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Resistance responses of rice to rice blast fungus after seed treatment with the endophytic *Achromobacter xylosoxidans* AUM54 strains

Manoharan Melvin Joe [a,c,*], Md. Rashedul Islam [b], Balathandayutham Karthikeyan [c], K. Bradeepa [c], Palanivel Karpagavinayaga Sivakumaar [c], Tongmin Sa [a,*]

[a] Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea
[b] Department of Biological Sciences, Inha University, Incheon 402-751, Republic of Korea
[c] Department of Agricultural Microbiology, Annamalai University, Annamalainagar 608002, India

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ABSTRACT

In the present investigation, we studied resistance imparted by seed treatment with an endophytic strain of *Achromobacter xylosoxidans*, AUM54, against rice blast caused by *Magnaporthe oryzae*. In vitro studies showed that *A. xylosoxidans* AUM54 was able to inhibit mycelial growth of *M. oryzae* by 11% and was able to increase rice germination and seedling vigor index of rice by 31 and 114%, respectively. AUM54 also showed better survivability in the spermosphere and spermoplane and was able to move systemically through the roots and stem. Among the evaluated carriers, liquid formulation amended with 2% glycerol sustained the maximum bacterial population (7.4 log cfu ml^{-1}) after six-months-storage at room temperature. Plants treated with *A. xylosoxidans* AUM54 followed by inoculation with *M. oryzae* showed a significant increase in the activities of defense related enzymes such as polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia-lyase (PAL) and chitinase. *A. xylosoxidans* AUM54 treatment was able to reduce blast disease incidence by 39% in treated rice plants. Additionally, inoculation with *A. xylosoxidans* AUM54 significantly enhanced the growth (3–13% plant height), and yield (11–31%) of inoculated rice plants under no-disease and disease conditions in the greenhouse experiments.

1. Introduction

Rice, a grain crop having the second-highest world production after maize (FAOSTAT, 2010), is the staple crop of economic importance in many countries (Yoon et al., 2011). Rice blast, caused by the fungus *Magnaporthe oryzae* (Hebert) Barr (anamorph *Pyricularia oryzae* Cavara) accounts for 10–15% of annual yield losses (Baker et al., 1997; Shirasawa et al., 2012). In India, the state of Tamilnadu ranks the second in rice productivity contributing to about 7% of the country's total production (ICRISAT-WWF Project, 2008). In this state, rice blast is one of the most devastating diseases on susceptible cultivars, causing a yield loss up to 90% (Mehrotra, 1998; Jaiganesh et al., 2007).

M. oryzae attacks rice plants at all stages of development and can infect leaves, stems, nodes and panicles (Sesma and Osbourn, 2004). It uses a hemibiotrophic infection strategy involving initial proliferation inside living host cells followed by a destructive necrotrophic mode (Park et al., 2009). Management of this disease includes either the use of fungicides or breeding of cultivars with major resistance genes (Manandhar et al., 1998; Shirasawa et al., 2012). The overuse of chemical fungicides for disease management has resulted in environmental pollution, residual hazards and ill health effects to the biotic community as a whole (Bonmann et al., 1992; Nandakumar et al., 2000). Though breeding of disease resistant cultivars is considered as a viable option, consumers and farmer's preference toward cultivation/consumption of a particular variety has laid down limitations in adopting this approach.

The use of biological agents in particular plant growth promoting rhizobacteria (PGPR) has been considered as an attractive viable option to the above control strategies. PGPR present in large numbers in association with roots are known to promote plant growth directly by improving the uptake of certain plant nutrients from the environment (Kloepper et al., 1991), and/or indirectly by preventing or lessening the deleterious effect of plant pathogens (Dobbelaere et al., 2003).

Endophytic bacteria by their ability to improve plant growth can be considered as PGPR, and their close interactions with plants make them an ideal candidate for enhancing plant growth and

* Corresponding authors. Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea. Tel.: +82 43263447.
E-mail addresses: micromelvin@gmail.com, mel_vin@sify.com (M.M. Joe), tomsa@chungbuk.ac.kr (T. Sa).

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productivity (Prasanna et al., 2012). Among these endophytic bacteria, diazotrophic endophytes are considered to be important because of the fact that these bacteria appear to provide more fixed nitrogen when compared to rhizospheric bacteria (Quispel, 1991) due to less competition for nutrients compared with rhizosphere bacteria (Reinhold-Hurek and Hurek, 1998). In addition to their diazotrophic nature, these bacteria are also known for other plant growth promoting (PGP) traits such as production of phytohormones, phosphate (P) solubilization, siderophore production, inhibition of ethylene biosynthesis and conferring resistance to plant species against pathogens (Jha and Kumar, 2009).

Among PGPR, diazotrophic endophytes belonging to *Achromobacter xylosoxidans* has been shown tremendous promise in terms of improvement of NO_3 uptake by roots and reduction of ethylene level via expression of putative 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Blaha et al., 2006; Jha and Kumar, 2009; Sgroy et al., 2009). *A. xylosoxidans* strain 31A was reported to tolerate salinity (Sgroy et al., 2009) and another endophytic *A. xylosoxidans* strain SF2 produces salicylic acids, which have been shown to inhibit the growth of pathogenic fungi and enhance the growth of sunflower crop under water stressed conditions (Forchetti et al., 2007).

Though *A. xylosoxidans* strains are reported to have multiple PGP characters, no effort has been made to evaluate its use as a biocontrol agent or its ability to induce systemic resistance in plants against diseases. Moreover, to the best of our knowledge no diazotrophic endophytic bacteria have been tried for the biocontrol of rice blast. In this study, we evaluated the antagonistic activity of *A. xylosoxidans* AUM54 against *M. oryzae*. We also determined the survivability of *A. xylosoxidans* AUM54 in the spermosphere and spermoplane and its ability to move systemically through the roots and stem. The ability of *A. xylosoxidans* AUM54 to survive in different carriers under room temperature was also evaluated. The strain was studied in vivo for its ability in promoting defense responses of rice plants and reducing disease incidence and promoting growth under no-disease and disease conditions in greenhouse.

2. Materials and methods

2.1. Microbial strains and growth conditions

A. xylosoxidans AUM54 was isolated from *Catharanthus roseus* (Karthikeyan et al., 2012) and the nucleotide sequence (16S rRNA) of this isolate was deposited to GenBank under the accession number JF827294. The isolate has been maintained in Johanna-Dobereiner Mannitol Vera-Baldani (JMV) agar (Baldani, 1996) slants at 30 ± 2 °C with monthly transfer.

The test pathogen *M. oryzae* isolate, AUP178, was from the collection of the Department of Microbiology, Faculty of Agriculture, Annamalai University and was maintained in potato dextrose agar.

2.2. Effect of A. xylosoxidans AUM54 on mycelial growth

The effect of *A. xylosoxidans* AUM54 on the mycelial growth of *M. oryzae* AUP178 was studied according to the methods of Filippi et al. (2011). Briefly, 5 mm disc of *M. oryzae mycelium* was transferred to the central part of a Petri dish containing potato dextrose agar (PDA). *A. xylosoxidans* AUM54 was streaked around *M. oryzae* mycelium. The evaluation was done by measuring the radial growth of *M. oryzae*.

2.3. Germination percentage and vigor index

The germination percentage was calculated from 5 to 7 days after sowing (DAS) using paper towel method (ISTA, 1976). The vigor index (*VI*) of the seedlings was measured at 21 DAS as suggested by Abdul-Baki and Anderson (1973) as follows:

$$VI = RL + SL \times GP$$

Where *RL* is root length (cm), *SL* is shoot length (cm) and *GP* is germination percentage.

2.4. Spermoplane survival and spermosphere population

The spermoplane survival and spermosphere population at an interval of 3, 5 and 7 days were determined according to the methods of Emmert et al. (1998), Ugoji et al. (2006), and Joe and Sivakumaar (2010) with required modifications. Seeds were surface sterilized and the efficiency of sterilization was tested by placing the seeds in nutrient and potato dextrose agar. For spermoplane population bacteria treated seeds were placed on water agar plates at the rate of 3–4 seeds per plate. The plates were sealed with Para-film and placed in dark at 24 °C. Spermoplane populations were sampled by aseptically removing seeds from the water agar and then placing each seed in 10 ml of phosphate buffer, followed by sonication for 30 s, serial dilution and plating on JMV agar. For spermosphere population assay, PVC tubes (5.0 cm diameter 12.0 cm long) were plugged in the bottom with cotton plug and filled with 90 g of sterile vermiculite. Ten grams of bacterized seeds was placed on the soil surface of each tube and then covered with 10 g of sterile vermiculite. Each tube received 25 ml of sterile water at planting and was incubated in the growth chamber under a 12 h photoperiod at 25 °C. At different intervals mentioned above, individual seedlings were removed from the tubes and 1 g of the vermiculite in close vicinity with the roots was removed and dissolved in 10 ml of phosphate buffer. The suspension was vortexed for 30 min at maximum speed, serially diluted and enumerated in JMV agar.

2.5. Systemic movement in rice plants

Systematic movement of *A. xylosoxidans* AUM54 in the rice plants was carried out according to the methods of Jaiganesh et al. (2007) with required modifications. Briefly, one hundred grams of surface sterilized seeds were treated with 1 ml culture filtrate (9 log cfu ml^{-1}), grown with rifampicin (30 mg l^{-1}) and ampicillin (20 mg l^{-1}) for 10 generations and tested for its ability to grow in antibiotic containing plates. Seeds treated with *A. xylosoxidans* AUM54 were air dried for 1 h in a laminar flow cabinet. Then, the seeds were taken with a sterile forceps and plated on moistened blotting paper in Petri dishes at 10 seeds per plate. The seeds were incubated for 10 days by moistening the plates daily with sterile water. After 10 days, the roots and shoots were separated and cut into bits of 1 cm size at a distance of 1, 3 and 5 cm from the tip of the root/shoot using sterile blades and the population of *A. xylosoxidans* AUM54 in root/shoot at different distances was estimated as described below.

One gram of shoot and root bits taken at different distances as described above from the rice plant was ground individually in a sterile pestle and mortar with 10 ml of phosphate buffer. The extract was then serially diluted and plated in JMV plates supplemented with antibiotics with the above mentioned concentrations.

2.6. Survivability in selected inoculant carriers

Talc based formulation was prepared based on the earlier reports of Bharathi et al. (2004) in which *A. xylosoxidans* was grown in JMV broth for 48 h, and the contents were mixed equally (v/v) with talc powder, $CaCO_3$ and carboxymethyl cellulose (CMC) under strict sterile conditions, and stored as mentioned below. Glycerol based liquid formulations were prepared based on the methods of Manikandan et al. (2010). Briefly, 2% glycerol was added in JMV broth and to this, 1 ml of log phase culture was inoculated and incubated at