J. Microbiol. Biotechnol. (2007), **17**(10), 1645–1654

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Influence of Plant Species and Environmental Conditions on Epiphytic and Endophytic Pink-Pigmented Facultative Methylotrophic Bacterial Populations Associated with Field-grown Rice Cultivars

MADHAIYAN, MUNUSAMY, SELVARAJ POONGUZHALI, AND TONGMIN SA*

Department of Agricultural Chemistry, Chungbuk National University, Cheongju 361-763, Korea

Received: April 5, 2007 Accepted: May 11, 2007

Abstract The total methylotrophic population associated with rice plants from different cultivars was enumerated at three different stages: vegetative, flowering, and harvesting. The bacterial population in the leaf, rhizosphere soil, endophytic in the stem and roots, and epiphytic in the florets and grains were determined from four rice cultivars, Il-mi, Nam-pyeoung, O-dae, and Dong-jin, sampled from three different field sites. The methylotrophic bacteria isolated on AMS media containing 0.5% methanol as the sole carbon source uniformly showed three distinct morphologies, which were recorded as separate groups and their distribution among the various samples was determined using the ecophysiological index. The growth stage at the time of sampling had a more significant effect on the methylotrophic population and their distribution than the field site or cultivar. A similar effect was also observed for the PPFMs, where their population in different plant parts increased from V10 to R4 and then decreased towards stage R9. A canonical discriminant analysis of the PPFM population from different parts of rice showed clear variations among the cultivars, sampled sites, and growth stages, although the variations were more prominent among the growth stages.

Keywords: Heterotrophs, methylotrophs, *Methylobacterium*, ecophysiological index, canonical discriminant analysis

The relationships of bacteria with plants have a long evolutionary history that probably began with the development of multicellularity. Holland *et al.* [11] stated that *Methylobacterium* is one such microbe that acts as little farmers, nurturing and protecting plants at every stage. The genus *Methylobacterium* includes strict aerobic, Gram-negative, facultative methylotrophic rods of *Alphaproteobacteria*, characterized by their ability to rely on methanol, a one-carbon compound, as the sole carbon and energy source, giving them a selective advantage for colonizing plants. Most species show pink to red pigmentation and are often referred to as pink-pigmented facultative methylotrophs (PPFMs). PPFMs are widely distributed in nature and are particularly known for their close association with plants [1, 19]. They are reported as the dominant phyllosphere population from more than seventy plant species tested [1]. One intriguing aspect of plant PPFM relationships is the possibility that PPFMs may provide cytokinins to the plant host or have cytokinin-like effects [16]. The direct mechanisms of the plant growth promotion occur through an increased nutrient uptake and production of phytohormones, etc., whereas indirect mechanisms include the suppression of phytopathogenic microorganisms through biocontrol or induction of plant defense enzymes [6, 14, 18, 23]. Recent evidences have suggested that PPFMs are more than a passive passenger on plants and are actively involved in plant growth promotion through direct and indirect mechanisms [12, 18, 31]. Since methylotrophic bacteria tend to form aggregates on the aerial parts of plants [35], the phenomenon of quorum-sensing (QS), which affects the multicellular behavior of bacteria in a community, gains importance. The presence of two QS systems with production of a novel long chained $C_{14:2}$ (*N*-tetra decenoyl) AHL molecule has been described in *Methylobacterium extorquens* AM1 [26] and a recent study has shown that the occurrence of QS systems in *Methylobacterium* is widespread [28].

As for studies on PPFMs, the results corroborate their occurrence or their plant growth promoting characteristics. A few reports have dealt with their population variance and dynamics in crop plants [1, 8, 25]. However, these studies have concentrated on the variation of the phyllosphere PPFM population. The leaf-inhabiting PPFMs were suggested probably to be the descendants of seedborne bacteria rather than from environmental sources [9]. However, in maize, it has been suggested that natural colonization occurred *via* air transfer of soil particles [29]. Plausible reports have also documented the occurrence of

*Corresponding author
Phone: 82-43-261-2561; Fax: 82-43-271-5921;
E-mail: tomsa@chungbuk.ac.kr

PPFMs in the rhizosphere or as endophytes [13, 27, 33]. Furthermore, the effects of plant species or different cultivars of the same species on the epiphytic or total rhizosphere population have been documented [5, 15]. However, no studies have yet dealt with the effect of biotic and abiotic factors on the interaction between PPFMs and plants. Accordingly, this study investigated the dynamics of a PPFM population associated with field-grown rice cultivars collected from three different sites to enumerate the PPFM population associated with different parts of rice and to assess the influence of plant factors: tissue type, cultivar and age. Samples were collected from four cultivars of rice plants grown at three different sites and the sampling performed at three stages of crop growth. The parts of the plants used for isolation were used rather than the terms epiphytes or endophytes. The total bacterial population and total methylotrophic population were also enumerated along with the PPFM population in this study.

MATERIALS AND METHODS

Description of Field Site, Plant, and Sampling

The rice plant and rhizosphere samples were obtained from research fields at three agricultural experiment stations located in the north and north-eastern parts of Korea. Three of the cultivars (Il-mi, Nam-pyeoung, and Dong-jin) were late maturing and one was an early maturing variety (O-dae). The field sites were located at the experimental stations of the Chungbuk Provincial Agricultural Research and Extension Services, Cheongwon (36° 58' 0N and 127° 57' 0E); the National Honam Agricultural Experiment Station, RDA, Iksan, (35° 54' 4N and 128° 54' 58E); and the National Yeongnam Agricultural Experiment Station, RDA, Milyang (35° 29' 36N and 128° 44' 56E) in the north and north-eastern parts of Korea. The mean temperature at the sampling sites was 25.4°C and the annual rainfall was 100.1–102.8 mm. The soil type was clay loam with a pH of 6.28–6.69. The seedlings were transplanted to the field sites on May 20–25, 2003, and the samplings taken at the vegetative (V10), flowering (R4), and reproductive (R9) phases [2] of plant growth. For each sampling, four plants and soil material from each cultivar were transferred immediately to presterilized bags, transported to the laboratory under ambient conditions, and processed within 12 h of removal from the ground.

Bacterial Isolations

The soil tightly adhering to the roots (10 g) was added to 90 ml of sterile distilled water (SDW) and shaken for 30 min at 150 rpm at 28°C to prepare a suspension. Ten-fold serial dilutions of the suspensions were then plated onto a nutrient agar (Nutrient broth, Difco, with 15 g/l agar) and ammonium mineral salts (AMS) agar with 0.5% (v/v) methanol [36] containing 10 μg/ml filter-sterilized cycloheximide in order to inhibit any fungal growth. To isolate the endophytes from the stem and root, the plant material was surface disinfected with 70% ethanol for 1 min, sodium hypochlorite solution (NaOCl, 2% available chlorine) for 5 min, and then rinsed twice with SDW. After the surface disinfection, the stem or root material was cut, triturated, and suspended in SDW. To isolate the leaf-associated bacteria, freshly emerging leaves (10 g) were collected and triturated, and a suspension was made in SDW. The epiphytic bacteria from the florets/rice grains were also isolated by placing 10 g of the appropriate tissue in 250-ml Erlenmeyer flasks containing 90 ml of SDW and agitating the flasks at 150 rpm at 22°C for 1 h. The leaf, root, stem, and floret/grain suspensions were then plated onto an NA and AMS agar as mentioned above, and incubated at 28°C for 3–10 days.

Estimation of Total Bacteria and Methylotrophic Population

The heterotrophs or the methylotrophs on NA or AMS plates were counted on day 7 and expressed in log CFU per g of soil or plant material used. The methylotrophic bacteria that utilized methanol as the carbon source on AMS plates could be divided into three different morphologies classes in all the samples. They were grouped as three classes: PPFMs, colonies of small to medium size (2 to 6 mm) with reddish pink pigmentation and well-defined margins; YPFMs, medium to large size colonies with yellow pigmentation, and NPFMs, white colonies without any pigmentation. To express the distribution of these three classes in each sample using a single number, this study used the ecophysiological index (EPI)=$-\Sigma(p_i \cdot \log_{10} p_i)$, (pi=population in class i/total population) to quantify the classes of organisms (i.e., subcommunities) with similar characteristics on the agar [3, 30]. Representative colonies from the higher dilutions were randomly chosen, subjected to single-colony isolation on AMS medium, and cryopreserved at −80°C in 50% glycerol for further studies.

Statistical Analysis

All analyses were carried out using the statistical analysis system (SAS) Version 9.1 (SAS Institute Inc., Cary, North Carolina, U.S.A.). Analysis of variance (ANOVA) for the PPFM population was carried out using the General Linear Model, GLM in SAS. The bacterial population data were log transformed before being subjected to further analysis. The means of the treatment results were subjected to ANOVA and presented using Fisher's protected least significant difference. The model adopted was A [log CFU (g/FW)]=C (cultivar) S (site) Sa (Sampling stage) C*S C*Sa S*Sa to check the effect of individual factors and the interactions between them. A canonical discriminant analysis (CDA) was carried out to discriminate the variations

among the provinces or varieties with reference to the PPFM populations. Given two or more groups of observations with measurements on several quantitative variables, CDA derives a linear combination of the variables that have the highest possible multiple correlation with the groups.

RESULTS

Cultivable Total Bacteria and Methylotrophic Bacterial Population

The distribution patterns of both heterotrophs and methylotrophs over the growth period of rice sampled at the V10, R4, and R9 stages were investigated in four cultivars collected from three sites. An analysis of the total population data for all the cultivars from the three different sites was conducted for each stage and the results on the mean population are discussed. The mean population of total bacteria was significantly different among the cultivars for all the plant parts, except for the florets and rhizosphere soil, whereas there were no significant differences among the three sites, except for the florets. Furthermore, the age or stage of the crop at the time of sampling also influenced the heterotrophic population associated with the leaf and root (Table 1). The methylotrophic population culturable on the AMS media showed the greatest variation among the sampled crop stages, for all the tissue types and rhizosphere soil, whereas no such variations existed between the cultivars or sites sampled. In general, the methylotrophic population increased from the early vegetative to the flowering stages, and then decreased during the end of the cropping season (Table 2), whereas no such variation was observed for the total heterotrophs. Thus, since the stage of the plant at sampling unambiguously had the most influence on the methylotrophic population associated with rice, the results are hitherto discussed with reference to the stage of the crop sampled.

Distribution of Methylotrophic Population

The populations counted on the AMS plates all showed three different morphologies, and hence the EPI was recorded to measure their distribution among the samples, as mentioned in Materials and Methods. The EPI values showed significant differences with respect to the stage of sampling, rather than the sampling sites or cultivars. The EPI values for the leaf, soil, and stem differed significantly among the different stages, whereas the root showed no significant variation. Higher values of EPI were recorded for leaf and soil than for the other tissues sampled, indicating that the methylotrophic population in the leaf and soil may have consisted of different types of bacteria other than PPFMs or *Methylobacterium*. Similarly, the EPI values for most of the tissues sampled were higher at the R4 stage (Table 3).

Variations in PPFM Population Associated with Rice

The PPFM populations recovered from the different sample groups were further concentrated and the mean population from the different cultivars and sites tabulated (Table 4). Whereas the methylotrophic population from the leaf and soil showed no greater variations, the PPFM

Table 1. Colonization of rice system by total culturable bacteria at three growth stages of different cultivars sampled from three sites.

Cultivar	Log CFU/g fresh weight					
	Leaf	Root	Soil	Stem	Floret	Grain
Cultivar*						
C1	7.10 ab	7.45 b	7.30 a	6.46 a	7.20 a	7.06 b
C2	6.91 b	7.41 b	7.32 a	6.54 ab	7.50 a	7.38 ab
C3	7.15 a	7.72 a	7.17 a	6.49 ab	7.36 a	7.22 a
C4	6.98 ab	7.68 a	7.37 a	6.56 a	7.24 a	7.26 ab
LSD ($P \leq 0.05$)	0.24	0.18	0.21	0.10	0.31	0.22
Sampled site‡						
S1	7.16 a	7.68 a	7.39 a	6.46 a	6.85 c	7.30 a
S2	6.83 a	7.46 b	7.43 a	6.56 a	7.33 a	7.31 a
S3	7.11 a	7.55 ab	7.06 b	6.53 ab	7.80 b	7.09 b
LSD ($P \leq 0.05$)	0.21	0.15	0.18	0.09	0.27	0.19
Growth stage of rice‡‡						
V10	6.54 c	7.51 b	7.57 a	-	-	-
R4	7.47 a	7.68 a	7.19 b	6.53 a	7.33	7.23
R9	7.09 b	7.51 b	7.12 b	6.50 a	-	-
LSD ($P \leq 0.05$)	0.21	0.15	0.18	0.07		

*Values represent mean population for rice cultivars collected from three sites at three growth stages. C1, Il-mi; C2, Dong-jin; C3, Nam-pyeoung; C4, O-dae; ‡Values represent mean population of four different cultivars collected at three growth stages. S1, Chungbuk; S2, Ho-nam; S3, Young-nam; ‡‡Values represent mean population of four different cultivars collected from three different sites. V10, vegetative; R4, flowering; R9, harvesting. Within each vertical column, values followed by the same letter are not statistically different according to Fischer's protected LSD ($P \leq 0.05$).

Table 2. Colonization of rice system by methylotrophic bacteria at three growth stages of different cultivars sampled from three sites.

Site	Log CFU/g fresh weight					
	Leaf	Root	Soil	Stem	Floret	Grain
Cultivar*						
C1	5.58 a	5.49 a	5.21 a	3.05 b	6.50 a	5.95 b
C2	5.83 a	5.50 a	5.40 a	3.42 a	6.77 a	6.04 b
C3	5.72 a	5.61 a	5.48 ab	3.31 ab	6.77 a	6.16 ab
C4	5.75 a	5.50 a	5.41 a	3.34 a	6.93 a	6.47 a
LSD ($P \leq 0.05$)	0.36	0.17	0.36	0.27	0.47	0.41
Sampled site‡						
S1	5.83 a	5.67 a	5.23 a	3.36 a	6.67 b	6.35 a
S2	5.89 a	5.59 a	5.35 a	2.90 a	6.37 b	5.68 b
S3	5.44 a	5.31 b	5.54 a	3.55 a	7.19 a	6.44 a
LSD ($P \leq 0.05$)	0.31	0.15	0.31	0.23	0.41	0.35
Growth stage of rice‡‡						
V10	5.28 c	4.97 c	4.67 c	-	-	-
R4	6.17 a	6.25 a	6.34 a	3.48 a	6.74	-
R9	5.71 b	5.35 b	5.11 b	3.08 b	-	6.16
LSD ($P \leq 0.05$)	0.39	0.15	0.31	0.19		

*Values represent mean population of rice cultivars collected from three different sites at three growth stages. C1, Il-mi; C2, Dong-jin; C3, Nam-pyeoung; C4, O-dae; ‡Values represent mean population of four different cultivars collected at three growth stages. S1, Chungbuk; S2, Ho-nam; S3, Young-nam; ‡‡Values represent mean population of four different cultivars collected from three different sites. V10, vegetative; R4, flowering; R9, harvesting. Within each vertical column, values followed by the same letter are not statistically different according to Fischer's protected LSD ($P \leq 0.05$).

population recovered from the soil remained lower compared with that for the leaf. The endophytic population from the roots or stem showed no greater variations. In general, the plant type was found to have an effect on the PPFM population, recording significant differences among the tissues sampled except for the floret and grain. However, the epiphytic population from grain revealed differences among the sampled sites, whereas the leaf population showed no significant variation between the sites (Table 4).

The variation in PPFM population throughout the crop growth period is shown in Fig. 1 for all the cultivars sampled. The leaf PPFM population increased from stage V10 to stage R4, and then declined during the end of the cropping season, irrespective of the cultivars sampled. The root PPFM population increased from V10 to R4, and then remained stable or declined towards the R10 sampling. However, the Nam-pyeoung cultivar showed an increase in the root PPFM population throughout the entire crop growth. The rhizosphere soil population showed a similar trend to that of the leaf, except for the cultivar O-dae, where the PPFM count declined from stages V10 to R4, and then subsequently increased at stage R10. The endophytic stem population increased from the earlier to the later stages of crop growth, whereas the epiphytic population recovered from florets and grains showed differences among the cultivars sampled (Fig. 1).

A further analysis examined how the population from different parts of the plant varied within and between the sampled cultivars, sites, and growth stages. The ANOVA was carried out taking into account the log transformed values of the colony forming units from all the sampled cultivars, sites, and growth stages. The data obtained are reported in Table 5, which shows the percent variance derived from the differences between cultivars, sites, or sampling periods taking into account the interactions within and between them.

As can be seen, 27.44% of the variance related to differences in the leaf population between the cultivars, whereas the value strongly increased to 167.84% among the sampled growth stages. The highest value of variance for differences in the leaf, soil, or stem populations was recorded for the sampled growth stages, whereas in roots it was among the sampled sites. The interactive effects between the sites and cultivars, the cultivars and growth stages and the sites, and growth stages remained significant

Table 3. Distribution of different groups of methylotrophs at different growth stages of rice crop as measured by the Shannon-Weaver index.

Growth stage	EPI (H)‡					
	Leaf	Root	Soil	Stem	Floret	Grain
V10	0.58 b	0.42 a	0.39 c	-	-	-
R4	0.73 a	0.46 a	0.74 a	0.37 b	0.49	-
R9	0.34 c	0.45 a	0.54 b	0.45 a		0.54
LSD ($P \leq 0.05$)	0.09	0.96	0.11	0.05		

‡Values represent mean population of four rice cultivars from three sites. V10, vegetative stage; R4, flowering stage; R9, harvesting stage. Within each vertical column, values followed by the same letter are not statistically different according to Fischer's protected LSD ($P \leq 0.05$).

Table 4. PPFM population densities in different plant tissues and rhizosphere soil sampled at three growth stages of rice from different sites.

Rice cultivar/ Sampling site	PPFM population (Log CFU g of sample)					
	Leaf	Root	Soil	Stem	Floret	Rice grain
C1[†]	4.41±0.15 b	2.74±0.10 b	2.49±1.44 b	2.23±0.85 b	6.28±0.03 a	5.59±0.16 a
C2	4.84±0.28 ba	2.86±0.22 ba	2.98±1.72 a	2.20±0.28 b	6.56±0.31 a	5.77±0.15 a
C3	4.73±0.20 ba	3.17±0.09 ba	2.98±1.72 a	2.22±0.34 b	6.37±0.14 a	5.70±0.31 a
C4	5.24±0.11 a	3.24±0.21 a	2.95±1.70 a	2.97±0.80 a	6.38±0.14 a	5.94±0.42 a
LSD ($P \leq 0.05$)	0.49	0.42	0.29	0.53	0.37	0.46
S1[‡]	4.97±0.21 a	3.05±0.03 a	2.80±1.62 b	2.67±0.04 a	6.31±0.09 a	5.54±0.20 a
S2	4.82±0.20 a	3.40±0.14 a	3.07±1.77 a	2.51±0.13 a	6.35±0.11 a	6.14±0.09 a
S3	4.61±0.15 a	2.56±0.12 b	2.67±1.54 b	2.04±0.05 b	6.54±0.26 a	5.56±0.33 b
LSD ($P \leq 0.05$)	0.43	0.36	0.25	0.46	0.32	0.40

[†]Values represent mean of three sampling sites and three growth stages. [‡]Values represent mean of four rice cultivars and three growth stages. Within each vertical column, values followed by the same letter are not statistically different. C1, Il-mi; C2, Dong-jin; C3, Nam-pyeoung; C4, O-dae; S1, Chungbuk; S2, Ho-nam; S3, Young-nam.

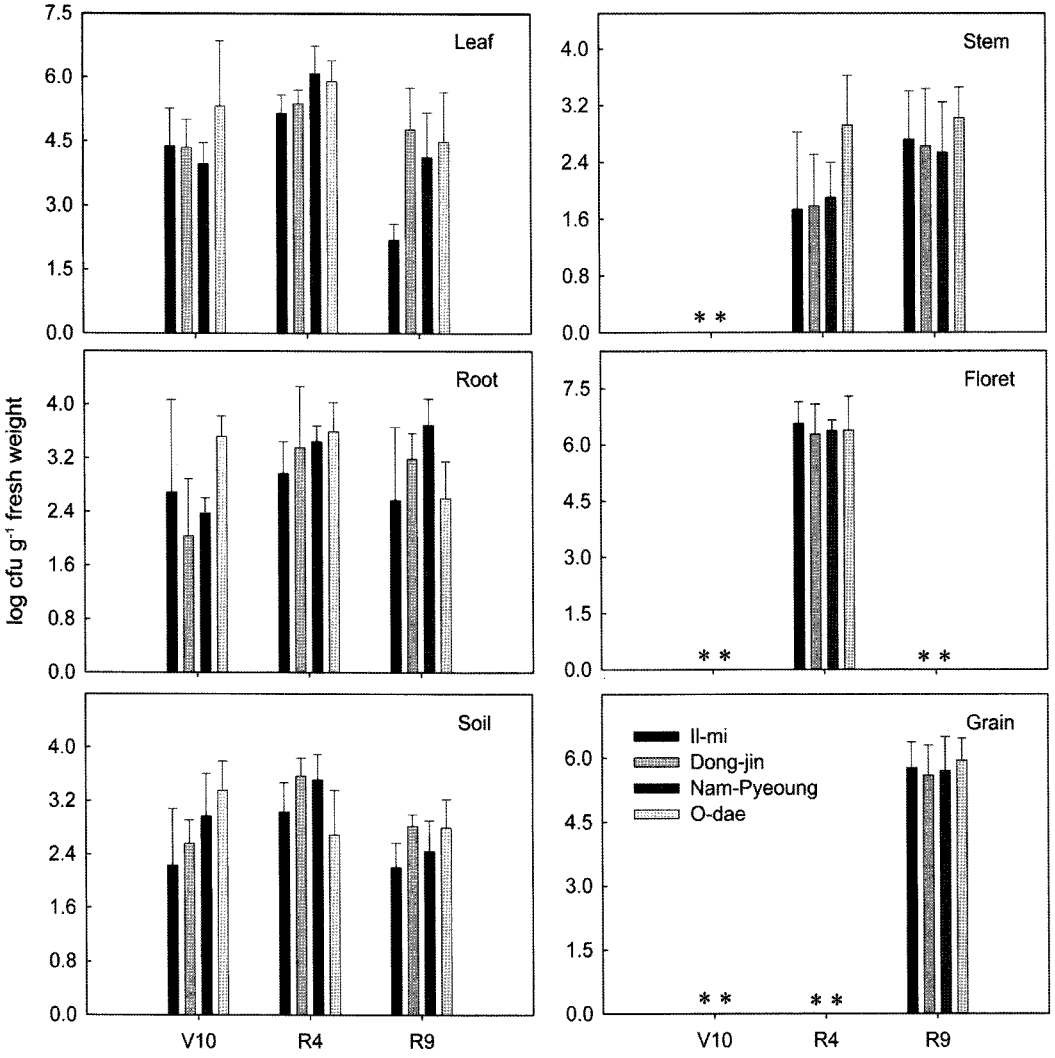

Fig. 1. Changes in the PPFM bacterial population associated with different rice cultivars at different growth stages.
The population represents the mean values of three plants collected from three different sites, and the bar represents the standard deviation of the mean (n=3). **, Not determined.

Table 5. ANOVA on PPFM bacterial population from different rice cultivars at three growth stages grouped according to sampled tissues.

Variance components	Source*					
	Leaf	Root	Soil	Stem	Floret	Grain
Among cultivars	27.44	13.73	16.66	21.60	2.50 (0.0834)	5.14 (0.0069)
Among sites	10.34	58.66	15.78	21.40	3.53 (0.0454)	37.06
Among samplings	167.84	38.48	39.42	62.45	–	–
Cultivar *vs* Site	56.61	22.47	2.56 (0.0275)	24.27	11.07	26.46
Cultivar *vs* Sampling	15.44	23.74	15.67	5.67 (0.0021)	–	–
Site vs Sampling	24.84	38.83	10.06	24.67	–	–
Cultivar *vs* Site *vs* Sampling	10.32	2.89 (0.0026)	6.93	13.86	–	–
Within tissues sampled	31.26	20.28	11.24	20.23	7.56	22.57

*Percent variation among the populations. In all cases except for the values given in parenthesis, the probability (P) of having a more extreme variance component than the observed value is $P<0.0001$.

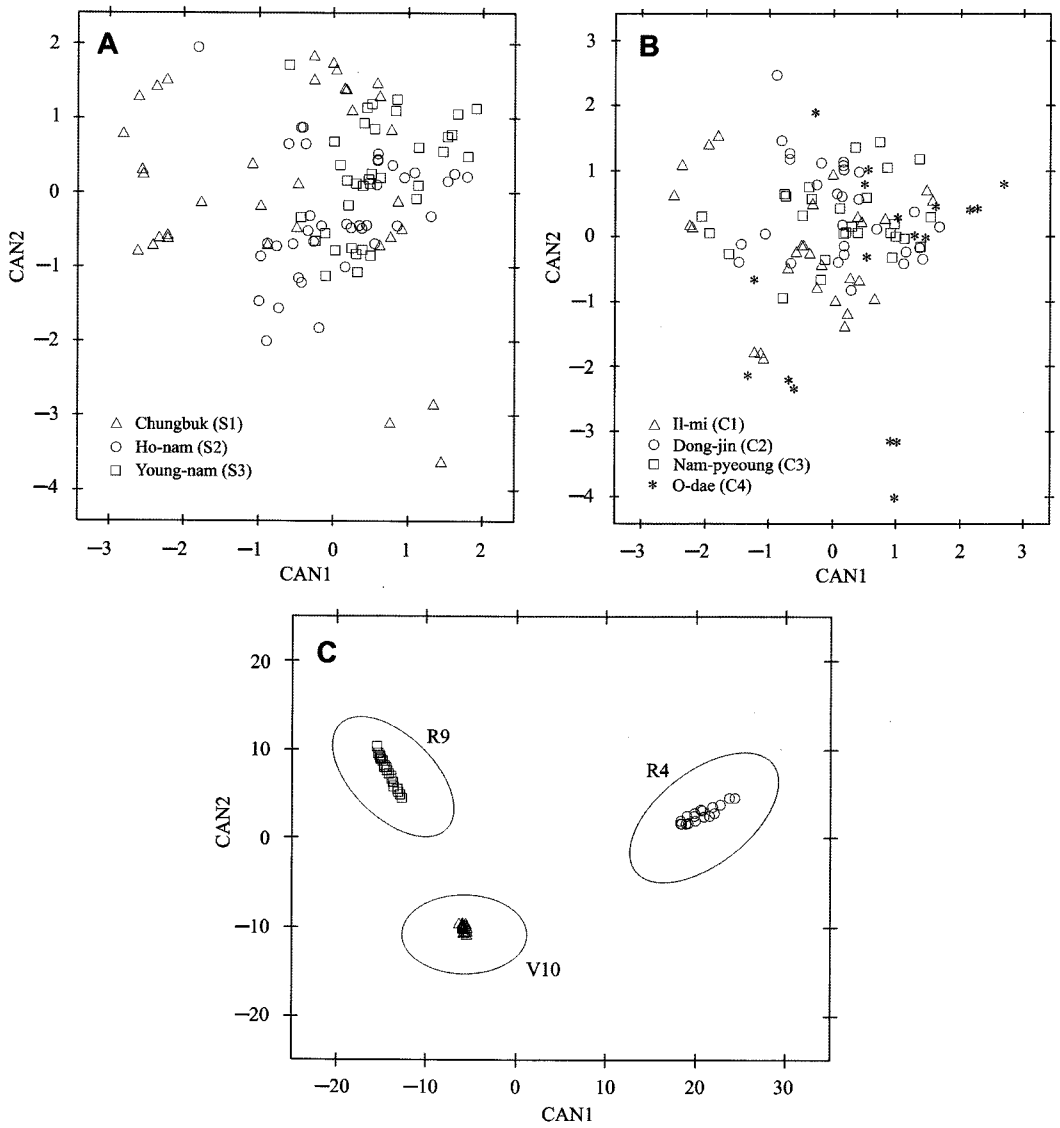

Fig. 2. Ordination plots of variables resulting from the first (CAN1) and second (CAN2) canonical functions for different (**A**) sites, (**B**) cultivars, and (**C**) growth stages.
The variables were generated based on the populations from different plant tissues and rhizosphere soil. V10, vegetative; R4, flowering; R9, harvesting stages.

($P<0.0001$). When the cumulative interaction is considered, 13.86% of the variance related to the stem population remained the highest, followed by 10.32% for the leaf population, 6.93% for the soil, and only 2.89% for the root population (Table 5). The variance within the tissues sampled was higher for leaf (31.26%) and grain (22.57%) populations and lower (7.56%) for the floret population. These results supported the idea that seasonal variation and the environment also seemed to play an important role in the fluctuations of the PPFM population in the different plant parts.

As revealed by the ANOVA, the main effects of the cultivars, growth stages, and sites and the interactive effect between them remained significant for the PPFM population from the different plant parts (Table 5). Although the effect of the growth stage was the highest for all the plant parts followed by the cultivar and the site, the interaction between these three factors revealed a different scenario. For example, the interaction between the cultivar and the sampled site affected the leaf population (56.61) more than the interaction between the cultivar and growth stage or the sampled site and growth stage. Surprisingly, the interaction of the growth stage and the cultivar or site remained significant for the PPFM population in the root, soil, and stem when compared with the interaction between the cultivar and the sampled site. The overall interaction between the three factors considered in this study remained significant for all the populations, with the stem population (13.86) being affected more (Table 5). Therefore, these results clearly show that soil, plant, and environmental factors, and their interactions within and between them, would seem to play a role in the PPFM dynamics associated with plants.

To look for the most discriminating factors influencing the PPFM population, CDA was applied to the population data separately for each group: the variety, site, and growth stage. The output data set on canonical variables was then used in conjunction with a %PlotIt macro to aid in the visual interpretation of the different classes (Fig. 2). The means of the canonical variable scores for each class were determined for first and second canonical functions (CAN). The CANDISC procedure for CDA performs a multivariate analysis of variance, providing four tests of hypothesis to compare the class means, and in the case of the sites, the Wilks' Lambda statistic remained significant ($P=0.001$). The CAN1 and CAN2 functions accounted for 83.15% and 16.85% of the total variation, respectively, and distinguished between the sites (Fig. 2A). The standardized canonical coefficients showed that the leaf, root, soil, and stem populations had the most influence on CAN1, whereas the floret and grain populations had the most influence on CAN2. Compared with the site S3, sites S1 and S2 had no significant differences in the PPFM population from the different plant parts. Thus, sites S1 and S2 may have had similar characteristics or conditions that favored the PPFM population, and this was also clearly evident from the analysis of the canonical variables, where S1 and S2 were more closely associated (Fig. 2A). The CDA of the cultivars or variety of rice crops did not reveal any clear differentiation among them. The Wilks' Lambda statistic showed no significant variations ($P=0.0129$). The standardized canonical coefficients showed that the floret and grain populations had the most influence on CAN2, whereas for the other populations it was CAN1. The mean values of the CAN1 had similar values for C2 and C3, hence, CAN2 was more efficient in separating these two groups (Fig. 2B). The rice-associated PPFM population showed significant variations among the growth stages, which was also obtained with the analysis of the canonical variables (Fig. 2C). The CAN1 function accounted for 79.83% of the total variance, whereas the remaining 20.17% was accounted for by CAN2. The CAN1 function was most important in separating stage R4 from stages V10 and R9, whereas CAN2 separated stages R4 and R9 from stage V10. The standardized canonical coefficients showed that the populations from all the samples, except those from the grain, had the most influence on CAN1.

DISCUSSION

Studies dealing with the exploration of rhizosphere microflora in rice for the most part investigated the anoxic interfaces or special functional groups of bacteria, such as methanogens and sulfate reducers, etc. [7, 20, 32]. This work for the first time reports the presence of methylotrophic bacteria in field-grown rice, and the variations in the PPFM population associated with the below and above ground plant canopy, exploring the interaction of environmental and physiological factors in the rice-PPFM association. The rice system hosted methylotrophic bacteria at all three stages of growth and in all the tissues sampled in this study. The overall prosperity of the plant-methylotroph association may be attributed to the unique ability of these bacteria to grow at the expense of methanol, a cell wall product from plants. Furthermore, the facultative methylotrophic characteristic of *Methylobacterium* to cometabolize methanol with an alternative carbon source benefits the bacteria during colonization [35].

Consistent with a previous report that the endophytic and epiphytic bacterial populations in soybean varied among the tissues sampled [17], the total heterotrophic and methylotrophic populations in rice also varied among the tissues sampled. The abundance of the PPFM population in the florets and grains remains unexplained, although it may have been related to methanol production and accumulation in the mature seeds [24]. Considering the different factors (i.e., plant genotype or cultivar, sampled sites, or growth

stage), the methylotrophic population and its distribution in rice showed greater and significant variations among the growth stages. Biotic interactions within the plants may have contributed to the substantially greater variability observed in the methylotrophic population than in the total bacterial population. However, the origins of the physiological stage-related differences from the different parts are likely complex and may include variability in the endogenous and exogenous plant-derived resources and increasing suitable conditions that favor a particular population.

The presence of PPFMs has been recovered from over 70 plant species from various families [10], and typically >80% of the viable bacteria recovered from leaves were PPFMs [8]. *Methylobacterium* represented the largest group of endophytic population in rice, persisting widely among several wild and cultivated varieties [4]. The surface and endophytic populations from the seeds of rice plants also include *Methylobacterium* as one of the dominant genera [22]. Consistent with these reports, the PPFM population present in all the samples tested in this study represented a greater proportion of the total methylotrophs. A high PPFM population was reported in white clover, with no changes or trends recognized over the sampling period [1]. However, red clover showed a clear increase in the bacterial count on the leaves, followed by a significant decrease in the late spring [25]. However, these studies only concentrated on the leaf PPFM population. In our study, we could observe the fluctuations or variations in PPFM populations in rhizosphere soil, as endophytes in stem and roots, and as epiphytes in floret and grains. Our results showed an increase in the number of PPFMs recovered from different parts of rice plants from V10 to R4 and that decreased towards the R9 growth phase. Increasingly suitable conditions for *Methylobacterium* species at the flowering stage might have led to relatively higher PPFM populations at R4. Field sites would play an important role in determining the microbial population and site factors that influence the composition of microbial communities [34].

In general, this study revealed that the plant genotype or the field sites had less effect on the PPFM dynamics than the growth stage, as there was a clear temporal variability. The minimal effect of the field sites may have been due to past agricultural practices or the past cropping history of the fields that could have masked the overwhelming effects of the field sites during the particular cropping period. Similarly, the effect of the plant type on a particular group of bacteria cannot be concluded by determining the population differences without studying the distribution of the different associated taxonomic groups. The temporal variability in the PPFM population could be associated with environmental variables such as the soil type or soil moisture, the temperature, or other climatic factors that vary on similar time scales. The CDA analysis also revealed that the difference in the rice-associated PPFM population was largely driven by the growth stage rather than the field site or plant type. Hence, it is noteworthy the the PPFMs may have been responding in concert to environmental characteristics that are temporally variable, although no specific parameters influencing the bacteria were identified.

In conclusion, the present results demonstrated that the growth stage of the plant at the time of sampling had a more significant effect on the rice PPFM population than the plant genotype or the sampled site. Furthermore, the presence of a definite population of different groups throughout the study suggests that bacterial populations other than PPFMs may also be present as efficient metabolizers of methanol from the plants. Therefore, the present work sheds more light on the dynamics of PPFMs in rice and the variation they undergo during plant development, as a foundation for better understanding of the mechanisms involved in plant-PPFM interactions.

Acknowledgments

M. Madhaiyan and S. Poonguzhali are indebted to the Korea Research Foundation (KRF), Republic of Korea, for a position as invited foreign scientist and PhD research grant, respectively. The authors are also grateful to Kyu-hoi Lee and Myoung-Su Park for their help and assistance in the sample collection. The authors also acknowledge the financial support from the Rural Development Administration (RDA), Republic of Korea.

References

1. Corpe, W. A. and S. Rheem. 1989. Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiol. Ecol.* **62:** 243–248.
2. Counce, P. A., T. C. Keisling, and A. J. Mitchell. 2000. A uniform, objective, and adaptive system for expressing rice development. *Crop Sci.* **40:** 436–443.
3. De Leij, F. A. A. M., J. M. Whipps, and J. M. Lynch. 1993. The use of colony development for the characterization of bacterial communities in soils and roots. *Microb. Ecol.* **27:** 81–97.
4. Elbeltagy, A., K. Nishioka, H. Suzuki, T. Sato, Y. I. Sato, H. Morisaki, H. Mitsui, and K. Minamisawa. 2000. Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. *Soil Sci. Plant Nutr.* **46:** 617–629.
5. Germida, J. J. and S. D. Siciliano. 2001. Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. *Biol.Fertil.Soils* **33:** 410–415.
6. Glick, B. R., C. B. Jacobson, M. M. K. Schwarze, and J. J. Pasternak. 1994. 1-Aminocyclopropane-1-carboxylate

deaminase mutants of plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2 do not stimulate canola root elongation. *Can. J. Microbiol.* **40:** 911–915.
7. Hengstmann, U., K. J. Chin, P. H. Janssen, and W. Liesack. 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. *Appl. Environ. Microbiol.* **65:** 5050–5058.
8. Hirano, S. S. and C. D. Upper. 1991. Bacterial community dynamics, pp. 271–294. *In* J. H. Andrews and S. S. Hirano (eds.), *Microbial Ecology of Leaves*. Springer-Verlag, New York, NY.
9. 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.
10. Holland, M. A. and J. C. Polacco. 1994. PPFMs and other contaminants: Is there more to plant physiology than just plant? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45:** 197–209.
11. Holland, M. A., R. L. G. Long, and J. C. Polacco. 2002. *Methylobacterium* spp.: Phylloplane bacteria involved in cross-talk with the plant host? p. 125–135. *In* S. E. Lindow, E. I. Hecht-Poinar, and V. J. Elliot (eds.) *Phyllosphere Microbiology.* APS Press, St. Paul, Minn.
12. Idris, R., M. Kuffner, L. Bodrossy, M. Puschenreiter, S. Monchy, W. W. Wenzel, and A. Sessitsch. 2006. Characterization of Ni-tolerant methylobacteria associated with the hyperaccumulating plant *Thlaspi goesingense* and description of *Methylobacterium goesingense* sp. nov. *Syst. Appl. Microbiol.* **29:** 634–644.
13. Idris, R., R. Trifonova, M. Puschenreiter, W. W. Wenzel, and A. Sessitsch. 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl. Environ. Microbiol.* **70:** 2667–2677.
14. Katiyar, V. and R. Goel. 2004. Improved plant growth from seed bacterization using siderophore overproducing cold resistant mutant of *Pseudomonas fluorescens*. *J. Microbiol. Biotechnol.* **14:** 653–657.
15. Kinkel, L. L., M. Wilson, and S. E. Lindow. 2000. Plant species and plant incubation conditions influence variability in epiphytic bacterial population size. *Microb. Ecol.* **39:** 1–11.
16. 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.
17. Kuklinsky-Sobral, J., W. L. Araujo, R. Mendes, I. O. Geraldi, A. A. Pizzirani-Kleiner, and J. L. Azevedo. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environ. Microbiol.* **6:** 1244–1251.
18. Lee, H. Y., K. H. Park, J. H. Shim, R. D. Park, Y. W. Kim, J. Y. Cho, H. B. Hoon, Y. C. Kim, G. S. Cha, H. B. Krishnan, and K. Y. Kim. 2005. Quantitative changes of plant defense enzymes in biocontrol of pepper (*Capsicium annuum L.*) late blight by antagonistic *Bacillus subtilis* HJ927. *J. Microbiol. Biotechnol.* **15:** 1073–1079.
19. Lidstrom, M. E. and L. Chistoserdova. 2002. Plants in the pink: Cytokinin production by *Methylobacterium*. *J. Bacteriol.* **184:** 1818.
20. Ludemann, H., I. Arth, and W. Liesack. 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* **66:** 754–762.
21. Madhaiyan, M., S. Poonguzhali, J. H. Ryu, and T. M. Sa. 2006. Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. *Planta* **224:** 268–278.
22. Mano, H., F. Tanaka, A. Watanabe, H. Kaga, S. Okunish, and H. Morisaki. 2006. Culturable surface and endophytic bacterial flora of the maturing seeds of rice plants (*Oryza sativa*) cultivated in a paddy field. *Microbes Environ.* **21:** 86–100.
23. Nautiyal, C. S., S. Mehta, and H. B. Singh. 2006. Biological control and plant-growth promotion by *Bacillus* strains from milk. *J. Microbiol. Biotechnol.* **16:** 184–192.
24. Obendorf, R. L., J. L Koch, R. J. Goreki, R. A. Amable, and M. T. Aveni. 1990. Methanol accumulation in maturing seeds. *J. Exp. Bot.* **41:** 489–495.
25. Omer, Z. S., R. Tombolini, and B. Gerhardson. 2004. Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). *FEMS Microbiol. Ecol.* **47:** 319–326.
26. Penalver, C. G. N., D. Morin, F. Cantet, O. Saurel, A. Milon, and J. A. Vorholt. 2006. *Methylobacterium extorquens* AM1 produces a novel type of acyl-homoserine lactone with a double unsaturated side chain under methylotrophic growth conditions. *FEBS Lett.* **580:** 561–567.
27. Pirttilä, A. M., H. Laukkanen, H. Pospiech, R. Myllylä, and A. Hohtola. 2000. Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by *in situ* hybridization. *Appl. Environ. Microbiol.* **66:** 3073–3077.
28. Poonguzhali, S., M. Madhaiyan, and T. M. Sa. 2007. Production of acyl-homoserine lactone quorum sensing signals is wide-spread in Gram-negative *Methylobacterium*. *J. Microbiol. Biotechnol.* **17:** 226–233.
29. Romanovskaya, V. A., S. M. Stolyar, Y. R. Malashenko, and T. N. Dodatko. 2001. The ways of plant colonization by *Methylobacterium* strains and properties of these bacteria. *Microbiology* **70:** 221–227.
30. Ruiz Palomino, M., J. A. Lucas Garcýa, B. Ramos, F. J. Gutierrez Manero, and A. Probanza. 2005. Seasonal diversity changes in alder (*Alnus glutinosa*) culturable rhizobacterial communities throughout a phenological cycle. *Appl. Soil Ecol.* **29:** 215–224.
31. Ryu, J. H., M. Madhaiyan, S. Poonguzhali, W. J. Yim, P. Indiragandhi, K. A. Kim, R. Anandham, J. C. Yun, K. H. Kim, and T. M. Sa. 2006. Plant growth substances produced by *Methylobacterium* spp. and their effect on tomato (*Lycopersicon esculentum* L.) and red pepper (*Capsicum annuum* L.) growth. *J. Microbiol. Biotechnol.* **16:** 1622–1628.
32. Scheid, D. and S. Stubner. 2001. Structure and diversity of Gram-negative sulfate-reducing bacteria on rice roots. *FEMS Microbiol. Ecol.* **36:** 175–183.

33. Schmalenberger, A. and C. Tebbe. 2002. Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar *Bosphore*. *FEMS Microbiol. Ecol.* **40:** 29–37.
34. Siciliano, S. D. and J. J. Germida. 1999. Taxonomic diversity of bacteria associated with the roots of field grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. *FEMS Microbiol. Ecol.* **29:** 263–272.
35. Sy, A., A. C. J. Timmers, C. Knief, and J. A. Vorholt. 2005. Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. *Appl. Environ. Microbiol.* **71:** 7245–7252.
36. 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.