*. It was also found that application of *Xoo* did not induced up-regulation of these defense genes in rice plants. Expression of *Xa1, OsPR1a* and *OsPR10a* was highly up-regulated after 16 hrs of inoculation in samples treated with *Pf*As1 + Zn (50 ppm). *OsPR1b* expressed only 96 h of inoculation. This perhaps is the first report to investigate the possible use of *Pf*As1 +Zn (50 ppm) –mediated induction of defense related gene expression for biological control and their probable role in protecting rice against *Xoo,* an important phytopathogenic bacteria.

Elicitation of induced systemic resistance (ISR) is a widespread phenomenon of many non pathogenic microorganism and biocontrol agent. For example, host is protected from the pathogen due to biocontrol agent by production of antibiotics, metabolites (Beneduzi *et al.* 2012, Bora *et al.* 2013) and/or by inducing plant defense mechanism (Bakker *et al.* 2007). Although ISR is known in the case biocontrol but the manner in which it does is not fully elucidated. Moreover, induced defense response is due to the biocontrol agent or due to interaction biocontrol agents with the pathogen is not yet elucidated. There are specific elicitors which are produced by the pathogen and percieved by signalling pathway during induced defense. These elicitors are of various types such as carbohydrate polymers, lipids, glycopeptides and glycoproteins. The *Pf*As1 produces antibiotics, lipopolysaccharides and iron regulated metabolites, which are known to induce systemic resistance (Bakker *et al.* 2007). Therefore, elucidating the molecular mechanism of resistance to BLB due to application of Zn *Pf*As1 appeared to be interesting.

The study revealed that effect of micronutrients can play significant role in enhancing antagonistic potential of *fluorescens*. This put forward the scope of further research in marinating consistency of field performance of bioagents through fortification of micronutrients. The expression profile of defense related genes in treated rice plants provide deeper insight into molecular mechanism of disease resistance induced by *P. fluorescens.*

ACKNOWLEDGMENTS

The authors express sincere gratitude to the Director of Post Graduate Studies, Assam Agricultural University and the Head, Department of Plant Pathology for providing necessary facilities and guidance to conduct the study. The authors acknowledge the Department of Agricultural Biotechnology, Assam Agricultural University and DBT-AAU Centre for providing laboratory facilities to carry out PCR analysis.

REFERENCES

Bora L C and Bora Popy. 2008. Vemicompost based bioformulation for management ofbacterial wilt of tomato in poly house. *Journal of Mycology and Plant Pathology* **38**(3): 527–30.

Bora Popy**,** Bora L C and Begum M. 2013. Ecofriendly management of bacterial wilt disease in brinjal through application of antagonistic microbial population. *Journal of Biological Control* **27**(1): 29–34

Bora Popy**,** Bora L C and Deka P C. 2016. Efficacy of substrate based bioformulation of microbial antagonists in the management of bacterial disease of some solanaceous vegetables in Assam. *Journal of Biological Control* **30**(1): 49–54

Bostock R M. 2005. Signal crosstalk and induced resistance: Straddling the line between cost and benefit. *Annual Review of Phytopathology* **43**: 545–58.

Deng H, Liu H, Li X, Xiao J and Wang S. 2012. A CCCH-Type zinc finger nucleic acid-binding protein quantitatively confers resistance against rice bacterial blight disease. *Plant Physiology* **158**: 876–89.

Fred A K, Kiswara G, Yi G and Kim K M. 2016. Screening Rice Cultivars for Resistance to Bacterial Leaf Blight. *Journal of Microbiology and Biotechnology* **26**(5): 938–45

Jeyalakshmi C, Madhiazhagan K and Rettinassababady C. 2010. Effect of different methods of application of *Pseudomonas fluorescens* against bacterial leaf blight under direct sown rice. *Journal of Biopesticide* **3**(2): 487–88.

Kumar A, Kumar A, Devi S, Patil S, Payal C and Negi S. 2012. Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Research in Science and Technology* **4**(1): 01–05.

Minorsky PV. 2008. On the inside. *Plant Physiology* **146**: 323–24.

Nino-Liu D O, Darnielle L and Bogdanove A J. 2006. *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Molecular Plant Pathology* **7**(5): 303–24.

Ponciano G, Yoshikawaa M, Leea J L, Ronaldb P C and Whalen M C. 2007. Pathogenesis-related gene expression in rice is correlated with developmentally controlled *Xa21*-mediated resistance against *Xanthomonas oryzae* pv. *oryzae*. *Physiological and Molecular Plant Pathology* **69**: 131–39.

Rani M U, Arundhathi K and Reddy G. 2012. Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. *African Journal of Biotechnology* **11**(32): 8085–91.