Bot. Bull. Acad. Sin. (2004) **45:** 315-324

Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp.

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(Received December 9, 2003; Accepted June 8, 2004)

Abstract. Pink-pigmented facultatively methylotrophic bacteria (PPFMs), persistent colonizers of plant leaf surfaces, belong to the genus *Methylobacterium* and are mostly transmitted through seeds. Plant growth-promoting activity of methylotrophic bacteria and their effects on disease suppression were evaluated on rice under greenhouse conditions. Rice seeds were inoculated with *Methylobacterium* sp. strain PPFM-Os-07 and seed germination was evaluated in terms of morphometric measurements, seedling growth, rate of germination (R_G), and seedling vigour index (SVI). Another experiment was carried out to study the induction of pathogenesis-related proteins (PR-proteins) in rice plants that were inoculated with methylotrophic bacteria by seed imbibition or foliar spray. In the third experiment, sixty-day-old rice plants grown in pots were challenge inoculated with *Rhizoctonia solani* strain TNAU-01. *Methylobacterium* inoculation promoted seed germination and plant growth. Increased plant height, number of tillers, plant biomass, and grain yield were observed. The average yield increases for seed imbibition and phyllosphere spray were, respectively, 22.1% and 24.3% greater than control. The bacteria also significantly reduced the sheath blight incidence when applied as either bacterial culture through seed imbibition and/or phyllosphere spray. The percent disease reduction recorded for seed imbibition alone and for combined applications of seed imbibition and phyllosphere spray were 17.8% and 23.5%. Rice plants sprayed with PPFM-Os-07 strain showed increased presence of PR-proteins and phenolic contents on day 1 after application. Maximum phenylalanine ammonia lyase (PAL) and peroxidase activity on day 4 and β-1,3-glucanase and chitinase activity on day 5 were recorded. The results suggest that *Methylobacterium* inoculation may alter rice susceptibility to *R. solani*. This work emphasizes the importance of evaluating induced systemic resistance while studying plant-associated growth promoting bacteria.

Keywords: *Methylobacterium* sp.; *Oryza sativa* L.; Induced systemic resistance; Pathogenesis-related proteins; *Rhizoctonia solani*.

Introduction

Methylobacterium spp. are a group of bacteria known as pink-pigmented facultative methylotrophs, or PPFMs (Austin and Goodfellow, 1979; Patt et al., 1976; Green and Bousfield, 1982, 1983). They are classified as alpha-*Proteobacteria* and are capable of growth on one-carbon compounds such as formate, formaldehyde, methanol, and methylamine as well as on a variety of C_2, C_3 and C_4 compounds (Lidstrom, 2001). They can be easily isolated from plant tissues using selective media containing methanol as the sole carbon source (Corpe, 1985) and identified by their pink color, which distinguishes them from the other unrelated methylotrophic organisms normally encountered on plant tissue. They are abundant and non-pathogenic, distributed ubiquitously in the plant phyllosphere, and have been isolated from more than 100 species of plants, ranging from liverworts and mosses to angiosperms and gymnosperms (Corpe and Basile, 1982; Dunleavy, 1990; 1998). Hirano and Upper (1992) found them to represent greater than 90% of the phylloplane microflora of *Phaseolus vulgaris* throughout the growing season. PPFMs show populations ranging from 10^4 to 10^7 colony-forming units per gram fresh weight of plant tissue, with the highest numbers present on actively growing and meristematic tissue (Dunleavy, 1988; Corpe and Rheem, 1989; Holland and Polacco, 1992; Hirano and Upper, 1992; Holland, 1997). They are seed- transmitted (Corpe and Basile, 1982) and in dry soybean seeds they number 10^5 per gram (Dunleavy, 1988; Holland and Polacco, 1992). Their slow-growing nature and distribution over the whole plant suggest that their numbers are regulated simply by dilution as the plant tissue expands away from growing points. Holland and Polacco (1992) reported surface sterilization of seeds or other plant tissues, routinely carried

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out in preparation for tissue culture, does not remove PPFMs. Confocal scanning laser microscopy showed *gfp*-tagged PPFMs to colonize the leaf surface by forming large aggregates and by occupying the grooves between the epidermal cells (Omer et al., 2004a). They were reported to colonize the rhizosphere as well as the phyllosphere. However the biological significance of these bacterial species is still unknown.

The practical significance of these bacteria, such as the biosynthesis of amino acids and single cell proteins and bioconversion of some substrates unusable by other organisms (such as methanol emitted by the stomata of plants) into products with economic value has brought them into prominence (Nemecek-Marshall et al., 1995). Basile et al. (1969) first demonstrated the growth enhancing effects of PPFMs on plants in a tissue culture system where they produced vitamin B_{12} and stimulated the growth of a liverwort, *Scapania nemorosa.* Interestingly they were also reported to produce growth hormones *viz*, cytokinins (zeatin, *trans*-zeatin and *trans*-zeatin riboside) (Koenig et al., 2002) and auxins (indole-3- acetic acid, indole-3- pyruvic acid and indole-3- butyric acid) (Ivanova et al., 2001; Omer et al., 2004b). They have also been shown to stimulate seed germination and plant growth (Corpe and Basile, 1982).

This study has been framed to understand the beneficial role of *Methylobacterium* on rice with the following objectives: 1) evaluation of the effect of PPFMs on rice seed germination, and 2) investigation of the effect of PPFM on induction of PR-proteins like phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5), peroxidase (E.C. 1.11.1.7), chitinase (E.C. 3.2.1.14) and β-1,3-glucanase (E.C. 3.2.1.39) and phenolics. These studies could ultimately lead to understanding the role of PPFMs in improving the performance of host plants, especially rice.

Materials and Methods

Bacterial Strains and Cultural Conditions

Methylobacterium sp. strain PPFM-Os-07 isolated from rice (*Oryza sativa* L.) cultivar Co-47 was used in the study (Madhaiyan, 2003). The reference culture *Methylobacterium extorquens* AM1 (isolated from air, AM1 stands for air-borne microbe) was kindly provided by M. A. Holland, Department of Biology, Salisbury University, Salisbury, MD 21801, USA. *Methylobacterium extorquens miaA* mutant was kindly provided by Joe C. Polacco, Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri 65211, USA. The sheath blight pathogen *Rhizoctonia solani* strain TNAU-01 was obtained from the Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore 641003, Tamilnadu, India. *Rhizoctonia solani* was maintained and grown in potato dextrose agar (PDA) before further use. Unless otherwise stated, methylobacteria were grown for 72 h on ammonium mineral salt (AMS) medium (pH 6.8) supplemented with 0.5% methanol and cycloheximide (30 μg ml^{-1}) (Whittenbery et al., 1970).

Experiment I

Testing of rice seed germination. Rice seeds were surface sterilized by heat treatment at 50°C for 48 h (M. A. Holland personal communication) for removal of seed borne methylotrophic bacteria, and they were imbibed in one of the following preparations for 5 h at room temperature with gentle shaking: T_1) PPFMs-Os-07 liquid culture; T_2) PPFMs-Os-07 spent medium; T_3) *miaA* mutant liquid culture; T_4) *miaA* mutant spent medium; T_5) *M. extorquens* AM1 liquid culture; T_6) *M. extorquens* AM1 spent medium; T_7) fresh AMS medium containing tetracycline (15 μg ml^{-1}), T_8) fresh AMS medium, and T_9) control. All cultures were grown to the stationary phase before they were used. After imbibition, the seeds (200 seeds/replicates) were drained and sown in sterile aluminum trays (20×20×10cm, 50 seeds/tray) containing fine, moist sand and then placed in a germination room and maintained at 20°C. Germination of rice seeds was recorded daily from day 5 to day 14, and the rate of germination (R_G) was calculated using the following formula.

$$R_G = \Sigma \frac{N_i}{D_i}$$

Where Ni is the number of germinated seeds in a given time, and Di is the time unit (day) (Hosseini and Jafari, 2002). For growth measurements ten seedlings from each treatment were taken randomly after 14 days of sowing. The shoot and root length of individual seedlings were recorded. After the fresh weight of each seedling was recorded, these were kept in the oven at 70°C for 48 h to determine their dry weight (Evans, 1972). The germination percentage of seeds was recorded, and the seedling vigour index (SVI) was calculated using the formula: SVI = % germination × seedling length (shoot length + root length) in cm. Results were expressed in whole numbers (Baki and Anderson, 1973).

Experiment II

Evaluation of pathogenecity related proteins and phenolics. Soil samples were collected from wet rice fields with the following physio-chemical properties: pH: 7.8, CEC: 26.4 cmol (p+) kg^{-1}, EC: 0.4 dSm^{-1}, organic carbon: 5.83 g kg^{-1}, available N: 220 kg ha^{-1}, available P_2O_5: 10 kg ha^{-1}, available K_2O: 260 kg ha^{-1} and available Ca: 11.42 cmol kg^{-1}, Zn: 2.80 ppm, Fe: 29.75 ppm, Mn: 3.9 ppm and Cu: 2.39 ppm. Samples were dried, passed through a 4 mm sieve, and mixed with farmyard manure in a 2:1 proportion. Rice seedlings were planted at the rate of four seedlings per hill and five hills per pot (40 L pots containing 20 kg soil mixture). In foliar application, 25 ml of liquid culture (1×10^9 CFU ml^{-1} of culture) per pot was sprayed 40 days after planting. From day 41 to day 48, the seedlings were evaluated for various PR-proteins *viz*, 1) chitinase, 2) peroxidase, 3) PAL, 4) β-1,3 glucanase, 5) PPO, and 6) phenolics.

Plant extraction. Rice leaves collected from all treatments were snap frozen and homogenized immediately with liquid nitrogen. One gram of homogenized sample was ex-

tracted at 4°C with appropriate buffer solutions, and the homogenate, centrifuged for 20 min at 10,000 rpm, was used for further studies. Sodium acetate buffer (0.5 M; pH 5.0), sodium citrate buffer (0.1 M; pH 5.0), sodium borate buffer (0.1 M; pH 7.0), and phosphate buffer (0.1 M; pH 7.0) were used to extract β-1,3-glucanase, chitinase, PAL, and peroxidase enzymes, respectively.

Phenylalanine ammonia lyase. PAL activity was measured following the method of Dickerson et al. (1984). The assay mixture, containing 100 µl of plant extract, 500 µl of 50 mM Tris HCl (pH 8.8), and 600 µl of 1 mM L-phenylalanine, was incubated for 60 min at room temperature, and the reaction was arrested by adding 2 N HCl. The assay mix was extracted with 1.5 ml of toluene by vortexing for 30 sec. Toluene was recovered after centrifuging at 1,000 rpm for 5 min. The absorbance of the toluene phase containing *trans*-cinnamic acid was measured at 290 nm against the blank of toluene. Enzyme activity was expressed as nmol *trans*-cinnamic acid released min^{-1} g^{-1} fresh weight.

Chitinase. For the colorimetric assay of chitinase, 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of plant extract, and 0.1 ml colloidal chitin (1 mg), prepared according to Berger and Reynolds (1958), were pipetted into a 1.5 ml Eppendorf tube. After 2 h at 37°C, the reaction was stopped by centrifugation at 1,000 *g* for 30 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass tube containing 30 µl of l M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme (Helicase, obtained from Sepracor, France) for 1 h (Boller and Mauch, 1988). The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig et al. (1959) using the internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as 1 nmol of GlcNAc equivalents released min^{-1} mg^{-1} of fresh weight.

β-1,3-glucanase. Enzyme activity was assayed by the laminarin-dinitrosalicylic acid method (Pan et al., 1991). The reaction mixture was prepared by mixing 62.5 µl of 4% laminarin and 62.5 µl of plant extract and incubating at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent (DNS, prepared by adding 300 ml of 4.5% NaOH to 880 ml containing 8.8 g of dinitro salicylic acid and 22.5 g potassium sodium tartrate) with subsequent heating for 5 min in a boiling water bath. The resulting colored solution was diluted with 4.5 ml of distilled water and vortexed. Products released were estimated for reducing groups at 500 nm. The enzyme activity was expressed as 1 nmol of reducing substances min^{-1} mg^{-1} of fresh weight.

Peroxidase. Peroxidase activity was determined at 30°C by a direct spectrophotometric method (Hammerschmidt and Kuc, 1982). The reaction mixture consisted of 0.5 ml plant extract, 1.5 ml of 0.05 M pyrogallol, and 0.5 ml of 1% hydrogen peroxide (H_2O_2). The reaction was incubated in a water bath, and absorbance at 420 nm was recorded at 30 s intervals for 30 min. The enzyme activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} of fresh weight.

Polyphenol oxidase. Plant sample (0.5 g) was ground in a prechilled pestle and mortar with 0.5 ml of extraction buffer (Tris-HCl, pH 7.5). The extract was taken in an eppendorf tube and centrifuged at 12,000 rpm for 20 min at 4°C. An aliquot of 0.2 ml supernatant mixed with 0.2 ml sample buffer (containing 1% SDS, 10% glycerol, 25 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.7 M mercaptoethanol), and 100 µl was loaded on to a native anionic polyacrylamide gel with 8% resolving gel and 4% stacking gel. Electrophoresis was carried out until the dye front reached the end of the resolving gel. After electrophoresis the gels were incubated in a solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in dark. Then 1-2 ml of 30% H_2O_2 was added dropwise with constant shaking until bands representing PPO activity appeared (Sindhu et al., 1984). After staining, the gel was washed with distilled water and photographed.

Phenolic compounds. Rice leaves (1.0 g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70°C (Swain and Hillis, 1959). One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteu reagent, and the solution was kept at 25°C. After 3 min, 1 ml of a saturated solution of Na_2CO_3 and 1 ml of distilled water were added, and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue color was measured using a Beckman DU64 spectrophotometer at 725 nm. The total soluble phenolic content was calculated by comparison with a standard curve obtained from a Folin-Ciocalteu reaction with phenol. Results were expressed as phenol equivalents in µg g^{-1} of fresh weight.

Experiment III

The PPFM strain—as seed imbibition, phyllosphere spray, or as combined application—was assessed for its efficiency in suppressing sheath blight of rice under pot culture conditions. Ten treatments *viz*, T_1) PPFM-Os-07 (seed imbibition); T_2) PPFM-Os-07 (phyllosphere spray on 30, 60 and 90 DAS); T_3) $T_1 + T_2$; T_4) *Rhizoctonia solani* (TNAU-01) alone; T_5) $T_1 + T_4$; T_6) $T_2 + T_4$; T_7) $T_1 + T_2 + T_4$; T_8) Carbendazim (2 g kg^{-1} of seeds); T_9) Fresh AMS medium, and T_{10}) control, were done to study the impact of PPFMs on rice.

For seed imbibition, the rice seeds were surface sterilized with 2% sodium hypochlorite (NaOCl) and soaked in a double volume of bacterial culture (1×10^9 CFU ml^{-1} of culture). After 12 h, the culture was drained, and the seeds were dried in shade for 30 min (Holland and Polacco, 1994) before being allowed to sprout for another 24 h prior to sowing (Vidhyasekaran and Muthamilan, 1999). After 25 days, the seedlings were pulled out from the pots and transplanted at the rate of four seedlings per hill and five hills per pot. For treatments that included phyllosphere spray, the cultures were applied using a hand sprayer (25 ml per pot; 1×10^9 CFU ml^{-1} of culture) at 30, 60, and 90 days after sowing (DAS). The water level was maintained

2 cm above the soil level in the pots throughout the experiment. Forty days after planting, sclerotia of *R. solani* were placed (2 per tiller) in the sheath of the rice plants. Five sheaths per hill and five hills per replication were used for inoculation. The inoculated portion of each plant was covered with absorbant cotton and tied with parafilm. It was then moistened with distilled water regularly to maintain high humidity. Development of symptoms was observed and recorded 7 days after inoculation on a scale graded from 0 to 5 (Sriram et al., 1997). Based on the grades, a disease index was calculated using the following formula: Disease index = total grade/no. of sheaths observed × 100/ maximum grade. Plant height and tiller number were measured 45 days after planting. Plants raised from seeds treated with carbendazim (2 g kg^{-1} of seeds as seed treatment and 0.1% foliar spray at 30 days after planting) served as a fungicide check, and foliar spray with distilled water served as control. The trial was laid out in a randomized block design with three replications.

Statistical Methods

A completely randomized design was used in all experiments. The mean, standard error (SE), and analysis of variance (ANOVA) were calculated, and means were separated by the LSD using an SAS package, Version 8.2 (SAS, 2001). Where appropriate, an arc sine transformation was used to normalize the data.

Results

Seed Germination Test

To determine whether methylotrophic bacteria can stimulate rice seed germination, we tested three strains of *Methylobacterium* sp. PPFM-Os-07, *M. extorquens* AM1 and *M. extorquens miaA* mutant as well as their spent media (Table 1). All the strains were found to have significant and consistent stimulatory effects on rice seed germination. However, the degree of stimulation obtained among strain PPFM-Os-07 and *M. extorquens* AM1 differed according to the strain employed. *Methylobacterium extorquens miaA* mutant stimulated better seed germination of non-heated seeds than the other two strains. Clarified spent media also registered better results than fresh media. In the case of heated seeds, the clarified spent medium was found better than bacterial inoculation. Clarified spent media grown with *miaA* mutant and PPFM-Os-07 strain produced 99% germination, followed by *M. extorquens* AM1 spent medium, which produced 97%. When the application of cells was considered, *miaA* mutant strain induced a higher germination percentage than the other two strains.

The inoculation of strain PPFM-Os-07 in rice proved beneficial in increasing the R_G and SVI over control. The R_G of rice seeds was calculated based on the germination counts recorded over time. The R_G of PPFM treated seeds ranged from 33.44 to 38.74, and in control it was 32.81 (Table 2). The difference in R_G among PPFM strains recorded could be related to the ability of individual strains to promote early germination of seeds. From the results, it is evident that the *M. extorquens* AM1 and PPFM-Os-07 strain was better in inducing early growth promotion than the *miaA* mutant. Though the SVI observed also followed the above trend, the PPFM-Os-07 strain was found better than the *miaA* mutant and control (Table 2).

Induction of Defense Enzymes

Almost all the enzymes and phenolics studied were higher in the *Methylobacterium* sp. strain PPFM-Os-07-treated plants than control. Increases in phenolic content were observed within 1 day after *Methylobacterium* treatment and peaked (255.36 µg of phenol. g^{-1} fresh weight) four days after application. In contrast, the concentration of phenolics in the control plant stood at 100.12 µg of phenol g^{-1} fresh weight on all days (Figure 1A). PAL and peroxidase activity were also significantly increased on day one after PPFM treatment, and the maximum PAL (150.78 nmol of *trans*-cinnamic acid $min^{-1}g^{-1}$ fresh weight) and peroxidase (1.91 absorbance units min^{-1} g^{-1} fresh weight) activities were observed on days 4 and 5, respectively (Figure 1B and Figure 1E). Chitinase and β-1,3 glucanase values

Table 1. Effect of seed imbibition of PPFMs on rice seed germination.

Treatments	Germination per cent	
	Non-Heated	Heated (50°C, 48 h)
PPFM-Os-07	80.83 (64.20)^{bc}	82.67 (65.44)^{c}
PPFM-Os-07 spent medium	81.17 (64.36)^{b}	99.00 (84.39)^{a}
M. extorquens miaA mutant	94.17 (76.79)^{a}	97.67 (81.50)^{b}
miaA mutant spent medium	81.00 (64.22)^{bc}	99.00 (84.39)^{a}
M. extorquens AM1	79.17 (62.92)^{bc}	80.50 (63.80)^{c}
M. extorquens AM1 spent medium	75.33 (60.30)^{cd}	97.50 (80.94)^{b}
Fresh AMS medium	76.17 (60.87)^{bcd}	61.17 (51.46)^{d}
Fresh AMS medium + tetracycline	76.33 (60.92)^{bcd}	61.67 (51.75)^{d}
Control	75.67 (58.86)^{d}	60.67 (51.09)^{d}
LSD (P≤0.05)	4.05	1.99

Values are the mean of three replications of 200 seeds each. Values in parentheses are arc sine transformed values. In the same column, significant differences according to LSD at P≤0.05 level are indicated by different letters.

Table 2. Germination rate and seedling vigour index of PPFM-treated rice seeds.

Treatments	Rate of germination (R_G)	Seedling vigour index (SVI)*
PPFM-Os-07	$37.51^{ab} \pm 0.61$	$4170^{a} \pm 33.00$
PPFM-Os-07 spent medium	$38.74^{a} \pm 0.58$	$4198^{a} \pm 88.62$
M. extorquens miaA mutant	$33.44^{c} \pm 0.62$	$2972^{cd} \pm 33.94$
miaA mutant spent medium	$33.68^{c} \pm 0.50$	$2992^{c} \pm 66.94$
M. extorquens AM1	$35.94^{b} \pm 1.00$	$4041^{b} \pm 19.33$
M. extorquens AM1 spent medium	$36.82^{b} \pm 1.22$	$4152^{a} \pm 81.08$
Fresh AMS medium	$32.42^{c} \pm 0.67$	$2875^{e} \pm 73.07$
Fresh AMS medium + tetracycline	$32.81^{c} \pm 0.90$	$2878^{de} \pm 55.62$
Control	$32.14^{c} \pm 0.27$	$2856^{e} \pm 21.68$
LSD (P≤0.05)	1.75	96.61

*Average of 10 rice seedlings. Each value represents mean of three replicates per treatment. In the same column, significant differences according to LSD at P≤0.05 level are indicated by different letters.

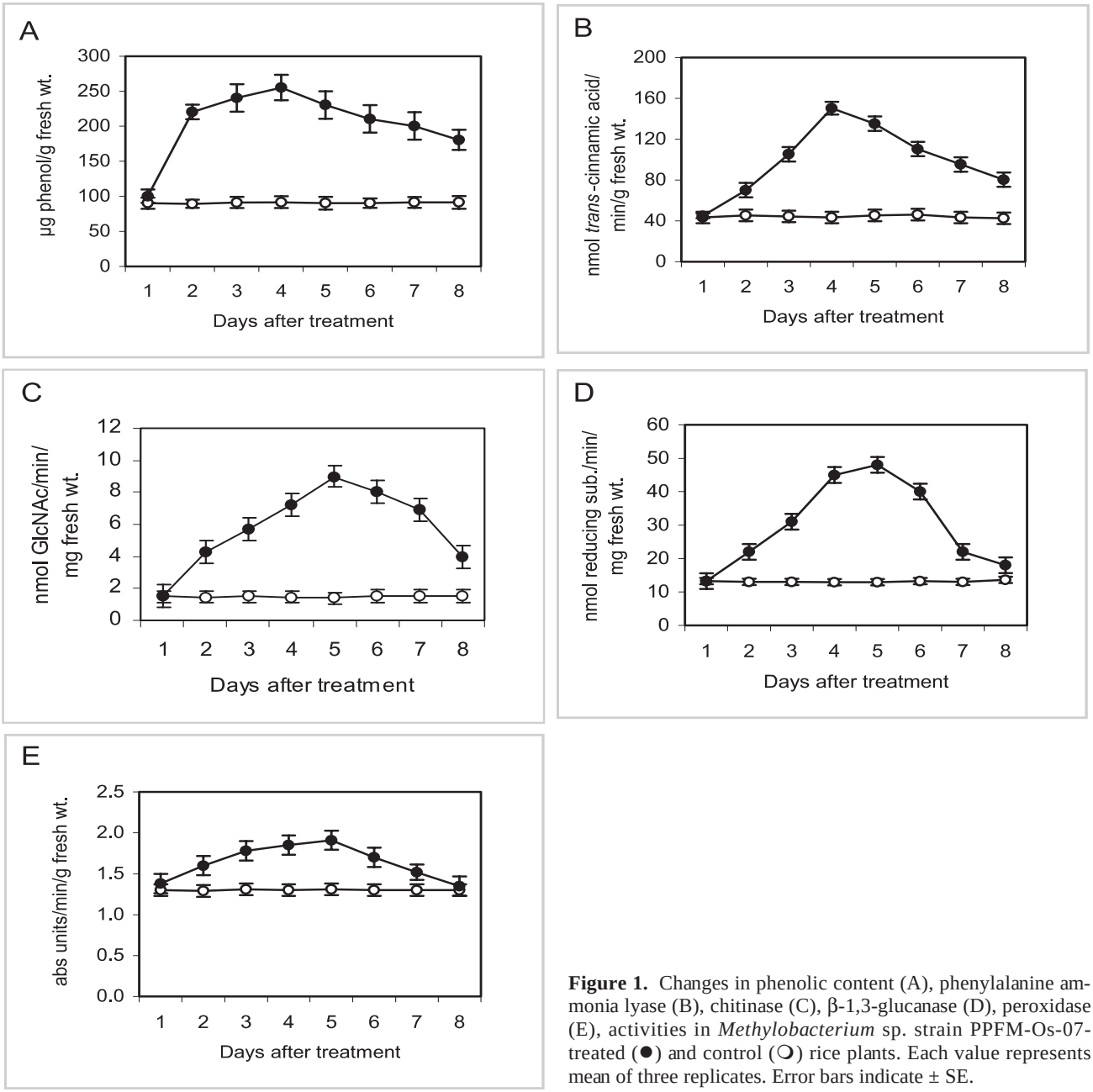

Figure 1. Changes in phenolic content (A), phenylalanine ammonia lyase (B), chitinase (C), β-1,3-glucanase (D), peroxidase (E), activities in *Methylobacterium* sp. strain PPFM-Os-07-treated (●) and control (❍) rice plants. Each value represents mean of three replicates. Error bars indicate ± SE.

stood equal to the control on day 1 and slowly progressed toward a peak on day 5 (9 nmol of GlcNAc equivalents min^{-1} mg^{-1} of fresh weight and 48 nmol of reducing substances min^{-1} mg^{-1} of fresh weight, respectively). The control values were well below treated values (Figure 1C and Figure 1D). The results on isozyme electrophoretic patterns of polyphenol oxidase revealed higher staining intensities of fractions in *Methylobacterium* sp. strain PPFM-Os-07-treated plants than control (Figure 2).

Biocontrol, Plant Growth and Yield Evaluation

Pot culture experiments established the efficacy of *Methylobacterium* strain in improving plant growth and inducing plant defense agents against *R. solani*. In general, the strain PPFM-Os-07 treatment led to significant increases in plant growth as measured by the number of tillers, plant height, and yield. Enhanced plant growth was observed in treatments involving both seed imbibition and phyllosphere spray compared to either treatment alone or to control. *Methylobacterium* sp. strain PPFM-Os-07-treated plants showed increased numbers of tillers and height when compared to untreated control (Table 3). This beneficial effect was not associated with carbendazim, fresh AMS medium, or control treatment. The increased plant growth ultimately resulted in a significant increase in yield. The combined application of seed imbibition and phyllosphere spray treatment resulted in a higher yield (55.44 g pot^{-1}) than other treatments. This was followed by plants challenged with *R. solani* but treated with carbendazim and combined application of seed imbibition and phyllosphere spray and individual inoculation of *Methylobacterium* strains. The per cent disease index (PDI) observed also showed marked differences among treatments. While the *R. solani*-challenged plants recorded an index value of 95.6%, the value recorded for carbendazim treatment and control were 0% and 25.3%, respectively. The combined application of seed imbibition and phyllosphere spray of PPFM-Os-07 strain recorded an index value of 17.8% (Figure 3).

Discussion

Like little farmers, microbes play an important role in seed germination and seedling establishment. The relationship between PPFM bacteria and plants illustrates this (Holland et al., 2002). Holland (1997) reported that PPFMs could be used as seed inoculum or in seed coatings designed to enhance germinability, storability, or vigour of seeds. In this study all three strains employed were found to have significant and consistent stimulatory effects on germination of both heat-treated and non-treated rice seeds. Our earlier studies also indicate the positive impact of *Methylobacterium* strains on sugarcane and cotton (Madhaiyan et al., 2004; Madhaiyan, 2003). The ability of

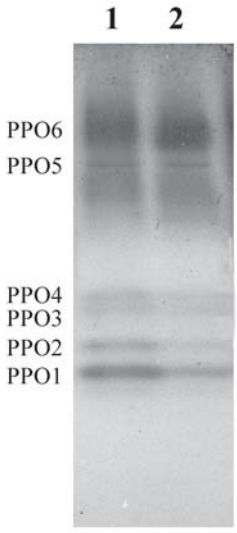

Figure 2. Polyphenol oxidase (PPO) isoforms separated by native polyacrylamide gel electrophoresis (PAGE) of soluble proteins from rice sheaths inoculated with *R. solani*. Crude enzyme extracts (50 µg protein) were loaded on 8% gel. After electrophoresis, the PPO isoforms were visualized in a staining solution containing 0.15% benzidine and 30% H_2O_2. Lane 1. *Methylobacterium* sp. strain PPFM-Os-07-treated plants at day 5 after *R. solani* challenge inoculation. Lane 2. Control plants without inoculation of *R. solani* TNAU-01.

Table 3. Effect of PPFM on growth and yield of rice under pot culture conditions.

Treatments	Mean shoot length (cm)	Mean plant biomass (g)	Mean no. of tillers/hill	Grain yield (g per pot)
PPFM-Os-07 (SI)	$77.63^{abc} \pm 1.24$	$12.02^{bc} \pm 0.01$	$12.7^{bc} \pm 0.57$	$50.36^{b} \pm 0.02$
PPFM-Os-07 (PS)	$77.25^{bcd} \pm 0.59$	$11.86^{cd} \pm 0.64$	$12.0^{c} \pm 0.47$	$51.21^{b} \pm 1.51$
SI + PS	$79.63^{a} \pm 2.18$	$12.96^{a} \pm 0.36$	$14.3^{a} \pm 0.19$	$55.44^{a} \pm 1.05$
R. solani TNAU-01	$70.31^{e} \pm 1.09$	$10.56^{fg} \pm 0.26$	$9.20^{e} \pm 0.09$	$0.00^{d} \pm 0.00$
SI + TNAU-01	$76.82^{cd} \pm 0.86$	$11.53^{cde} \pm 0.47$	$11.0^{d} \pm 0.24$	$41.36^{c} \pm 0.88$
PS + TNAU-01	$75.36^{d} \pm 0.17$	$11.23^{de} \pm 0.11$	$12.7^{bc} \pm 0.38$	$42.26^{c} \pm 0.12$
SI + PS + TNAU-01	$79.36^{ab} \pm 0.94$	$12.52^{ab} \pm 0.25$	$13.3^{b} \pm 0.42$	$51.42^{b} \pm 1.0$
Carbendazim	$71.25^{e} \pm 1.06$	$11.08^{ef} \pm 0.27$	$13.0^{b} \pm 0.24$	$51.62^{b} \pm 0.61$
Fresh AMS medium	$70.21^{e} \pm 0.10$	$10.52^{fg} \pm 0.20$	$9.30^{e} \pm 0.14$	$41.82^{c} \pm 1.09$
Control	$67.23^{f} \pm 0.58$	$10.27^{g} \pm 0.36$	$9.20^{e} \pm 0.19$	$41.21^{c} \pm 0.57$
LSD (P≤0.05)	2.17	0.65	0.75	1.87

Each value represents mean of three replicates per treatment. In the same column, significant differences according to LSD at P≤0.05 levels are indicated by different letters. SI, seed imbibition and PS, phyllosphere spray.

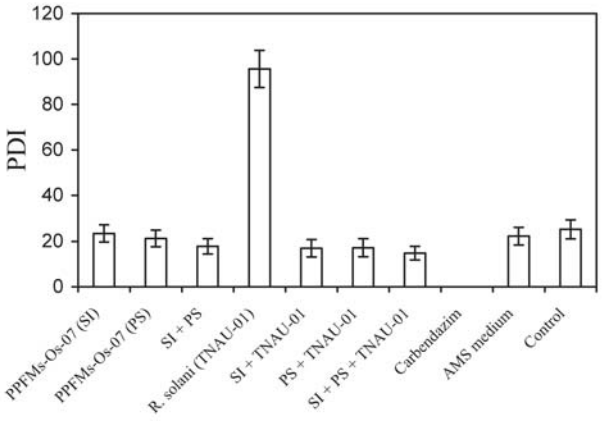

Figure 3. Per cent disease index for various treatments applied with *Methylobacterium* sp. strain PPFM-Os-07 and carbendazim for the control of *R. solani* TNAU-01. Each value represents mean of three replicates per treatment. In the same column, significant differences according to LSD at P≤0.05 level (11.56) are indicated by different letters. SI, seed imbibition and PS, phyllosphere spray. Error bars indicate ± SE.

spent culture media to promote better seed germination than the bacterial cells could be due to a diffusible substance secreted into the medium by the methylotrophic bacteria (Koenig et al., 2002). However, confirmation of this will require an understanding of the nature of the substances, other than plant growth hormones, available in the spent culture.

When plants are invaded by microorganisms or damaged by mechanical injuries, major physiological changes and plant defense enzymes are generally induced. The defense related enzymes, including PR-proteins, are reported to accumulate in plants with the onset of induced systemic resistance. Treatment with various biotic and abiotic inducers leads to increased activities of chitinase, PAL, β-1,3-glucanase, peroxidase, and PPO. However the extent and course of increase varies according to the inducer and host plant (Schmid and Uldrich, 1994). Fluorescent pseudomonads have been reported to induce systemic resistance in several crops by activating defense related genes (M'Piga et al., 1997; Xue et al., 1998). Maurhofer et al. (1994) reported that systemic resistance induction by fluorescent pseudomonads in tobacco plants was associated with accumulation of β-1,3-glucanase and chitinase enzymes. These defense proteins have the potential to hydrolyze the major components of fungal cell walls *viz*, chitin and β-1,3 glucans, respectively (Ham et al., 1991; Ren and West, 1992). Peroxidases have been implicated in a number of physiological functions that may contribute to resistance, including oxidation of hydroxy cinnamyl alcohol into free radical intermediates, phenol oxidation, polysaccharide cross linking, cross linking of extension monomers (Schmid and Feucht, 1980), lignification (Walter, 1992), and in the deposition of phenolic material into plant cell walls during resistant interaction (Graham and Graham, 1991).

To date, no reports are available on the part played by PPFMs in inducing systemic resistance in plants. Through this study we report this phenomenon in rice plants inoculated with strain PPFM-Os-07. The results of the present study reveal that PPFM-Os-07 strain can induce various defense related proteins and synthesis of phenolics. The defense enzymes and phenolics observed in PPFM-treated rice plants showed a higher level of expression than control plants. Increased enzyme activities observed in plants in this study indicate the induction of these enzymes in response to *Methylobacterium* inoculation. However, the recognition of PPFMs as non-pathogens by the plant could be related the plant's ability to deal with their own waste products generated during its growth process (Holland, 1997). Holland (1997) speculated that the plant depends on the bacteria for the remova1 of metabolic waste products generated during its growth. Methanol, a waste product of plants, is a fitting example of this kind of relationship, degraded by PPFMs into simpler compounds, such as ammonium, which eventually are returned to the plant (Holland and Polacco, 1992; Stebbins et al., 1991). Further one could speculate on the possibility that PPFMs produce chemical substances (Koenig et al., 2002), which might act as signal molecules indicating the existence of such beneficially active waste managers in the phyllosphere and triggering the utilization or deactivation of those signal molecules (Holland, 1997).

Induced systemic resistance activity of rice plants in response to methylotrophic bacteria suggests the possibility that PPFMs might be used as a means of biological control of disease. However, this requires further in vitro and in vivo studies for successful large-scale use.

Acknowledgments. We thank Mark A. Holland for his valuable suggestions, guidance, and correction of the manuscript. This work was partially supported by Korea Science and Engineering Foundation through the Research Centre for the Development of Advanced Horticultural Technology at Chungbuk National University.

Literature Cited

Austin, B. and M. Goodfellow. 1979. *Pseudomonas mesophilica*, a new species of pink bacteria isolated from leaf surfaces. Int. J. Syst. Bacteriol. **29:** 373.

Baki, A.A. and J.D. Anderson. 1973. Vigour determination in soybean seed by multiple criteria. Crop Sci. **13:** 630-632.

Basile, D.V., L.L. Slade, and W.A. Corpe. 1969. An association between a bacterium and a liverwort, *Scapania nemorosa*. Bull. Torrey Bot. Club **96:** 6711-6714.

Berger, L.R. and D.M. Reynolds. 1958. The chitinase system of a strain of *Streptomyces griseus*. Biochem. Biophys. Acta. **29:** 522-534.

Boller, T. and F. Mauch. 1988. Colorimetric assay for chitinase. Meth. Enzymol. **161:** 430-435.

Corpe, W.A. 1985. A method for detecting methylotrophic bacteria on solid surfaces. J. Microbiol. Methods **3:** 215-221.

Corpe, W.A. and D.V. Basile. 1982. Methanol-utilizing bacteria associated with green plants. Dev. Indust. Microbiol. **23:**

483-493.

Corpe, W.A. and S. Rheem. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. Microbiol. Ecol. **62:** 243-248.

Dickerson, D.P., S.F. Pascholati, A.E. Hagerman, L.G. Butler, and R.L. Nicholson. 1984. Phenylalanine ammonia-lyase and hydroxycinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. Physiol. Pl. Path. **25:** 111-123.

Dunleavy, J.M. 1990. Urease production by *Methylobacterium mesophilicum*, a seed transmitted bacterium ubiquitous in soybean. Presented at 3rd Biennial Conf. Mol. Cell. Biol. Soybean; Ames, Iowa, July, pp. 23-25.

Dunleavy, J.M. 1988. *Curtobacterium plantarum* sp. nov. is ubiquitous in plant leaves and is seed transmitted in soybean and corn. Int. J. Syst. Bacteriol. **39:** 240-249.

Evans, G.C. 1972. The Quantitative Analysis of Plant Growth. Blackwell Science Publication, Oxford.

Graham, M.Y. and T.L. Graham. 1991. Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytopthora megasperma* f. sp. *glycinea* wall glucan. Plant Physiol. **97:** 1445-1456.

Green, P.N. and I.J. Bousfield. 1983. Emendation of *Methylobacterium* Patt, Cole and Hanson 1976, *Methylobacterium rhodinum* (Heumann 1962) comb. Nov. corig; *Methylobacterium radiotolerans* (Ito and Iizuka 1971), Comb. nov. corrig., and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. Int. J. Syst. Bacteriol. **33:** 875-877.

Green, P.N. and I.J. Bousfield. 1982. A taxonomic study of some Gram- negative facultatively methylotrophic bacteria. J. Gen. Microbiol. **128:** 623.

Ham, K.S., S. Kauffmann, P. Albersheim, and A.G. Darvil. 1991. A soybean pathogenesis-related protein with β-1,3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal cell walls. Mol. Pl. Microbe Interact. **4:** 545-552.

Hammerschmidt, R. and J.A. Kuc. 1982. Lignification as a mechanism for induced systemic resistance in cucumber. Physiol. Pl. Pathol. **20:** 61-71.

Hirano, S.S. and C.D. Upper. 1992. Bacterial community dynamics. *In* J.H. Andrews and S.S. Hirano (eds.), Microbial Ecology of Leaves, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 271-294.

Holland, M.A. 1997. Occams razor applied to hormonology. Are cytokinins produced by plants? Plant Physiol. **115:** 865-868.

Holland, M.A., R.L.G. Long, and J.C. Polacco. 2002. *Methylobacterium* spp.: phylloplane bacteria involved in cross-talk with the plant host?. *In* S. E. Lindow, E. I. Hecht-Poinar, and V. J. Elliot (eds.), Phyllosphere Microbiology, APS Press, St. Paul, Minn., pp. 125-135.

Holland, M.A and J.C. Polacco. 1994. PPFMs and other contaminants: Is there more to plant physiology than just plant? Ann. Rev. Plant Physiol. Plant Mol. Biol. **45:** 197-209.

Holland, M.A. and J.C. Polacco. 1992. Urease-null and hydrogenase-null phenotypes of a phylloplane bacterium reveal altered nickel metabolism in two soybean mutants. Plant Physiol. **98:** 942-948.

Hosseini, S.Z. and M. Jafari. 2002. Investigation on effect of salinity stress on germination of three accessions of tall wheat grass (*Agropyron elongatum*). Paper no. 2289, 17th WCSS, 14-21 August 2002, Thailand.

Ivanova, E.G., N.V. Doronina, and Y.A. Trotsenko. 2001. Aerobic methylobacteria are capable of synthesizing auxins. Microbiology **70:** 392-397.

Koenig, R.L., R.O. Morris, and J.C. Polacco. 2002. tRNA is the source of low-level *trans*-Zeatin production in *Methylobacterium* spp. J. Bacteriol. **184:** 1832-1842.

Lidstrom, M.E. 2001. The aerobic methylotrophic bacteria. *In* M. Dworkin (eds.), The Prokaryotes, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 223-244.

M'Piga, P., R.R. Belanger, T.C. Paulitz, and N. Benhamou. 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiol. Molec. Pl. Path. **50:** 301-320.

Madhaiyan, M. 2003. Molecular aspects, diversity and plant interaction of facultative methylotrophs occurring in tropical plants. Ph.D. dissertation, Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India.

Madhaiyan, M., K. Hari, S. Seshadri, SP. Sundaram, and T.M. Sa. 2004. Association of methylotrophic bacteria with sugarcane clone Co86032 (*Saccharum officinarum* L.) improves plant growth and yield (communicated).

Maurhofer, M., C. Hase, P. Meuwly, T.P. Metraux, and G. Defago. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHAO: influence of the *gacA* gene and of pyoverdine production. Phytopathology **84:** 139-146.

Nemecek-Marshall, M., R.C. MacDonald, J.J. Franzen, C.L. Wojciechowski, and R. Fall. 1995. Methanol emission from leaves: enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development. Plant Physiol. **108:** 1359-1368.

Omer, Z.S., R. Tombolini, and B. Gerhardson. 2004a. Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol. Ecol. **47:** 319-326.

Omer, Z.S., R. Tombolini, A. Broberg, and B. Gerhardson. 2004b. Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. Plant Growth Regul. **43:** 93-96.

Pan, S.Q., X.S. Ye, and J. Kuc. 1991. Association of β-1,3-glucanase activity and isoform pattern with systemic resistance to blue mould in tobacco induced by stem injection with *Pernospora tabacina* or leaf inoculation with tobacco mosaic virus. Physiol. Molec. Pl. Pathol. **39:** 25-39.

Patt, T.E., G.C. Cole, and R.S. Hanson. 1976. *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. Int. J. Syst. Bacteriol. **26:** 226-229.

Reissig, J.L., J.L. Stromer, and L.F. Leloir. 1959. A modified colorimetric method for the estimation of N-acetyl sugars. J. Biol. Chem. **217:** 959-962.

Ren, Y.Y. and C.A. West. 1992. Elicitation of diterpene biosynthesis in rice (*Oryza sativa* L.) by chitin. Pl. Physiol. **9:** 1169-1178.

SAS, Institute Inc. 2001. SAS user's guide, Version 8.2, SAS Institute Inc., Cary, North Carolina, USA.

Schmid, P.S. and W. Feucht. 1980. Tissue specific oxidation of browning of polyphenols by peroxidase in cherry shoots. Gartenbauwissenschaft **45:** 68-73.

Schmid, P.S. and W.R. Ullrich. 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. Physiol. Mol. Plant Pathol. **45:** 291-304.

Sindhu, J.S., S. Ravi, and J.L. Minocha. 1984. Peroxidases isozyme patterns in primary trisomics of pearl millet. Theor. Appl. Genet. **68:** 179-182.

Sriram, S., T. Raguchander, P. Vidhyasekaran, S. Muthukrishnan, and R. Samiyappan. 1997. Genetic relatedness with special reference to virulence among the isolates of *Rhizoctonia solani* causing sheath blight in rice. J. Plant Dis. Protect. **104:** 260-271.

Stebbins, N., M.A. Holland, S.R. Cianzio, and J.C. Polacco. 1991. Genetic tests of the roles of the embryonic ureases of soybean. Plant Physiol. **97:** 1004-1010.

Swain, T. and W.E. Hillis. 1959. The phenolic constituents of *Prunus domestica* I. The quantitative analysis of phenolic constituents. J. Sci. Food Agric. **10:** 63-68.

Vidhyasekaran, P. and M. Muthamilan. 1999. Evaluation of powder formulations of *Pseudomonas fluorescens* Pf1 for control of rice sheath blight. Biocon. Sci. Technol. **9:** 67-74.

Walter, M.H. 1992. Regulation of lignification in defense. *In* T. Boller. and F. Meins (eds.), Genes Involved in Plant Defenses, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 327-352.

Whittenbury, R., S.L. Davies, and J.F. Wilkinson. 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Microbiol. **61:** 205-218.

Xue, L., P.M. Charest, and S.H. Jabaji-Hare. 1998. Systemic induction of peroxidases, β-1,3-glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathology **88:** 359-365.

水稻（*Oryza sativa* L.）品種 Co-47 可被 *Methylobacterium* 菌促進生長及誘導出系統性的抗病狀態

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粉紅色的兼性甲醇菌 (PPFMs)，及 *Methylobacterium* 屬，為植物葉表之常住客，大多經由種子傳播。本文檢驗溫室中此菌對水稻生長之促進及對疾病之抑制。水稻種子以 *Methylobacterium* sp. 菌株 PPFM-Os-07 接種，然後評估種子發芽情形，項目有：形態判定，幼菌生長，發芽速率 (R_G)，及種子活力指標 (SVI)。另一實驗則檢驗水稻浸水含甲醇菌或葉面噴酒甲醇菌然後看病菌相關蛋白質 (PR-proteins) 之誘導情形。第三實驗，發芽 60 天之盆栽水稻以 *Rhizoctonia solani* 菌株 TNAU-01。甲醇菌之接種促進種子發芽，稻苗生長，增加株高，抽穗數，植體生物量，及稻穀產量。平均增產，經由浸泡方式或葉面噴酒方式，可分別達 22.1% 或 24.3%。甲醇菌也顯著地減少鞘銹病之發生，不論是上述那一種處理方式：如單獨浸泡處理可減小 17.8% 之感病；如浸泡和葉面噴酒併用則可減少 23.5%。水稻植物噴酒 PPFM-Os-07 菌株後 1 天就發現 PR-proteins 及 phenolic contents 增加。最高量之 phenylalanine ammonia lyase 及 peroxidase 活性出現在第 4 天；β-1,3-glucanase 及 chitinase 活性則出現在第 5 天。以上結果顯示接種甲醇菌可改變水稻對 *R. solani* 之感病性。本文強調當研究和植物密切接觸之細菌的助長情形時應同時探討是否出自於誘導系統性抗病之效果。

關鍵詞：甲醇菌；水稻；誘導產生之系統性抗病；病菌相關蛋白質：*Rhizoctonia solani*。