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PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF *Methylobacterium* sp. STRAINS AND FOLIAR-APPLIED METHANOL ON GROWTH AND DEVELOPMENT OF RICE *Oryza sativa* L.

by

CHARLES M. MALITI

A dissertation submitted to the Graduate Faculty of Biology in partial fulfillment of
the requirement for the degree of Doctor of Philosophy,
The City University of New York.

2000

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Physiological and Biochemical Effects of *Methylobacterium* sp. Strains and Foliar-Applied Methanol on Growth and Development of Rice *Oryza sativa* L.

by
Charles M. Maliti

Advisor - Professor Dominick V. Basile

Phylloplane saprophytic microflora and epiphytic microorganisms during their vital life sustaining activities secrete biologically active substances that directly or indirectly influence the biochemical and physiological processes of host plant tissue. Although these compounds are likely to be in micro-concentrations they could have significant beneficial or detrimental effects on growth and metabolic processes of the host plant.

This study was designed to evaluate the effect of some *Methylobacterium sp.* strains on growth and development of rice at three levels, tissue culture, seedlings *in vitro* and in mature plants grown in simulated natural conditions in a greenhouse. The experiments were carried out to evaluate the promise of several Pink-pigmented facultative methylotrophic bacteria (PPFM) strains potential as phylloplane microbial factors that could significantly increase growth, development and absolute rice grain yield. As a follow up study, *in vitro* experiments were carried out to determine if the observed growth stimulatory effect resulting from foliar methanol treatment was partly mediated by some of the PPFM strains such as the Q5, originally isolated from rice leaf.

Data and findings were analyzed qualitatively and quantitatively using descriptive statistics, Pearson's correlation, Students *t*-test and analysis of variance (ANOVA). Qualitative findings revealed that there is a wide range of facultative methylotrophic bacteria strains on the leaf surface of rice plants. Inoculation of two isolated ***Methylobacterium*** sp. strains designated Q4 and Q5 onto seed embryo-derived calli significantly stimulated cell proliferation and differentiation in tissue culture. Inoculation of either the Q4 or Q5 strain on 9 day old axenic rice seedlings significantly increased seedling growth in height, leaf development, root growth and biomass ***in vitro***. Qualitative evidence derived from tissue culture experiments indicated that the Q4 and Q5 strains plant growth and development stimulatory properties were mediated by microbial derived cytokinin and auxin-like growth regulators.

Evaluation of the physiological effects of foliar-applied aqueous methanol revealed that the stimulatory effect of methanol is partly mediated by methylotrophic bacteria on rice leaf surface. Significant increases in leaf chlorophyll content and biomass accumulation in tropical and temperate rice cultivars were observed in treated plants. However the response was not cultivar specific. Indirect evidence from ***in vitro*** experiments showed that rice plants and their PPFM strains such as Q4 and Q5 constitute a group of mutualistic cobionts. The physiological partnership appeared to be based on production and release of methanol from plants tissues in micro-quantities, and the utilization of plant-derived methanol as source of carbon by PPFM including Q4 and Q5.

Taxonomic characterization of the Q4 and Q5 based on morphological characteristics, carbon-compound utilization properties, resulted in identification and placement of the two strains in the 7 and 35^{e} strain category in the genus ***Methylobacterium*** sp. The findings from the ***in vitro*** and glasshouse experiments have shown that the inoculation of rice plants in the field with either Q4 or Q5 PPFM strain via seed coating technique, coupled with application of methanol treatments on rice foliage is a viable alternate technology that could significantly increase rice production.

To
my parents, Elijah Maliti Kibwaa and Susan W. Maliti, who
carefully looked after my education and from whom I inherited
the interest in science

my sister Mary M. Maliti who unselfishly
encouraged me in my work all along the way

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my mentor/advisor Dr. Dominick V. Basile for his valuable time, advise and constant support throughout this research project and preparation of this thesis. I am deeply indebted to Dr. Dwight Kincaid for his advise on statistical analysis of the data, as well as for his encouragement, support and for having faith in me all along the way.

I am thankful to Dr William A. Corpe, for his helpful suggestions, enthusiasm and constant guidance on microbiological aspects of this project. I would like to thank the members of my advisory committee, Dr Thomas Jensen, Dr Jack Valdovinos and Dr Philip Ammirato for their encouragement and support.

I gratefully acknowledge Dr. Joseph Rachlin, Dr. Thomas Borgese, Dr. Barbara Warkentine, Dr. Martin Muntzel, Dr. Garbriel Aisenberg and Dr Eleanor Wurtzel for offering many helpful suggestions. I am sincerely grateful to Mr David Cain for his excellent work and for maintaining all the experimental plants. I also thank Mr. Michael Baxter, Dr. Antonios Pappantoniou, Mr. Steve Trimboli and Ms Kathy Lieberman for their technical support during my research.

My great appreciation goes to my family, particularly my late father Elijah Maliti who spend much of his time up to the last Sunday of his life trying to teach me the value and love for work. I will always be thankful to my mother Susan W. Maliti and my sister Mary M. Maliti who tirelessly supported me along the way and more so at times when I was streamlining technical or personal glitches. I would like to express special thanks to Drs. Manitha Weerasuriya, Pattan Dial, Harish Ratnayaka, Clarence Branch and Mr. Rajendra Ghabaran for all their encouragement. My sincere thanks also go to Joan Reid of Graduate Center CUNY, Dolores Vitanza, Patricia Carver and all graduate students for their codiality and support. I am indebted to the Departments of Biological Sciences, Lehman College and Graduate Center, for financial support during my stay at Lehman College, CUNY.

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INTRODUCTION

The aerial parts of green plants provide a favorable habitat for a great number of microorganisms including numerous strains of bacteria. These microbial cobionts which establish colonies on the aerial parts (phylloplane) can be beneficial to the host plant, while in other cases they are potential pathogens, capable of being parasitic and causing disease. Recent reports suggest that some of the microorganisms, benefit crops through stimulation of growth of the host plant or by inhibiting the establishment and growth of pathogenic strains (Kloepper *et al.,* 1989).

Within this large group of the phylloplane microorganisms are the aerobic, heterotrophic, pink-pigmented facultative methylotrophic bacteria (PPFM), which are probably distributed ubiquitously on green plants (Corpe and Basile 1982; Corpe and Rheem 1989). Isolation of a beneficial PPFM strain that stimulated growth of a leafy liverwort *Scapania nemorosa* in axenic culture has been reported (Basile *et al.*, 1969). However for the first time we report a PPFM-induced stimulation of growth of rice *Oryza sativa* L., an economically important food crop. This is in spite of several reports which indicate that some of the saprophytic microflora which are in close contact with the host plants during the entire vegetative period secrete biologically active metabolic products which have considerable effect on plant physiological activities (Klincare *et al.* 1971; Kloepper *et al.*,1989; Holland and Polacco 1994; Costacurta and Vanderleyden 1995).

As a follow up study to the findings of Basile *et al.,* (1969) on leafy liverwort-PPFM interaction, the primary objectives of this study was to carry out a survey of the *Methylobacterium* sp. strains associated with rice *Oryza sativa* L. and other members of grass family (Poaceae), the principal aim being to isolate PPFM strains with the potential of stimulating growth of *japonica* and *indica* rice tissue culture and seedlings *in vitro*. Investigations on the effects of

Methylobactrerium sp. strains on rice were given priority mainly because rice is a major cereal crop that supports the nutritional requirements of over 60% of the human population (Yamada and Loh 1984).

Rice *Oryza sativa* is an economically important food crop, that has been cultivated for many centuries, and has formed the basis for the rise of civilizations and establishment of center of cultures throughout the history of mankind. The plant is taxonomically classified in the genus *Oryza* within the grass family Poaceae. There are over 20 species of *Oryza* native to Asia and Africa. The two species that are mostly cultivated are *O.sativa* L., which is grown world wide and *O. glaberrima* Steud., which is confined to areas of West Africa.

Within the species *Oryza sativa* L. the most abundant cultivated varieties (cultivars) belong to the subspecies *indica* and *japonica*. A third subspecies *javanica*, which is tall and grows vigorously, is exclusively grown in Indonesia. The *indica* cultivars are slow growers, requiring 160 -170 days to mature (Ashraf *et al.*, 1994). Their ecogeographic distribution is within the tropical and subtropical areas which includes Southeast Asia, Africa, and Latin America. Morphologically the *indica* cultivars have tall weak stems, long drooping thin, pale green leaves, and relatively large lax panicle. Physiological traits include occurrence of lodging, 0.3 harvest index, adaptation to low fertility soil conditions and photoperiod sensitivity.

The *japonica* cultivars are primarily grown in the temperate zones such as Japan, Korea, China, Taiwan and the USA (California, Louisiana, Texas, Arkansa) and Australia. Relative to the *indica* cultivars, the *japonica* cultivars have short stiff stalk, dark green, upright leaves. The main physiological traits include resistance to lodging, good response to application of fertilizers, early maturing fast growers and considerably higher yields per given area than the *indica* cultivars (Ashraf *et al*, 1994). To evaluate the consistency of results in this study, the reported experiments were conducted using several cultivars representing the two main subspecies of rice *indica* and *japonica*.

The productivity of cereal crops is influenced by physiological determinants and the genetic make of the plant. The main physiological characteristics that determine grain yields of a cereal crop in the field are (1) dry matter economy of the crop, (2) components of the grain, (3) water economy of the crop, and (4) nitrogen economy of the crop (Slafer *et al.*, 1994). The latter two factors have minimal effect on rice productivity because rice as a crop is mainly grown in semi-aquatic conditions. In addition, on the basis of plant nutrients requirement, the crop can grow and produce good yields in relatively poor soils, where other cereals such as wheat, corn and barley cannot grow successfully.

Evidently, it is the first two factors, namely, the dry matter economy (above the ground biomass) and components of the grain (partitioning of the biomass into reproductive sinks), that affect rice yields. The grain component is analyzed quantitatively in terms of the number of harvested grains per M^2 area from the crop in the field and on the basis of the mean weight of individual grains. Therefore, it is noteworthy that total biomass is a reliable and major indicator of the potential productivity of a given rice cultivar, as demonstrated by Guangman and Jiling (1989). Their report showed that high yielding semi dwarf or intermediate height cultivars had about 40% higher total biomass accumulation relative to the tall low yield cultivars. Guided by this finding, the experiments described in this study were also designed to evaluate the effect of isolated ***Methylobacterium*** sp strains and foliar- applied methanol on vegetative (above the ground) biomass accumulation and root dry matter accumulation, the former being the main indicator of potential yield.

Aqueous methanol was selected as a potential growth stimulating compound for several reasons. First, foliar application of aqueous methanol to C_3 plants exposed to high temperature and full sunlight in arid conditions is reported to have increased the growth and harvest yield of some plants such as tomato (*Lycopersion esculentum.*), soy bean (*Glycine max*), and *Hibiscus esculentus*. (Nonomura and Benson 1992a; Rowe *et al.*, 1994; Li *et a.,l* 1995 and Dorcus and Vivekanandan 1996). The suggested possible mechanisms attributed to these physiological responses of plants to methanol treatment include inhibition of photorespiration, induced rapid assimilation of methanol as a substitute for CO_2 and systemic methanol-induced increase in metabolic rate in the whole plant with concomitant increase in the carbon dioxide conversion efficiency.

Methanol was also selected as a potential growth stimulating compound on the basis of the hypothesis that its application on rice foliage could result in increased population or distribution of the PPFM strains on the leaf surface. The hypothesis was based on several reports which have demonstrated that methanol is used by methylotrophic bacteria including the PPFM as the primary source of carbon. Since a strain of PPFM was shown to enhance growth of leafy liverwort (Basile *et al.*, 1969), it was thus reasonable to speculate that the beneficial effects of the methanol treatments could be mediated by PPFM strains on the leaf surface. Although there is no direct evidence, it is probable that a mutualistic relationship exists, whereby methylotrophic microorganisms benefit by utilizing the methanol produced and released by green plants, while in turn the microbes secrete biologically active compounds that are physiologically beneficial to the host plant.

In summary this study was carried out first to determine the independent effect of each of the isolated ***Methylobacterium*** sp strains designated Q4 and Q5 on growth and development of rice in tissue culture and seedlings ***in vitro***. Secondly, the experiments were carried out to evaluate the effect of combined treatment of seedlings with Q4 or Q5 cobionts and foliar- applied methanol on seedling growth. Thirdly, greenhouse based experiments were conducted to determine quantitatively the effects of the treatments on rice crop physiological productivity parameter based on (a) dry matter economy (dry matter accumulation and partitioning of the biomass to the vegetative structures) and (b) yield component, [primarily the partitioning of the biomass to grains within reproductive structures (panicle)] of the plant.

The knowledge generated by this study will facilitate the evaluation of the potential of rice-***Methylobacterium*** sp strains interaction as a new agrotechnique which can be purposefully modified to increase rice grain yield. This agrotechnique would be of great benefit, particularly in parts of the world where other technologies cannot be implemented due to economic or environmental considerations.

CHAPTER ONE

Isolation of *Methylobacterium* sp. Strains that Stimulate Growth And Development Of Rice Tissue Culture and Seedlings *In vitro*.

SUMMARY

A survey of several diverse species of green plant has shown that pink-pigmented facultative methylotrophic bacteria (PPFM) of the genus *Methylobacterium* comprise a prominent portion of the normal microflora inhabiting their surfaces. Although a PPFM isolate capable of stimulating growth and development of a leafy liverwort *Scapania nemorosa* in axenic culture has been reported previously. In the present study we report the isolation two new strains of PPFM designated Q4 & Q5 capable of significantly stimulating growth and development of rice, an economically important food crop. They were isolated using a selective agar-solidified methanol-ammonium salts medium. The two rice cultivars used in this study *japonica* cv. CR76 and *indica* cv. A301 were cultured axenically either as callus or seedlings. That the plant cultures were initially bacteria-free was confirmed by absence of any other microbial growth when homogenized tissues were inoculated onto peptone-enriched nutrient broth. Co-incubation of Q4 and Q5 PPFM strains with embryo-derived rice calli on hormone-free medium resulted in continued callus growth. The Q4 and Q5 significantly ($p < 0.05$) inhibited plantlet regeneration from rice calli subcultured on regeneration MS media. A 0.5 Phi (ϕ) coefficient indicated a strong association between the inoculation of the Q4 or Q5 PPFM strain and inhibition of regeneration of plantlets. These results mimicked those produced by medium supplemented with auxin and cytokinin. Co-culture of Q4 & Q5 strains with 5 day old *japonica* cv. CR76 and *indica* cv. A301 rice seedlings *in vitro* resulted in a significant ($p \leq 0.05$) stimulation of growth parameters such as root development, vegetative growth, leaf development and biomass productivity of plants in 9 days. The results of the cobiotic experiments indicate that if Q4 and /or Q5 were inhabiting rice plant surfaces in the field cultivation, they could significantly stimulate the plants growth and development.

INTRODUCTION

Rice *Oryza sativa* is an most important staple food crop and the only cereal that is grown exclusively for human food consumption. Approximately 150 million hectares of land world wide (11% of worlds arable land) are devoted exclusively to rice production (Neue 1993). It is the main source of carbohydrates and proteins for over 2.3 billion people primarily the low-income populations (Yamada and Loh 1984).

Over 90% of the world's rice crop is harvested in Asia. China is the leading producer, with an annual output of 35.3% of the total world production, the other major producers are India (20.4%), United States (1.4%), Africa (2.1%), Latin America (3.7%), Brazil (2.1%), Thailand (4.3%) and Philippines (1.9%) (Yamada and Loh 1984). In the USA, rice is primarily grown as a cash crop. Approximately 60% of the total annual harvest [1.8 million metric tons (MMT) in 1978] is exported to the world market. Recent statistics show that although the USA produces 1.4% of the annual global output, it is the leading expoter and the most reliable supplier of rice in the world market. In other regions such as Asia or Africa the rice produced is consumed locally.

To meet the requirements of the descendants of today's rice consumers, annual rice production must increase by 47% from 518 million tons (1989) to 760 million tons in 2020 as forecast by International Rice Research Institute (IRRI report 1989). In many parts of the world, a combination of feasible technologies will continually be required to sustain the present annual rice production level and possibly to increase the crop yields in order to meet the basic needs of future generations. The challenge of increasing the rice productivity by 47% within a period of 25 years, requires a diversification of agricultural systems and incorporation of appropriate biotechniques.

It is evident that the production of sufficient food of minimal acceptable quality to each individual is one of the most urgent problems of our time. In less developed countries there are at least 460 million human beings that are malnourished. In developed countries food shortages are less severe. However approximately 3% of the population live below maintenance level and have limited access to quality food. It is clear that progress in dealing with malnutrition will depend on action on many fronts in national and local situations. Minimal increase in rice production can play a major role in alleviating malnutrition and hunger.

Although major advances have been made towards improvement of rice production within the last four decades mainly through conventional breeding techniques and improved agronomic practises, there are limitations associated with these approaches. For instance in the 1960s a 40% increase in rice production was registered, as well as 30% in the 1970's and a 3.1% in the 1980's. Collectively the major increases in rice production were attributed to the development of a two new, hybrids Taichung Native 1 (TN1) and IR8 both achieved through conventional cross-breeding. The TN1 which is a dwarf cultivar has a high grain/straw ratio and high responsiveness to fertilizer application. In 1962 the International Rice Research Institute (IRRI) in Philippines developed a semi dwarf strain of *indica* rice (IR8) that responded to fertilizer application with greater yields than TN1.

In spite of this general improvement in rice production over the last three decades as a result of improvement in rice varieties, crop management and resource management technology, these techniques have some limitations. For instance 30 years after the release of IR8 by IRRI no new variety has ever surpassed the IR8 in yield potential. Currently most of the modern rice cultivars developed for local climatic regions world wide carry the genes for the desirable traits introduced from IR-8 and TN1.

Considering the limitations of conventional breeding and the degradation of aquatic environments as a result of the use of chemical fertilizers, this study was designed to evaluate the potential of plant-microbe interactions with a view of identifying possible strains of microbes that have the potential of stimulating rice growth, development and yields. A general review of previous research findings indicated that several micro-organisms benefit agricultural crops in terms of productivity and general plant health through biogeochemical recycling of nutrients (Smith and Read 1996; Stacey *et al.*, 1992). Extensive studies also have demonstrated the importance of symbiotic relationships between the host plant and the microflora inhabiting the rhizosphere (Glick 1995; van Peer and Schippers 1989; Kloepper *et al.*, 1988). However the physiological significance of the saprophytic phylloplane microbial life remains poorly understood. On some of the tropical rain forest leaves, microbial life forms a 50 µm thick layer (Holland and Polacco 1994). In addition, of all the phylloplane microbes, the pathogenic or partially parasitic micro-organisms have received more attention. This study was thus designed to determine if there are any positive physiological effects confered by the presence of the non-pathogenic phylloplane PPFM cobionts on rice growth.

In general, most of the microbes inhabiting the phylloplane surfaces of green plants are saprophytic, feeding primarily on materials leached from the leaf (Tukey 1971). A study conducted by Basile *et al.*, (1969) demonstrated that some phylloplane pink-pigmented facultative methylotrophic bacteria (PPFM) strains, isolated from surfaces of a leafy liverwort, *Scapania nemorosa*, benefited the host plant by stimulating its growth. Subsequent studies also demonstrated that bacteria with similar features (PPFM) could be selectively isolated from most, if not all, of the field collected green plants including monocots, dicots (angiosperms), gymnosperms, lower vascular and non vascular plants (Corpe 1985; Corpe and Rheem 1989).

The PPFM were found to be a significant part of the microbes living on the phylloplane, particularly the young leaves of the plants (Basile *et al.*, 1969; Corpe and Basile 1982; Corpe 1985). The basis for the reported microbial stimulation of leafy liverworts growth is not known, but the findings by Nishio, Yano and Kamikubo (1975) and Toraya *et al.*, (1975) indicated that PPFM isolates produce vitamin B-12, which directly or indirectly affects plants growth and development. Other reports indicate that some phylloplane bacteria strains produce auxins and cytokinins (Klincare *et al.*, 1971; Rodrigues *et al.*, 1972).

So far there are no reports on any study of the effects of different strains of *Methylobacterium* sp. on growth and development of an economically important food crop such as rice. However, recent experiments carried out under controlled conditions showed that some PPFM strains can stimulate growth of *Streptocarpus proxis* a vascular plant in tissue culture (Corpe and Basile 1982).

The research reported in this chapter (one) was therefore designed to evaluate the effects of specific strains of *Methylobacterium* sp. on growth and development of rice. The study consisted of two parts; an evaluation of the quantitative and qualitative effects of isolated pink-pigmented facultative methylotrophic bacteria, a group of saprophytic phylloplane bacteria on the growth and development of rice tissue culture and young seedlings *in vitro* and Second an evaluation of the feasibility of activating this rice-PPFM interaction to serve as an alternate approach towards the improvement of rice crop productivity.

MATERIALS AND METHODS

Plant material, growth media and callus induction

Mature seeds of rice *Oryza sativa* L. *Japonica* cv. CR76 and *indica* cv. A301, stored in a dry and aerated place at room temperature, were used as the initial explant materials for the experiments. The seeds were kindly provided by Y.L. Wu & D. S. Mikkelson Department of Agronomy and Range Science, University of California, Davis. The mature seeds were dehusked and surface sterilized for 1 minute in 95% ethanol. Further sterilization was effected by soaking and constantly shaking the seeds for 30 minutes in 100 ml of 35% of sodium hypochlorite (appropriately diluted commercial bleach), with 0.02% tween 20, a surfactant as recommended (Nabor *et al.*, 1983; Rance *et al.*,1994). The surfaced sterilized seeds were washed by rinsing five times in sterile distilled water.

Callus was initiated by incubating the seeds in the dark at 27°C on modified Murashige and Skoog [(MS) 1962] basal media [The salt stock solutions prepared as 100x were added as 10 ml per 1000 ml of medium . The stock concentrations per liter were: NH_4NO_3, 165.0 g; KNO_3, 190.0 g; $MgSO_4.7H_2O$, 37.0 g; $MnSO_4.H_2O$, 37.0 g; $ZnSO_4.7H_2O$, 0.86 g; $CuSO_4.5H_2O$, 0.0025 g; $CaCl_2.2H_2O$, 44.0 g; KI,0.083 g; $CoCl_2.6H_2O$, 0.0025 g; KH_2PO_4, 17.0 g; H_3BO_3, 0.620 g; $NaMoO_4.H_2O$, 0.025 g; $FeSO_4.7H_2O$, 2.784 g; NaEDTA, 3.724 g)]. The media was supplemented with thiamine (0.4 mg/l), vitamin stock 10.0 ml (prepared as 5 mg nicotinic acid and 5 mg pyridoxine per 100 ml water) myo-inositol (0.1 g/l), sucrose (30 g/l). 2,4-Dichlorophenoxyacetic acid (2,4-D), a growth regulating hormone was added at a final concentration of 3.5 mg/l. The pH was adjusted to 5.7. The medium was solidified in 1.2% agar (w/v) and autoclaved for 20 minutes at 121.1°C, 17 psi. The medium was then distributed at a rate of 25 ml per sterile culture jar. The initial callus was subcultured regularly at interval of 4 - 5 weeks on Gamborg's medium

(B5) containing 2.0 mg/l 2,4-D and 0.1 mg/l BAP. The calli generated, was used as the explant material during the subsequent experiments. The calli was used in testing, selection and isolation of ***Methylobacterium*** sp. strains that stimulate growth and development of rice *in vitro*, following the protocol described by Basile *et al.*, (1969).

Isolation of methylotrophic bacteria strains

The methylotrophs were isolated from various species of green plants, collected from the New York metropolitan area and from Harriman State Park, Rockland County, Westchester, New York. The plant materials included liverworts (Bryophyta), clover (Leguminoseae/Fabaceae) and several specimens from the grass family (Poaceae). All the materials were handled aseptically following described aseptic techniques described by Corpe and Rheem (1989). Three replicates for each plant species were prepared. The methylotroph strain designated Q4 was isolated from rice tissue culture in our laboratory, where it was originally a contaminant. The Q4 strain was selected on the basis of its ability to stimulate the growth (cell proliferation/callus induction) of the contaminated rice tissue (calli) and also its ability to stimulate (induce) root formation (tissue differentiation/organogenesis). Some of the methylotrophic strains were isolated from the young leaves of the plants using surface impression technique on a modified buffered ammonia salts solution supplemented with methanol (MeAmS) (Corpe and Basile 1982). The leaves (whole or segments) were rinsed using sterile water and pressed firmly onto the surface of solidified media in petri plates and incubated at 27°C. The composition of the modified MeAmS-agar per liter was 40 ml of Na_2HPO_4 +KH_2PO_4 (1M; pH7.0); $(NH_4)_2SO_4$, 1.0 g; $MgSO_4$, 0.3 g; $CaCl_2.2H_2O$, 0.05 g; $(NH_4)_6MoO_{24}.4H_2O$, 0.2 mg and $FeSO_4$. $7H_2O$, 1.5 mg. 10 ml of 1000x Hutners "metal 49" micronutrients solution (*Basile 1978 micronutrients*

II). The pH of the media was adjusted to 7.0 and Difco Bacto agar was added at a concentration of 1.5 g/100 ml of media (Corpe 1985). The prepared solidified agar-basal salt media was heat sterilized in an autoclave at 121°C, and 17 psi for 20 minutes. Filter sterilized aqueous methanol (Sigma Chemical Co., St Louis, MO,USA), was added to the basal media as a 20% (v/v) solution to give a final concentration of 1.0% (v/v). Sterile cycloheximide an antibiotic (Sigma Chemical Co., St Louis, MO, USA) prepared as 0.5 mg/50 ml, was added aseptically to the cooled media to give a final concentration of 10.0 µg/ml, as described by Corpe *et al* 1985. The antibiotic inhibits the growth and spreading of fungi over the agar surface, which is a major component of the heterotrophic micro-flora on green leaves. The prepared media contained methanol as the sole carbon source. Consequently methylotrophs were the only known microbes that could grow on this media. Bacterial colonies growing on this media were selected, isolated and tested to determine their ability to stimulate growth and development of *japonica* cv. CR76 and *indica* cv. A301 calli and seedlings *in vitro*.

Investigations on the effects of methylotrophic bacteria strains on callus proliferation

Japonica cv. CR76 and *indica* cv. A301 callus pieces (1.5 cm^3) obtained from subcultured calli were transfered onto modified Murashige and Skoog media (1962) devoid of all exogenous phytohormones. To determine their growth stimulative effects, the selected methylotrophic bacteria isolates were inoculated onto the media adjacent to the axenic *Japonica* cv. CR76 and *Indica* cv. A301 calli, as described by Basile *et al.*, 1969; Corpe and Basile 1982; Basile *et al.*, 1985. Among the strains of methylotrophic bacteria were several PPFM isolates. 40 replicates of the two rice cultivar *Japonica* cv. CR76 and *Indica* cv. A301 were established; 20 control sets were uninoculated with the methylotrophs. The relative increase in calli size over a period of 60 days was used as an indicator of the growth stimulative properties of the PPFM and the other methylotrophic bacteria isolates.

Investigations on the effects of methylotrophic bacteria strains on plantlet regeneration

Surface sterilized mature seed embryo of *japonica* cv. CR76 and *indica* cv. A301 were used in initiate callus in a modified MS medium. The seeds contain meristematic cells competent to express totipotency, an essential property in callus induction and regeneration. The embryogenic callus was isolated and used in the plantlet regeneration studies. Embryogenic cell callus is compact, nodulated and milky white to yellow in texture under light microscope (Bhaskaran and Smith 1988; 1990; Smith 1992; Heyser and Nabor 1982). The non-embryogenic callus is loose, crystalline and yellow to brown in color. The non-embryogenic callus has low shoot initiation frequency (Abe and Futsuhara 1985; Ammirato P.V. 1983; Heyser *et al.*,1983). During the plantlet regeneration experiment, the embryogenic callus was partially desiccated to enhance regeneration (Rance *et al.*,1994). The calli was transfered onto MS plantlet regeneration media in 20.0 cm glass test tubes and capped. The regeneration media contained 0.5 mg/l naphthalene acetic acid (NAA) and 5 mg/l 6-benzyl-aminopurine (BA) phytohormones. Half of the calli in test tubes were inoculated with one of the isolated strains PPFM phenotypes designated Q4 and Q5. All of the calli was incubated in darkness in a growth chamber at 22°C for 10 days. The calli were then incubated on tissue culture room shelves at 21°C ± 0.5 day and night temperature and 16 hour/ 8 hour, day/night photoperiod, following the protocol described by Rance et al 1994. The day 4000 lux at the surface of the growth media, was provided by 40W wide spectrum fluorescent lamps (Sylvania

Cool White 40W F48T12/CW. USA). The relative humidity inside the test tubes was 100%. The relative number of regenerated plants was determined in both the control and the experimental group. The data of regenerated plantlets was analyzed statistically and a comparison was drawn to determine the affect of the Q4 and Q5 PPFM strains on the efficiency of rice plantlet regeneration.

Experiments on the comparative effects of several methylotrophic strains on the growth of seedlings *in vitro*

Several strains of methylotrophic bacteria were used in a comparative investigation of the effects of the PPFM and other *Methylobacterium* sp. on rice growth and development *in vitro*. The strains, isolated from leaves of various plants (experiment 2), were placed into four categories based on their relative colony pigmentation namely, QP (pink), QW (white), QY (yellow), and QPP (pale pink) and physical/morphological properties of cells of each colony. Other pink-pigmented methylotrophic bacteria strains tested in this study were designated Q4 and Q5. Each of the bacteria free rice seedlings was established in a 5.0 ml of modified basal salts MS media in a 2.5 cm x 20.0 cm long glass test tube. The seedlings were germinated from mature seeds that were dehusked and surface sterilized for 1 minute in 95% ethanol. Further sterilization was achieved by soaking and constant agitation of the seeds in 35% of sodium hypochlorite solution for 30 minutes and five times washing in sterile water. After germination all contaminated seedlings were discarded prior to the onset of this study.

The seedlings were established in aseptically prepared modified basal salts MS medium (devoid of vitamins, sucrose and growth regulators) with all additives being autoclaved. The seedlings were grown at constant 24°C day /night temperature and at 16 hour/8 hour, day/night photoperiod. On day 4 after germination, the experimental sets of the seedlings were inoculated with the isolated methylotrophic bacteria strains (QP, QPP, QW, QY, Q4, and Q5), following a randomized block experimental design. The seedlings were harvested 5 days after inoculation of the methylotrophic bacteria strains. The shoot growth (cm) root development (cm), number of leaves and biomass productivity (mg) of the seedlings were determined. To measure the biomass productivity (dry weight) of the harvested seedlings, plant materials were placed in glassine envelopes and brought to dryness in an oven for 48 hours at 70°C. The samples were allowed to equilibrate with the laboratory atmosphere and weighed immediately. The comparative effects of the bacterial strains were determined statistically (Student's *t*-tests using *Bonferroni procedure* for multiple comparisons). In this test, the computed t-statistic was compared to the tabulated t-value of $t\ \alpha/c$ for unpaired one-tailed test, where c is the number of comparisons. The p-value was determined for the samples mean separations.

Experiments on the effects of the isolated Q4 and Q5 strains on growth and development of young seedlings *in vitro*

Bacteria free rice seedlings, germinated from surface sterilized seeds, were used in this experiment. The seedlings were established in a modified MS basal salt media containing 10 ml of 100x Hutners "metal 49"; *Basile micronutrients II* (Basile 1978). The medium was prepared without vitamins, sucrose and growth regulators and was autoclaved for 20 minutes at 121°C and 17 psi.

In a randomized block experimental design (Gomez and Gomez 1984), sets of 5 day old seedlings were inoculated separately with one of the PPFM phenotype designated Q4 and Q5 selected in the comparative study. The seedlings were incubated and grown at constant 24°C day /night temperature and at 16 hour/8 hour, day/night photoperiod. The source of light was 40W wide spectrum fluorescent lamps (Sylvania Cool White 40W F48T12/CW. USA). The seedlings were harvested 9 days after inoculation of the plants with approximately 5000 bacterial cells per seedling with either the Q4 or Q5 strain. The growth and development of the seedling was analyzed based on shoot development (height cm), number of leaves and biomass (mg). The comparative effects of the bacterial strains was determined through analysis of variance (ANOVA), and F-tests and p-values were determined for mean separations. The mean values were reported as X (± SE). All the tests performed were one-tail. Statistical significance was determined at $p = 0.05$ or $p = 0.01$. To confirm the non contamination status of the *in vitro* culture, the media in which the seedlings were growing was inoculated onto Difco nutrient broth and incubated in the dark at 30°C for 72 hours.

Statistical analyses

Each experiment was repeated at least twice or was conducted following a randomized block experimental design as described by Gomez and Gomez (1984). The treatment consisted of at least three replications. The comparative effects of the isolated *Methylobacterium* sp strains (designated Q4, QPP, Q5, QW, QP & QY) on root development (cm), linear shoot growth (cm), relative number of leaf development, biomass productivity (mg) and plantlet regeneration from callus were analyzed. Computer based statistics and graphics packages [Statview ™ 512+ version 1.1 (Abacus Concepts Inc. Calabasas, California) and JMP: statistics made visual ™ version 3.0 (SAS Institute Inc. Cary, North Carolina)] were used in analyzing the data collected. The entire data sets obtained in each experiment were used. The graphics (figures) were generated in Cricket Graph. version 1.3.2 (Cricket Software. Malven, Pennsylvania) and transformed to publication quality in Canvas ™ version 6.07 (Daneba Software Systems Inc. Miami, Florida). F-test in a multi-comparison one factor ANOVA and/or unpaired one-tail Student's *t*-test were used to determine the level of statistical significance of the results and for mean separations.

Photography

Photomacrographs representing the colonies of the isolated *Methylobacterium* sp. strains and the various effects of the isolated Q4 and Q5 PPFM phenotype on callus induction, plantlet regeneration, and development of rice seedlings in vitro and in the greenhouse were taken with either a Polaroid Microcam camera fitted with 337, high speed, panchromatic, medium contrast B&W film, for low light situations and subjects that require filtration for contrast enhancement, or with a Kodak 35 mm color film using an Olympus OM-4 camera, fitted with a Vivitar 28 mm - 135 mm macro-zoom lens.

RESULTS

Qualitative analysis of callus induction

Induction of callus is an important step during the process of regeneration of plantlets from genetically or biotechnologically modified plant tissue. Successful callus induction is also the single most critical step in micropropagation techniques. Repeated application of the recommended 3.5 mg/l 2,4-D concentration (Smith 1992) resulted in slow and poor callus induction from mature seed embryo of *indica* cv. A301 and *japonica* cv CR76. A preliminary experiment, carried out to determine the most effective concentration of 2,4-D growth regulator, demonstrated that a modified MS-agar media supplemented with 5.0 mg/l 2,4-D promoted more rapid callus formation relative to 3.5 mg/l 2,4-D. A 0.5 cm^2 callus developed after 4 weeks incubation in the dark at 23°C.

Further incubation of the calli in the dark at 23°C to week 6 from the onset of the experiment resulted in the formation of distinct embryogenic calli and non-embryogenic calli (Fig. 1.1A). The embryogenic callus is compact and nodulated in texture and milky white to yellow in pigmentation, while the non-embryogenic callus is loose and crystalline in texture and has yellow to brown pigmentation (Fig. 1.1A). The non-embryogenic calli has low shoot initiation frequency (Abe and Futsuhara 1885). The embryogenic calli initiated from the mature rice seed embryo grew rapidly when it was subcultured on solidified Gamborg's (B5) media. The tissue formed from cell proliferation formed numerous embryo-like globular structures (Fig. 1.1B). [These nodulated globular structures formed plantlets (Fig. 1.11C) when the calli were transfered to MS regeneration media supplemented with 0.5 mg/l NAA and 5.0 mg/l BA phytohormones.]

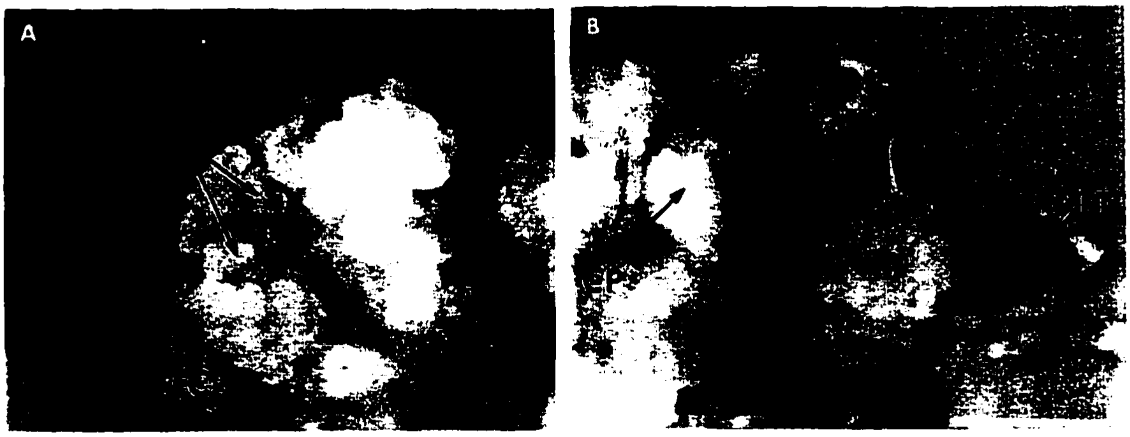

Figure 1.1 Growth and development of callus initiated from mature seed embryo of *indica* rice (cv. A301) cultured in MS medium containing 5.0 mg/l 2,4-D. **A:** Embryo-like callus (EC) derived from seed embryo after 4 weeks growth on solidified MS media containing 5.0 mg/l 2,4-D **B:** Growth and development of the rice calli subcultured on B5 medium containing 2.0 mg/l 2,4-D and 0.1 mg/l BAP. IC-initial callus, RT-developing root, CP-cell proliferation.

[In a separate experiment hundreds of embryos were formed when about a 5.0 mm^3 embryogenic calli were subcultured in 250 ml of MS cell suspension media supplemented with 2.0 mg/l 2,4-D. Apparently this is an excellent technique for micropropagation of genetically transformed rice cells.]

Methylotrophic bacteria strains

The leaf surface of all the green plants collected from the Harriman State Park, New York, and Lehman College area were colonized by numerous methylotrophic bacteria strains. The isolated strains had varying colony pigmentations and were categorized into several groups based on colony pigment phenotypic characteristic. The colonies were classified into the following groups pink (QP), yellow (QY), pale pink (QPP) and white (QW). The colonies also showed typical Methylobacterium strains properties, including the ability to utilize methanol as a carbon source and rod (bacillus) morphometric structure of cells. These bacterial strains were isolated and established on MeAmS media through leaf surface impression technique (Fig. 1.2 A). Some of the strains were further subcultured in pure strands on MeAmS, among these were the PPFM designated Q4 and Q5 (Fig. 1.2 B). The cycloheximide antibiotic, applied at the rate of 10 µm/l successfully inhibited the growth of fungi on the MeAmS media.

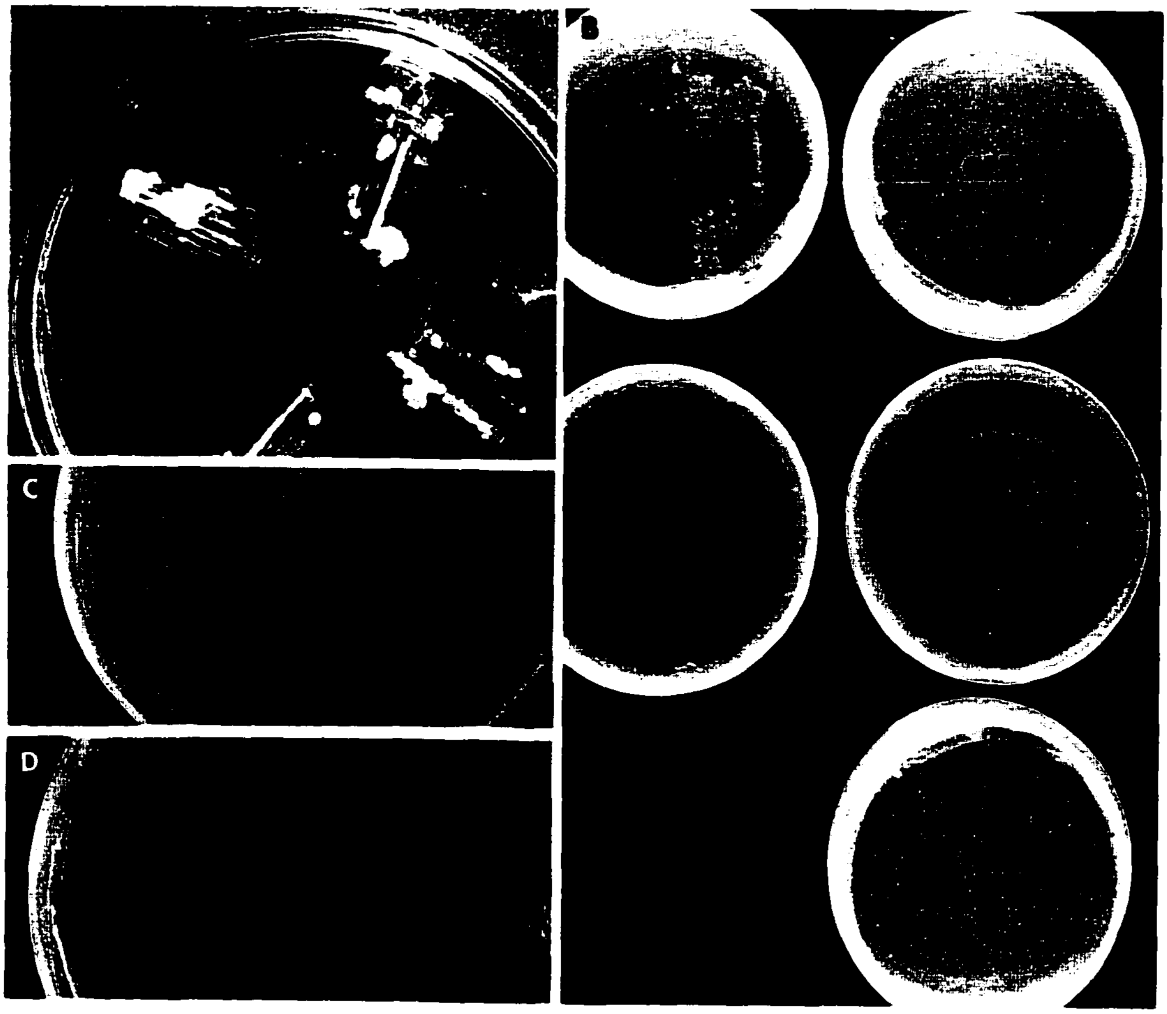

Figure 1.2 Methylotrophic bacteria colonies isolated from leaf segments of grasses (Family Poaceae) by surface impression technique on buffered MeAmS-agar media, supplemented with 10.0 ug/ml cycloheximide. **A:** Pigmented colonies developed after 14 days incubation at 30°C were pink (P), yellow (Y), pale pink (PP) and white (W). **B:** Pure cultures of the isolated ***Methylobacterium*** sp. strains Q4, Q5, QY, and QW grown on MeAmS-agar media. **C & D:** Pure Q4 and Q5 isolates cultured on peptone beef extract-nutrient broth, absence of other heterotrophic colonies indicates contamination free status of the strains.

Comparative effect of several *Methylobacterium* sp strains on seedlings growth and development *in vitro* (preliminary experiments)

a) Japonica; (Oryza sativa L. cv. CR76)

A comparison of means was carried out to determine the positive, neutral or negative effects of each of the isolated strains on growth parameters, stem growth (SG), root development (RT), leaves development (LV), and dry weight (DW). The results showed that some of the strains had a positive growth stimulatory effect (Fig. 1.3).

The Q4 and Q5 strains enhanced growth by greater percent relative to the other isolated strains (Fig. 1.3, & 1.5), on the basis of the measured growth parameters. In 9 day old *japonica* cv. CR76 seedlings, the Q4 strain stimulated biomass productivity significantly by 18.8% [$t = 2.598$, $p = 0.03$ (Student's *t*-test)], 5 days after the inoculation of the cobiont (Fig. 1.3). The mean dry weight (biomass) of the Q4 strain treated plants was 19.00 mg (± 0.58) relative to the control at 16.0 mg (± 1.00). The QP strain stimulated seedlings growth ($t = 2.132$, $p = 0.05$), in unpaired one-tail Student's *t*-test. The biomass of the QP treated seedlings was 19.33 mg (± 1.20), representing a 20.8% increase, over the control. QY strain enhanced growth significantly ($t = 2.096$, $p = 0.04$), by 17.2%. The dry weight of 9 day old, QY treated plants was 18.74 mg (± 0.85), relative to the control 16.0 mg (± 1.00). Even though the inoculation of QW strain resulted in 12.5% increase in biomass productivity, the increment was not significant at $p \leq 0.05$. Analysis of shoot growth showed that two of the isolated strains stimulated stem growth. A significant 11.8% ($t = 2.061$, $p = 0.04$, Student's *t*-test) stimulation in QY treated seedlings (24.52 cm ± 0.94) was recorded over the control. In seedlings inoculated with the QP strain, a significant ($t = 3.247$, $p \leq 0.01$) 11.6% stimulation of shoot (stem) growth was recorded over the control at 21.9 cm ± 0.20 (Fig. 1.3). The effects of QW strain was not significant. However the higher mean value indicated a relatively positive enhancement of stem growth (Fig. 1.3).

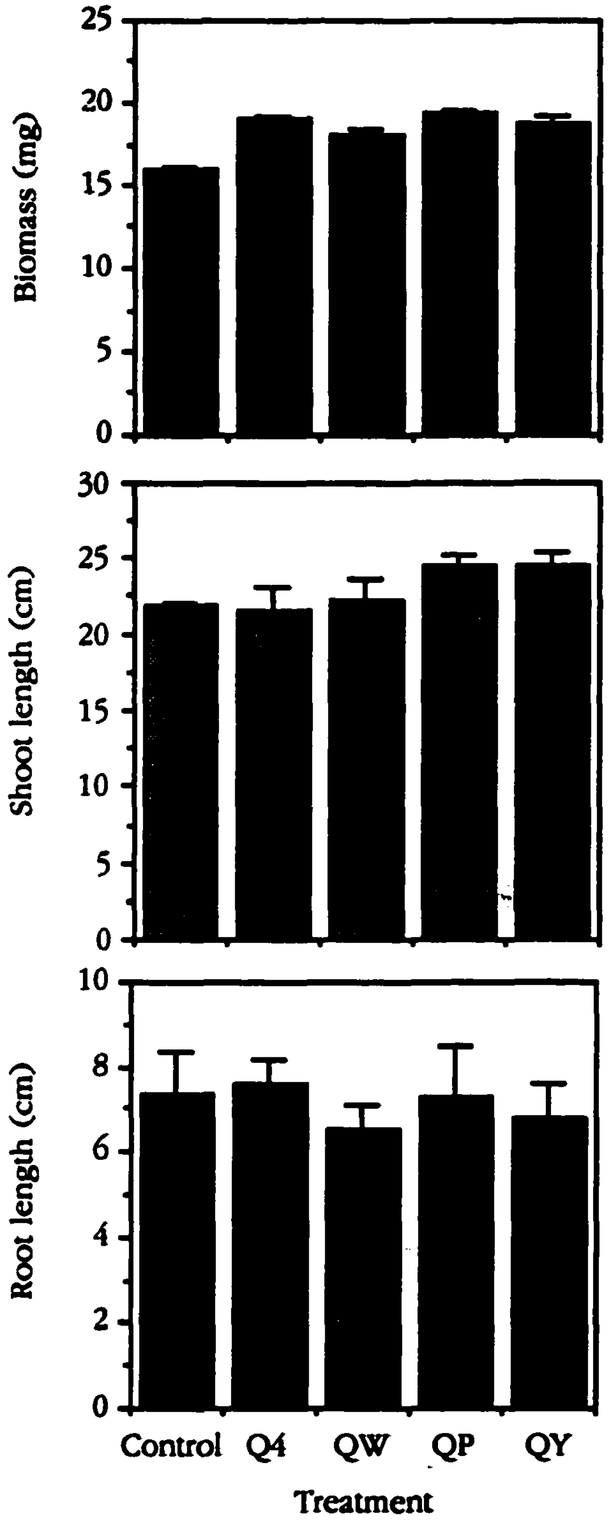

Figure 1.3 Growth and development of 14 day old *japonica* rice seedlings (cv.CR76) *in vitro*, 5 days after the inoculation of *Methylobacterium* sp. strains designated Q4, QW, QP and QY. Error bars represent ± SE of means. All seedlings were grown on MS basal-salts media devoid of sucrose and growth regulators, pH 5.7

Further analysis of the isolated ***Methylobacterium*** sp strains effects showed that Q4 strain relatively stimulated root development by 3.7%. Although the effect was not statistically significant [t = 1.600, p = 0.09 (Student's *t*-test], the average length of the root system was 7.60 cm (± 0.12) compared to the control (7.33 cm ± 0.12). The root system development in seedlings inoculated with QP (7.30 cm ± 0.24) was not different from the control seedlings. QY and QW strains inhibited root development (Fig. 1.3), QY (6.76 cm ± 0.37) by 7.8%. and QW (6.50 cm ± 0.40) by 11.3%. It is clear that while some strains may stimulate growth with regard to one growth parameter, the presence of the cobiont may inhibit other developmental parameters of the host plant. The mechanisms regulating this physiological processes are not understood. However, one possibility is an alteration of phytohormones within the host plants meristems as a result of physical presence of the microbes.

Quantitative analysis of the pooled data of the isolated Q4, QW, QP, and QY designated M-trophs demonstrated a cumulative significant [F = 8.635, p = 0.01 (oneway ANOVA)] stimulatory effect (Fig. 1.4). The mean biomass productivity for the M-trophs was 18.77 mg (± 0.39). It was significantly different from the control where 16.00 mg (± 0.10) of total biomass were recorded. The standard deviation (Diamond signs) and standard error bars (Fig. 1.4A) were not overlapping. This showed that the sample results can be extrapolated to explain growth variations that may occur in plants populations that are inhabited by these microbes in nature. In addition, a logistic regression analysis (Fig. 1.4B) clearly demonstrated that 9 day old seedlings having a biomass of over 18 mg and selected from the samples have a high probability (p = 0.92) of having been inoculated with the isolated M-trophs (*Methylobacterium* spp) strain. This finding has an implication on the cobiotic relationship between host rice plants and their microbial flora. It suggests that in nature the stimulatory effect of some strains may coumouflage inhibitory effect of other strains.

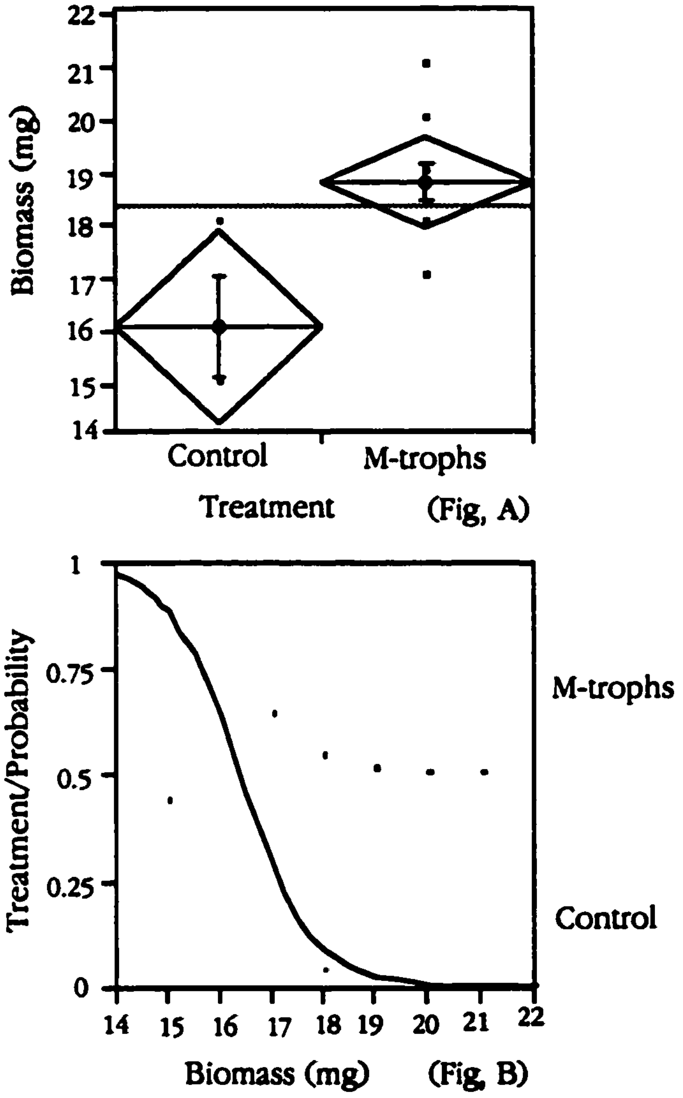

Figure 1.4 Biomass productivity of *japonica* seedlings (cv. CR76) *in vitro* after inoculation of several *Methylobacterium* sp strains (Q4, QP, QW, & QY) **A:** pooled effect of the four strains (m-trophs) **B:** Logistic regression, an analysis of the pooled effect of the methylotrophic bacterial strains, showing that any seedling selected at random and having a biomass of over 18 mg had a probability of 0.958 of having been inoculated with a growth stimulating *Methylobacterium* sp strain. (Control n = 3, M-trophs n = 14). The diamonds indicate projected population standard deviations (SD) and the error bars represent ± SE of means.

The effect of Q4, QW, QP, and QY *Methylobacterium* sp strains on the process of leaf development of 10 day old seedlings was determined at day 6 after inoculation of the cobionts. Some of the strains significantly stimulated leaf development in axenically grown seedlings. The Q4 PPFM enhanced leaf development by 50.0% (significant at p = 0.01, t = 2.666, in unpaired one-tail Student's *t*-test). The number of leaves developed by Q4 treated seedlings 6 days after inoculation was 3.00 relative to the control 2.00, in both cases the SE was ± 0.00. The QW (2.67 ± 0.33) and QY (2.4 ± 0.25) strains increased the rate of leaf development in rice seedlings by 33.5% (significant at p = 0.05, t = 2.000) and 20.0% (not significant, p = 0.13, t = 1.230) respectively, 6 days after the inoculation of the cobionts (Fig. 1.5A). Though the QP strain (2.33 ± 0.33) exhibited a similar pattern, the strain enhanced leaf development by 15.0% relative to the control (2.00 ± 0.00), however the stimulation of development was not significant (t = 1.00, p = 0.186).

The mean values for the rate of leaf development in the pooled Q4, QW, QP & QY (M-trophs) data at day 6 was 2.57 (± 0.14) while the mean value for the control was 2.00. Analysis of the pooled data (M-trophs vs control) showed a significant difference [t = 1.879, p = 0.04, (Student's *t*-test]. On the basis of leaf development curves (Fig. 1.5A: effects of each strain & Fig. 1.5B: pooled data), it is evident that the four isolated *Methylobacterium* sp strains positively affected leaf development in *japonica* cv.CR76. Though the effects of some of the isolated strains (QP & QY) were not significant, the positive patterns were similar to the effects of Q4 and QW. The criteria for the selection of Q4 strain for the consecutive studies was its cumulatively higher rate (%) of stimulating stem growth (shoot growth in cm), leaf development and biomass productivity (mg) in 10 day old seedlings *in vitro* compared to QW, QP & QY in this preliminary study.

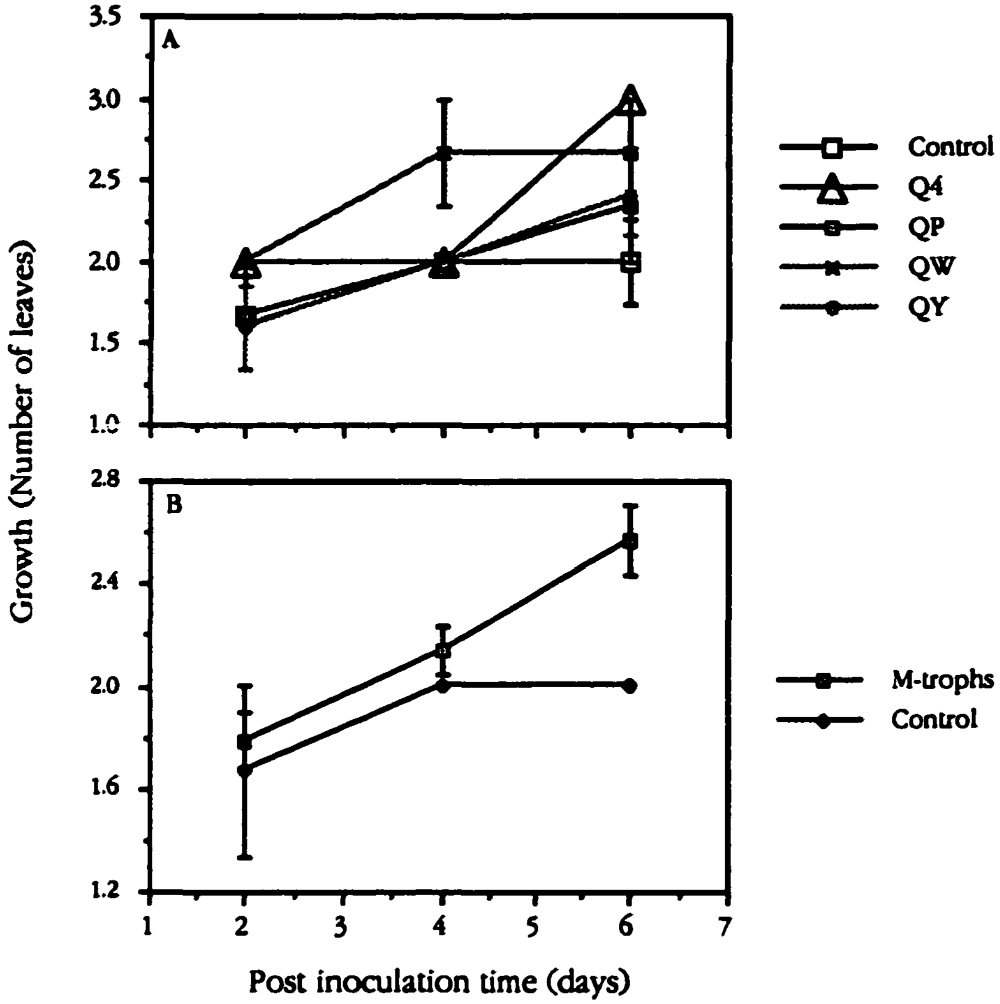

Figure 1.5 Number of leaves in *japonica* seedlings (cv. CR76) as a function of post-inoculation time with several isolated *Methylobacterium* sp strains (Q4, QP, QW, & QY). **A:** the independent effect of each of the strains. **B:** pooled effect of the four strains (M-trophs) is significantly different from the control at P < 0.05 at day 6, (Control n = 3, M-trophs n = 14). Error bars represent ± SE of means.

b) Indica; (Oryza sativa L. cv. A301

A significant increase in measured stem (shoot) growth (FG), root development (RT), leaves development (LV) and biomass productivity [dry weight(DW)], as a result of inoculation of a strain of bacterial cobiont to a host plant in axenic culture, is a reliable indicator of the cobionts potential growth stimulatory effect. In this experiment, the Q5 strain enhanced biomass productivity in *Indica* cv.A301 treated seedlings (14.67 mg ± 0.33) by 16.02% over the control at 12.67 mg (± 1.76), [t = 1.114, p = 0.16 (Student's *t*-test)]. The stimulation of biomass productivity was detected 5 days after the inoculation of the cobiont (Fig. 1.6). No significant increase in biomass accumulation was observed in seedlings inoculated with the QPP strain.

Analysis of stem (shoot) growth (cm) showed a greater positive effect of Q5 PPFM inoculant compared to the QPP strain (Fig. 1.6). The Q5 PPFM strain enhanced shoot growth by 16.3% [t = 0.988, p = 0.18 (Student's *t*-test)] the mean values were 19.3 cm (± 1.42) relative to the 16.6 cm (± 2.24) recorded in the controls. QPP increased shoot growth by 14.5% [t = 0.894, p = 0.20 (Student's *t*-test)] the mean values were 19.08 cm (± 1.65). Though the results were not significant (p = 0.21) the pattern of the stimulation of growth rate was consistent and similar to the the pattern observed in *japonica* cv.CR76 seedlings inoculated with the Q4 strain.

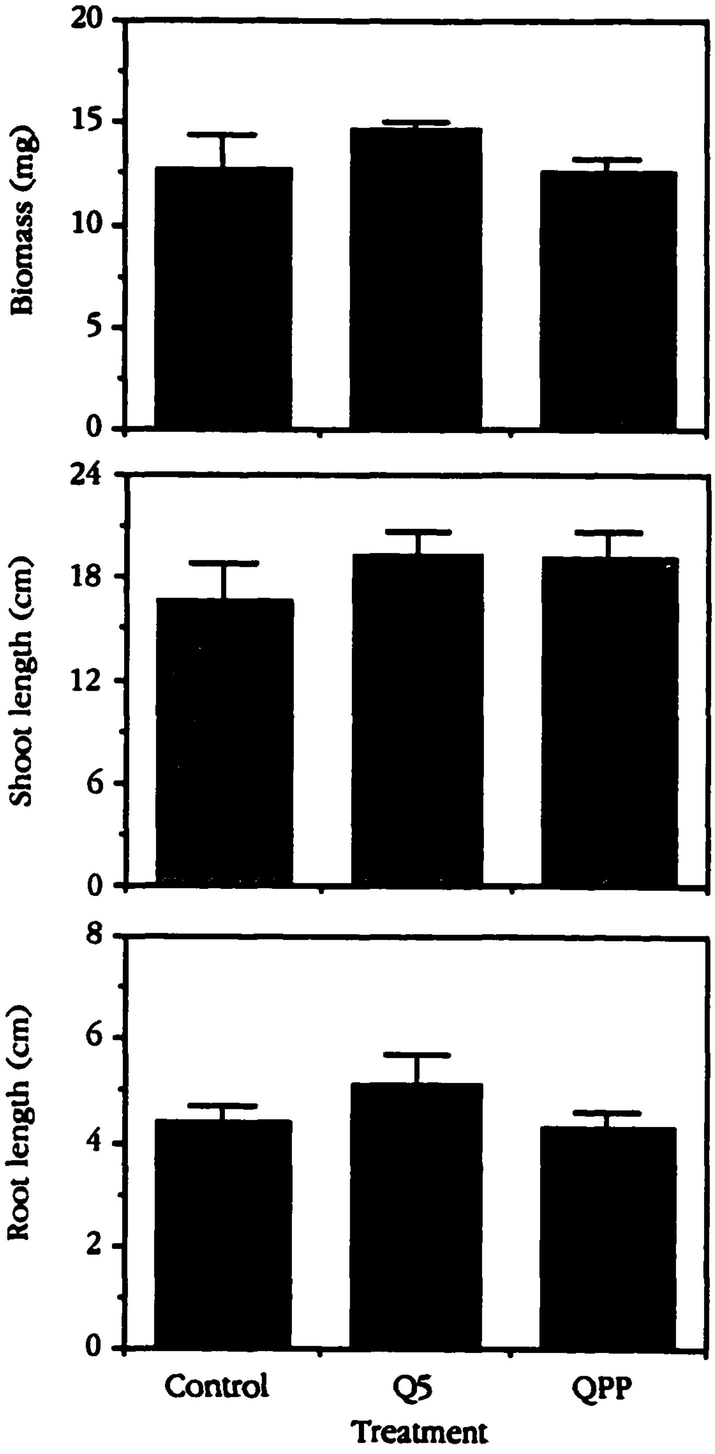

Figure 1.6 Growth of 4 day old *indica* seedlings (cv. A301) *in vitro*, 5 days after the inoculation of *Methylobacterium* sp strains designated Q5 and QPP. Error bars represent ± SE of means. All the seedlings were established on MS basal-salts media, pH 5.7.

Further analysis showed that Q5 strain enhanced root development by 17.5%, however the increase was not statistically significant ($t = 1.112$, $p = 0.16$, Student's *t*-test). The average length of the main root in treated plants was 5.1 cm (± 0.62) compared to the control 4.35 cm (± 0.29). Also the inoculation of QPP strain had no significant effect on root development.

Overall, the growth stimulatory effect of the Q5 PPFM strain on the measured growth parameters (biomass, stem growth and root development) was greater compared to the effect of QPP strain. Even though the stimulation of growth was not significant in some of the measured parameters, the relative greater stimulation of growth was the basis for the selection of Q5 PPFM isolate for further testing. The follow up experiments involved extensive randomized block design experiments with large sample sizes as well as an analysis of the effect of the isolated strains on callus induction, plantlet regeneration from calli and growth and development of seedlings *in vitro*.

Quantitative analysis of the pooled data of the Q5 and QPPP isolated strains (M-trophs) indicated that the significant stimulatory effect of Q5 PPFM strain on measured growth parameters (FG, DW & RT) is camouflaged by the non significant effect of QPP strain on root development and biomass productivity (Fig. 1.7A). The overlapping of standard deviation (Diamond signs) and standard error bars suggests that the effectiveness of a selected bacterial strain can be masked by other microbial strains in a pooled sample data. Evidently this is likely to be the case that exists in nature. However a logistic regression analysis (Fig. 1.7B) showed that seedlings selected at random from the samples and having a biomass of over 16 mg, had a very high probability (0.87) of having been inoculated with the isolated either the Q5 or the QPP strain. This finding suggests that, although pooled data can demonstrate the coumoflaging effects of some strains, the stimulatory effect of each individual strain is likely to be expressed effectively.

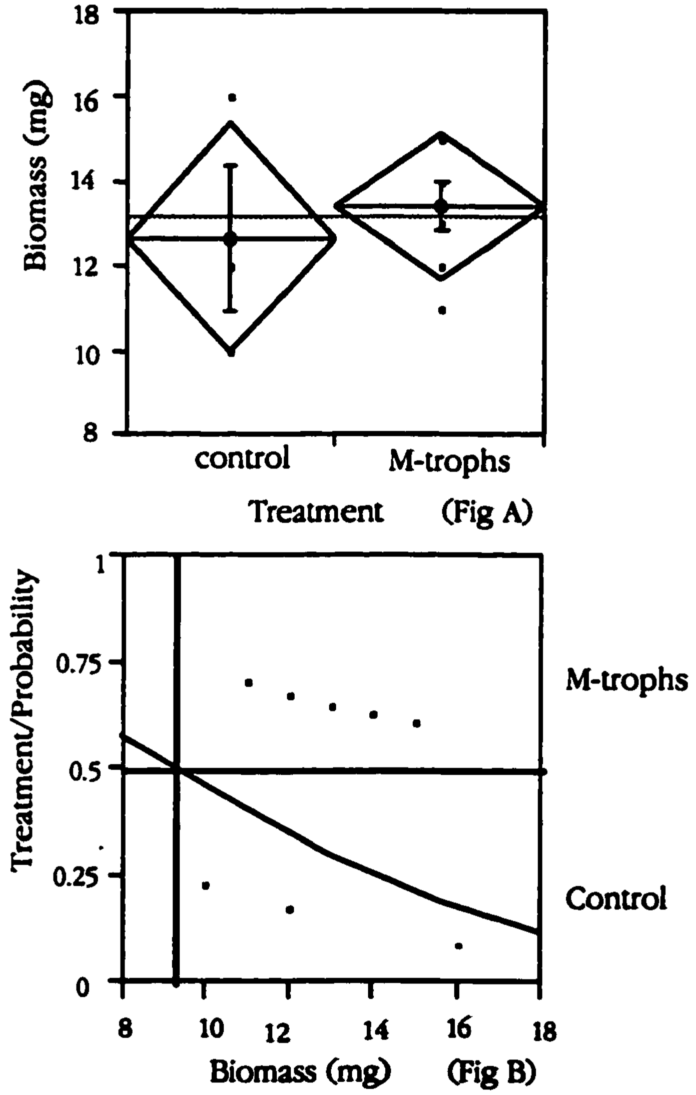

Figure 1.7 Biomass productivity of *indica* seedlings (cv. A301) after inoculation of Q5 and QPP strains. **A:** pooled effect of the Q5 and QPPP strains. The diamonds indicate projected population standard deviations (SD) and the error bars represent ± SE of means. **B:** Logistic regression, an analysis of the pooled effect of the methylotrophic bacterial strains.

Evaluation of the effects of Q5 and QPP strains on leaf development in *indica* cv. A301 at day 6 after inoculation of the cobionts showed that the strains had a stimulatory effect. The Q5 PPFM enhanced leaf development by 16.7% [2.33 ± 0.33, t = 1.00, p = 0.19 (Student's *t*-test)]. The QPP strain increased leaf development by 12.5% [2.25 ± 0.25, t = 0.845, p = 0.22)] (Fig. 1.8A). In spite of the enhanced leaf development, the stimulatory effects were statistically non significant at $p < 0.05$. The mean values for the leaf development of the pooled Q5 & QPP data (Fig. 1.8B) at day 6 was 2.29 (± 0.18) relative to the control (2.00 ± 0.00). On the basis of leaves development curves, (Fig. 1.8A: independent effect of each strain &Fig. 1.8B: pooled data), it is evident that the two isolated PPFM strains positively affected leaf development. Though the results were not significant (t = 0.98, p = 0.17), the criteria for the selection of Q5 for the consecutive studies was its relatively greater capacity (%) of stimulating stem (shoot) growth (height in cm), leaves development, and biomass productivity (mg) in rice seedlings *in vitro* compared to QPP in this preliminary study.

Leaf development is an important factor in plant growth, since leaves have a major role in the absorption of the photosynthetically active radiation (PAR). Leaves convert about 5% of the solar energy reaching the earth,s surface (1.3 kW/m^2) into carbohydrates (Taiz and Zeiger 1991). Therefore, rapid development of leaves would be beneficial to the plant, as it would increase the efficiency of the photosynthetic apparatus. Based on the results obtained in this preliminary study, the Q4 and Q5 PPFM phenotypes enhanced leaf development and the overall plant growth by greater percentages relative to the other isolated strains (Fig. 1.6 & Fig. 1.8). Consequently, they were selected for further testing in a more extensive study involving callus proliferation and plantlet regeneration *in vitro*.

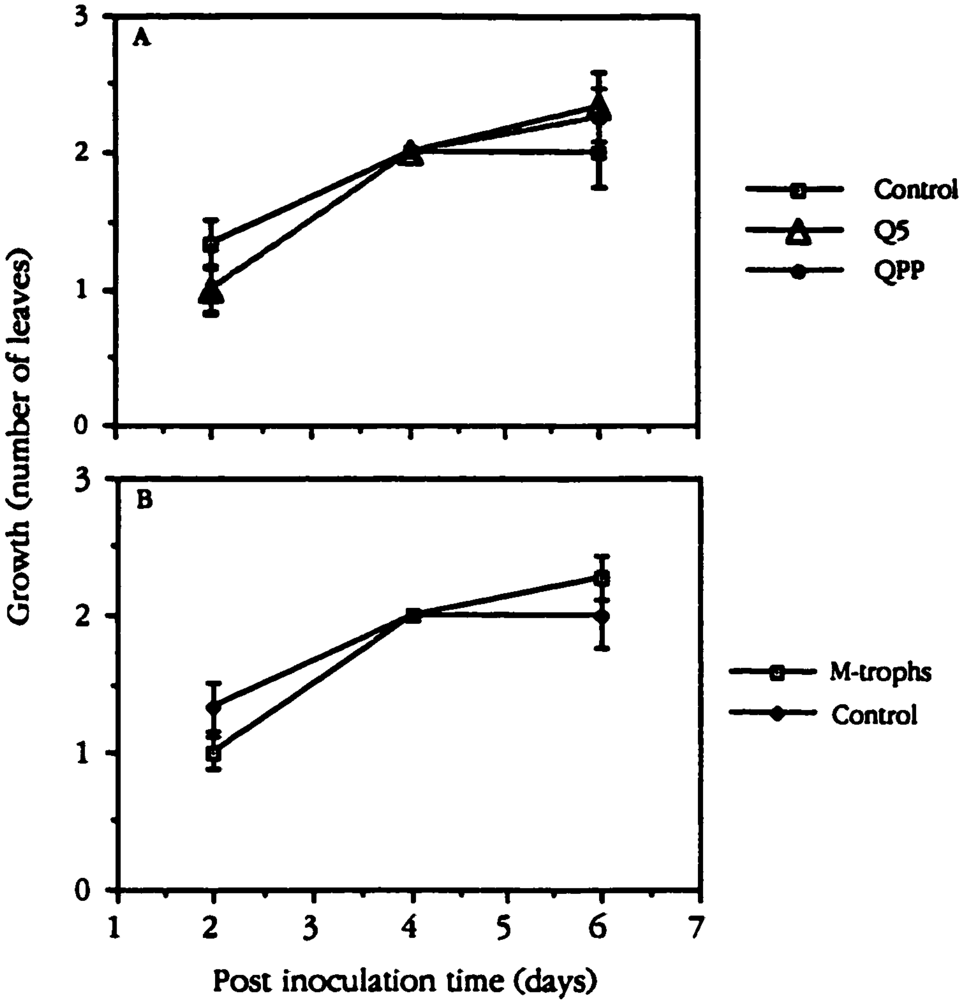

Figure 1.8 Number of leaves in *indica* (cv. A301) as a function of post inoculation time of Q5 and QPP strains. A: the independent effect of each strain, B: pooled effect of the two strains (Control n = 3, M-trophs n = 7). The error bars represent ± SE of means.

Qualitative analysis of the effect of Q4 strain on callus proliferation and organogenesis

The presence of Q4 cobiont on *indica* cv. A301 tissue culture, where it was originally a contaminant, significantly stimulated cell proliferation (callus induction) and organogenesis (root formation). Active cell proliferation and root development from the callus was observed at week 8 after the subculture of initiated callus, presumably during week 8 of the cobiotic association between the

Q4 strain and the calli (Fig. 1.9C & Fig. 1.19D). The callus had been initiated in a Murashige and Skoog (MS) media supplemented with 5.0 mg/l. 2,4-D.

In a separate, randomized experimental design, where the rice calli had been sub-cultured on MS media devoid of growth regulators, the callus inoculated with the Q4 strain exhibited cell proliferation even though the MS media was devoid of phytohormones [Fig. 1.9B(3)]. The results mimicked the cell proliferation observed when rice calli was subcultured on MS media containing the 5.0 mg/l 2,4-D growth regulator [Fig. 1.9A &Fig. 1.9B(2)]. These results suggest that the Q4 PPFM strain synthesized and released a phytohormone-like compound with auxin and/ or cytokinin-like properties. The callus grown on a media devoid of growth regulators and the Q4 strain exhibited minimal cell growth, cell death marked by dark spots, a standard indicator of the accumulation of phenolic compounds was visible at the callus edge [Fig. 1.9B (1)].

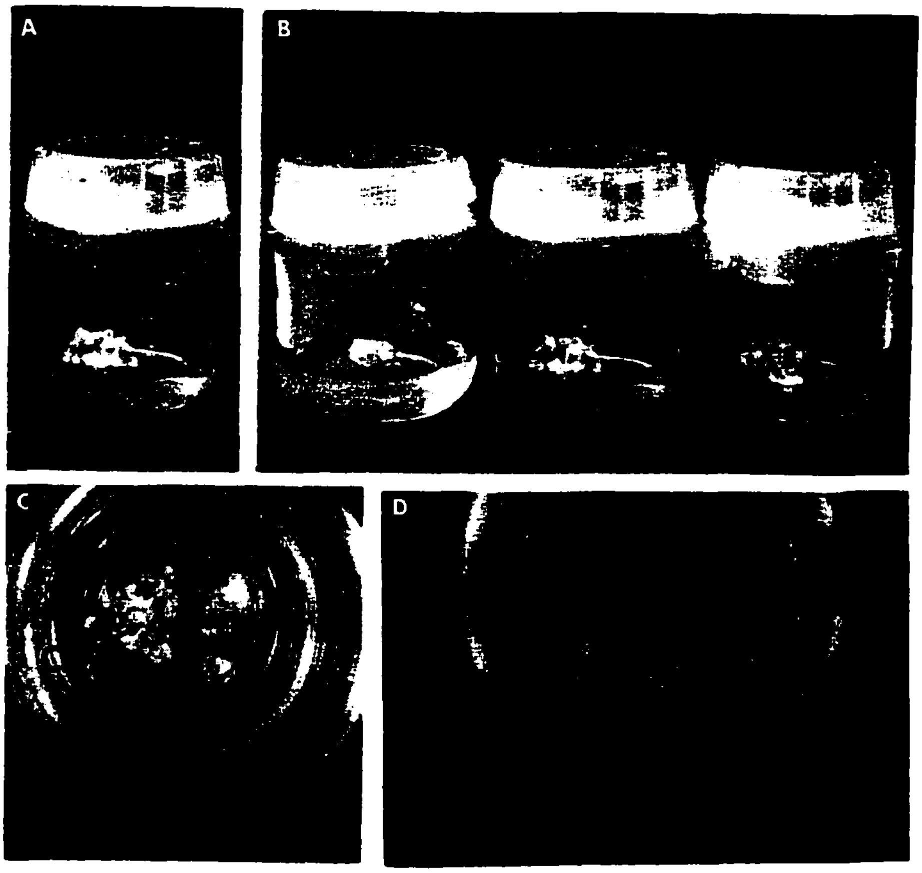

Figure 1.9 The effect of Q4 strain on *indica* (cv. A301) on callus growth and differentiation (organogenesis) **A:** Callus induction from mature rice seed embryo cultured on modified MS basal medium containing 2,4-D 3.5 mg/L. (CP = cell proliferation) **B:** Relative effect of Q4 strain and 2,4-D growth regulator on cell proliferation in subcultured calli (1 = control, lacking Q4 and 2,4-D growth regulator, 2 = treated with 2,4-D 3.5 mg/L, 3 = inoculated with Q4) **C:** The effect of Q4 strain on cell proliferation (CP) in MS basal media devoid of exogenous growth regulators. **D:** Q4 induced cell proliferation (CP) and root formation (RT)

Qualitative and quantitative analysis of the effect of Q4 and Q5 strains on plantlet regeneration *in vitro*

Embryo derived 0.4 - 0.5 cm^2 globular callus formed within 4 weeks when mature rice seeds were cultured on a MS media supplemented with 5.0 mg/l 2,4-D. The *japonica* cv.CR76 formed callus at 25% faster rate relative to *indica* cv A301. At week 6 after the initiation of the callus, 0.8 - 1.0 cm^2 white to pale-yellow embryogenic callus tissue had formed. These calli were partially desiccated to enhance regeneration capacity as recommended by Rance *et al* 1994. Calli initiated in MS supplemented with 5.0 mg /l 2,4-D showed no signs of organogenesis or generalized morphogenetic development. Ten days after the embryogenic callus was transfered onto MS regeneration culture media containing 0.5 mg/l NAA and 5.0 mg/l BA, embryo-like structures were formed (Fig. 1.10B). The relative number of the embryo-like cellular structures formed was greater in control cultures devoid of Q4 or Q5 cobionts. In the calli inoculated with the bacteria strains, the low rate of embryo formation was manifest in the lower number (%) of plantlets regenerated, as demonstrated in the calli inoculated with Q4 strain [*indica* cv A301. (Fig. 1.10E)] and calli inoculated with Q5 [*japonica* cv. CR76. Fig. 1.10D)]. The calli inoculated with the Q4 or Q5 cobionts exhibited continous unorganized growth [cell proliferation (Fig. 1.10C, &Fig. 1.10E)]. Relative quantitative differences between the Q4 and Q5 strains were observed based on the level of inhibition of plantlet regeneration. The Q5 strain inhibited plantlet regeneration in cv. CR76 (Table 1.1) to a greater extend relative to the Q4 strain.

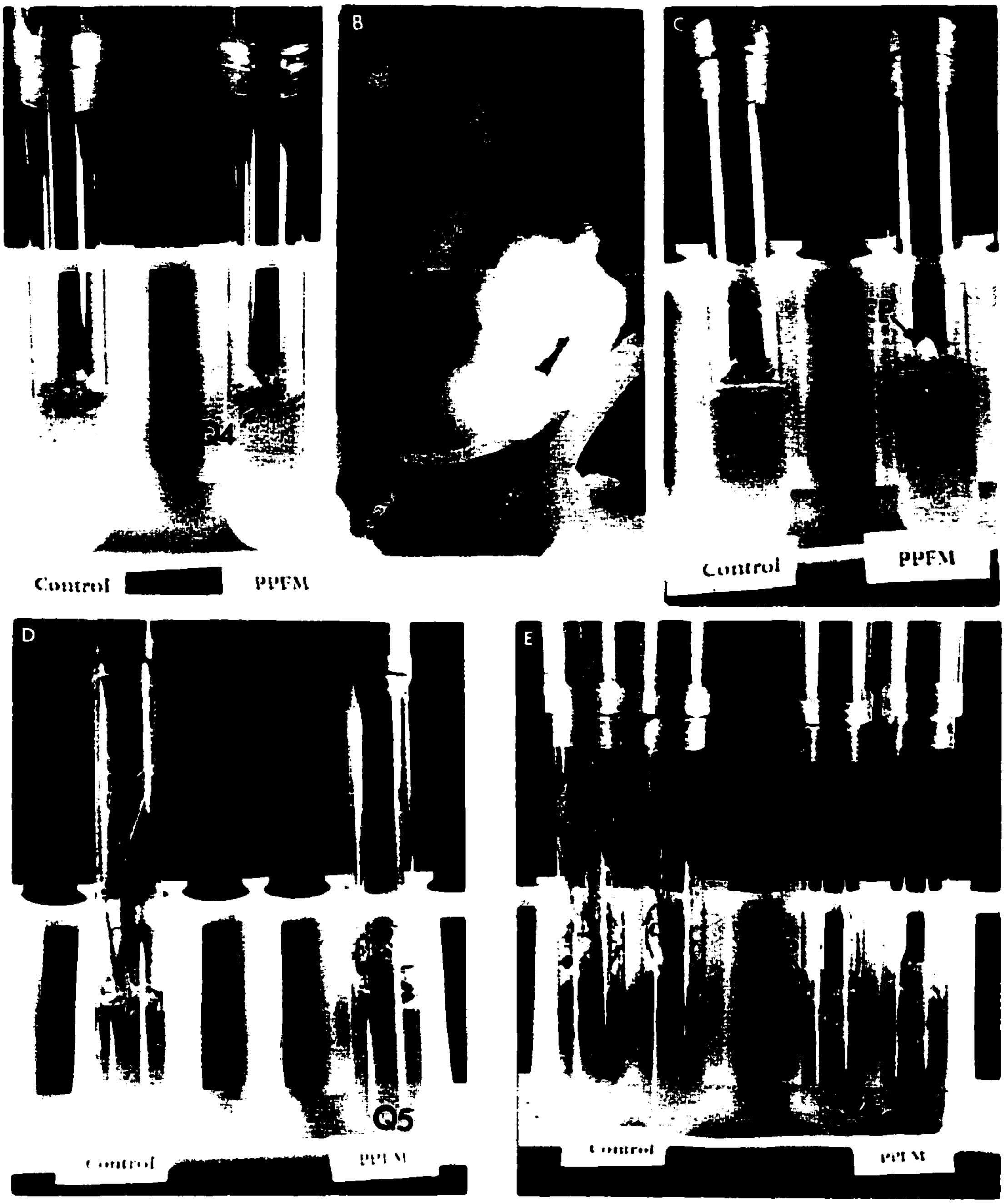

Figure 1.10 Inhibitory effect of Q4 and Q5 strains, on seedling regeneration. **A:** Embryogenic callus inoculated with the Q4 strains (pink- pigmented colonies at the arrow) and subcultured in MS regeneration medium. **B:** Embryo (Em) formed by calli after 10 days growth in regeneration medium **C:** Plantlet regeneration and Q4 induced cell proliferation (CP) after 10 days culture in dark **D:** Regenerated *japonica* plantlets (cv. CR76) in the presence of Q5 cobiont, after 14 day growth period in a 2000 Lux lighted culture room. **E:** Regenerated ***indica*** plantlets (cv. A301) CP indicates Q4 induced cell proliferation.

Analysis of the plantlet regeneration data showed that the Q4 and the Q5 PPFM strains inhibited plantlet regeneration in the two rice cultivars (Table 1.1 & 1.2). In *japonica* cv. CR76, 85 plantlets were regenerated from 8 sets of 5 mm^2 control calli in MS regeneration media devoid of Q4 or Q5. In contrast, 30 plantlets formed in calli inoculated with the Q4 strain and 7 plantlets in calli inoculated with the Q5 strain (Table 1.1). In a similar pattern, 80 plantlets were regenerated from *indica* cv. A301 calli control (calli devoid of Q4 or Q5) and 28 plantlets from the calli inoculated with the Q4 strain (Table 1.1)]. The results show that the Q4 and the Q5 strains have inhibitory effect on plantlet regeneration.

A Chi-square (X^2) test of the association between plantlet regeneration and the presence of Q4 or Q5 cobionts showed that the two strains inhibit plantlet regeneration, while at the same time inducing callus growth (cell proliferation) in rice tissue culture (Table 1.2). In *japonica* cv. CR76 there was a significant relationship between the low rate (inhibition) of plantlet regeneration and the presence of the Q5 strain cobiont. The computed $X^2 = 4.0$ value at $p < 0.025$ was located within the critical region in a one-tail test, as shown on the computation below. The tabulated X^2 value is 3.84 at α .05. Consequently the null hypothesis (H^o) stating that the % of experimental sets expressing morphogenetic development (organogenesis) in form of plantlets/roots is the same in calli devoid of Q5 or inoculated with the Q5 cobiont was rejected at 5% significance level ($p < 0.05$).

Table 1.1 The effect of Q4 and Q5 strains on the plantlet regeneration and callus induction in *indica* cv. A301 and *japonica* cv. CR76, after 7 weeks subculturing period in a solidified MS plantlet regeneration media at pH 5.7, supplemented with 0.5 mg/l NAA and 0.5 mg/l BA.

Set	CR 76			A 301	
	Control	Q4	Q5	Control	Q4
1	plantlet (3) callus (+)	callus (+++)	callus (++$^{\bullet\bullet}$)	Plantlet (41)	plantlet (4) callus (++$^{\bullet}$)
2	plantlet (7) callus (+)	callus (+++)	Callus (+++$^{\bullet\bullet\bullet}$)	plantlet (15)	callus (++)
3	plantlet (14)	callus (++)	callus (++$^{\bullet\bullet}$)	callus (++$^{\bullet\bullet\bullet}$)	plantlet (11) callus (++)
4	callus (++$^{\bullet}$)	plantlet (1) callus (++)	callus (+++$^{\bullet}$)	callus (+$^{\bullet\bullet\bullet}$)	callus (+++$^{\bullet\bullet}$)
5	callus (++$^{\bullet}$)	callus (++)	callus (+++$^{\bullet\bullet}$)	callus (+$^{\bullet\bullet\bullet}$)	callus (++$^{\bullet}$)
6	callus (++) root dev	plantlet (8) callus (++)	callus (+++$^{\bullet\bullet}$)	callus (+)	plantlet (9) callus (++)
7	callus (+) root dev	callus (++)	plantlet (1) callus (+++)	callus (++$^{\bullet\bullet\bullet}$)	plantlet (5) callus (++)
8	plantlets (54)	plantlet (21) callus (++)	plantlet (6) callus (+++)	plantlet (24)	callus (++)
Total plantlets regenerated	85	30	7	80	28

The relative inhibitory effect of Q4 and Q5 *Methylobacterium* sp. strains on plantlet regeneration is indicated by their stimulatory effect on callus induction (+++, heavy; ++, moderate; +, low). The level of activation of chloroplasts is indicated by the qualitative intensity of chlorophyll synthesized by the plant tissue ($^{\bullet\bullet\bullet}$ Heavy; $^{\bullet\bullet}$ moderate; $^{\bullet}$ low). The number in parentheses indicates the number of plantlets regenerated from the initial (1.0 x 0.8 x 0.3) cm callus.

Table 1.2 Two by two contingency table of the frequencies of rice plantlet regeneration in a MS plus 0.5 mg/l NAA and 5.0 mg/l BA culture media in presence or absence of Q4 or Q5 PPFM strains.

(i) *Japonica* cv CR76 + Q5 cobiont

Experimental set observations	Q5 PPFM inoculation	Control	Total
Plantlets/roots	2 (25%)	6 (75%)	8
No Plantlets/roots	6 (75%)	2 (25%)	8
Total	8 (100%)	8(100%)	n = 16

(ii) *Japonica* cv.CR76 + Q4 cobiont

Experimental sets status	Q4 PPFM inoculation	Control	Total
Plantlets/roots	3 (37.5%)	6 (75%)	8
No Plantlets/roots	5 (62.5%)	2 (25%)	8
Total	8 (100%)	8(100%)	n = 16

(iii) *Indica* cv. A301 + Q4 cobiont

Experimental sets status	Q4 PPFM inoculation	Control	Total
Plantlets/roots	4 (50%)	3 (37.5%)	8
No Plantlets/roots	4 (50%)	5 (62.5%)	8
Total	8 (100%)	8(100%)	n = 16

The a, b,c, and d values used in the computation $X^2$of value were derived from this table based on the measured parameters shown below

I. Plantlets &/ or roots frequency
i) in Q5 or Q4 inoculated calli = a
ii) in control calli = b

II. No plantlets &/ or roots frequency
i) in Q5 or Q4 inoculated calli = c
ii) in control calli = d

The computed X^2 value in the three [(i), (ii) & (iii)] experimental sets (Table 1.2) showed that the inoculation of either the Q4 or Q5 strain onto embryogenic callus grown in a regeneration media had an inhibitory effect on plantlet regeneration. The effect was significant ($p \leq 0.05$) in *japonica* cv. CR76 inoculated with the Q5 cobiont. The results recorded in *japonica* cv. CR76 + Q4 cobiont and *Indica* cv. A301 + Q5 cobiont sets exhibited a similar inhibitory pattern, but the X^2 values for the experimental sets [Table 1.2. (ii) & (iii)] were not significant at $p < 0.05$, as indicated by the X^2 values computed

(i) *Japonica* cv. CR76 + Q5 cobiont.

$$X^2 = \frac{n\,(ad - bc)^2}{(a+c)\,(b+d)\,(a+b)\,(c+d)}$$

$$= \frac{16\,[(2)(2) - (6)(6)]^2}{(2+6)\,(6+2)\,(2+6)\,(6+2)}$$

$= 4.0$ The df defined by $(c-1)(r-1) = (2-1)(r-1) = 1$

The tabulated X^2. value at α .05 = 3.84.

(ii) *Japonica* cv. CR76 + Q4 cobiont.

$$X^2 = \frac{n\,(ad - bc)^2}{(a+c)\,(b+d)\,(a+b)\,(c+d)}$$

$$= \frac{16\,[(3)(2) - (6)(5)]^2}{(3+5)\,(6+2)\,(3+6)\,(5+2)}$$

$= 2.48$ (not significant)

The tabulated X^2. value at α .05 = 3.84.

The computed X^2 value for (iii) Indica cv. A301 + Q4 cobiont = 0.25
The df in all the sets was given by (c -1) (r -1) = (2 -1) (r -1) =1, and the
c = number of columns (treatment sets)
r = number of rows (experimental status)

Although data analysis and X^2 test shows that the presence of Q5 cobiont significantly inhibited rice plantlets regeneration at α .05 in *japonica* cv. CR76, the computed Phi (ϕ) coefficient, which determines the level of the strength of association between variables, indicated that there was a strong association at the α .05 significance level.

$$\text{Phi } (\phi) = \frac{ad - bc}{\sqrt{(a+c)\,(b+d)\,(a+b)\,(c+d)}}$$

$$= \frac{[(2)(2) - (6)(6)]}{\sqrt{(2+6)\,(6+2)\,(2+6)\,(6+2)}}$$

$$= 0.5$$

The Phi coefficient has a 0 -1 range, the values close to 0 indicate weak or zero association while values close to 1 indicate strong association.

In the *japonica* cv CR76 - Q5 interaction, the computed phi coefficient (0.5), which is a measure of the strength of association between the two variables (cobiont Q5 & plantlets regeneration capacity) showed that there is a strong association between the presence of the Q5 strain and inhibition of rice plantlets regeneration from tissue culture (callus) This phenomenon strongly suggests that the Q5 strain or compounds synthesized and released by the Q5 strain altered the phytohormones balance within the regeneration media in the callus was subcultured in. This phenomenon may account for the observed Q4 or Q5 induced stimulation of growth and development of intact seedlings *in vitro*. It is noteworthy that these rarely detectable microbes have the potential of affecting the process of experimental or commercial micropropagation of plants through inhibition of regeneration.

Although regenerated cereal plantlets frequently do not successfully survive transfer to soil (Lazzeri and Lorz 1990), about 65% of the plantlets regenerated in this study were successfully established in soil in growth chamber at 100% humidity and constant 23 °C day and night temperature regime. However, the number of successful plants decreased to 33 % when the rice plants (Fig. 1.11D & Fig. 1.11E), were transfered to simulated natural environmental conditions in a greenhouse. The plants were similar in morphology to seed-grown rice plants. Fourteen weeks after being transplanted in pots in greenhouse, all the *indica* cv. A301 regenerated plants (control and initially inoculated with the Q4) grown in the greenhouse (Fig. 1.11E) were found to be fertile.

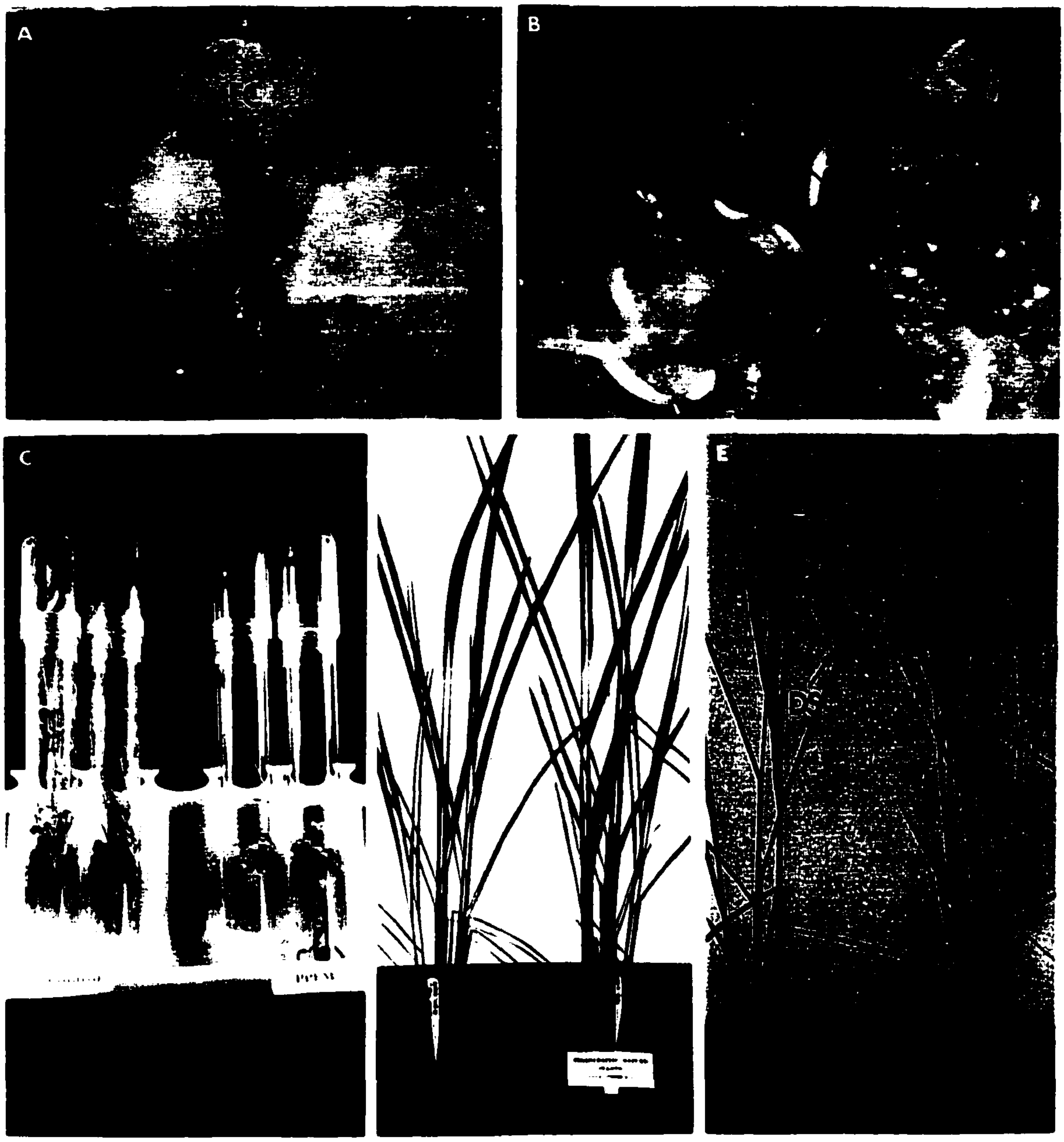

Figure 1.11 Embryogenic callus induction, plantlet regeneration and establishment of mature fertile *indica* (cv. A301) plants **A:** Compact, white to pale yellow globular embryogenic callus (EC) generated after 4 weeks subculturing of seed embryo derived calli in B5 media. **B:** Leaf primordia (LV) at early stages of plantlet regeneration, at day 14 **C:** Plantlet regeneration from the embryogenic callus in MS media and inoculated with Q4 strain. **D:** Regenerated mature plants grown in the greenhouse. **E:** Mature fertile regenerated plants at flowering and seed development stage (DS).

Physiological effects of Q4 and Q5 strains on rice seedlings growth *in vitro*

Axenic seedlings were established through germination of surface sterilized seeds in agar-MS basal salts media. The contamination free status of the seedlings and media was confirmed by the absence of microbial growth after streaking of the MS media on which the seedlings were established onto nutrient broth (peptone beef-extract) followed by 72 hrs incubation at 30°C. Data analysis showed that Q4 significantly stimulated growth and development of 5 day old *japonica* cv. CR 76 seedlings 9 days after inoculation (Table 1.3). The inoculation of Q4 onto *japonica* cv. CR76 seedlings resulted in a 20. 3% increase in biomass productivity [significant at p = 0.01, F = 7.688, in one factor ANOVA] over the control. A significant [F = 5.636, p = 0.02 (in one factor AOVA)], 16.5% stimulation of leaf development was also recorded (Table. 1.3). Although the stimulation of shoot (stem) growth was not significant (F = 2.27, p = 0.14), a 6.1% enhancement of stem growth (height in cm) was recorded in the Q4 treated seedlings over the control (Table 1.3). The relative stimulation of stem growth in the 10 day old seedlings was higher 5 days after inoculation of the Q4 cobiont. A similar pattern of stimulation of growth and development by Q5 was observed in *Indica* cv. A301 (Fig. 1.12). Evidently, the observed stimulation of growth and development was directly or indirectly mediated by the Q4 or Q5 strains.

Table 1.3 The effect of Q4 PPFM cobiont on several growth parameters of 14 day old *japonica* seedlings (cv. CR76) *in vitro*, 9 days after inoculations.

Development property	Control (Mean±SE)	Q4 (PPFM) (Mean±SE)	F-test	pvalue
Biomass	11.51±0.49	13.85±0.71*	7.69	0.010
Stem growth	28.72±0.81	30.46±0.82	2.27	0.142
Leaves number	2.06± 0.06	2.40±0.13*	5.63	0.024

* denotes value that is significantly different from the control at $p \leq .05$

The stem growth represents shoot height in cm and biomass was measured in mg. The n values were control = 16 and Q4 = 15. The df for the comparison and separation of means = 29.

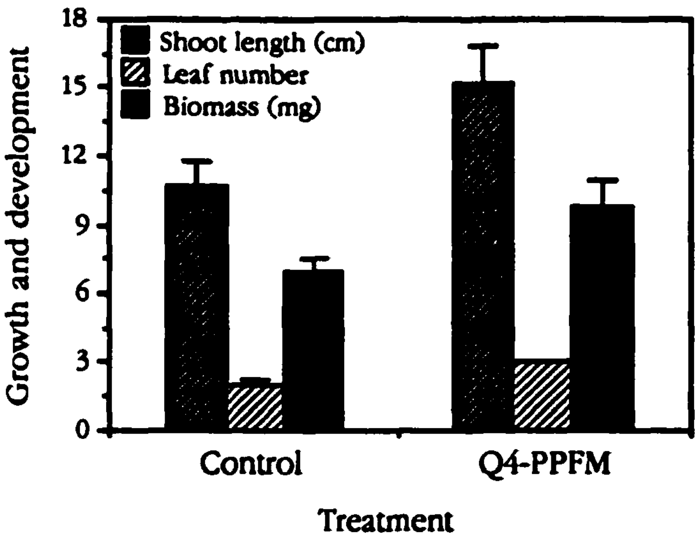

Figure 1.12 Growth and development of 14 day old *indica* seedlings (cv. A301) *in vitro*, 9 days after inoculation of Q5 strain. The shoot (stem) growth indicates seedlings height in cm. The n values were control = 5 and Q5 (PPFM) = 5. The df for the comparison and separation of means in an unpaired one-tail *t*-test = 9. Significant differences were observed on comparison and separation of the control and treatment sample means, p 0.05 (stem growth in cm), $p \leq 0.01$ (leaves number) and $p \leq 0.05$ (biomass in mg). Error bars represent ± SE of means.

Evaluation of biomass productivity, which is a reliable indicator of plant growth and response of plants to changes in nutritional, biotic and abiotic environmental conditions, showed that Q5 strain had stimulatory effect on *indica* cv. A301 seedlings growth. Significant increases in the measured growth and development based on dry weight (biomass), leaf development and shoot (stem) growth were observed in 14 day old seedlings 9 days after inoculation of the Q5. The treatment of the *indica* cv. A301 with Q5 resulted in a 41.2% increase in biomass productivity over the control (Fig. 1.12). The Q5 strain also enhanced leaf development significantly by 36.4 % [F = 16.00, p = 0.003, in one factor ANOVA]. In *Indica* cv. A301, the 42.6% increase in shoot growth over the control at 10.66 cm was significant at p = 0.04 [F = 2.375, one factor ANOVA (Fig. 1.12)]. However, in *japonica* cv. CR76 seedlings, the Q5 modulated stem (shoot) growth was not significant [F = 2.277, p = 0. 14, (Table. 3]. The results reported here using large sample sizes confirmed the preliminary observations and results obtained during the comparative evaluation of growth stimulatory effect of five (QP, Q4, QPP, QW, & QY) isolated *Methylobacterium* spp strains. The two strains (Q4 and Q5) were selected during the preliminary study for further testing out of the total of the five strains primarily due to their relatively superior (not necessarily significant at p ≤ 0.05) growth and development stimulatory effect.

DISCUSSION

Successful induction of callus is one of the critical stages in micropropagation of rice, particularly from genetically transformed cells. In most of the cultivars, rice regeneration of plantlets *in vitro* from wildtype cells or biotechnologically modified cells is at present slow and inefficient and therefore it remains to be one of the major obstacles in the improvement of nutritional value of rice grains.

The results and data presented in this study have shown that a modified MS-agar media supplemented with a 5.0 mg/l 2,4-D phytohormone induced rapid callus formation from mature seed embryo of *indica* cv. A301 and *japonica* cv. CR76 when compared to the recommended 1.0 mg/l - 3.5 mg/l 2,4-D concentration range (Smith 1992). The 5.0 mg/l, 2,4-D concentration was selected after a preliminary test. The growth regulators matrix test was carried out due to poor callus induction results following the repeated application of the recommended 3.5 mg/l 2,4-D (Smith 1992). Qualitative evidence showed that relatively higher concentration of 2,4-D (5.0 mg/l) is more effective in initiating callus particularly in *indica* cv. A301, which is reported to have poor calli induction vigor (Heyser *et al.*, 1983, Baskaran and Smith 1990). The incubation of the calli in the dark at 23°C for a period of 6 weeks resulted in the formation of embryogenic calli and non-embryogenic calli (Fig. 1.1A). The embryogenic callus had a characteristic compact-nodulated texture and was milky white to yellow in pigmentation, while the non-embryogenic callus was identified on the basis of its loose-crystalline texture and yellow to brown pigmentation (Fig. 1.1A). The non-embryogenic calli has low shoot initiation frequency (Abe and Futsuhara 1885). The embryogenic calli initiated from the mature rice seed embryo grew rapidly when it was subcultured on solidified Gamborg's (B5) media. The tissue formed from cell proliferation formed numerous embryo-like globular structures

(Fig. 1.1B). The formation of plantlets (Fig. 1.11C) when the calli were transfered to MS regeneration media supplemented with 0.5 mg/l NAA and 5.0 mg/l BA phytohormones confirmed the totipotency of the induced calli.

This study has also revealed that exposure of rice calli on either solidified media or cell suspension to natural or artificial light leads to inhibition of cell growth and proliferation. The calli exposed to light turned brown due to the accumulation of phenolic compounds. Overall this led to decreased formation of embryo. Based on these findings, it is evident that successful callus induction and continous production of embryogenic callus of either *japonica* cv. CR76 or *indica* cv. A301 requires consistent subculturing in darkness. This technique proved to be fast and reliable for the clonal multiplication of totipotent cell lines of *indica* cv. A301. The technique might be applied to other important *indica* cultivars, which are reported to have poor regeneration frequency (Rance *et al.*, 1994). Also evident is the importance of the early visual selection of regenerable totipotent callus.

The experiments on the isolation of methylotrophic bacteria strains showed that the phylloplane of all the plants collected from the Harriman State Park, Rockland County, New York were colonized or were in a mutualistic relationship with various methylotrophic bacteria strains. The isolated strains had distinct colony pigmentation (Fig. 1.2A), including pink (QP), yellow (QY), pale pink (QPP), and white (QW). Although the association of the *Methylobacterium* sp. with plant families has been studied by several authors (Basile 1969; Austin, Goodfellow and Dicknison 1978; Corpe and Basile 1982; Yoshimura 1982), for the first time we have now reported the isolation of two PPFM designated Q4 and Q5 strains that quantitatively and qualitatively stimulate significantly ($p \leq .05$) the growth and development of rice seedlings *in vitro* (Table 3).

Qualitative data from this study suggests that PPFM are the major microflora on the leaves of young 3-4 day old plants in the grass family (Poaceae). The older leaves ≥ 6 days were colonized by relatively a higher number of the white (QW) strains of the *Methylobacterium* sp. compared to other pigmented strains such as the pink (QP) and yellow (QY) pigmented strains. It is highly likely that the PPFM strains require a high concentration of methanol which declines in older leaves as a result of reduced rate of demethylation of pectins (Corpe 1985, McFeeters and Armstrong 1984). It also likely that the PPFM cannot compete aggressively for limited nutrients since they are slow growers. These two factors may account for the observed decline of PPFM strains in older leaves relative to the other methylotrophic bacterial strains.

The results reported here are consistent with the findings of Austin, Goodfellow and Dickinson (1978). In their study based on numerical taxonomy of phylloplane bacteria isolated from *Lolium perenne*, they showed that pink chromogens (PPFM micro-organisms) constituted a relatively large percent of the isolated phylloplane microflora in young leaves. Therefore appears that a positive correlation exists between the population of the PPFM micro-organisms and the release of methanol from young developing leaves (MacDonald and Fall 1992). Since several studies have demonstrated that PPFM utilize plant derived methanol as the primary source of carbon (Corpe and Basile 1982), the evidence derived from the demonstrated qualitative and quantitative stimulation of growth and development of *japonica* cv. CR76 and *indica* cv. A301 rice seedlings *in vitro* as shown in this study suggests that an extra-and /or intracellular symbiotic or mutualistic association exists between rice seedlings the Q4 and Q5 PPFM cobionts. It is also likely that the association demonstrated by this study also exists in other green plants that are colonized by these strains in their natural environment.

The findings of this study provide further evidence in support of several studies reported in recent years that have clearly demonstrated that the actual magnitude of the plant growth, form and function is a dynamic process that is only partly dependent on the signals transcribed from DNA. These studies have also shown that overall growth parameters such as shoot (stem) growth (FG), root development (RT), leaves development (LV) and biomass productivity [dry weight(DW)], are modulated by abiotic and biotic environmental factors. Among these biotic factors are the phylloplane and the rhizosphere bacterial cobionts, which frequently have beneficial or detrimental effects on growth. The observed relative increase in biomass (dry matter) accumulation and root development following the inoculation of either Q4, QP, QY QPP or Q5 bacterial cobiont onto axenic seedlings revealed the cobionts potential growth stimulatory effect (Fig. 1.3 & Fig. 1.6). However the comparison of mean values showed that the stimulatory effect of some of the strains was not statistically significant at $p \leq .05$ reference point.

Although some of the isolated strains were capable of stimulating seedlings growth as demonstrated by the measured growth parameter, the mechanisms regulating this physiological processes are not well understood. However it is logical to hypothesize that the physical presence of the PPFM microbes, on phylloplane may lead to change in phytohormones concentrations within the host plants apical meristems. It is also likely that the Q4 , Q5 and other inoculants secreted microbial metabolites, that altered biochemical processes within the plant tissues significantly thereby accounting for the observed increase in growth.

This study also suggests that the overall effects of microbial flora in nature on host rice plants is an aggregate of microbial mitigated physiological affects induced by numerous strains. However the consideration of all growth rate parameters in measured in this study indicates that the growth stimulatory effect of Q4 strain was relatively greater compared to the QP, QY and QW strain. This was the basis of selecting Q4 strain for further testing in a highly replicated (randomized block experimental design) and large sample size *in vitro* study.

The Q4 and Q5 strain also significantly stimulated leaf development *in vitro*. Leaf development is an important factor in plant growth, primarily due to their major role in the absorption of the photosynthetically active radiation (PAR) in the 400 nm-700 nm waveband. Leaves convert about 5% of the solar energy reaching the earths surface 1.3 kW/m^2 into carbohydrates (Taiz and Zeiger 1991). It is therefore evident that rapid development of leaves would be beneficial to the plant, particularly by increasing the efficiency of the photosynthetic apparatus.

The the preliminary study showed that Q4 and Q5 strain could stimulate leaf development and the overall plant growth by greater percent relative to the other isolated strains (Fig. 1.6 & Fig. 1.8). In addition to the other growth parameters that were measured, the Q4 and Q5 strains were selected for further experiments involving callus induction and plantlet regeneration *in vitro* on the basis of their ability to stimulate leaf development.

There are several possible mechanisms through which these microbial strains could modulate the process of plant growth and development as illustrated in figure 1.13. These mechanisms are outlined below and afterwards each is discussed in details

a) the sequestering of mineral nutrients from the media or from soil where plants are established in soil.

b) the synthesis of phytohormones or phytohormone-like compounds which are released into the plant transport vascular system or into tissue culture medium.

c) the synthesis and release of microbial derived enzymes which positively facilitate the biochemical and physiological processes in the host plant tissues.

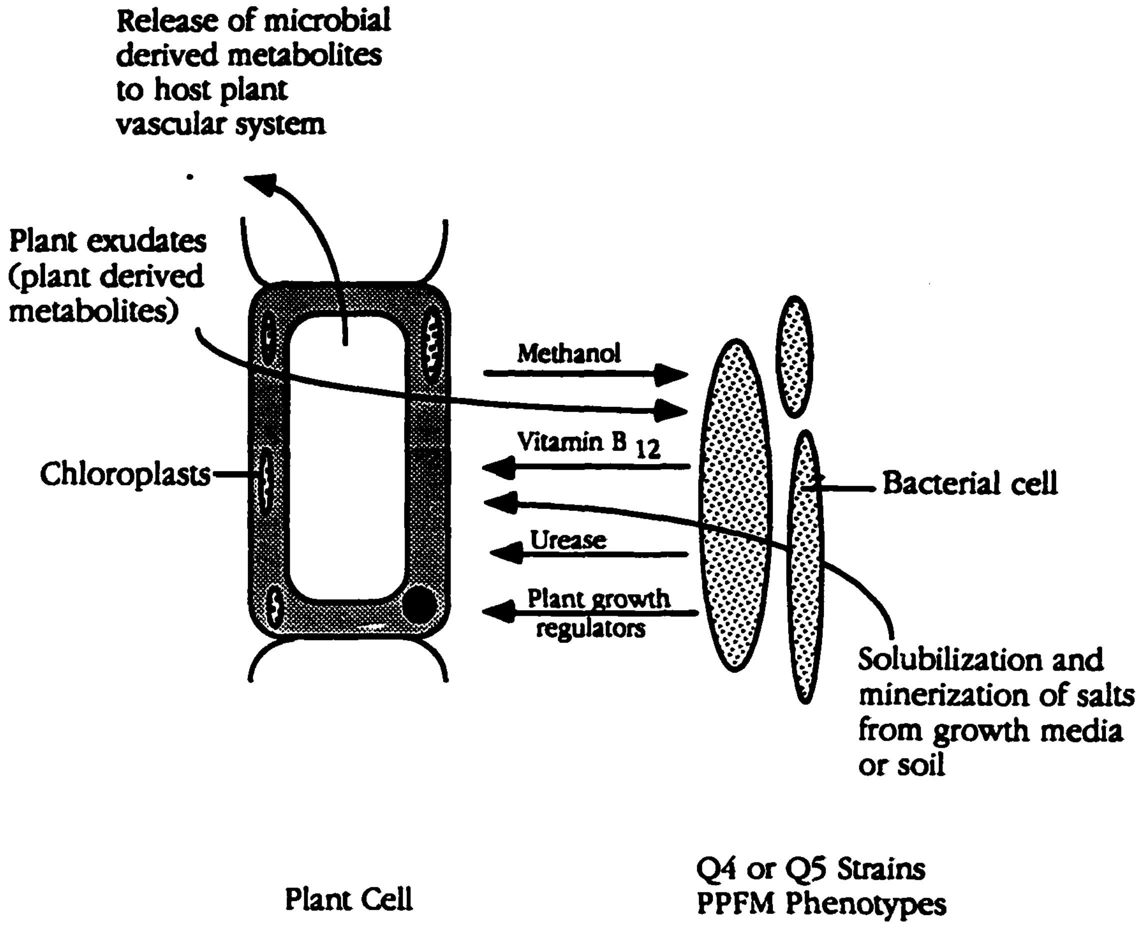

Figure 1. 13 Possible biochemical interaction mechanisms and levels of exchange of metabolites between rice tissues and Q4 or Q5 bacterial cells.

The mechanism of sequestering mineral nutrients (macro/micronutrients) typically involves the synthesis of molecules that facilitate the uptake of the ions. It is reported that the process occurs through the synthesis of siderophores that sequester ions such as iron from the media and make available to the plant cells. This mechanism has been reported in rhizosphere bacteria in soil grown plants (Bar-Ness *et al.*, 1992).

A couple of years ago the mechanisms involving the synthesis of cytokinin and auxin-like compounds by some PPFM strains were reported (Rodrigues *et al*, 1972). It is possible that, the released phytohormone-like compounds can supplement the endogenously synthesized phytohormones and thereby activate the growth of the rice or host plant at various stages of its development. The exogenous growth regulators could also affect the level of endogenous auxins directly by modifying enzyme synthesis and indirectly via effectors (Gasper *et a.,l* 1996). It is therefore possible that during the process of induction of callus from mature seed embryo, natural, synthetic or microbial derived phytohormones could suppress the process of differentiation and thus promote cell proliferation of the embryogenic totipotent cells in the embryogenic plant within the rice seed.

The third possible mechanism could involve the synthesis and release of urease enzyme (Fig. 1.13). The synthesis of the microbial urease enzyme has been reported in several PPFM strains (Holland and Polacco 1994). It was further demonstrated that the microbial derived enzyme had the capacity of supporting urease deficient (mutants) soy bean plants. This could be one of the mechanisms through which the isolated Q4 and Q5 strains modulated the development of the rice seedlings *in vitro.* Urease is essential in nitrogen metabolism in plants. Collectively, the observed modulation of rice growth may have been effected through a combination of these mechanisms.

During the callus induction and organogenesis experiments, the 2,4-D growth regulator initiated the reverting of the mature seed embryo tissue to callus (dedifferentiated state). Normally exogenously applied auxins are capable of inducing dedifferentiation of plant tissue and therefore altering the genetically pre-programmed physiological development of the plant tissue through stimulation of cell wall elongation and cell division.

The formation of roots by the rice callus in the presence of the Q4 strain cobiont suggested that the Q4 isolate synthesized and secreted a cytokinin or a molecular compound with cytokinin properties. Though cytokinins stimulate cell division and callus growth (Miller *et al.*, 1956, 1961; Binus 1994), it is the balance between the exogenous cytokinins and auxins that regulates cellular differentiation, cell enlargement, morphogenesis of shoot and root, and chloroplast maturation in tissue culture (Costacurta and Vanderleyden 1995). Traces of cytokinin in auxin rich media, which translates into high ratio of auxin to cytokinin (A/C = 114), induces root formation from callus (Skoog and Armstrong 1970; Taiz and Zeiger 1991). Intermediate ratios (2 ≥ A/C ≤ 5) induce callus formation (cell proliferation) while low ratios (A/C < 2) promote formation of shoot (Skoog and Miller 1965). It is therefore possible that the Q4 strain synthesized and released low levels of cytokinin or an analog with cytokinin activity, which generated the high ratio of auxin (2,4-D.)/cytokinin required for root initiated and observed in the MS media supplemented with 5.0 mg/l 2,4-D (Fig. 1.9). The proliferation of cell and tissue growth observed in the MS media devoid of all growth regulator, can be attributed to the presence of trace level of the cytokinin or cytokinin-like compound. Cytokinins promote cell division and this may account for the observed callus growth in the MS media devoid of all growth regulators [Fig. 1.9 B(3)]. The callus growth observed in rice tissue inoculated with the Q4 or Q5 strain and established on the media devoid of growth regulators mimicked the cell proliferation observed in the tissue grown in the media supplemented with 5.0 mg/l 2,4-D [Fig. 1.9B (2)]. The findings from this study indicate that the existence of a mutualistic or symbiotic relationship between the Q4, and the Q5 PPFM strains with rice or other host green plants in nature may be partly due to the physiological effect of the microbial derived phytohormone or phytohormone-like compounds. This factor may account for the Q4 and Q5 growth stimulating properties observed in 9 day old and 14 day old rice seedlings.

Qualitative and quantitative analysis showed that the Q4 and Q5 strains had some effects on plantlet regeneration. Several reports so far indicate that rapid regeneration of plants from cell or tissue culture *in vitro* is one of the critical process in the genetic improvement or genetic engineering of food crops and other plants with economic value. The findings of these experiments have clearly demonstrated that the Q4 and Q5 strains have the capacity of promoting rice callus induction (Fig. 1.9) as well as the development of rice seedlings *in vitro*. These findings are important, particularly with regard to the evaluation of the feasibility of utilizing this microbial property, to promote the growth of rice plants grown in the field. The results on plantlet regeneration experiments indicate that minor contamination of laboratory or commercial based rice regeneration studies by either Q4 or Q5 strains can have a negative effect by inhibiting plantlet regeneration (Table 1.2 & Fig. 1.10). It is note worthy that Q4 and Q5 strains are slow growers and therefore they may not be detected early enough as contaminants in tissue culture studies. The development of techniques of detecting and preventing contamination by these strains in regeneration experiments requires further investigation.

The regeneration capacity results of *japonica* cv. CR76 and *indica* cv. A301 reported in this study support the findings of Rance *et al.*, (1914), where partial desiccation of mature embryo-derived calli enhanced regeneration ability of *indica* cv. TN1, IR72 and IR 64. In addition, these results indicate that the effectiveness of partial desiccation as a protocol of enhancing regeneration is independent of the rice cultivar used. Though the mechanism of how partial desiccation enhances regeneration is not well understood, Kermode *et al.*, (1989) showed that, during seed development, desiccation induces changes in translatable mRNAs and elicited modifications in protein synthesis. Recent studies by Rance *et al.*, (1994) have further demonstrated that partial desiccation of mature embryo-derived calli resulted in differences in soluble protein pattern in a SDS-PAGE analysis. The patterns revealed the induction of two major proteins, having molecular weight of 22 kDa and a 26 kDa. Therefore it is apparent that partial desiccation induces the expression of development genes.

In these callus induction and regeneration experiments, a considerably short period of time, 59 - 62 days of *in vitro* culture of the plant materials starting from mature seeds to the transfer of the green regenerated plantlets to soil was recorded. This period of time is shorter than the 84 - 118 days reported for *indica* cv. IR39385 by Hartke and Lorz (1989). The time also compares favorably to the shortest recorded time in regeneration studies 48 - 50 day by Rance *et al.*, (1994) for *indica* cv. TN1, IR72 and IR64. The regenerated plants were initially acclimatized in growth chamber at 100% humidity and in low range 23 - 24°C day/night temperature regime. The initial survival rate of regenerated rice plantlets was 65% following their transfer to soil. The potted seedlings, acclimatized to a greenhouse environmental conditions through gradual decrease of the humidity to 80%, developed to be mature fertile plants (Fig. 1.11E). Their characteristics and morphology was identical to the seed-grown plants.

The quantitative analysis of the physiological effect of the Q4 and Q5 strains on rice seedlings growth and development *in vitro* using large sample sizes confirmed that the strains had the capacity of stimulating the growth and development of inoculated seedlings. This finding provided further evidence for the potential critical role which these microbes may play in promoting growth, biomass production and yields of field grown rice plants.

It is evident from these results that shoot (stem) growth (FG), biomass [dry weight (DW)] and leaves development (LD) and other growth parameters are modulated by the inoculation of these two PPFM strains. The mechanisms through which these strains modulate the process of plant growth and development in rice are poorly understood. The proposed mechanisms include mineralization of organic phosphorous compounds or solubilization of inorganic phosphorous (Subba 1982). Also reported is the synthesis of siderophores that can sequester iron from the soil or media and make it available to the plant cells (Bar-Ness *et al.*, 1992). In this study the observed heavy Q4 and Q5 strains colonization of rice root system *in vitro* is suggestive. Even though in general phosphate solubilizing or mineralizing strains have been found to have the capacity to promote plant growth, increased availability of phosphates to the plant or the detection of increased levels it tissues has not been demonstrated (Subba 1982).

Indirect evidence based on Q4 and Q5 stimulation of rice callus induction and inhibition of plantlet regeneration suggests that the strains modulate plant growth through the synthesis of growth regulators. This observation supports the findings of Rodrigues *et al.*, (1972) where some PPFM strains were reported to have the capacity of synthesizing auxin and cytokinin like phytohormones. In other reported studies, results of field trials with bacterial strains (*Azotobacter chroococcum and Bacillus megaterium*) in the formerly Soviet Union showed that the inoculation of field grown plants with these microbial species increased the yields of many crops by 10-20% in approximately 70% all field trials (Kloepper *et al.*, 1989). The initial indicators of the beneficial effect of these bacterial inoculate were manifested in form of increased seedling emergence, vigor, seedlings weight and root development. The experiments carried out with the Q4 and Q5 PPFM inoculants have showed similar pattern of growth promotion properties characterized by increased seedling vigor (shoot growth) and significant seedling biomass productivity.

A fourth mechanism contributing towards the enhanced rice seedlings growth could involve the synthesis and release of vitamin B_{12} or related compounds by the Q4 and Q5 strains (Fig. 1.13). A recent study reported by Basile *et al.*, (1985) demonstrated that low concentrations of exogenously applied vitamin B_{12} (7.4 µM) could significantly enhanced growth of two species of leafy liverwort *in vitro*. Since vitamin B_{12}-producing PPFM have been successfully isolated from field collected liverworts (Basile *et al.*,1969; Corpe and Basile 1982; Corpe *et al.*,1984). The evidence reported in the findings of Basile *et al.*, (1985) study suggests that the reported significant stimulation of growth and development of *Scapania nemorosa* by an epiphytic PPFM (Basile *et al.*, 1969), could have been mediated by PPFM-synthesized vitamin B_{12}. Even though this is indirect evidence, the presence of B_{12}-dependent enzymes in higher plants such as potatoes (*Solanum tuberosum*) and beans (*Phaseolus vulgaris*) both of which are flowering plants (Poston 1977, 1978), suggests that these plants are dependent on microbially synthesized vitamin B_{12}. Further, the abundance of B_{12}-dependent enzymes in angiosperms suggests that these plants are possibly dependent on the ubiquitously distributed PPFM.

Other possible mechanisms may involve the synthesis of methylotroph derived urease. Recent studies have shown that most if not all of the PPFM strains synthesize the enzyme which is reported to have the ability of supporting the growth of urease deficient (mutant) soybeans (Holland and Polacco 1994). Over all, the modulation of growth of rice plants inoculated with the Q4 or Q5 strain is probably not a product of one mechanism, but rather the cumulative effect of two of more synergistically operating mechanisms.

In summary, the isolated Q4 and Q5 PPFM strains can significantly increase shoot growth rates, seedling vigor, root development, leaf development and biomass productivity rice. The stimulation of growth by the other isolated strains was not significant in most of the growth parameters measured. Although the effectiveness of the PPFM bacterial inoculant is dependent on multiple biotic and abiotic factors, of great significance is the colonization of host plants phylloplane or rhizosphere by other microorganisms which are likely to positively or negatively modulate the effectiveness of the Q4 and Q5 inocula in the natural environment. Evidence gained so far suggests that the mechanisms involved in the Q4 and Q5 growth and development promotion properties may be mediated through plant growth regulators, increased nutrients uptake and utilization of microbial derived enzymes. Further research is required to quantitatively test critically each one of these mechanisms.

CHAPTER TWO

Taxonomic Characterization of Two Methylotrophic Bacteria Strains that Stimulate Growth and Development of Rice Seedlings *In vitro.*

SUMMARY

Methylotrophic bacteria constitute a significant part of rice and other green plants leaf microflora. Among these, are strains of pink-pigmented facultative methylotrophs (PPFM), which represent a distinct taxon in the genus *Methylobacterium.* A PPFM strain that stimulated growth of a leafy liverwort *Scapania nemorosa* in axenic culture has been reported. The strain was originally identified as *Pseudomonas extorquens.* Later studies reclassified the strain as *Methylobacterium extorquens* placing the strain in the genus *Methylobacterium.* In the past there has not been any report on PPFM-induced or stimulation of growth and development of an economically important crop plant. In this study, we are reporting the isolation, taxonomic characterization and microscopy profile of two PPFM strains designated Q4 and Q5. These strains stimulated the growth and development of two rice cultivars; *japonica* cv. CR76 and *indica* cv. A301 *in vitro.* An analysis based on utilization of several carbon-sources coupled with light and electron microscopy studies have placed the Q4 and Q5 isolates into the genus *Methylobacterium.* Based on biochemical properties, the strains are similar to unassigned strains of *M.mesophilicum* identified as 7 and 35^{e}. Microscopy studies shows that Q4 and Q5 isolates have rod shape morphology and gram-negative staining properties. Unlike the type species *M.mesophilicum,* the closest possible species group, the Q4 and Q5 strains metabolize acetate as source of carbon and in addition they have a distinct inability to assimilate citrate. Significant stimulation of growth and development of rice seedlings at $p \leq 0.05$ by Q4 and Q5 strains was recorded. Analysis of biomass accumulation in seedlings inoculated with strains showed that the Q4 and Q5 cobionts increased the dry matter accumulation by 20.3% and 41.2% respectively over the controls. The enhancement of rice plants growth *in vitro* by the PPFM has greater economic implication. It represents a viable model for the improvement of growth and yield of rice in the field.

INTRODUCTION

Plant tissues *in vivo* and *in vitro* are frequently colonized by taxonomically a wide range of microorganisms. Though most of these microbial cobionts are found in the rhizosphere, there is also a wide spectrum of microflora located in the phylloplane of green plants. The abundance and distribution of phylloplane microbes varies. The highest density is reported in the tropics. Studies have shown that microbial life on the surface of leaves in some rain forests forms a layer that is 50 um thick (Holland 1994). Some of these microbes are physiologically beneficial to the host plant while others are neutral or significantly detrimental, particularly the pathogenic strains. Among the phylloplane microflora are the pink-pigmented facultative methylotrophs (PPFM) in the genus *Methylobacterium*. The PPFM are chemoheterotrophic (saprophytic) microflora that ubiquitously inhabit the phylloplane surfaces of most if not all green plants (Corpe 1985). Most of the phylloplane saprophytes, including the PPFM, feed primarily on materials leached from the leaf (Klincare *et al.*, 1971). In some plant species the amount of organic and inorganic compounds leached from a leaf can be substantial percent of the total plant biomass. For example, in a study of soybean *Phaseolus vulgaris*, 6% of the dry weight of leaves, primarily in form of carbohydrates, was leached out in a period of 24 hours (Tukey 1971).

There are several reports on beneficial plant-*Methylobacterium* species interactions. Evidence obtained some time ago (Basile *et al.*, 1969) showed that presence of some strains of PPFM, isolated from surfaces of a leafy liverwort, *Scapania nemorosa*, was correlated with stimulation of plant growth and development when inoculated as a cobiont on the media adjacent to tissue culture of leafy liverwort *in vitro*. Subsequent work showed that bacteria with similar features, that is, pink-pigmented facultative methylotrophic (PPFM), could be selectively isolated from any field collected green plant (Corpe and Basile 1982; Corpe 1985;

Corpe and Rheem 1989). The wide spread distribution of this group of bacteria was later confirmed by reports on immunochemical taxonomic studies by Corpe and Jensen (1991), which showed that all the pink-pigmented facultative methylotrophic bacterial strains isolated from over 60 green plant species were of the genus *Methylobacterium*. Although the stimulation of growth of liverworts has been demonstrated repeatedly, the mechanism or the basis for the PPFM strains stimulation of the leafy liverworts growth is not known. However the findings by Nishio, Yano and Kamikubo (1975) and Toraya *et al.*, (1975) showed that PPFM isolates synthesized and/or accumulated vitamin B_{12} (cyanocobalamine). Subsequent findings showed that the application of exogenous vitamin B_{12} stimulates the development of two species of leafy liverworts in axenic culture (Basile *et al.*, 1985).

The second possible mechanism may involve the utilization of the microbial derived enzyme urease. There are reports that some phylloplane PPFM bacteria produce urease, a nickle containing metalloenzyme which is essential in nitrogen metabolism in plants. The enzyme degrades ureides and urea, the major transport forms of nitrogen in plants. This was demonstrated by the inhibition of urea accumulation in the embryogenic axis of soybean by PPFM urease activity (Holland and Polacco 1992). Other possible mechanisms may involve synthesis and secretion of microbial derived phytohormones such as auxins and cytokinins (Polacco and Holand 1994; Klincare *et al.*, 1971; Rodrigues *et al.*, 1972). These metabolites may be responsible for the observed promotion of growth.

Research on the utilization of bacterial strains requires appropriate description and or taxonomic identification of the strains. However, the taxonomic characterization of bacterial strains is a tedious and uncertain process that demands great input of time and labour. Furthermore, the development of new knowledge has led to reclassification of bacterial isolates, sometimes leading to a shift of an isolate from one genus to a new one. For example, the first *Methylobacterium* strain described in the literature was isolated by Bassalik in 1913 from earthworm contents and was named *Bacillus extorquens*. In a later development, Bhat and Barker (1948) assigned the *B. extorquens* isolate of Bassalik to the genus *Vibrio* and its name being *Vibrio extorquens*. In 1959 the same species was transferred to genus *Pseudomonas* by Krasil'nikov. Later Bassalik *et al.*, (1960) transfered the species to genus *Flavobacterium*. Finally the species was reclassified as *Pseudomonas extorquens* by Doudoroff and Palleroni (1974) in the 8th edition of *Bergeys Manual of Determinative Bacteriology*. In a similar development the strain that stimulated the growth and development of the leafy liverwort and reported by Basile *et al.*, 1969 was originally characterized as a *Pseudomonas extorquens* (Bassalik) species. Today, after numerous reviews, the *Pseudomonas extorquens* has been placed in the genus *Methylobcterium*. In spite of the fluidity of taxonomic characterization of phylloplane microbes, it is evident that successful follow up studies can only be carried successfully where isolated strains with specific properties have been characterized to genus or species level (Green and Bousfield 1988).

The research reported here is not designed to determine the mechanisms by which PPFM stimulated growth in leafy liverworts. Rather, it is first to report the isolation of two strains of PPFM phenotype that stimulate growth of rice *Oryza sativa* an economically important plant and secondly, to report the taxonomic characterization of the two strains designated Q4 and Q5, based on microscopic studies and their ability to utilize of specific organic chemical compounds. The two strains, Q4 and Q5, were found to stimulate growth and development *in vitro* of seedlings and explanted tissue from two rice cultivars, *indica* cv. A301 and *japonica* cv. CR76.

MATERIALS AND METHODS

Experiments on establishment of axenic rice seedlings

Axenic rice seedlings were germinated from surface sterilized mature rice seeds from the *japonica* cv. CR76 *indica* cv. A301 cultivars. To achieve the axenic conditions, the seeds were dehusked and surface sterilized for 1 minute in 95% ethanol, which also hardens the testa, an essential property that inhibits over-sterilization which would kill the embryo. Further sterilization was effected by treatment of the seeds with 35% of sodium hypochlorite containing 0.02% tween 20, a surfactant followed by washing of the seeds in sterile distilled water.

The seedlings were germinated in modified Murashige and Skoog [(MS) 1962] basal media devoid of vitamins, sucrose and growth regulators. The pH was adjusted to 5.7. The medium was solidified in 1.2% agar (w/v) and autoclaved for 20 minutes at 121.1°C, 17 psi. The medium was then distributed at a rate of 25 ml per sterile culture jar. Axenic 5 day old seedlings were inoculated selectively with pure strains of the isolated methylotrophic bacteria strains based on techniques described by Basile *et al.*, 1969; Corpe and Basile 1985. The contamination free status of the seedlings was tested by streaking media in which the seedlings were established in on a beef extract enriched nutrient broth.

Isolation of methylotrophic bacteria from green plants leaf surfaces

The methylotrophs were isolated from leaves of various species of green plants, collected from the New York metropolitan area and from Harriman State Park, Rockland County, New York. All the plant materials were handled using sterile techniques. The methylotrophs were isolated from the surface of young leaves using the surface impression technique, on a modified buffered ammonia salt solution supplemented with methanol (MeAmS) (Corpe and Basile 1982). The leaf segments were rinsed using with water and pressed firmly onto the surface of solidified media in petri plates and incubated at 30°C. Six strains were selected for further testing based on their phenotypic pigmentation of the colonies. One of the methylotrophic strains designated Q4 was isolated from rice tissue cultures where it was originally a contaminant. The Q4 strain was selected on the basis of its ability to stimulate the growth differentiation of the contaminated undifferentiated rice tissue. Other strains of methylotrophs were isolated from the young leaves of the plants,

Filter sterilized aqueous methanol (Sigma Chemical Co., St Louis, MO,USA) was added to autoclaved basal media as a 20% (v/v) solution to give a final concentration of 1.0% (v/v). Sterile antibiotic cycloheximide (Sigma Chemical Co., St Louis, MO, USA) prepared as 0.5 mg/50 ml was added aseptically to the cooled media to give a final concentration of 10.0 ug/ml (Corpe *et al.*, 1985). The antibiotic inhibits the growth and spreading of fungi over the agar surface, which are a major heterotrophic microflora on green leaves. The relative stimulatory effect on seedling growth and development was used as the criteria for the selection of the two strains that were used as cobiont inoculants in subsequent experiments.

Experiments on the effects of selected methylotrophic strains on seedling growth *in vitro*.

CR76 and A301 calli pieces (1.5 cm^3) were subcultured onto modified MS media (1962) devoid of all exogenous phytohormones. Axenic seedlings were germinated in a MS basal salt media devoid of growth regulators and sucrose, a carbon source. The selected methylotrophic isolates were inoculated on media adjacent to a callus or onto the leaves of the axenic seedlings. The effect of the selected strains on seedling growth and development *in vitro* was based on dry matter accumulation, number of leaves and linear shoot growth of the seedlings and was analyzed statistically. The relative increase in size of calli, expressed as cm^3 /60 days or development of roots from the calli inoculated with the bacterial cobionts was used as the measure of growth and differentiation stimulatory effect.

Characterization of the growth stimulating Q4 and Q5 *Methylobacterium* sp. strains

The two PPFM phenotype strains designated Q4 and Q5 that stimulated growth and development of seedlings were classified and differentiated from other possible microorganisms on the basis morphology and differential gram-staininging technique. Furthermore, the strains were characterized on the basis of their utilization of carbon sources following the protocols described by Green and Bousfield (1982, 1983, 1988). The carbon sources included, D-glucose, L-arabinose, sodium citrate, acetate, ethanol, methanol (positive control), fructose and beef extract-peptone enriched nutrient broth. Sterile stock solutions were pipetted into ammonium-salts agar to provide a final concentration of 1% w/v. The carefully mixed agar was poured into sterile petri plates. Single colonies of each isolate (72-96 hr) were transfered into a sterile 2.5 ml buffered saline to provide a faintly turbid suspension. Inocula from the suspension were were streaked as a single line from the center to the periphery of the plates with different carbon sources or none (controls). Morphology, relative growth and the pigmentation of the Q4 and Q5 colonies were determined over a 21 days period of incubation at 30°C.

RESULTS

Methylotrophic bacterial strains isolated from green plants.

Several methylotrophic bacteria strains designated Q4, QPP, Q5, QP, QY and QW were isolated from green plant leaf samples collected from the New York metropolitan area and from Harriman State Park, Rockland County (Fig. 2.1). The isolated methanol-utilizing microorganisms were isolated on a selective MeAmS media after 21-28 days incubation on methanol-agar plates at 30°C. The maximum specific growth of colonies was reached after 28 days. The isolated strains which had varying pigmentation were grown and maintained on methanol-agar and nutrient-agar media. These strains were grouped into several categories based on phenotypic pigmentation of the colonies (Table 2.1). Pure cultures of each isolated strain representing the six categories were isolated and further purified through repeated alteration of single-colony transfer from petri plate containing selective MeAmS-agar to liquid broth media. The contamination free status of the pure strains was confirmed (absence of any other colonies) when representative colonies were plated on nutrient broth (Fig. 2.1).

Table 2.1 Phenotypic classification of methanol-utilizing bacteria strains isolated from several plant species in Harriman state park and New York metropolitan area.

Category	Pigmentation of colonies
Q4	light pink
QPP	pale pink
Q5	pink
QP	dark pink
QY	yellow
QW	white

The Q4 strain was isolated from rice callus culture where it was a contaminant

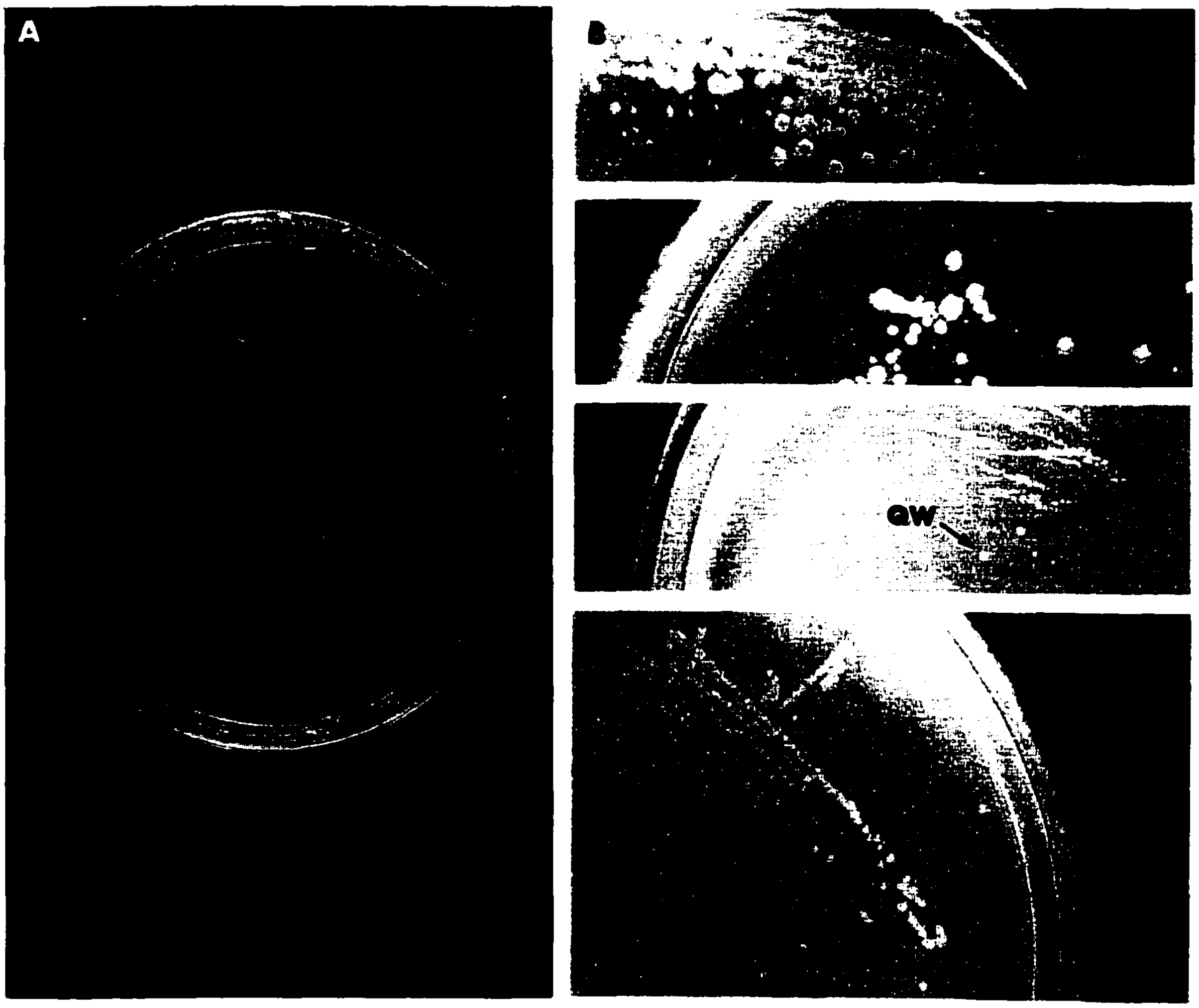

Figure 2.1 Methylotrophic bacterial strains isolated from plants, by leaf impression technique on a selective MeAmS medium. **A:** Colony pigmentation of strains Pink (p), yellow (y) and white (w).

B: Pure Q4, QY, QW and Q5 methylotrophic strains growing on peptone, beef extract nutrient broth, the purity test was confirmed by absence of any other microbial contaminants.

Prior to the inoculation of the Q4 and Q5 isolates onto rice seedlings their contamination free status was confirmed by the absence of any other microbial colonies after streaking of the macerated MeAmS media on which either the Q4 or Q5 were growing on and the pure strain colonies onto nutrient broth enriched with peptone and beef extract. In the preceding study (Chapter 1), Q4 and Q5 were selected for all the subsequent experiments due to their superior growth and development stimulating properties. A common feature of all the isolates was their ability to grow on methanol as the sole source of carbon.

Effects of Q4 and Q5 Methylobacteria strains on seedling growth and development *in vitro*

The six differentially pigmented isolates Q4, QPP, Q5, QP, QY and QY were tested for their growth and development stimulating properties. Q4 was selected by default because it was initially isolated from rice tissue culture where it had stimulated callus proliferation and root formation even though it was a contaminant. Of the other five isolates, Q5 was selected due to its relatively superior growth stimulating properties (Chapter 1) after its inoculation as a cobiont on axenic seedlings. The Q4 and Q5 treated seedlings had greater growth vigor relative to the uninoculated control seedlings.

Further analysis was performed as unpaired one-tail *t*-tests designed to test if biomass accumulation in Q4 or Q5 treated plants was higher than the control. The data revealed that the Q4 and Q5 strains significantly ($t = 2.095$, $p = 0.03$ and $t = 2.773$, $p = 0.01$ respectively) enhanced dry matter accumulation in treated seedlings relative to the control *in vitro* (Table 2.2). Increased dry matter (biomass) accumulation was observed in the two cultivars (Table 2), for instance, the biomass of A301 seedlings inoculated with Q5 was 9.74 (±1.22) relative to 6.90 (±0.58) recorded in the control. The increase in dry matter accumulation in Q4 or Q5 inoculated seedlings was 20.3% in CR76 and 41.2% in A301 over the control.

Table 2.2 The effects of isolated Q4 and Q5 strains on dry matter accumulation (mean ± SE) of 14 day old seedlings 9 days after inoculation.

Cultivar	Control (Mean ± SE)	Q4 or Q5 (Mean ± SE)	df	*t*-test	p-value
japonica cv. CR76	11.51± 0.49	13.85 ± 0.71**	29	2.773	0.0048
indica cv. A301	6.90 ± 0.58	9.74 ± 1.22*	8	2.095	0.034

** denotes value that is significantly different from the control at $p \leq 0\ .01$, while* denotes value that is significantly different from the control at $p \leq 0\ .05$ in a unpaired one-tail *t*-test

The CR76 cultivar was inoculated with Q4, while A301 was inoculated with Q5. The CR76 n values were Control =16 and Q4 (PPFM) =15. A301 n values were Control = 5 and Q5 (PPFM) = 5.

Microscopy profile and taxonomic characterization of Q4 and Q5 strains.

The two isolates that stimulated the growth and development of rice seedlings *in vitro* were both methanol-utilizers and pink-pigmented. Based on standard differential gram staining technique, the two strains, designated Q4 and Q5 were identified as gram-negative, aerobic and rod shaped (Fig. 2.1). Thus the two strains were phenotypicaly very similar to many of the strains of PPFM bacteria described by Green and Bousfield (1982, 1988). Electron-microscopic examination of the Q4 and Q5 strains revealed the presence of extracellular viscous material surrounding the cells (Fig. 2.3). Probably, this is a simple homopolysaccharide of sugars such as glucan and mannan or a heteropolysaccharide polymer comprised of a mixture of monosaccharide forms reportedly produced by methylotrophs (Hug *et al.*,1978; Hou *et al.*, 1978). From these characteristics, it is evident that the isolated Q4 and Q5 strains are members of the large group of ubiquitous microorganisms known as the pink-pigmented facultative methylotrophs in the genus *Methylobacterium*. In addition the growth of the strains on MeAmS culture media containing 10.0 ug/ml cycloheximide antibiotic that inhibits the growth of fungus as well as the pink pigmentation of the colonies confirmed that these were methylotrophic bacteria strains.

The isolate's ability to utilize one-carbon and multiple-carbon compounds was based on eight compounds that were tested . As shown in Table 2.1, the Q4 and Q5 strains utilized L-arabinose, D- glucose, fructose, ethanol, and methanol (MeAmS). Under the same culture conditions, citrate and acetate were not assimilated. It is noteworthy that the two isolates were able to grow successfully when cultured in nutrient broth enriched with beef extract and peptone. The finding indicate that the isolates can thrive on a wide spectrum of carbon compounds.

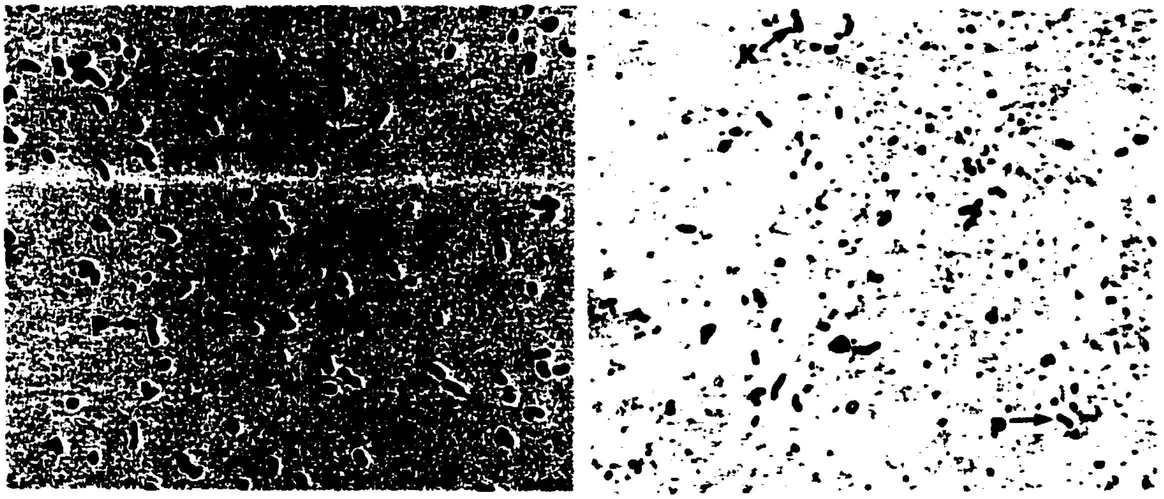

Figure 2.2 Photomicrographs of Q4 (A) and Q5 (B) strains showing morphological structure of cells including polar growth (P) and cellular branching (K). Each strains cell magnification = 600X.

Table 2.3 Utilization of carbon source by Q4 and Q5 strains that stimulate the growth and development of rice seedlings *in vitro* compared to the results reported for other *Methylobacterium* species, by Green and Bousfield (1988).

Strains	**Carbon sources**								
	L-Arabinose	D-glucose	Fructose	Citrate	Ethanol	Acetate	AmS	MeAmS	Nutrient agar
Q4	++++	++++	++	-	+	-	-	++	+++
Q5	++++	++++	++	-	+	w	-	++	+++

The level of growth in ammonium salt media supplemented with various carbon sources at pH 7.0, (++++ = heavy, +++ to ++ = moderate, + = low, w = very low, - = no growth). The relative growth of colonies was used as the basis for the characterization of PPFM. The colony growth was determined after 14 days incubation at 30°C.

Table 2.4 Reference table of carbon utilization by species in the genus *Methylobacterium* adopted from Green and Bousfield (1988).

Species/ strain	**Carbon sources**						
(Green & Bousfield) (1988)	L-Arabinose	D-glucose	Fructose	Citrate	Ethanol	Acetate	Nutrient agar
M. rhodinum	-	w	+	+	+	+	+
M. organophilum	-	+	+	-	+	+	+
M. radiotolerans	+	+	-	+	v	+	+
M. fujisawaense	+	+	v	+	v	+	+
M. mesophilicum	+	+	-	+	+	-	-
N-2^c	+	+	+	+	-	-	+
7 and 35^e	+	+	+	-	+	+	+

+ = growth; v = variable; w = weak growth; - = no growth.

The table shows some of the features that distinguish among *Methylobacterium* species and some unassigned strains, based on grown on specific carbon source substrates 14 days after incubation at 30°C as reported by Green and Bousfield (1982,1988)

Figure 2.3 Electron micrographs (TEM) of Q4 strain (**A**) magnification = 7,046X and Q5 strain (B) magnification = 4,081X. Cells are enclosed in an extracellular matrix (E), W = cell wall. The cells were grown on a selective Methanol Ammonium Salts media. All cell preparations were stained with 1% uranylacetate (negative staining technique).

The species within the genus *Methylobacterium* are differentiated mainly by the pattern of the compounds they are able to utilize as carbon and energy sources (Table 2.4). These compounds range from one-carbon compounds such as methanol to multi-carbon compounds such as glucose. All the carbon utilization tests shown in table 2.3 were carried out as described by Green and Bousfield (1988). The tests (Table 2.3) were designed to facilitate the placement of the Q4 and Q5 isolates within one of the categories of *Methylobacterium* species or strains.

DISCUSSION

The isolated pink-pigmented facultative methylotrophic (PPFM) bacteria strains designated Q4 and Q5 were capable of utilizing methanol a one-carbon compounds as a source of carbon. An analysis based on utilization of carbon compounds led to the placement of these isolates under the species *M. mesophilicum*, within the category of unassigned strain 7 and 35^{e} (Table 2.3 and Table 2.4). In recent years, several studies, including reports on DNA/DNA homologies and electrophoretic comparison of total soluble proteins of representative PPFM strains by Hood *et al*, (1987, 1988), has led to validation of eight species of the genus *Methylobacterium*. These species are *M. organophilum* (the type species), *M. rhodinum, M. mesophilicum, M. radiotolerans, M. rhodesianum, M. zamanii, M.fujisawaense and M. extorquens*. All the species have a characteristic property of being able to utilize methanol as carbon source. A characteristic feature of these strains based on evidence from several studies suggests that one way in which methanol is assimilated by the microorganisms is via the serine pathway (Anthony. 1975; Lindstrom 1991). The main products are poly-ß-hydoxybutyrate, acetyl-CoA and carbon dioxide. These primary products from the microbial metabolic processes may be assimilated by the host plant cells. In addition, similar to other PPFM phenotypes, the Q4 and Q5 bacterial strains could utilize a wide spectrum of multi-carbon compounds such as arabinose, fructose and glucose (Table 2.3). Studies by Green and Bousfield (1981, 1982, 1983) showed that most but not all PPFM bacteria strains can grow on nutrient agar and further reported the isolation of a bacterial PPFM phenotype strain capable of utilizing methane as sole carbon source. It important to note that, in order to minimize over extending of this study, tests on Q4 or Q5 ability to assimilate methane were not carried . However since these strains stimulate growth and development of rice seedlings (Table 2.2), it would be interesting to isolate methane utilizing strains and inoculate the strains onto rice seeds in the field. World wide most of the rice is grown in semi-aquatic conditions were methane is produced in large quantities. Since methane has far greater greenhouse effect than carbon dioxide (Neue 1993). There is a potential role of PPFM strains as pollution bioregulators.

The characterization of PPFM bacteria remains uncertain and challenging at the species level primarily because isolates assigned to the genus *Methylobacterium* although regarded as gram-negative are often stained gram-variable. In addition, the morphological properties of the strain cells, which range from rods to branched rods that exhibit polar growth has added more uncertainty to the already checkered taxonomic history. To date there are eight validated species of the genus *Methylobacterium* including the formerly *Pseudomonas extorquens* which was transfered to this genus by Bousfield and Green (1985) due to its nomenclatural similarity to the previously validated seven species. The taxonomic fluidity within the genus *Methylobacterium* has equally made the taxonomic characterization of Q4 and Q5 difficult.

Q4 and Q5 strains were selected primarily because of their capacity to stimulate growth of rice seedlings. There appears to be several mechanism through which some of the PPFM, possibly including Q4 and Q5, enhance their host plants growth and development. One of the possible mechanisms that has been gaining more support proposes that the enhancement of growth is mediated by microbial derived enzyme urease (Holland and Polacco 1992, 1994). In their study, Holland and Polacco (1992) demonstrated that developing seeds produce an abundant embryo specific urease which accumulates to 0.1% of the total seed dry weigh, a significantly very high level. Secondly, the two authors were able to show that a PPFM contaminant in a urease deficient double mutant of soybean could produce sufficient levels of urease in either callus or young leaves. This accounted for up to 15 - 40% of urease activity observed in wild type plants. A confirmatory test, showed that killing of the PPFM cobiont in soybean tissue culture leads to a decrease in the level of urease activity. In the confirmation test, soybean callus maintained for 3 weeks on a medium containing an 100 ug/ml cefotaxime antibiotic, showed a 30% decrease in urease activity than the control. As further evidence, an assay for urease activity of soybean leaves showed that the reduction of the number of PPFM in the tissue was correlated with the loss of urease activity. This is an important finding because is suggests that the mechanism operates both at cellular level in tissue culture and at organismic level in intact plant leaves (Holland and Polacco 1992). It is noteworthy that, so far, all PPFM bacteria strains that have been isolated produce urease.

Emerging evidence in the literature suggests that biochemical activities in many plants might be due to presence of naturally associated non-pathogenic bacteria (Basile *et al.*, 1969; Klincare *et al.*, 1971; Rodrigues-pereira *et al.*, 1972; Miller and Donnelly 1987; Holland and Polacco 1992). These associations are not exceptional examples of plant-microbe mutualistic interaction but are indicators of a broad general biological phenomenon. The stimulation of growth and development of rice seedlings development *in vitro* by Q4 and Q5 PPFM phenotypes suggests that in nature, the interaction of PPFM with rice plants would be beneficial.

The first description of PPFM association with plants was demonstrated in tissue culture of leafy liverwort by Basile *et al.*, (1969). Since then PPFM have been isolated from from more than sixty species of vascular and non-vascular plants (Corpe and Rheem 1989). These observations have led to the speculation that the PPFM bacteria are universally associated with plants. However the exact nature of the mutualistic relationship is still unclear. Other supporting studies have shown that plants produce substantial levels of free methanol (Corpe and Rheem 1989; MacDonald and Fall 1992). The reports indicate that there is a constant availability of methanol, a vital resource exploitable only by the PPFM including the Q4 and Q5 strains. The methanol, although it is accessible to other phylloplane bacteria, the non-methylotrophic strains are unable to utilize it as a source of carbon. It is noteworthy that *Methylobacterium* species such as the PPFM, unlike other leaf microflora have capacity to thrive on methanol, a one-carbon compound.

The biological phenomenon in whereby some microbial inoculants confer metabolic and or physiological benefits to the host plant is not just limited to PPFM. It is a broad and diverse biological phenomenon as shown in other bacterial and mycorrhizal inoculations. For instance recent studies have shown that natural inhabitants of plant surfaces which are either saprophytes, epiphytes or endophytes enhance adaptation of tissue culture propagules to environmental stress (Herman 1996a, 1996b,). Further, these studies have shown that *in vitro* co-culture of plant tissue explants with beneficial microorganisms induces developmental and metabolic changes in the derived plantlets *in vitro* and in the field. The microbes enhances plantlets tolerance to abiotic and biotic stress (Nowak 1998).

In the past decades, plant associated microorganisms were always considered to be problem causing contaminants particularly in most of the *in vitro* tissue cultures (Cassel 1991; Leifert *et al.*, 1994). This study, which has highlighted two PPFM strains designated Q4 and Q5, together with other similar studies (Basile *et al.*, 1969, 1985; Corpe and Basile 1982; Holland and Polacco 1992) clearly demonstrates that even in one genus such as the *Methylobacterium,* there are numerous strains of microbial strains that have beneficial effects on their host plants. In the field, some of these plant-beneficial microorganisms such as the endophytic vesicular mycorrhiza and perhaps the PPFM can improve plant performance under stress environment and consequently enhance yields (Kloeper *et al.*, 1989; Glick 1995; Lazarovits and Nowak 1997). For instance, experimental evidence shows that bacterization and arbuscular mycorrhization of potato plants *Solanum tuberosum*, through bacterial and mycorrhizal inoculations, a process now refered to as "biotization" (Nowak 1998) will be the technology of choice in commercial micropropagation. Evidence derived from "biotization" experiments by Nowak (1998) shows that, in plantlet regeneration experiments, bacteriarized potato plantlets had significantly better survival rate than the non-bacteriarized control. In our own experiments we have observed that Q4 and Q5 inoculated rice seedlings had greater seedlings vigor relative to non-inoculated seedling. The significance of the stimulation of growth and development of rice plants by the Q4 and Q5 PPFM phenotypes has greater economic implication. The enhancement of interaction between these Q4 and Q5 PPFM represents a viable model for the improvement of yields and physiological performance of rice in the field. The characterization of these strains provides a baseline for the search of similar strains that could have the potential of stimulating the growth, development and yields of other major cereals such as wheat and barley in temperate countries and sorghum in the tropics.

In summary, it is evident from the findings of this study that the leaf surfaces of the members of the grass family (Poaceae) are colonized by a wide range of facultative methylotrophic bacteria as indicated by the variation of the pigmentation of the isolated colonies. Stimulation of rice seedlings growth *in vitro* by some of the isolates shows that phylloplane saprophytic microflora and other epihpytic microorganisms during their vital life sustaining activities secrete biologically active substances which directly or indirectly enhance biochemical and physiological processes of host plant tissue. Though this compounds are likely to be in micro-concentrations they appear to have significant effect on the metabolic processes of the host plant. Results obtained from this study and other similar studies suggests that green plants and their PPFM bacteria such as the Q4 and Q5 strains constitute a group of mutualistic cobionts that have co-evolved a physiologically meaningful partnership. This study, in addition to characterizing the Q4 and Q5 PPFM phenotype strains, also emphasizes the potential beneficial role of non-pathogenic bacterial strains, *in vitro* and *in vivo*. It is possible that *in vivo*, the Q4 and Q5 microbes occupy microsites on the host plant, thus making them unavailable to microbial pathogens. In terms of taxonomy, the PPFM are currently firmly placed in the genus *Methylobacterium*. Based on morphological characteristics, carbon-compounds utilization properties, biochemical and physiological features, the Q4 and Q5 isolates have now been identified as being similar to 7 and 35^{e} strain. However, the taxonomic position of PPFM as a group and its constitutive members, including the Q4 and Q5 strains will still remain open to discussion until when DNA fingerprinting and/ or DNA/DNA homology with the relevant type strain is carried out as a confirmatory test.

CHAPTER THREE

Physiological Effects of Phylloplane *Methylobacterium* sp. Strains and Foliar-Applied Methanol on Vegetative and Reproductive Parameters of Rice

SUMMARY

The phylloplane of green plants is habited by numerous species of microorganisms. A series of experiments were conducted to determine if the treatment of rice seedlings *in vitro* with the two recently isolated Q4 and Q5 strains of the *Methylobacterium* sp. coupled with the treatment with foliar-applied methanol would result in superior stimulation of growth and development . Rice *Oryza sativa* L. cultivars grown in the tropical and temperate climates and representing the two main subspecies *indica* and *japonica* were selected for the experiments. The data collected and analyzed using descriptive statistics, Students t-test and ANOVA., showed that inoculation of the Q4 on cv. CR76 resulted in significant 20.3% ($F = 7.688$, $p = 0.009$) and 16.5% ($F = 5.636$, $p = 0.02$) increase in biomass and leaf development over the control respectively. The treatment of seedlings with the Q5 strain coupled with foliar-applied methanol stimulated biomass accumulation by 41.2% in cv. A301 over the control, also a 12.0% increase in biomass accumulation was recorded in seedlings inoculated with the Q5 cobiont and unexposed to methanol treatments. Enhanced partitioning of fixed carbon in reproductive structures, the panicle and grains following foliar methanol treatment was demonstrated in greenhouse grown plants. Analysis of growth and yield parameter in cv. IR 36, revealed that the absolute grain yield increased significantly ($t = 3.353$, $p = 0.0012$) by 38.1% in treated plants over the control. Significant higher total leaf chlorophyll content and stimulation of early flowering were reported in treated plants. Increased photosynthesis and the assimilation of methanol as a carbon source are possible mechanisms that could account for the increases in biomass accumulation. This study shows that inoculation of rice phylloplane with either Q4 or Q5 *Methylobacterium* sp. strains coupled with foliar-applied methanol has beneficial economic implication. It is a feasible model for the improvement of vegetative dry matter component and absolute grain yield of rice crop.

INTRODUCTION

Rice is an important food crop and it is the main source of calories for over 60% of the total world population. However the present levels of production cannot meet present and predicted future needs. The primary means of increasing yields have to date have mainly focused on improved agricultural practices and the development of higher-yielding varieties through convectional breeding programs. The agricultural practices that require expensive equipment may be impractical in less developed countries. In addition the copious use of chemical fertilizers is less cost effective, has negative effects on the environment and frequently disrupts the relatively stable interdependence of organisms in ecosystems.

The significant increases in yields to date are attributed to breeding programs carried out by IRRI and others. The increase in yields are as a result of development of hybrids that have superior leaf light harvesting capacity and higher grain yield index. These two parameters (light harvesting capacity and grain yield index) are primarily influenced by the morphology of a mature rice plant. The first semi dwarf cultivar developed by hybridization of *japonica* and *indica* was the short stature (83-85 cm) and high tillering Taichung Nativel (TN-1) in 1956 by Taiwan Agricultural Experiment Station (Huang *et al.*, 1972).

The conventional breeding programs dating back to 1960's have resulted in the development of *japonica-indica* hybrids with high grain yield potential mainly through alteration of the mature plant morphology. The single most important achievement of the breeding program has been the development of the high-yielding intermediate height or the elite-semidwarf varieties (Dalrymple 1986). The hybrid produce grains of superior milling quality relative to the tall traditional varieties. The semi dwarf hybrids adapt well to varied ecogeographic regions and also respond to fertilizer application positively. The increase in yields in dwarf cultivars is due to high grain/straw ratios (Yamada and Loh 1984).

So far the conventional breeding efforts for the last three decades have not resulted to the development of any hybrid with superior semi dwarf morphological and physiologically photosynthetic index characteristics of the high-yielding IR 8 variety developed in 1960 by IRRI. The desirable characteristics of this elite-semidwarf cultivar include responsiveness to nitrogenous fertilizers, short stature sturdy stem which confers lodging resistance, a high tillering capacity, and dark green erect leaves, which utilize solar energy more efficiently (Khush 1984; Guangman and Jiling 1989). Other desirable physiological traits include enhanced ability to produce a greater amount of dry matter (total biomass production), and a harvest index of 0.5, which implies that the hybrids has a yield potential of 8-9 t/ha. These critical physiological properties of the developed hybrid cultivars confer enhanced photosynthetic efficiency, sink capacity, and translocation capacity.

At the molecular level, the morphological modification breakthrough which resulted in doubling of yields involved the change of plant type from tall variety to semi dwarf through the transfer of a single recessive gene Sd_1 from the Taiwans semidwarf-DGWG variety to Peta, a tall variety, resistant to diseases. The incorporation of the Sd_1 gene in the resultant hybrid (IR-8) led to short plant stature (Khush 1984). The short stiff culm (stem) makes the rice plant such as the IR8 cultivars and Basmati 385 to be more resistant to lodging and also to be tolerant to nitrogen fertilizer application, in contrast the tall Basmati 370, Peta and Binato varieties that are more susceptible to lodging and are less responsive to nitrogen fertilizer (Majid and Habib 1986). A gene or a block of genes close to the Sd_1 gene and that is linked and transfered together confers high tillering capacity and erect leaves. These two traits are highly desirable and are associated with increase in rice yields productivity (Khush 1984).

Overall, it is noteworthy that the conventional breeding programs and recent genetic engineering techniques have not produced a variety with greater yield potential than the elite-semidwarf IR 8 variety developed by IRRI in 1960. While recent biotechniques have have been able to confer to the semi dwarf variety desirable traits such as resistance to diseases and pest, the challenge of increasing yields is as serious as it was in 1960.

It is in the light of this challenge that this study was undertaken to evaluate the physiological effect of aqueous methanol, a volatile organic compound (VOC) produced by plants, on the growth and yields of rice plants first *in vitro* and secondly in simulated field conditions. Why was methanol selected as a possible organic molecule that could possibly contribute towards increasing rice yields?

There are several reasons: First, foliar application of nutrient-supplemented aqueous methanol to C_3 plants exposed to high temperature and full sunlight in arid conditions is reported to have increased the growth and harvest yield of some plants such as tomato (*Lycopersion esculentum.*), soybean (*Glycine max*), and *Hibiscus esculentus* (Nonomura and Benson 1992a; Rowe *et al.*, 1994; Li *et al.*, 1995; Dorcus and Vivekanandan 1996). In addition, treatment of desert crops with methanol has been reported to decrease daily water requirements. In other reports, methanol treatments were found to have no effect on growth and yields in some plant species such as peppermint (*Metha piperita*), a crop grown for commercial oil production (Mitchell *et al.*, 1994), and in sour orange tree (*Citrus aurantium;* Rutaceae). In all studies, the effect of foliar-applied methanol on rice growth and development has not been reported, in spite of the critical role of rice as a major cereal crop that supports the nutritional requirements of over 60% of the total world human population.

Physiological basis of increasing grain yield potential

The productivity of a crop is influenced by the genetic make up of the plant and physiological determinants. The main physiological characteristics that determine the grain yields of a crop in the field are (1) dry matter economy of the crop, (2) components of the grain, (3) water economy of the crop, and (4) nitrogen economy of the crop (Slafer *et al.*, 1994).

Dry matter economy, primarily refers to the above ground biomass productivity at maturity and the partitioning of the biomass into reproductive sinks (grains). The grain component is analyzed quantitatively in terms of the number of harvested grains per M^2 area from the crop in the field and on the basis of the mean weight of individual grains. Water economy of the crop is based on the efficiency of water use and the total amount of water utilized per plant during the growing season. The nitrogen economy is assessed on the basis of the efficiency of nitrogen uptake and utilization.

The total biomass is a reliable and major indicator of the potential productivity of a given rice cultivar. Guangman and Jiling (1989) demonstrated that the high yielding semi dwarf or intermediate height cultivars have about 40% higher total biomass accumulation relative to the tall low yield cultivars. In addition to biomass productivity, other factors that have contributed towards improvement in the yields of the hybrids include modification of canopy architecture through selective breeding programs. Canopy with a superior light-intercepting characteristics is associated with improvement in net photosynthesis and improved nutrient uptake (Jiang *et al.*, 1988; Kuronda *et al.*, 1989; Arjunan *et al.*, 1990). In general plant, growth represents the excess of photosynthesis over respiration. Therefore an increase in net photosynthesis is positively correlated to increase in plant productivity. The canopy architecture particularly in form the of erect leaves is important in biomass production. Reports indicate that the rice cultivars with droopy leaves such as IR4 and IR57 have relatively lower capacity for biomass production relative to cultivars with erect leaves such as IR 8 and IR36 (Akita 1989).

The second objective of this study was to measure the effect of foliar-applied methanol and selected *Methylobacterium* sp cobionts on biomass accumulation in rice plants and, furthermore the partitioning of dry matter to the reproductive structure, specifically absolute grain yield. Several studies reported so far have demonstrated the existence of growth stimulating strains of the genus *Methylobacterium*, particularly the pink-pigmented facultative methylotrophs (Basile *et al.*, 1969; Corpe and Basile 1982). In the preceding study, reported in chapter one, and also published in the abstracts of the 96th and 97th general meeting of American Society for Microbiology in 1996 and 1997 respectively, two *Methylobacterium* strains designated Q4 and Q5 significantly stimulated growth of rice seedlings *in vitro*, particularly the accumulation of biomass and the development of leaves. Based on the findings of our previous study, the experiments described here were also carried out to determine the cumulative effect of isolated *Methylobacterium* sp strains designated Q4 and Q5 and foliar-applied methanol on morphological development of rice and, secondly, to quantify the effects of the treatment on rice crop physiological productivity parameter based on (a) dry matter economy (dry matter accumulation and partitioning of the biomass to the vegetative structures) and (b) yield component, primarily the partitioning of the biomass to grains within reproductive structures (panicle) of the plant. An additional goal of this study was to measure changes in chlorophyll synthesis within the rice leaves following the treatment with the foliar-applied aqueous methanol during the active vegetative growth phase of the rice plants.

MATERIALS AND METHODS

I. METHANOL TREATMENTS AND *IN VITRO* EXPERIMENTS

Plant material, *Methylobacterium* sp. strains and methanol solutions

All the plants used in this study were started from *japonica* cv. calrose (CR)76 and *indica* cv. A301 seeds kindly provided by Y.L. Wu and D.S. Mikkelson. Department of Agronomy and Range Science, University of California Davis. The seeds used in these experiments were harvested from stock grown and maintained in the research glasshouse at Lehman College, The City University of New York.

For the in vitro experiments, the seeds were dehusked and surface sterilized through 1 minute treatment in 95% ethanol followed by 30 minutes treatment in 35% v/v sodium hypochloride supplemented with 0.03% v/v Liqui-Nox [(laboratory detergent) Alconox Inc, New York, New York USA]. The seeds were then rinsed five times in sterile distilled water. Seeds of the same size and quality were used in all the experiments. All seedlings for the *in vitro* experiments were established in MS basal salt media devoid of a carbon source and growth regulators.

The two *Methylobacterium* strains inoculated onto the germinated axenic seedlings were the Q4 and Q5. The Q4 strains was selected because of its ability to promote somatic embryogenesis in rice callus cultures, where it was originally a contaminant. Q5 was selected due it superior growth stimulatory effect in a comparative study of several strains isolated from diverse species of green plants. In an earlier study (Maliti *et al.*, 1997) the Q4 &Q5 strains stimulated cell proliferation in rice tissue culture and the development of rice seedlings *in vitro*.

The methanol treatment solutions (pH 6.5), ranging from 0% to 40 % in 5 % increments, were supplemented with 0.2 % w/v glycine and 0.5% w/v disodium glycerophosphate as recommended for plants grown indoors (Nonomura and Benson 1992).

Experiments to determine the most effective concentration of the foliar-applied methanol treatments

To determine the effective concentration of foliar-applied methanol concentrations ranging from 0% to 40% on rice growth and development, seedlings of cv. A301 were grown in the greenhouse and dry matter (biomass) accumulation as a function of methanol treatments was measured. Data collected was used to select the most effective methanol treatment. Prior to the initial treatments, 10 day old rice seedlings were transplanted into 300 ml polyethylene pots containing soil mixture formulated as 2: 0.5: 5 w/w ratio of premium-organic top soil (Earthgrow, Inc, Lebanon, CT)., VitaHume compost manure (Hyponex Corporation, Maryville, OH), and water garden (aquatic plants) soil (Earthgrow Inc, Lebanon, CT) respectively. The poted plants were suspended in large 800 ml polyethlene pots to provide the shallow aquatic environment required for the successful growth of rice.

In a randomized experimental design the rice seedlings were foliar-treated with the methanol solutions (3.0ml/plant) at day 1 of week 2 and day 1 of week 3, during a 14 week growth period, seedlings in the control group were foliar-sprayed with distilled water, supplemented with 0.2 % w/v glycine and 0.5% w/v disodium glycerophosphate at pH 6.5 (0% methanol). Data for the measurements of growth patterns based on, leaves development (number) was collected at week 1, 5 and 9 after initial treatment. Commercial chemical fertilizer (20 : 20 : 20.) N : P : K., prepared as 15 g /liter stock was applied to each plant at rate of 20 ml /plant during week 6. Dry matter accumulation (mg) was measured at the end of 14 weeks growth period. The optimal concentration of methanol solution determined through statistical analysis of the data from this experiment was used in the foliar-treatment of plants in the subsequent greenhouse and *in vitro* experiments.

Experiments on the effects of concomitant treatment of seedlings with Q4 or Q5 strain and foliar-applied methanol on seedlings growth

In an *in vitro* study, axenic rice seedlings were germinated from surface sterilized seeds in 25 mls of MS basal salt media devoid of growth regulators in 2.5 cm x 20.0 cm glass caped test tubes. The 5 day old seedlings were inoculated with 1.0 ml of approximately 272,000 cells/ml stock of either the Q4 or Q5 strains (*Methylobacterium* sp.) isolated in a previous study. On day 7 after germination (2 days after the inoculation of the Q4 or Q5 cobiont), the seedlings were subdivided into homogeneous groups and separately treated with the optimum methanol solution (25%), in a randomized complete block experimental design. The seedlings were harvested at day 14, (9 days after inoculation of either Q4 or Q5 and 7 days after methanol treatments).

The independent effect of either the Q4 or Q5 cobionts, or the effect of independent methanol treatments as well as the combined effect of cobiont (Q4 or Q5) and methanol treatments on seedlings growth and development was analyzed on the basis of biomass accumulation (mg), leaf development (number) and shoot growth (cm) of the seedling *in vitro*.

II. GROWTH CHAMBER EXPERIMENTS

Plant material and growth chamber conditions

Experimental plants were germinated from surface sterilized mature seeds of *Oryza sativa* L. cv. A301, cv. Calrose (CR)76, cv. IR36 and garden pea *Pisum sativum* L. cv Alaska. The germinated seedlings were grown in pasteurized soil mixture containing 2: 0.5: 5 ratio of premium-organic top soil (Earthgrow, Inc, Lebanon, CT)., VitaHume compost manure (Hyponex Corporation, Maryville, OH), and water garden soil (Earthgrow.,Inc, Lebanon, CT) respectively. The garden pea plants were watered regularly while the rice plants were maintained in a semi-aquatic conditions through out the growth period. An 18 hour photoperiod was provided by fluorescent light supplemented by incandescent lights. Temperatures were set at 24 ± 2°C day and 23 ± 2 °C night, and 80%- 85% relative humidity was maintained through out the growth period .

Experiments on the effects of Q4 and Q5 strains on seedlings survival and vigor during the early stages of development.

a) Seed viability/seedlings survival

Some of the seed transmitted microorganisms play an important physiological role during the early stages of seedling development after germination. For instance cobiont *Rhizobium* sp in symbiotic relationship with legumes such as soybean *Glycine max* (L.) actively fix nitrogen from the atmosphere and as a result increase the nitrates available to the host legume plant. In a recent study, Polacco and Holand (1994) demonstrated that mutant soy bean plants deficient in urease (an enzyme required for nitrogen metabolism) could physiologically thrive as wild type by relying on PPFM derived microbial urease. Since nitrogen metabolism process is a critical in all green plants, it is thus conceivable that absence or a decrease of naturally occuring PPFM cobionts may significantly affect the development of plants that fully or partially depend on the PPFM derived metabolites. In a relevant study, a decrease in the population of seed microflora after long term storage of seeds in various plant species has been reported (Klincare *et al.*, 1971). The implication of these agropractices that lead to the decrease of seed microflora on the physiology of the host plant has not been reported.

The experiments reported here were carried out to evaluate the effect of Q4 and Q5 strains on the seed viability, and seedling survival during the early stages growth (first 21 days) after germination. The experiments were designed to simulate the observed decrease in seed microflora population after long term seed storage. The reduction of seed-associated microflora was effected through the previously described seed surface sterilization technique.

To determine seed viability and seedlings survival, 7 sets of surface sterilized seeds were prepared for each of the plant material, (cv. A301- 33 seeds/set., cv. CR76 - 22 seeds /set., cv. IR 36 - 38 seeds/set, and cv. Alaska- 32 seeds/set). The sets of each plant material were treated with one of the following aqueous mixtures in a completely randomized experimental design,

i). Control (sterile distilled water)

ii). Q4 bacterial cells suspension

iii). Q5 bacterial cells suspension

iv). MeE [a microfilter sterilized extract from the culture media (MeAmS) in which either Q4 or Q5 had been grown in to a stationary phase after 14 days incubation at 30°C].

v). Me [a microfilter sterilized unused PPFM culture media (MeAmS)]

vi). B12 (5mg vitamin B12/ml/set) prepared as a 250mg/50 ml stock from commercial 250 mg cyanocobalamin.

The imbibed seeds were allowed to germinate in sterile filter papers, in sterile petri plates, sealed with parafilm, and placed in the dark in a 24 ± 2°C growth chamber. At day 3 and 5, the relative seed viability and seedling survival was determined and reported as % of the total number of seeds. The overall effect of the Q4 and Q5 strains was evaluated based on pooled data of all the germination frequency of three rice cultivars and garden pea seeds. The data analyzed statistically (*t*-test) for separation of means, was reported as seedlings survival (%) at day 3 and 5.

b) Seedling vigor and growth

To determine the progressive growth of seedling, 4 day old seedlings of same quality from the sets of the rice and garden pea seeds, germinated in Q4 or Q5, Me, MeE , B12 or the control solutions were transplanted into polythene pots (4.5 x 5.5 x 6) cm containing pasteurized soil mixture. The seedlings were grown and maintained in a growth chamber adjusted to a 24 ± 2°C day/night temperature regime, 80% relative humidity and 16 hour /8 hour, day/night photoperiod. The sources of day light were 40W wide spectrum florescent lamps (Sylvania Cool white 40W F48T12/CW.USA) and 100W Incandescent light (Tungsram USA 100W, GEC, Cleveland, Ohio, USA).

Shoot growth (cm) and leaves development (number) of rice plants were recorded at day 10, 17 and 24 after germination. The growth and development pattern of the garden pea seedlings was monitored over the entire plants life cycle (8 weeks). The growth parameters recorded and analyzed were seed viability, flowering time (days after germination), dry matter accumulation (biomass) and partitioning of biomass to reproductive structures (g) based on grain yields.

III. GLASSHOUSE EXPERIMENTS

Plant materials and glasshouse conditions

The rice cultivars used in this study were cv. CR76 and cv. A301 (USA cultivars, kindly provided by Y. L. Wu and D. S. Mikkelson. Department of Agronomy and Range Science, University of California Davis from University of California, Davis)., cv. Basmati 370 (Indian cultivar. from Kenya Seed Company, Nairobi, Kenya.), cv. Pishori (East African cultivar, from Kenya Seed Company, Nairobi, Kenya.) and cv. IR 36 (IRRI Philippines, kindly provided by Dr Eleanor Wurtzel, Lehman College, The City University of New York.) All the seeds were germinated in petri plates lined with moist filter paper in a 24 ± 2°C growth chamber. The 3 day old seedlings were transfered into 75 cm x 35 cm x 6cm flatbed trays containing a 2 : 1 of premium-organic top soil (Earthgrow, Inc, Lebanon, CT), and VitaHume compost and manure (Hyponex Corporation, Maryville, OH). All the seedlings were maintained in a 100% relative humidity mist room at 25 ± 1°C day/night temperature regime for a period of 10 days, after which they were transfered into a regular greenhouse room.

The growth conditions in the glasshouse were 23°C - 24°C (night) / 24°C-27° C (day) temperature regime, 60% -70% relative humidity, and 14 hour/ 10 hour, light/ dark photoperiod. The light intensity at the surface of the pots during the experimental period was maintained at 44000 lux by supplementing natural day light with 60 W wide spectrum fluorescent lamps (Philips 60W, Denver, Mass., USA). Foliar-treatment of plants with methanol solutions was effected with a hand held linear polyethylene plant garden sprayer model S-69 (The Agfa Corporation, Miami lakes, Florida, USA).

The effects of methanol treatment on leaf chlorophyll content, and methylotrophs population on leaf surface.

Young seedlings (10 day old) were transplanted into 300 ml polyethylene pots containing 2: 0.5: 5 ratio of premium-organic top soil, VitaHume compost and manure, and water garden soil, respectively. The 300 ml pots containing the rice plants were suspended in 800 ml polyethlene pots to provide the shallow aquatic environment required for growth of rice.

The rice plants were assigned to blocks in a complete randomized experimental design. Foliar treatments of the plants with 0% (control) or 25 % v/v aqueous methanol (Sigma Chemical Co. St Louis, MO, USA.) were repeated at intervals of 3 weeks. The foliage of the rice was sprayed to drip. The growth, development, and colonization by methylotrophs was monitored over a period of 14 weeks after germination.

a). Growth analysis

To determine the relative growth of the treated plants, the replicates from each of the established 5 rice cultivar blocks were harvested after a period of 14 weeks. Shoot and root biomass (g) were determined after drying the plant samples in glassine envelopes at 70°C for 48 hours. Prior to measurement of plant material weight, all samples were allowed to equlibrate with the laboratory atmosphere. The data collected was analyzed statistically, Analysis of variance (anova) carried out, F tests or *t*-tests and their corresponding p-values were determined for mean separations.

b). Chlorophyll content analysis

The average leaf chlorophyll content of the treated plants was determined in the harvested first two leaves from each of the 12 plant replicates in each treatment block. The chlorophyll and other leaf pigments were extracted by immersing three randomly selected leaf discs about (1.62 cm^2) in 96% ethanol for

2 days in small tightly sealed vials, placed in the dark as described by Wintermans and De Mots 1965. Spectrophotometric procedure was followed to determine the total chlorophyll extracted and its component pigments (*chlorophyll a and b*). The concentrations were determined, at three wavelengths [649 (*chlorophyll a*), 654 (total), 665 (*chlorophyll b*)] The leaf chlorophyll content expressed as (ug/ cm^2) was adjusted for dilutions and leaf surface area following the technique described by Winterman and De Mots (1965).

c). Methylotrophs population and distribution studies

Surface impression and standard plate count techniques were used to determine the relative effect of methanol solution treatments on population and distribution of PPFM and other methylotrophs on leaves surface. The young leaves (first or second leaf) of the greenhouse grown plants were collected at day 0, 5, 10, 15, 20 and 25 after the initial treatment. All the treatment solutions were supplemented with 0.5% w/v glycerophosphate and 0.2% w/v glycine and adjusted to pH 6.5. All the leaf material were handled asceptic techniques.

i) Surface impression technique.

Leaf segments of the first or second leaf at the apex of the shoot were measured to determine their surface area. The segments were then pressed on the surface of a selective MeAmS-agar medium (Corpe and Basile 1982) in petri plates. The constituents of the MeAmS media included 1% v/v Methanol ammonium basal salts, Hutners "metal 49" (Basile 1978 micronutrient II) and 1.0 ug/ml of filter sterilized cyloheximide. All the plates were incubated at 27°C for 21 days, to allow the development of the slow growing methylotrophs colonies. The distribution and the number of pink, yellow white and other methylotrophic colonies on the adaxial and abaxial surface of the leaf segments were determined, phenotypically described and expressed as number of colonies/cm^2 of fresh leaf.

ii) Standard plate count technique.

The first two leaves at the apex of the shoot of treated plants were aseptically excised, placed in a sterile polythene bags and immediately refrigerated at 4 -11°C. The surface area of each leaf set was measured and the methylotrophic bacteria population on the plants phylloplane was determined using the selective MeAms-agar medium. The leaves material were prepared following a modified procedure described by Corpe and Rheem (1989). The leaves were ground aseptically in 3.0 ml of sterile 0.05 M phosphate pH 7.0 buffer, in a sterile mortar and pestle with 2.0 - 3.0 g sterile sand granules. The ground leaf material, were centrifuged for 5 minutes at 1000 x g to remove the leaves debri and sand granules. The supernatant was serially diluted and surface plated on MeAmS-agar selective medium containing 1.0 ug/ml cycloheximide. The plates, sealed with parafilm were incubated at 27°C for 21 days before the final count. The total population of methylotrophs was determined and expressed as number of colonies/ fresh leaf area (cm^2).

Quantitative effects of methanol treatments on flowering time and the partitioning of biomass to vegetative and reproductive structures (grain yield).

The seedlings of temperate cultivar (cv. CR76., cv. A301) and tropical cultivars (cv. IR 36., cv. Pishori cv. Basmati 370) were grown in a greenhouse to maturity in 2 blocks of 12 replicates. The plants were grown in a established in a mixture of 2: 0.5: 5 ratio of premium-organic top soil, VitaHume compost and manure, and water garden soil. All plants were maintained in a shallow semi-aquatic conditions in a simulated tropical microclimate. Commercial chemical fertilizer, NPK (20 : 20 : 20.) prepared as 15 g /liter stock was applied to each plant at rate of 25 ml /plant during week 4, 8 and 12. All plants were foliar-treated with the methanol solutions between 11:30 am and 2.00 pm at week 4 and week 11 of the growth cycle after seeding.

The tiller and leaf development growth parameters of cv. CR76., cv. A301 cv. IR 36., cv. Pishori and cv. Basmati 370 were measured during week 1, 9 and 17 after the initial treatment. Dry matter accumulation was determined at week 17 in all cultivars except cv. IR36.

The cv. IR36 plants were grown to reproductive stage (week 17-18). The main factors essential in determining % yield of each plant were recorded: tillering number, number of panicles per plant and the absolute grain weight per plant. The grains were harvested, when 75% of the total seed coats per panicle changed color from green to brown, as recommended by International Rice Research Institute (IRRI). In these experiments, the data from the greenhouse measurements was evaluated through analysis of variance (anova). In addition, F tests or *t*-tests between pairs of treatments and their corresponding p-values were determined for means separation and comparisons.

RESULTS

Greenhouse Experiments I: Methanol Treatments

Establishment of the most effective methanol treatment

The effect of foliar-methanol treatments on of 3 week old seedlings growth as indicated by biomass accumulation and leaf development increased progressively from low concentration (0%) and reached the peak at 25% concentration. Application of higher concentrations (30% - 40%) progressively inhibited biomass accumulation (Fig. 3.1 & Fig. 3.2) relative to the plants treated with 25% concentration, however the mean biomass values were relatively higher than the mean of the control.

The analysis of the effect of treatments at week 1 showed that all methanol concentrations slowed down overall shoot growth (height in cm) relative to the control (Table 3.1). At week 5, the 5% to 20% methanol treatments showed minimal stimulation of growth, however the increases were not significantly different from the control. During the same period, the 25% methanol treatment stimulated shoot growth, for instance at week 5, a significant ($t = 3.412$, $p = 0.003$) shoot growth in cm was observed in treated plants (198.93 ± 5.42) relative to the control (156.77 ± 10.40). Higher methanol concentrations (30% and 40%) treatments enhanced growth but not significantly suggesting that these concentrations were probably toxic to the plant tissues. The growth patterns during week 9 were similar to that observed during week 5. However, the 15%, 20%, 25% and 30% treatments significantly stimulated shoot growth at $p = 0.05$ (Table 3.1) in the greenhouse grown rice seedlings.

Table 3.1 Growth of cv. A301 seedlings as a function of treatment with 0% to 40% foliar-applied methanol concentrations. Growth measurements were based on increase in shoot length (height cm), over a period of 9 weeks.

Week	Treatment (MeOH conc %)	Shoot height (Mean ± SE)	df	*t*-Test	*P* value
1	0	36.50 ± 0.74	6	-	-
	5	33.10 ± 1.30*	5	2.364	.0118
	10	30.66 ± 1.94*	6	2.813	.0078
	15	32.00 ± 1.67*	5	2.598	.0124
	20	32.98 ± 1.02*	5	2.863	.0077
	25	33.08 ± 1.00*	5	2.657	.0111
	30	32.32 ± 1.67*	5	2.393	.0178
	40	32.20 ± 0.95*	5	3.640	.0019
5	0	156.77 ± 10.40	6	-	-
	5	158.12 ± 16.18	5	0.072	.4719
	10	162.66 ± 12.20	6	0.367	.3599
	15	162.38 ± 10.67	5	0.635	.2692
	20	177.32 ± 19.57	5	0.967	.1770
	25	198.93 ± 5.42*	5	3.412	.0029
	30	180.97 ± 15.46*	5	1.333	.1048
	40	171.05 ± 13.79*	5	0.841	.2092
9	0	208.36 ± 13.91	6	-	-
	5	236.30 ± 40.32	5	0.698	.2497
	10	223.77 ± 13.07	6	0.807	.2175
	15	238.88 ± 9.39	5	1.753	.0537
	20	261.72 ± 22.86*	5	2.061	.0319
	25	249.65 ± 6.64*	5	2.532	.0140
	30	371.83 ± 34.76*	5	1.796	.0500
	40	225.10 ± 10.85	5	0.924	.1875

* Mean is significantly different from the control at $p \leq 0.05$

The control and methanol solutions were enriched with 0.2% w/v glycine and 0.5% w/v disodium D-L-glycerophoshate per liter. Treatments were applied as foliar spray on young rice plants during week 3 after seeding. Growth of the seedlings was measured during week 1, 5, and 9 after treatment.

Leaf development in 12 week old seedlings grown, 9 weeks after treatments in general showed that methanol treatments enhanced the process of leaf formation. However the leaf development stimulation following low methanol concentrations treatments (5% to 10%) and high concentration treatment (40%) was not significant at $p = 0.05$, relative to the control (Table 3.2). The treatment with 15%, 20%, and 30% resulted in significant ($p \leq 0.05$) stimulation of leaf development. The greatest level of stimulation of growth based on leaf number [significant at $p = 0.013$ ($t = 2.605$)] was observed in seedlings treated with 25% concentration (Table 3.2), the increase in the rate of leaf development within the 9 week period represented a 21.6% increase in the number of leaves developed in the treated plants over the control.

Table 3.2 Comparative effects of foliar-applied methanol on leaf development in cv. A301 seedlings. The number of leaves was determined at week 9 after treatment.

Treatment (MeOH conc %)	Leaf number (Mean ± SE)	df	*t*-Test	*P* value
0	22.71 ± 1.36	6	-	-
5	24.83 ± 2.12	5	0.867	.2022
10	24.00 ± 1.63	6	0.605	.2781
15	26.00 ± 1.03*	5	1.872	.0439
20	27.17 ± 1.89*	5	1.955	.0382
25	27.60 ± 1.12*	5	2.605	.0131
30	27.00 ± 1.97*	5	1.837	.0467
40	24.67 ± 1.84	5	0.871	.2013

* Mean is significantly different from the control at $p \leq 0.05$

The rate of tiller development in 12 week old seedlings 9 weeks after foliar-treatments was significant ($t = 2.602$, $p \leq 0.012$) in the plants treated with 25%

methanol compared to the control (Table 3.3). Even though the low methanol concentrations (5%, 10% 15% and 30%) showed a correspondingly increasing stimulation of tiller formation, these increments were not statistically significant at $p = 0.05$ (Table 3.3). Treatments with high methanol concentration (30% and 40%) showed no significant stimulation of tiller formation. It is noteworthy that the stimulation of tiller formation by methanol solutions above the 25% concentration, decreased proportionaly with increase in the concentration of the methanol treatment used (Table 3.3), possibly due to increasing toxicity of methanol to the plant tissues.

Table 3.3 Comparative effects of foliar-applied methanol treatments on tiller development in cv. A301 plants, 9 weeks after treatment.

Treatment (MeOH conc %)	Tiller number (Mean ± SE)	df	*t*-Test	*P* value
0	2.57 ± 0.20	6	-	-
5	2.67 ± 0.21	5	0.325	.3736
10	2.71 ± 0.29	6	0.407	.3452
15	3.17 ± 0.31	5	1.664	.0622
20	3.00 ± 0.37	5	1.069	.1541
25	3.33 ± 0.21*	5	2.602	.0123
30	3.00 ± 0.26	5	1.326	.1059
40	2.67 ± 0.21	5	0.325	.3756

* Mean is significantly different from the control at $p \leq 0.05$

The overall response of seedlings to the 0% to 40% methanol concentration treatments was best analyzed on the basis of dry matter accumulation. During the active growth period of plants, dry matter (biomass) accumulation indicates the rate at which carbon is assimilated through photosynthetic process. The partitioning of dry matter to plants vegetative structures (biomass productivity), also refered to as growth, represents the excess of the carbon assimilated during photosynthesis over the carbon lost during cellular respiration.

The mean dry weight (biomass ± SE) of 12 week old seedlings, 9 weeks after treatment with 25% methanol spray, showed that the treatment significantly ($t = 2.008$, $p = 0.035$) stimulated seedlings biomass accumulation (Fig. 3.1 & Table 3.4). Even though in some cases small stimulation of growth based on biomass data was recorded in the seedlings treated with the lower methanol concentrations (5%, 10%, 15% and 20%) and also in the seedlings treated with the higher concentrations (30% and 40%) the mean values were not statistically significant (Table 3.4).

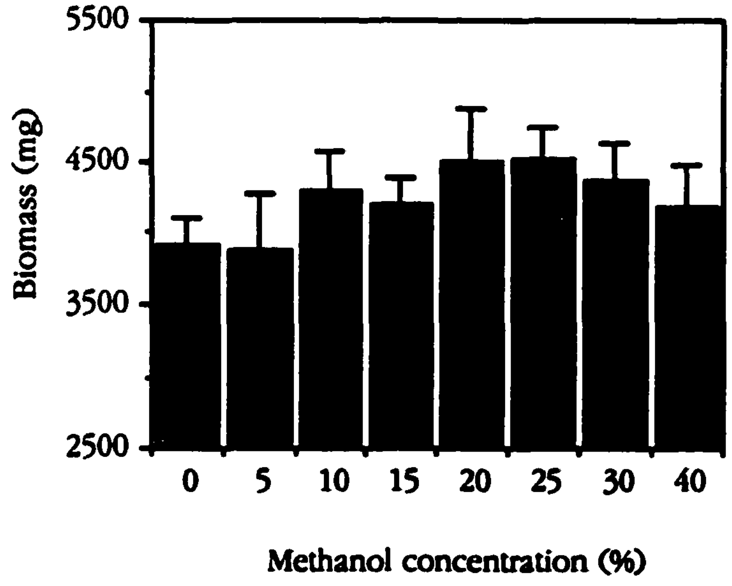

Figure 3.1 The effect of several methanol treatments (0% to 40%) on dry matter (biomass) accumulation in the shoot vegetative structures of cv. A301 seedlings. The control and methanol solutions were enriched with 0.2% w/v glycine and 0.5% w/v disodium D-L-glycerophoshate per liter. Error bars represent ± SE of means.

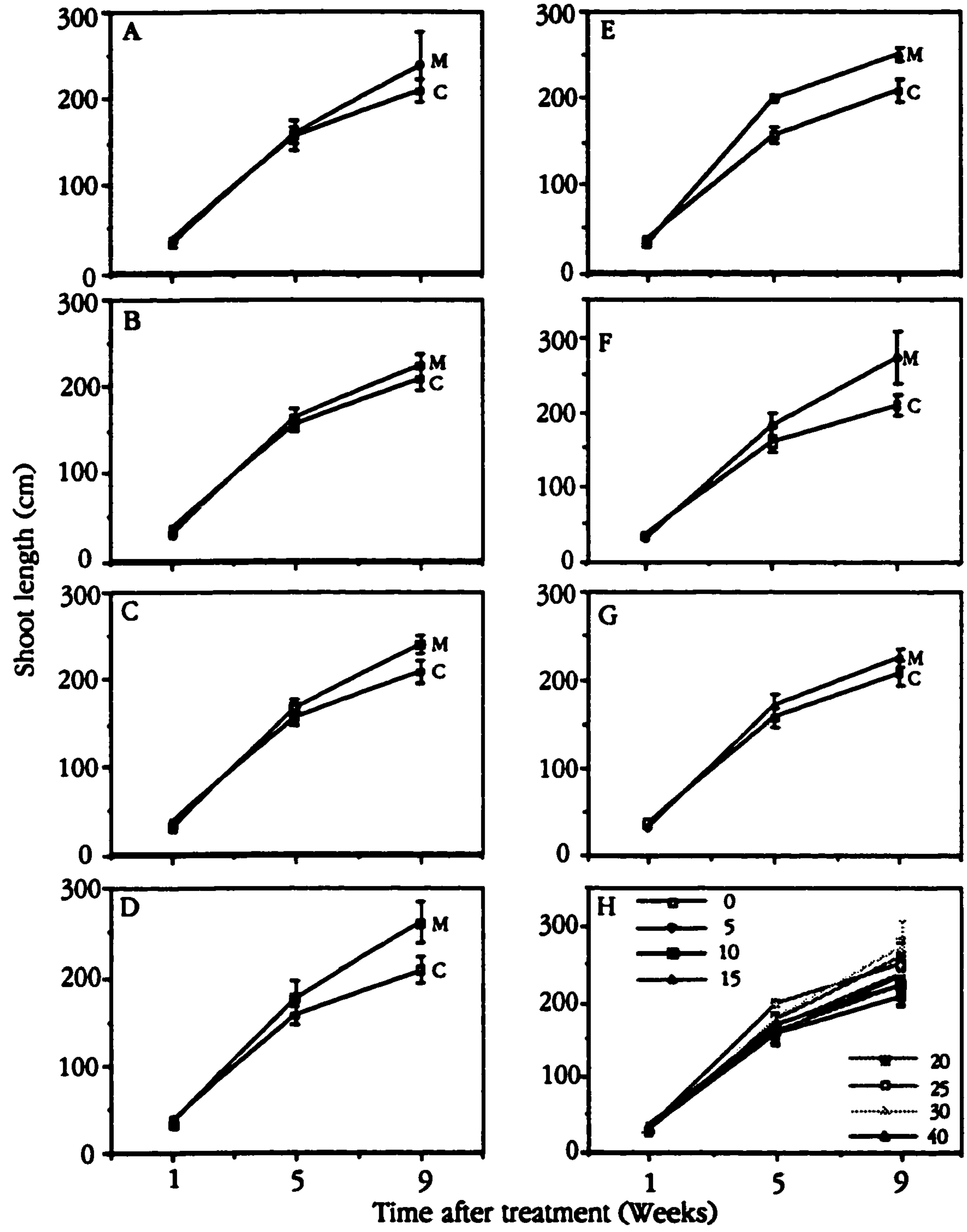

Figure 3.2 Shoot length of cv. A301 seedlings as a function of foliar-applied methanol concentrations ranging from 0% to 40%. A-G different concentrations of methanol are A , 5%. B, 10%. C, 15%. D, 20%. E, 25%. F, 30% and G, 40%. H represents pooled data for the A - G. In each graph C = control and M = methanol treated plants. Error bars represent mean ± SE. Where error bars are not visible, the error is too small to be shown on the scale of the graph.

Table 3.4 Growth stimulatory effect of foliar-applied 25% methanol on biomass accumulation (mean ± SE) in cv. A301 seedlings relative to higher and lower concentrations, 9 weeks after the initial treatment in a greenhouse.

Treatment (MeOH conc %)	Biomass (mg) (Mean ± SE)	df	*t*-Test	*P* value
0	3908.29 ± 202.64	6	-	-
5	3873.00 ± 406.30	5	0.081	.4683
10	4290.00 ± 280.84	6	1.102	.1460
15	4195.50 ± 196.05	5	1.009	.1672
20	4492.50 ± 386.47	5	1.398	.0948
25	4518.33 ± 227.75*	5	2.008	.0350
30	4359.50 ± 279.02	5	1.335	.1044
40	4186.00 ± 268.74	5	0.839	.2096

* Mean is significantly different from the control at $p \leq 0.05$

The analysis of the comparative effects of several methanol concentration 0% to 40% treatments on growth and development of seedlings based on shoot growth (cm), leaf development (number) and dry matter accumulation (biomass) in vegetative structures showed that 25% methanol treatment was the most effective treatment because it was only concentration that significantly stimulated all the three measured growth parameters at $p \leq 0.05$. On the basis of these results, the 25% methanol solution was the concentration used for all subsequent studies to study the effects of foliar-methanol treatment on growth, development and yields of rice plants *in vitro* experiments and in the greenhouse.

In vitro Experiments

Superior seedlings growth as a result of combined treatment with Q4 or Q5 strain inocula and foliar-applied methanol *in vitro*

The results obtained from *in vitro* experiments showed that the inoculation of 5 day old seedlings with either Q4 or Q5 strains stimulated growth and development of the treated seedlings over the control. *Indica* cv. A 301 data analysis revealed that Q5 strain stimulated biomass accumulation significantly ($t = 2.095$, $p = 0.034$), in an unpaired one tail Student's *t*-test (Fig. 3.3). The biomass accumulation increased by 41.2% in treated plants. The inoculation of Q5 also enhanced shoot growth (length in cm) significantly, in the same cultivar by 42.6% over the control (Fig. 3.4). The cv. A301 seedlings treated with plain 25% methanol solution in absence of any PPFM strain *in vitro* exhibited a relatively minimal stimulation of biomass productivity of 17.3% (Fig. 3.3) however the results were not significant at $p \leq 0.05$. The combined treatment of seedlings with foliar-applied 25% methanol together with the Q5 strain resulted in a significant [$t = 2.39$, $p = 0.05$ (Student's *t*-test)] 53.2% increase in biomass productivity (Fig 3.3), as indicated by mean values of 6.90 mg ± 0.58 in control relative to 10.56 mg ± 0.27 in Q5-MeOH 25% treated plants.

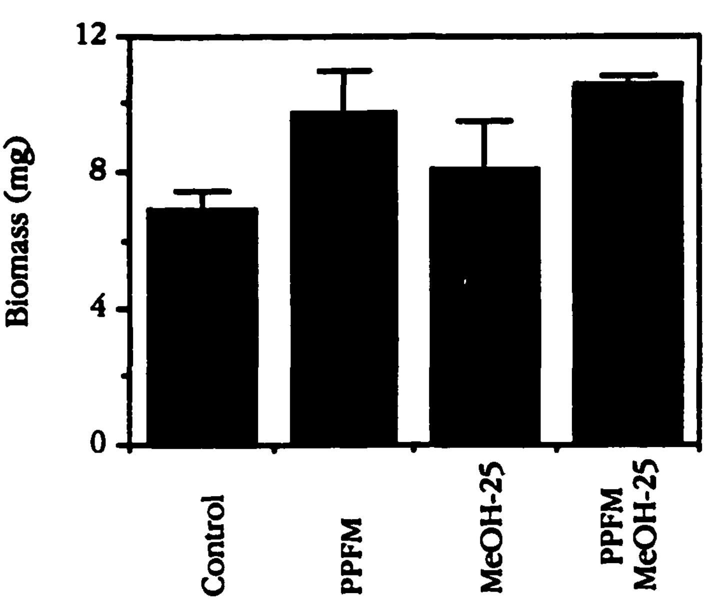

Figure 3.3 Comparative effect of independent and combined treatments with Q5 strain (PPFM) inoculation or foliar-applied 25% methanol (MeOH-25) on biomass accumulation in 14 day old *indica* cv. A301 seedlings *in vitro*. Combined treatment with Q5 and methanol is indicated by (PPFM MeOH-25). All seedlings were foliar-sprayed with methanol at day 2 after inoculation with Q5. Error bars indicate ± SE.

The inoculation of Q5 strain on seedlings also stimulated shoot growth (length in cm) significantly ($t = 2.318$, $p \leq 0.049$) by 42.1%, while treatment with 25% aqueous methanol stimulated shoot growth by 40.6%. A highly significant ($t = 3.374$, $p = 0.01$) 89.7% increase in shoot growth was recorded in seedlings treated subjected to combined treatment with Q5 strain inocula and 25% methanol solution (Fig. 3.4). The superior stimulation of biomass productivity and shoot growth following a combined treatment of seedlings with the isolated Q5 strain and foliar-applied methanol (Fig. 3.3 & 3.4) clearly demonstrated the beneficial effect of the presence of the Q5 cobiont (a PPFM strain), when plants are treated with foliar methanol spray. In nature all green plants leaf surfaces have been found to be colonized by PPFM (Corpe and Rheem 1989). These two observations, may explain the results obtained by Nonomura and Benson (1992), in which several agricultural crops such as watermelon and cabbage showed increase in growth rate and yield after treatment with methanol solutions in arid conditions.

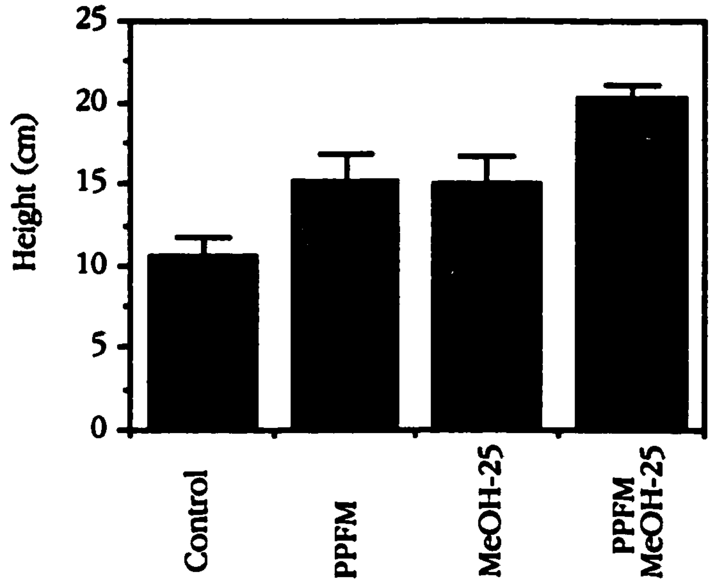

Figure 3.4 The effect of Q5 (PPFM) strain and foliar-applied methanol (MeOH-25) on shoot growth (cm) in 14 day old *indica* cv. A301 seedlings *in vitro*, 9 days after inoculation with Q5 strain or foliar treatment with 0.5 ml of 25% methanol solution. PPFM MeOH-25 represents the effect of combined treatment of seedlings with the Q5 strain and 25% methanol.. Error bars indicate ± SE.

The analysis of several measured growth parameters revealed that the response of cv. CR76 seedlings to the inoculation of Q4 strain was similar to the growth response patterns observed in cv. A301 cultivar following the inoculation of Q5 cobiont. The Q4 strain significantly stimulated biomass productivity (t =

2.773, p = 0.005) and leaf development (t = 2.374, p = 0.012) in cv. CR76 seedlings (Fig. 3.5 & Fig 3.6).

The biomass of 14 day old seedlings treated with the Q4 strain recorded as 13.85 ± 0.71 mg represented a 20.3% increase over the uninoculated controls whose biomass was 11.51 ± 0.49 mg. The recorded biomass of seedlings treated with foliar-applied 25% methanol solution was greater based on the mean relative to the control, however the differences between the mean values were not significant. Seedlings subjected to combined treatment with Q5 strain and 25% methanol registered a 9.5% (statistically non significant) increase in biomass productivity over the control.

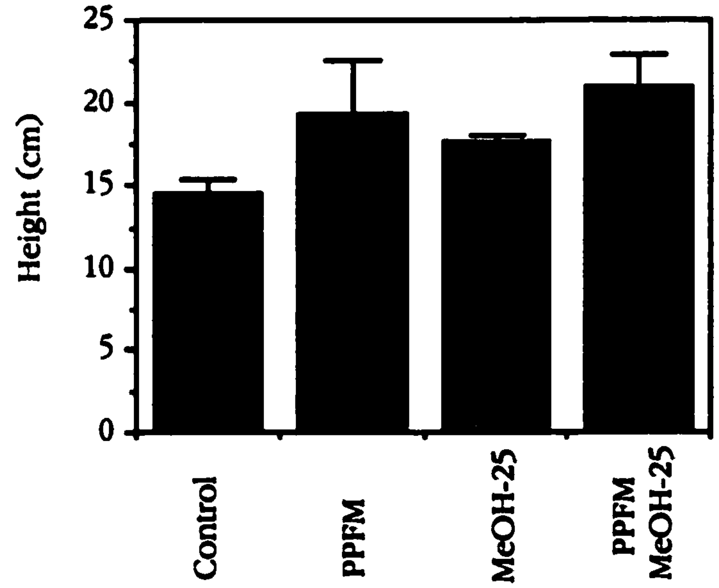

Figure 3.5 Effects of Q4 strain and foliar-applied methanol on shoot (stem) growth in 14 day old *japonica* cv. CR76 seedlings in vitro, 9 days after inoculation with Q4 and 5 days after foliar treatment with 0.5 ml of 25% methanol. solution. PPFM MeOH-25 represents the effect of combined treatment of seedlings with the Q4 strain and 25% methanol. Error bars indicate ± SE.

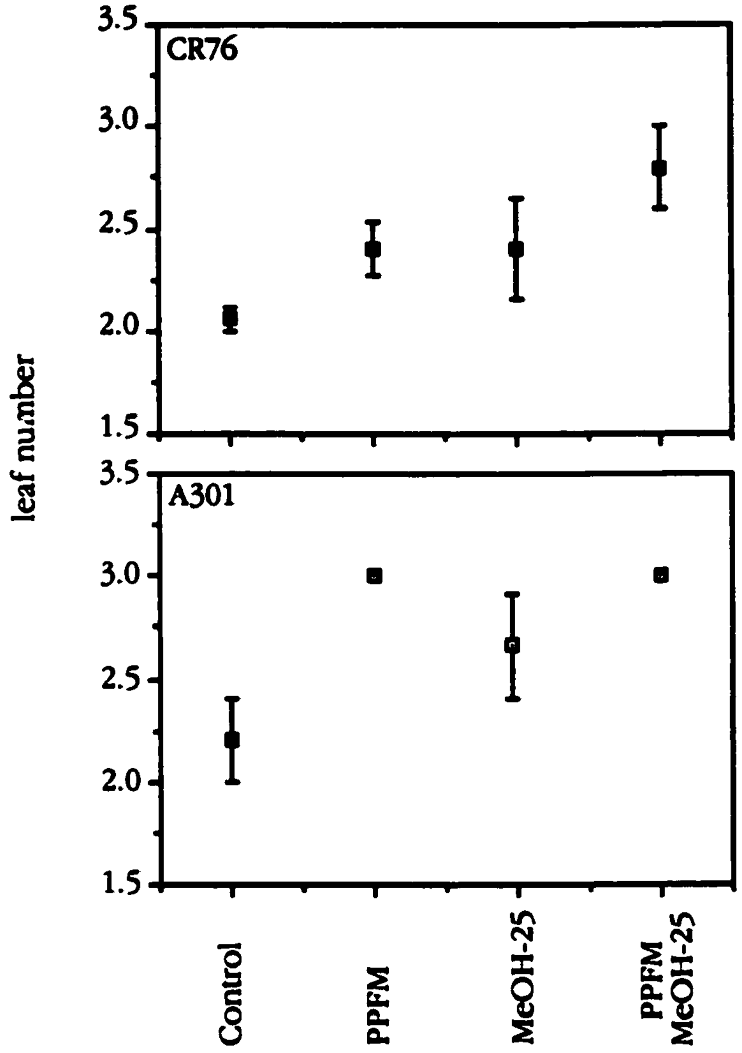

Figure 3.6 Leaf development in 14 day old *japonica* cv. CR76 and *indica* cv. A301 seedlings *in vitro*, 9 days after inoculation with Q4 strain (PPFM) on cv. CR76 and with Q5 strain on cv. A301. The effect of the combined treatment with Q4 or Q5 strain and the 25% Methanol solutions is indicated by PPFM MeOH-25. The solid square represents the mean and vertical bar ± SE of means. Where vertical bars are not indicated (A301), the standard errors were too small to be shown on the scale of the graph.

The process of leaf development in 14 day old cv. CR76 and cv. A301 seedlings was significantly ($p = 0.05$) enhanced by the inoculation of Q4 and Q5 strains respectively. A 16.5% increase in the rate of leaf development was observed in Q4 treated cv. CR76 plants over the control (Fig. 3.6). In addition, a highly significant ($t = 3.227$, $p = 0.01$) 35.9% stimulation of leaves development process was observed in cv. CR76 plants subjected combined treatment with Q4 bacterial strain and methanol (Fig. 3.6). The observed stimulation of the process of leaf development in cv. CR76 and cv. A301 represents an important finding because there exists a direct correlation between the number of leaves developed and the rate of photosynthetic process in green plants. In general the increase in the number of leaves resulted in an increase in the photosynthetic surface area.

Growth chamber experiments

The effect of Q4 and Q5 strains on rice and *Pisum sativum* (dicot) germination and seedlings survival

a) Treatment-response effects in rice cultivar, monocots

A study of the effects of several treatments including treatment with either Q4 or Q5 strains on the rate of seed germination at day 3 showed that the inoculation of the bacterial strains delayed the process of seeds germination relative to the control (Fig. 3.7), the effect was more evident in the pooled data (Fig. 3.8). However the differences between the seed germination and seedling survival in the treatments and the control were not significant at $p \leq 0.05$ (Fig. 3.8).

Pooled data of the treated rice seeds, cv. (A301, CR76 and IR36) and the garden pea cv. Alaska showed latency in seed germination and seedlings survival at day 3 in all treated seeds relative to the control (Fig. 3.8). However this pattern was reversed by day 5, in cv. A301, cv. Alaska pea and cv. CR76. This pattern was also observed in the pooled data (Fig. 3.8). Even though in some cases the mean values were higher in treated seeds, the results were not statistically significant. Based on the samples analyzed it was evident that these strains may not affect seed germination process. Germination is defined as the bursting of seed coat followed by emerging of either radicle or plumule or both.

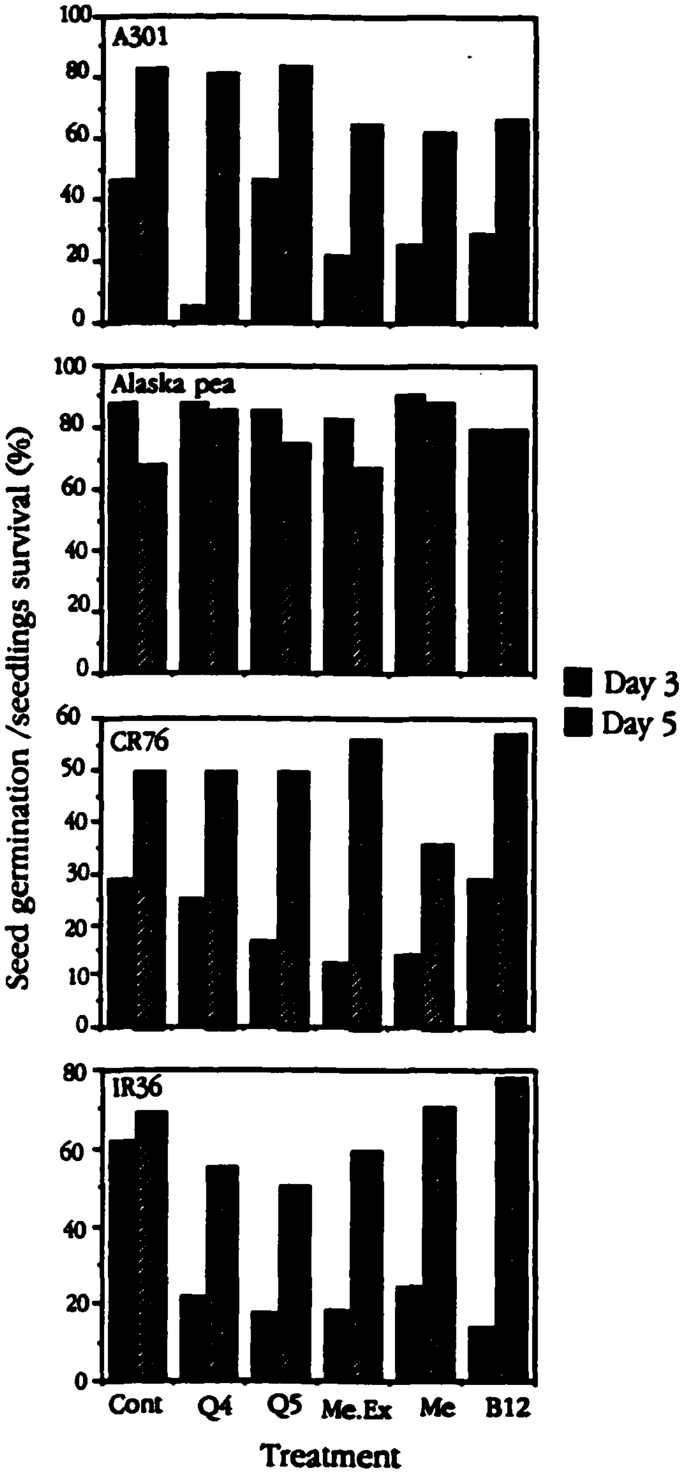

Figure 3.7 Comparative effects of several treatments including inoculation of Q4 and Q5 strains on seed germination and seedlings survival (%) of three rice cultivars and a garden pea at day 3 and 5 after seeding. Cont = control, Me.Ex = Q4 or Q5 media extract in which the strains had been cultured in to log phase. Me = sterile PPFM culture media and B12 = 5mg vitamin B12/ml/ seed set.

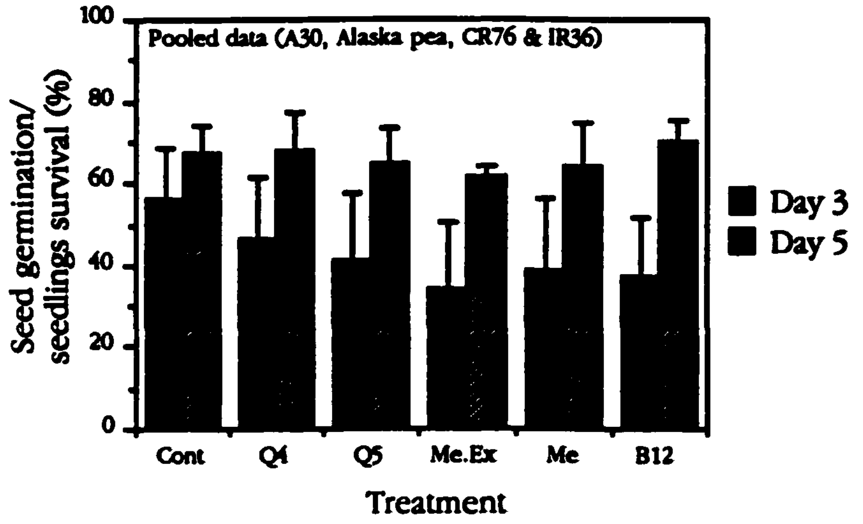

Figure 3.8 Comparative cumulative (pooled) effects of Q4, Q5 and several other treatments on seed germination and seedlings survival (%) of three rice cultivars and a garden pea at day 3 and 5 after seeding. Cont = control, Me.Ex = Q4 or Q5 media extract in which the strains had been cultured in to log phase, while Me = sterile PPFM culture media and B12 = 5mg vitamin B12/ml/ seed set. Error bars indicate ± SE.

In cv. CR76 seedlings, measurements of shoot length during day 10, 17 and 24 showed that at this stage of growth, seedlings germinated from seeds treated with Q5, B12 or Me.Ex showed significantly ($p \leq 0.05$) higher growth vigor compared to the control (Table 3.5). At day 10, Q4 strain, increased seedling growth vigor on the basis of shoot growth by 11.9% however the mean value was not significantly different from the control.

Leaf development stimulation by day 10 was significant in seedlings germinated from seeds treated with Q5 strain. In the other treatments the number of leaf mean values were higher relative to the control, but the increases were not statistically significant (Table 3.6).

Table 3.5 Shoot growth (length in cm) over a period of 24 days as affected by several treatments including the inoculation of Q4 and Q5 strains on cv. CR76 seeds, germinated and grown in a pasteurized soil in a growth chamber.

Cultivar	Day	Treatment	Shoot length (Mean ± SE)	df	*t*-Test	*P* value
CR76	10	control	3.12 ± 0.34	11	-	-
		Q4	3.49 ± 0.31	7	.763	.2270
		Q5	6.21 ± 0.26*	7	6.618	.0001
		B12	4.60 ± 0.46*	7	2.670	.0077
		Me.Ex	4.95 ± 0.46*	7	3.291	.0020
CR76	17	control	9.93 ± 0.94	11	-	-
		Q4	10.34 ± 1.20	6	.267	.3960
		Q5	17.26 ± 0.88*	7	5.401	.0001
		B12	14.35 ± 1.13*	7	2.280	.0060
		Me.Ex	14.86 ± 1.52*	7	2.932	.0045
CR76	24	control	22.25 ± 1.11	11	-	-
		Q4	22.01 ± 1.88	6	.113	.4560
		Q5	28.70 ± 0.94*	7	4.150	.0003
		B12	27.94 ± 1.82*	7	2.820	.0061
		Me.Ex	27.08 ± 1.76*	7	2.440	.0130

* Mean is significantly different from the control at $p \leq 0.05$

The Q4 and Q5 were applied as "seed dressing" inoculations, other seed sets were imbibed in vitamin B12 (5.0mg/ml) solution, or in filter sterilized media extract (MeEx) in which Q4 and Q5 had been grown to log phase

Table 3.6 The effects of several treatments including Q5 strain inoculant on cv. CR76 leaf development (number), over a period of 24 days after seeding in pasteurized soil in a growth chamber.

Cultivar	Day	Treatment	Leaf number (Mean ± SE)	df	*t*-Test	*P* value
CR76	10	control	12.09 ± 0.88	10	-	-
		Q5	14.13 ± 0.52*	7	1.807	.0442
		B12	13.88 ± 0.88	7	1.460	.0897
		Me.Ex	12.13 ± 0.85	7	.027	.4890

* Mean is significantly different from the control at $p \leq 0.05$

The Q5 was applied as "seed dressing" inoculant, other seed sets were imbibed in vitamin B12 (5.0mg/ml) solution, or in filter sterilized media extract (MeEx) in Q5 had been grown to log phase

The A301 is a late maturing rice cultivar relative to cv. CR76 which matures earlier during a growth cycle. In a pattern similar to its slow growth and development process, the response of cv. A301 seedlings to treatments was similarly slow. At day 10, the shoot length of the cv. A301 seedlings treated with either Q4, Q5 or B12 was not significantly different from the the control (Table 3.6).

At day 24, seedlings germinated from seeds inoculated with Q4 strain showed a 5.6% increase in shoot growth relative to the control, while a 3.1% increase in shoot growth was recorded in seedlings germinated from seeds inoculated with Q5 strains. In both cases the enhanced growth rate was not significant (Table 3.7). In a similar pattern leaf development was not significantly affected by any of the treatments (Table 3.8). This pattern of growth and the slow treatment-response effect observed in cv. A301 seedlings may be attributed to the dwarf morphology of the A301 rice cultivar.

Table 3.7 Shoot growth (length in cm) over a period of 24 days as affected by several treatments including the inoculation of Q4 and Q5 strains on cv. A301 seeds, germinated and grown in a pasteurized soil in a growth chamber.

Cultivar	Day	Treatment	Shoot length (Mean ± SE)	df	*t*-Test	*P* value
A301	10	control	4.14 ± 0.28	11	-	-
		Q4	4.34 ± 0.38	7	.447	.3290
		Q5	4.03 ± 0.17	7	.316	.3780
		B12	3.66 ± 0.22	7	1.248	.1140
A301	17	control	11.99 ± 0.38	11	-	-
		Q4	12.69 ± 0.57	6	1.061	.1530
		Q5	11.85 ± 0.55	7	.226	.4140
		B12	10.98 ± 0.35*	7	1.867	.0391
A301	24	control	20.32 ± 0.49	11	-	-
		Q4	21.46 ± 0.75	6	1.320	.1020
		Q5	20.94 ± 0.65	7	.770	.2260
		B12	19.58 ± 0.34	7	1.101	.4260

* Mean is significantly different from the control at $p \leq 0.05$

The Q4 and Q5 were applied as "seed dressing" inoculations, other seed sets were imbibed in vitamin B12 (5.0mg/ml) solution.

Table 3.8 The effects of several treatments including Q4 and Q5 strain inoculant on cv. A301 leaf development (number), over a period of 24 days after seeding in pasteurized soil in a growth chamber.

Cultivar	Day	Treatment	Leaf number (Mean ± SE)	df	*t*-Test	*P* value
A301	10	control	11.67 ± 0.63	10	-	-
		Q4	11.29 ± 0.89	7	.356	.3630
		Q5	11.75 ± 1.08	7	.071	.4720
		B12	11.38 ± 0.68	7	.306	.3816

* Mean is significantly different from the control at $p \leq 0.05$

The Q4 and Q5 were applied as "seed dressing" inoculations, the other seed sets were either imbibed in vitamin B12 5mg/ml solution.

b) Treatment-response effect in a garden pea cultivar, a dicot.

Garden pea plants, germinated from the seeds inoculated ("seed dressed") with either Q4 or Q5 strain cell suspensions, exhibited positive growth and development response as indicated by early flowering capacity, biomass productivity and absolute grain yield (Table 3.9).

Data analysis showed that inoculation of the microbial strains onto seeds in form of "seed dressing" could induce early flowering in mature plants (Table 3.9). Flowering occured 5 days earlier in plants treated with the Q4 strain, at day 29.75 ± 1.83 relative to the control which flowered on day 34.67 ± 2.15, the mean values differences were significant ($t = 1.739$, $p \leq 0.05$) in an unpaired one-tail Student's *t*-test. Plants germinated from seeds treated with Q5 strain exhibited 3 days earlier flowering relative to the control, however the, Q5 induced early flowering was not significant. Treatment of seeds with MeAmS basal salts prior to germination had no significant effect on flowering period.

Table 3.9 Measured growth and development parameters of garden pea *Pisum sativum*, cv. Alaska pea as affected by treatment with Q4 and Q5 strains "seed dressing" inoculated at seeding or imbibition of seeds in MeAmS basal salts.

Growth parameters	Treatment	Mean ± SE	df	*t*-Test	*P* value
Flowering Time (Days)	control	34.67 ± 2.15	11	-	-
	Q4	29.75 ± 1.83*	11	1.739	.0480
	Q5	32.08 ± 1.65	11	.952	.1756
	Me.	37.86 ± 2.04	6	.986	.1691
Biomass (g)	control	1.27 ± 0.098	11	-	-
	Q4	1.34 ± 0.132	11	.391	.3499
	Q5	1.23 ± 0.094	11	.335	.3703
	Me.	1.24 ± 0.093	5	.224	.4127
Yield (g)	control	0.50 ± 0.039	11	-	-
	Q4	0.60 ± 0.056*	11	1.376	.0914
	Q5	0.47 ± 0.065	11	.404	.3449
	Me.	0.41 ± 0.053*	6	.409	.0968

* Mean is significantly different from the control at $p \leq 0.05$

The Q4 and Q5 were applied as "seed dressing" inoculations, other seeds sets were imbibed in a filter sterilized methanol ammonium basal salts medium (Me). Growth and development of plants was monitored over the entire growth cycle.

The pre-germination treatment of seeds with the isolated Q4 or Q5 bacterial strains resulted in an enhanced growth and development of garden pea plants. Even though significant stimulation of early flowering was observed, the 5.5% increase in biomass productivity and the 20% increase in yields over the controls were not significant at $p \leq 0.05$. (Table 3.9). Pre-treatment of seeds with either the Q5 strain or the bacterial culture basal salt media (Me.) showed no stimulatory effect on both biomass accumulation and absolute yields.

Greenhouse Experiments II: Rice Vegetative Growth and Yields

Effects of methanol treatments on mature plants leaf chlorophyll content.

a) In tropical rice cultivars.

Analysis of leaf chlorophyll content (ug/cm^2) showed that foliar-treatment of rice plants with methanol significantly increased chlorophyll *a* content ($t = 2.215$, $p = 0.05$ in Basmati 370 and ($t = 11.986$, $p = 0.001$) in Pishori cultivars in unpaired one-tail: Students *t*-test, (Table 3.10). The increases in chlorophyll *a* contents were 4.9% in Basmati 370, 25.8% in Pishori. In IR36 cultivar, the recorded 1.5% increase in leaf chlorophyll *a* in treated plants was not statistically significant ($p = 0.362$). However, the response pattern was similar to that observed in the Basmati 370 and Pishori cultivars (Table 10).

In all the three tropical cultivars tested (Fig. 3.9), it was found that the total chlorophyll content was relatively higher in methanol treated plants than in the controls.

Table 3.10 Effect of foliar-applied methanol (MeOH), on leaf chlorophyll content ug/cm^2: mean with SE, in three tropical rice cultivars. Column *n* is the sample size

Cultivar	Chlorophyll	Control Mean ± SE	n	MeOH Mean ± SE	n	*t*-test	df	*P* value
Basmati	*a*	29.83 ± 0.41	6	31.30 ± 0.53*	6	2.215	10	0.0511
370	*b*	8.92 ± 0.19	6	9.17 ± 0.18	6	0.902	10	0.1940
	total	38.90 ± 0.50	6	40.25 ± 0.68	6	1.596	10	0.0709
IR 36	*a*	41.97 ± 1.25	7	42.59 ± 1.22	7	0.361	12	0.3620
	b	12.05 ± 0.56	7	12.03 ± 0.42	7	0.030	12	0.4883
	total	53.83 ± 1.62	7	54..26 ± 1.45	7	0.197	12	0.4238
Pishori	*a*	28.22 ± 0.26	8	35.49 ± 0.55**	8	11.986	14	0.0001
	b	7.79 ± 0.15	8	9.80 ± 0.22**	8	7.589	14	0.0001
	total	35.95 ± 0.40	8	45.15 ± 0.53**	8	13.915	14	0.0001

* Mean is significantly different from the control ($p \leq 0.05$)

** Mean is significantly different from the control ($p \leq 0.001$)

- The cultivars represented three geographical regions Basmati 370 (India), IR 36 (Philippines), and Pishori (Kenya, East Africa).
- The treatments supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l were foliar-applied to young rice plants during week 4 and week 11 after seeding. The chlorophyll content was determined during week 4 after the second treatment.
- p-values were derived from unpaired one-tail comparison of means

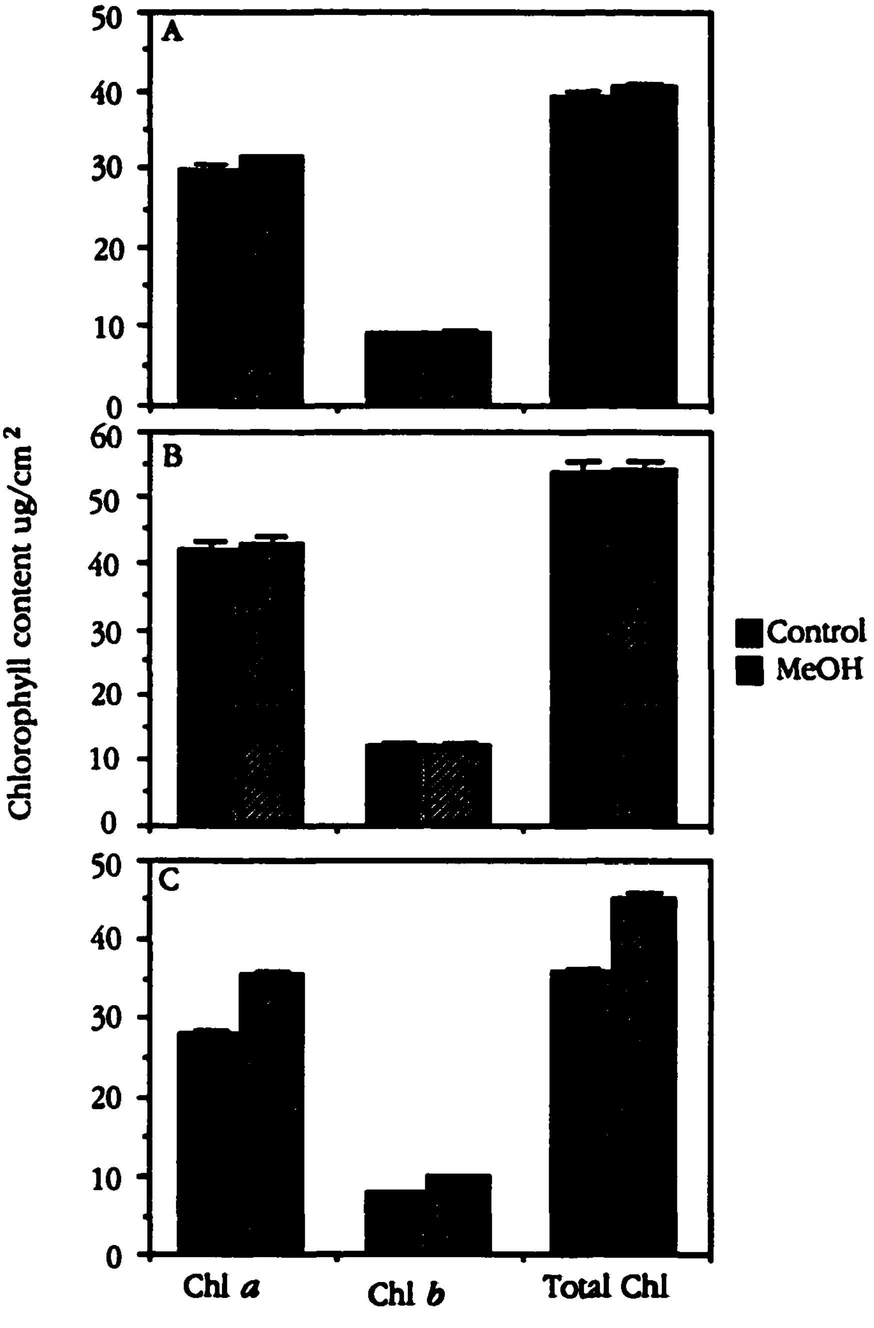

Figure 3.9 Comparison of total chlorophyll (Total Chl), chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) content (ug/cm^2) in the leaf of tropical cultivars, following treatment with foliar-applied methanol.. A. = Basmati 370., B. = IR36., and C. = Pishori (Basmati 217). Where error bars are not visible, the ± SE at $p \leq 0.05$ is too small to be shown on the scale of the graph.

b) In temperate rice cultivars.

The cv. CR76 and cv. A301 plants treated with foliar-applied methanol had significantly higher leaf chlorophyll *a* and total chlorophyll content (ug/cm^2) than the controls (Fig. 3.10). The 55.1% increase in leaf chlorophyll *a* in treated cv. CR76 plants to 34.88 ± 2.30 ug/cm^2 relative to 22.48 ± 0.54 ug/cm^2 in control was significant [F = 27.38, df = 12, p = 0.0002 (one factor ANOVA)]. However in cv. A301, the observed 5.6% increase in leaf chlorophyll *a* content in treated plants to 38.54 ± 1.05 ug/cm^2 relative to 36.49 ± 1.05 ug/cm^2 in control was not statistically significant p = 0.285.

In cv. CR76, the total chlorophyll content in treated plants significantly increased by 59.98% [F = 299.68, df = 12, p = 0.001 (one factor ANOVA)], compared to the control. In cv. A301. the observed 5.49% increase in total chlorophyll content in treated plants was not significant (p = 0.313). Further analysis revealed that there was a significant 65.08% increase [F = 31.99, df = 12, p = 0.001 (one factor ANOVA)], in leaf chlorophyll *b* content in treated CR76 cultivar. In cv. A301 the observed 7.61% increase in leaf chlorophyll *b* content was not statistically significant.

In addition, further observation suggested that the the mature plant morphology predetermines the effect of foliar-applied methanol on leaf chlorophyll content. The slow response of the semi-dwarf temperate cv. A301 was similar to slow response observed in cv. IR36, a semi-dwarf tropical cultivar. In contrast, significant effects at $p \leq 0.05$ were observed in the medium height tropical and temperate cultivars, cv. Basmati 370, cv. pishori and cv. CR76.

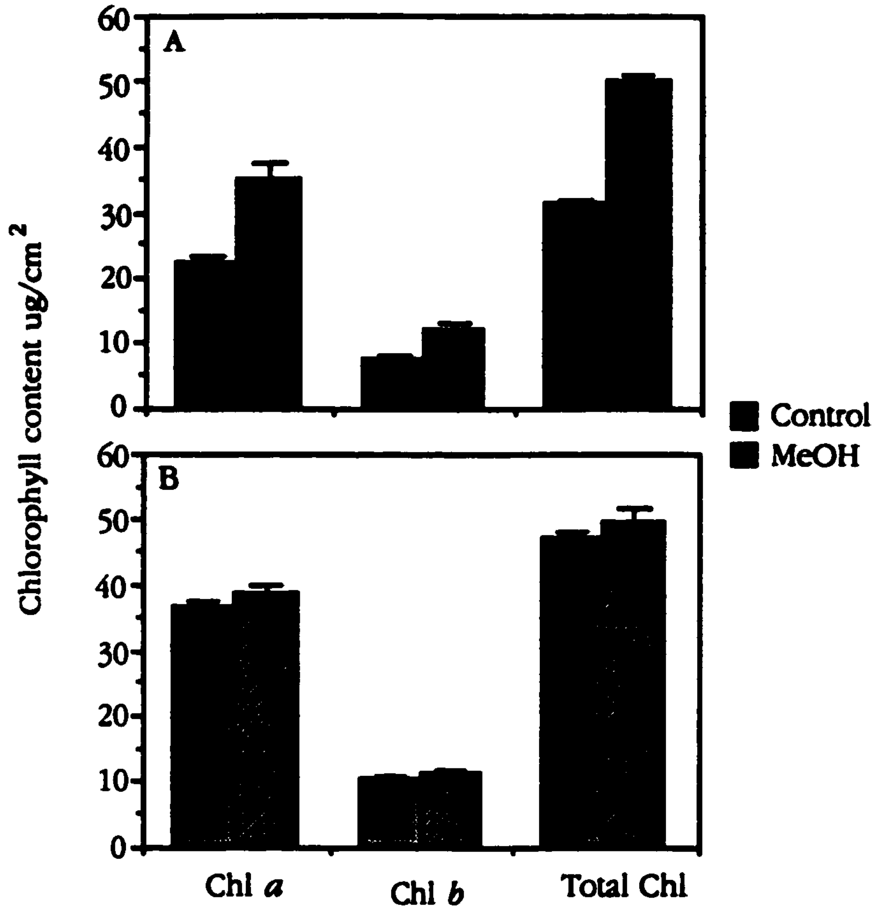

Figure 3.10 Relative total chlorophyll (Total Chl), chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) content in temperate cultivars, after treatment with foliar-applied methanol. A = CR76 and B = A301. Where error bars are not visible, the ± SE at $p \leq 0.05$ is too small to be shown on the scale of the graph.

The data analysis also showed that the response of rice to foliar-applied methanol is not subspecies specific. Of the five cultivars treated, two tropical cultivars (Basmati 370 & Pishori) of the *indica* subspecies and one temperate cultivar (CR76) of the *japonica* subspecies showed significant increase in leaf chlorophyll content in response to foliar-applied methanol.

The effects of methanol treatments on methylotrophic bacteria population on leaf surface

The normal microflora developing on healthy rice plants leaf surface is diverse and varied in composition. Using the reported MeAMS-agar selective media, formulated to favor maximum growth of methylotrophic bacteria strains, an increase in the relative number of PPFM on leaf surface of plants treated with the foliar-applied methanol was observed on day 15 after treatment (Table 3.11). Although the results (based on standard plate count) were not significant, this is mainly attributed to the small sample sizes in each treatment group. In all the three cultivars approximately 95% of the heterotrophic plate count was comprised of the PPFM.

Table 3.11 The population of methylotrophic bacteria (PPFM) colonies on leaf surface per cm^2 at day 5 and 15 after treatment, ± SE represents standard error of the mean. The n = 4 in control and MeOH samples for each cultivar, in the pooled data n = 12 in the control and treatment groups.

Cultivar	Day	Control	MeOH	*t*-Test	df	*p*-value
IR36	5	433.50 ± 21.42	501.33 ± 74.46	2.314	6	0.059
	15	526.83 ± 86.19	1030.20 ± 212.16	2.068	6	0.084
CR76	5	545.19 ± 111.69	391.17 ± 81.09	0.511	6	0.628
	15	398.31 ± 104.55	553.35 ± 126.99	0.942	6	0.382
A301	5	592.17 ± 65.84	497.25 ± 53.55	2.103	6	0.080
	15	402.39 ± 80.58	244.29 ± 44.88	2.065	6	0.085
Pooled data	5	437.07 ± 44.37	527.85 ± 46.92	1.441	22	0.164
[IR36,CR76,A301]	15	550.80 ± 60.69	666.57 ± 125.46	1.054	22	0.303

The PPFM colonies were determined using standard plate count technique.

The cv. A301 plants were infected by mites (pest) during the experiments, the infection is likely to be responsible for the decline in PPFM population on the leaf surface on day 15 in both the control and experimental group.

Results based on leaf surface impression experiments showed that foliar-methanol treatments of the three rice cultivars significantly stimulated methylotrophic bacteria colonies population shift on day 10 in IR36 cultivar and on day 5 in CR76 cultivar (Fig. 3.11). In A301 cultivar, a gradual increase in methylotrophic bacteria population was observed (Fig. 3.11), although statistically non significant, the positive shift reached peak at day 10 and the trend was similar to that observed in IR36 and CR76 cultivars.

In addition, the data obtained from leaf surface impression experiments showed that PPFM colonies averaged 75% of the total methylotrophic bacteria count/cm^2 of rice leaf surface. Other isolated colonies were yellow-orange pigmented and white. In some of the plates fungal growth was observed despite the addition of cycloheximide (antifungal compound) to the MeAmS-agar medium

The findings on positive PPFM population shift in greenhouse grown plants treated with the foliar-applied are important. In a related study, the results on *in vitro* experiments (axenic) showed that combined treatment of rice seedlings with foliar-applied methanol and Q4 or Q5 (PPFM) strains resulted in superior stimulation of growth than either treatment alone as shown by higher mean values (Fig. 3.3, Fig 3.4 & Fig. 3.5). The growth and development stimulatory effect following methanol treatment of rice plants in greenhouse (Fig. 3.14) and *in vitro* experiments (Fig. 3.3), is partly mediated by inoculated PPFM (Q4 or Q5) strain or by the PPFM that colonize rice plant leaf surfaces in nature.

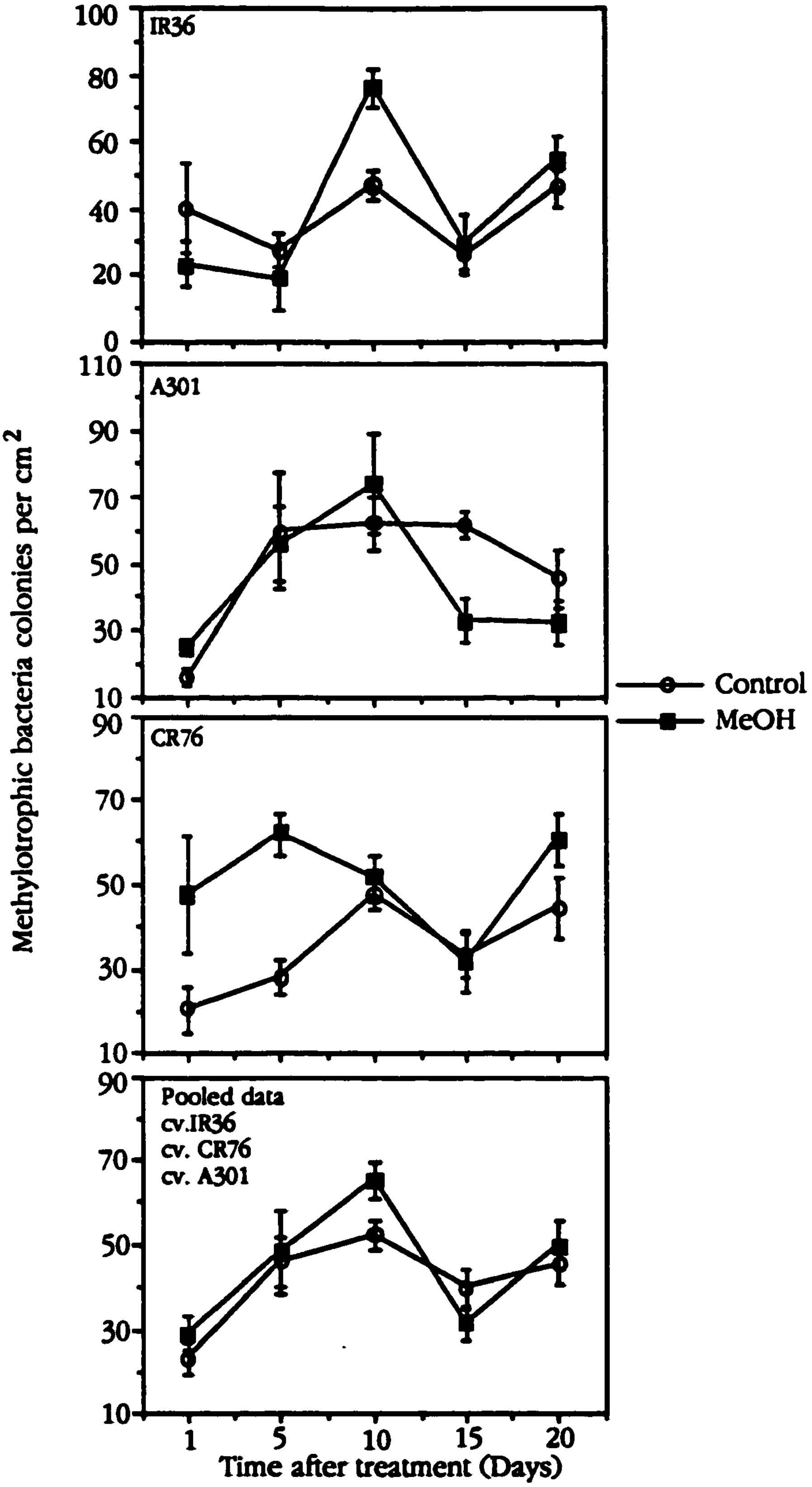

Figure 3. 11 The population of methylotrophic bacteria on leaf surface mean ± SE per cm^2 as a function of time after treatment. The colonies were isolated by leaf surface impression technique on selective MeAMS-agar media.

The effects of methanol treatments on partitioning of biomass to vegetative and reproductive (grain) structures

a) Quantitative experiments: temperate rice cultivar

I) Vegetative structures

In CR76 and A301 temperate cultivars, the growth stimulatory effect of methanol treatment was more evident on week 17 (Fig. 3.12 & Fig 3.13). In CR76 cultivar, data analysis showed that there was significant stimulation of growth and development in the greenhouse grown mature plants as indicated by shoot growth [length in cm (F = 55.525, df = 27, p = 0.001), leaf number per plant (F = 47.402, df = 27, p = 0.001), and tiller number (F = 30.655, df = 27, p = 0.001),in treated plants relative to the control group (Fig. 3.12).

In the elite A301 semi-dwarf cultivar methanol treatments significantly stimulated leaf development during week 17 (F = 7.656, df = 26, p = 0.005), and the shoot growth (F = 4.712, df = 26, p = 0.039) as well as during week 9 (Fig. 3.13). In contrast, the tiller development (number) process in the same cultivar was not significantly (p = 0.361) affected by the treatments (Fig. 3.13). However, a minimal stimulation of tiller formation during week 17 was observed in treated plants relative to the controls.

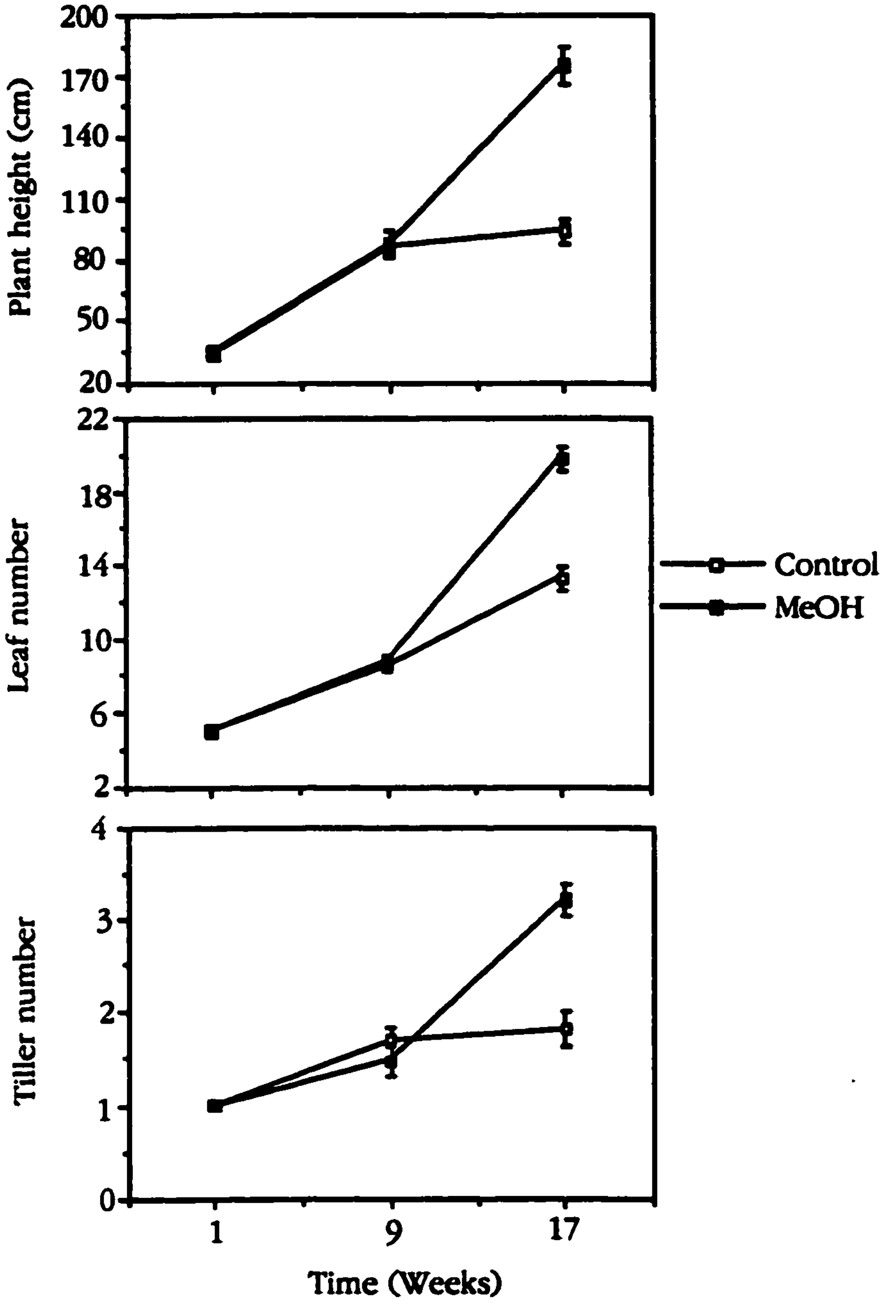

Figure 3.12 The effect of foliar-applied methanol on tillering capacity, leaf development and shoot growth (plant height in cm) of greenhouse grown cv. CR76, a temperate cultivar. Error bars indicate ± SE of means at $p \leq 0.05$.

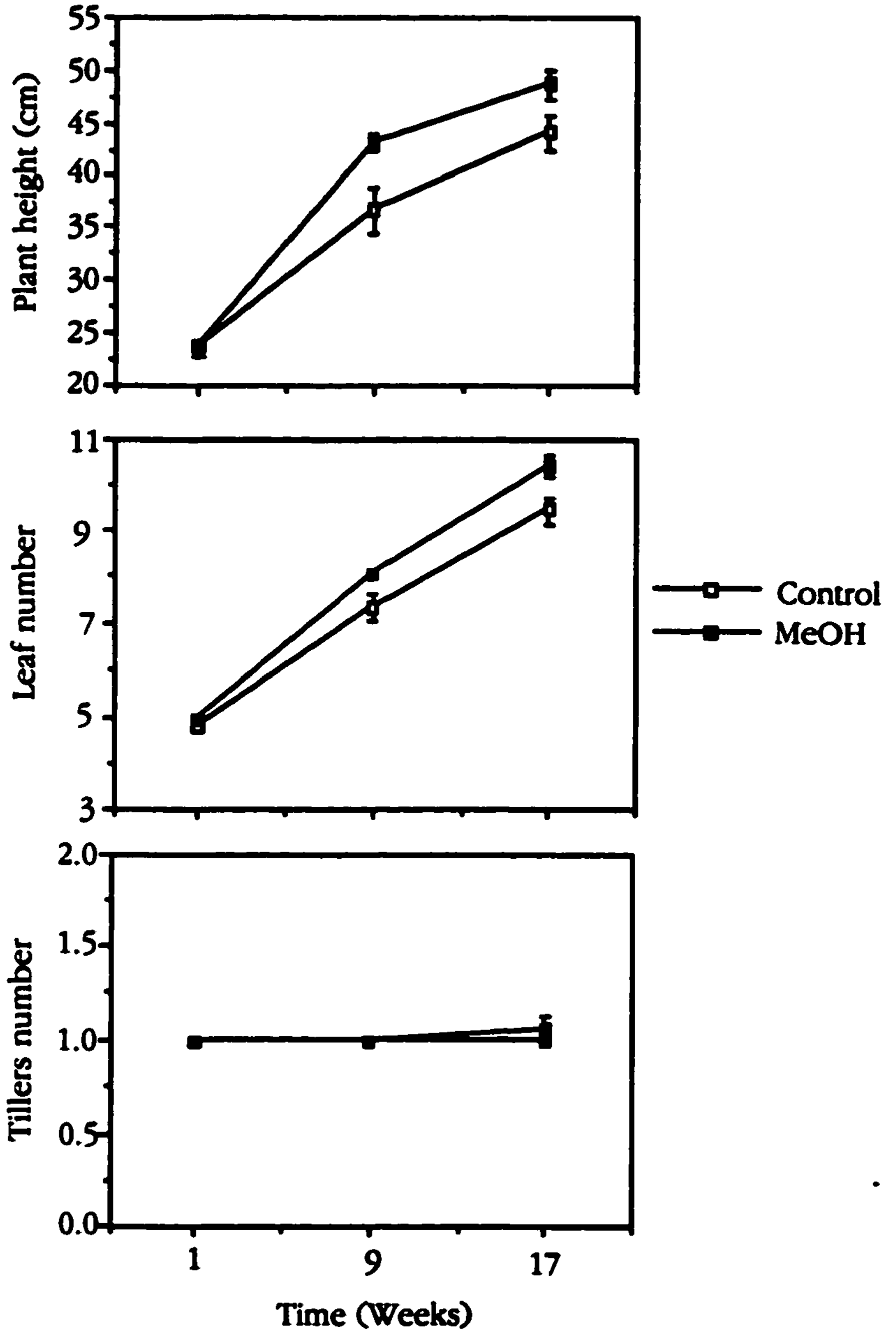

Figure 3.13 The effect of foliar-applied methanol on tillering capacity, leaf development and shoot growth (shoot length) of greenhouse grown cv. A301, a temperate cultivar. Error bars indicate ± SE of means at $p \leq 0.05$.

II) Biomass productivity

Analysis of biomass accumulation in vegetative structures showed that methanol treatment stimulated the process of dry matter accumulation in rice plants significantly (Table 3.12). In treated cv. CR76 plants, a significant ($p \leq 0.01$, Students *t*-test) 25.6% increase in dry matter accumulation was recorded in shoot structures and 17.9% in root structures, over the control.

Table 3.12 Effect of foliar-applied methanol treatment on shoot and root biomass in g (mean ±SE) of greenhouse grown cv. CR 76, a temperate cultivar

Growth parameter	Control (Mean ± SE) n = 13	MeOH (Mean ± SE) n = 12	*t*-Test	df	*P* value
Shoot biomass	1.21 ± 0.04	1.52 ± 0.06••	4.253	23	0.0001
Root biomass	0.56 ± 0.03	0.66 ± 0.03•	2.470	23	0.0100

• Means is significantly different ($p \leq 0.05$)
•• Means is highly significantly different ($p \leq 0.001$)

The p-values were based on unpaired one-tail comparison of means, and all the treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l. The treatments were foliar-applied to young rice plants during week 4 and week 11 after seeding.

Analysis of biomass productivity in cv. A301 vegetative structures showed that the response pattern of this cultivar was similar to that of treated cv. CR76 plants. The foliar-treatment of the cv.A301 plants with methanol stimulated dry matter accumulation in the vegetative structures (Table 3.13). A significant 17.6% increase ($t = 2.257$, $df = 26$, $p = 0.045$) in dry matter accumulation was recorded in shoot (vegetative) structures. However the observed 17.5% increase in dry matter (biomass) accumulation in root structures of treated plants was not statistically significant ($p = 0.087$).

Table 3.13 Effect of foliar-applied methanol treatment on shoot and root biomass in g (mean ± SE) of greenhouse grown cv. A301, a temperate cultivar

Growth parameter	Control (Mean ± SE) n = 13	MeOH (Mean ± SE) n = 15	*t*-Test	df	*P* value
Shoot biomass	0.603 ± 0.04	0.709 ± 0.03*	2.257	26	0.0445
Root biomass	0.269 ± 0.03	0.316 ± 0.02	1.400	26	0.0867

* Means is significantly different from the control ($p \leq 0.05$)

The p-values were based on unpaired one-tail comparison of means, and all the treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l. The treatments were foliar-applied to young rice plants during week 4 and week 11 after seeding.

The comparison of biomass accumulation pattern in vegetative and root structures of the two temperate CR76 and A301 exhibited some similarities (Fig. 3.14). It was found out that biomass accumulation and its partitioning into the vegetative shoot and root structure increased significantly in methanol treated plants (Fig. 3.14). On average, the mean values were lower for the elite semi-dwarf A301 cultivar relative to the medium height CR76 cultivar. However, consistency in the pattern of the dry matter accumulation in treated versus control plants was evident. In both cv. CR76 and cv. A301, the rate of partitioning of dry matter to the photosynthetic shoot (vegetative) structure and the root structure was at the ratio of 2 : 1. On average the roots network was relatively greater in treated plants compared to the controls. In general the foliar-applied methanol affected development of both the root system and the shoot vegetative structure.

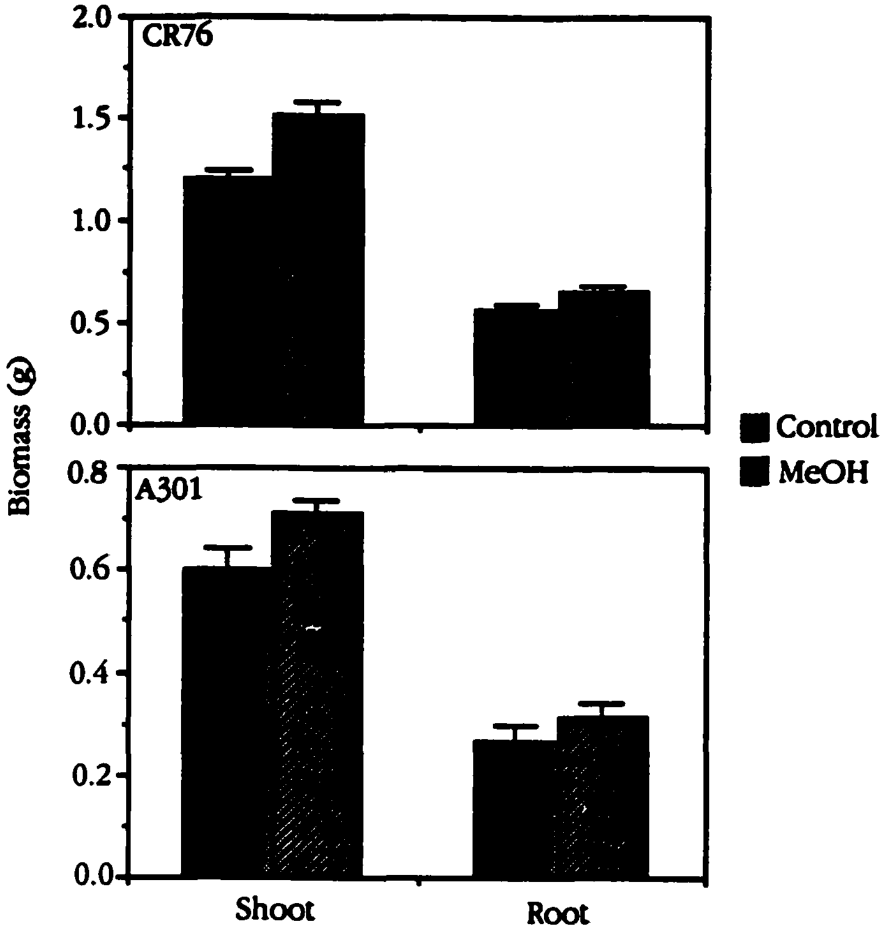

Figure 3.14 A comparison of the effect of foliar-applied methanol treatments on biomass accumulation in vegetative (shoot) and root structure in two temperate cultivars, cv. CR76 and cv. A301. Error bars indicate ± SE of means.

b)Quantitative experiments: tropical rice cultivars

I) Vegetative structures

The stimulatory effects of foliar-methanol treatments on growth and development of the three tropical cultivars IR36, Pishori and Basmati 370 was more evident on week 17. In general, the growth stimulatory induced partly by methanol treatments was exhibited in the three growth parameters measured, namely shoot growth (length in cm), leaf development (number) and tiller number per plant (Fig. 3.15, 3.16 & 3.17). In the elite semi-dwarf IR36 cultivar, significant [$p = 0.05$ (unpaired one tail Student's *t*-test)] increases in all the three measured growth parameters were observed in the treated plants during week 17 (Fig. 3.15).

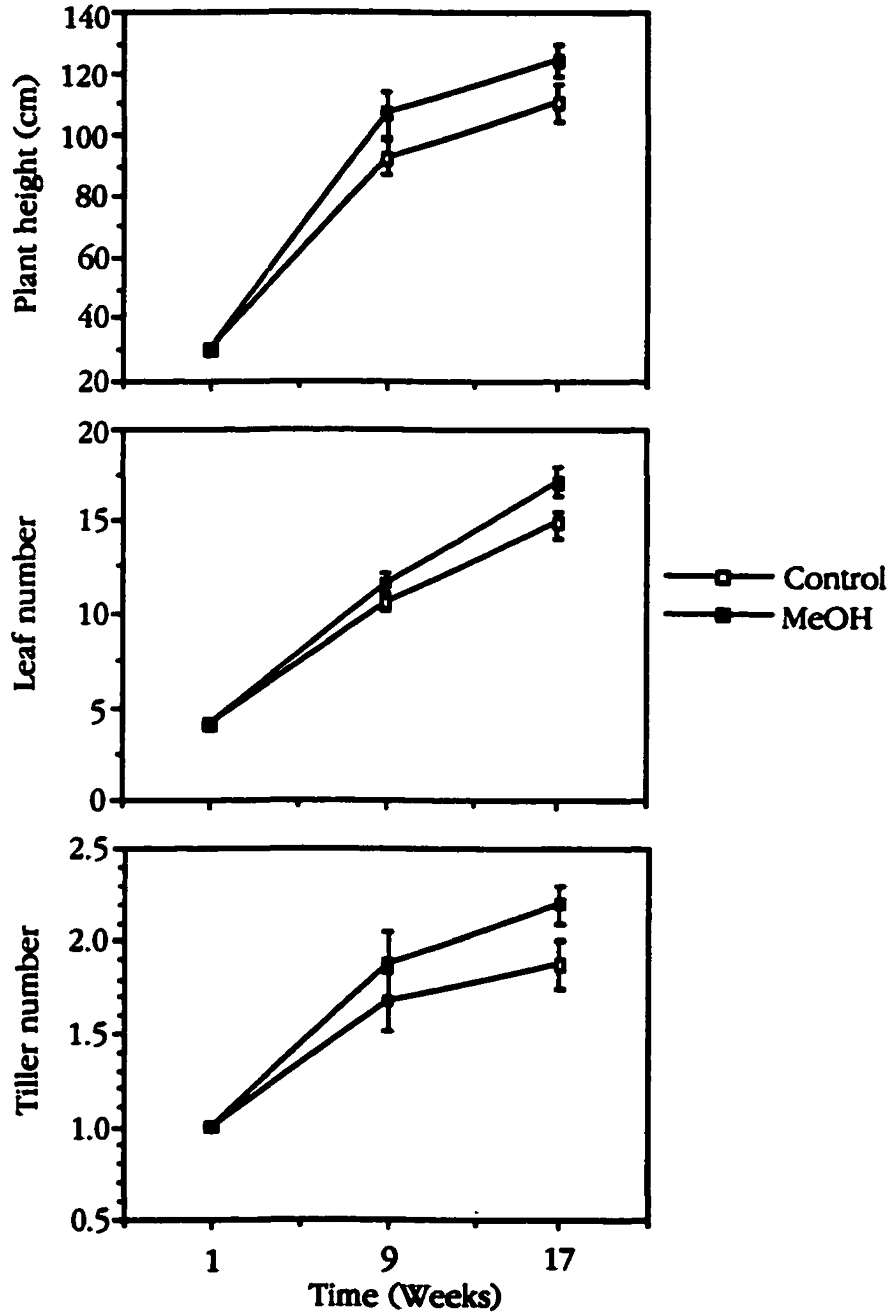

Figure 3.15 The effect of foliar-applied methanol on tillering capacity, leaf development and shoot growth (shoot length) of greenhouse-grown cv. IR36, a tropical cultivar. Error bars indicate ± SE of means.

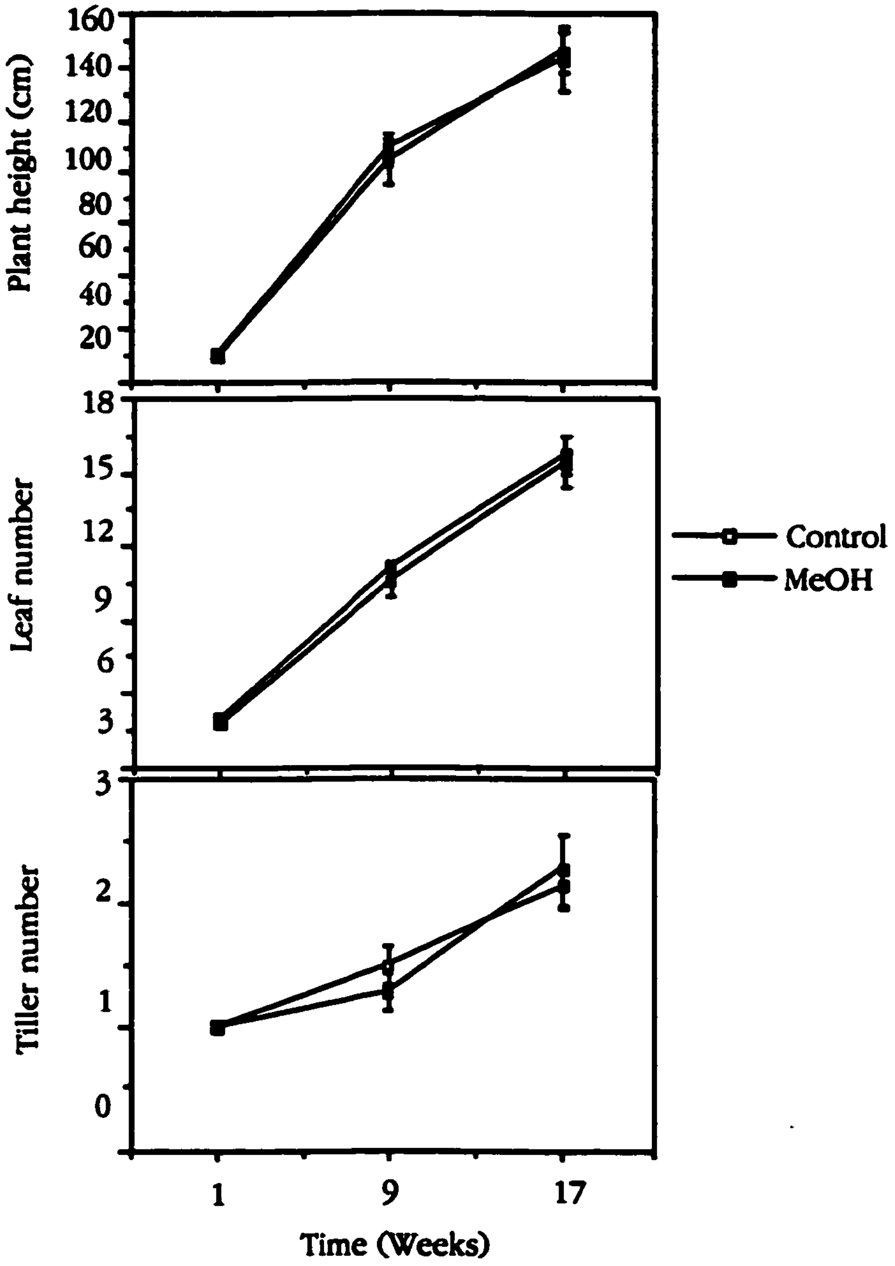

Figure 3.16 The effect of foliar-applied methanol on tillering capacity, leaf development and shoot growth (shoot length) of greenhouse-grown cv. Pishori (Basmati 217), a tropical cultivar. Error bars indicate ± SE of means.

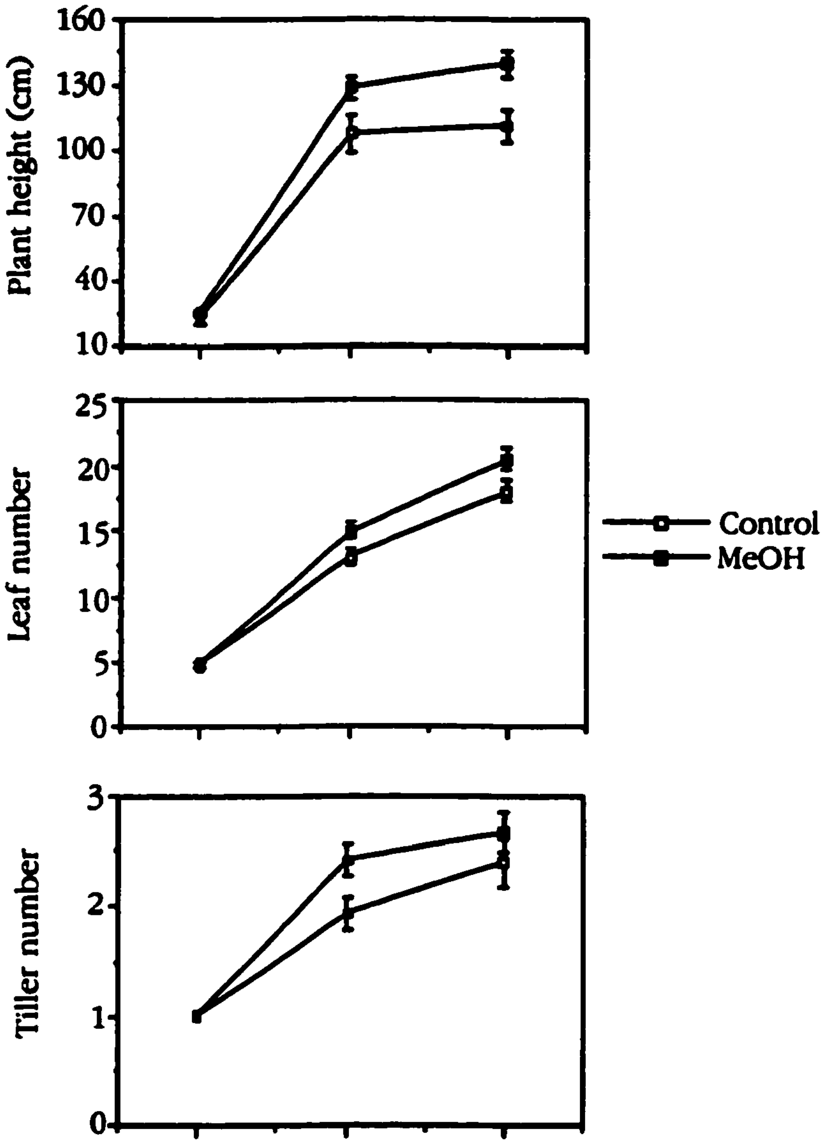

Figure 3.17 The effect of foliar-applied methanol on tillering capacity, leaf development and shoot growth (shoot length) of greenhouse-grown cv. Basmati 370, a tropical cultivar. Error bars indicate ± SE of means.

The foliar-methanol treatments significantly stimulated leaf development (t = 2.080, df = 28, p = 0.023), tiller development (t = 5.914, df = 28, p = 0.012) and shoot growth (t = 1.727, df = 28, p = 0.048) in the elite semi-dwarf cv. IR36 cultivar. In the medium height cv. Basmati 370 significant stimulation of shoot growth in cm (F = 8.004, df = 23, p = 0.009), tiller development (F = 5.988, df = 23, p = 0.023) and leaf development (F = 4.172, df = 23, p = 0.052) were recorded (Fig. 3.15 & 3.17). In the tall height cv. Pishori, a minimal non significant stimulation of leaf and shoot growth was observed (Fig. 3.16). The growth pattern was however similar to that observed in cv. IR36 and cv. Basmati 370. The slow response of the Pishori cultivar may be attributed to its relative long growth cycle. Similarly the development of tillers (number) in treated cv. Pishori plants was not significant (p = 0.718). However, a minimal stimulation of tiller formation was observed in the three cultivars at week 17 relative to the controls.

II) Biomass productivity

In the elite semi-dwarf IR36 cultivar, significant increases in biomass accumulation in shoot vegetative structures (t = 1.762, p = 0.044) and root structures (t = 2.262, p = 0.015) were recorded in the plants treated with foliar-applied methanol (Fig. 3.18). In the treated cv. IR36 plants the biomass productivity in shoot vegetative structures was 9.8% higher than in the control plants (Table 3.14). In the treated medium height Basmati 370 cultivar, a significant 9.2% increase in root structures biomass accumulation (t = 1.888, p = 0.035) was recorded over the control, but the increase was not significant in the shoot vegetative structures (Table 3.16). In the tall Pishori cultivar, there was no significant increase in shoot (vegetative) and root biomass accumulation in treated plants, however a 9.2% increase in root biomass was recorded (Table 3.15).

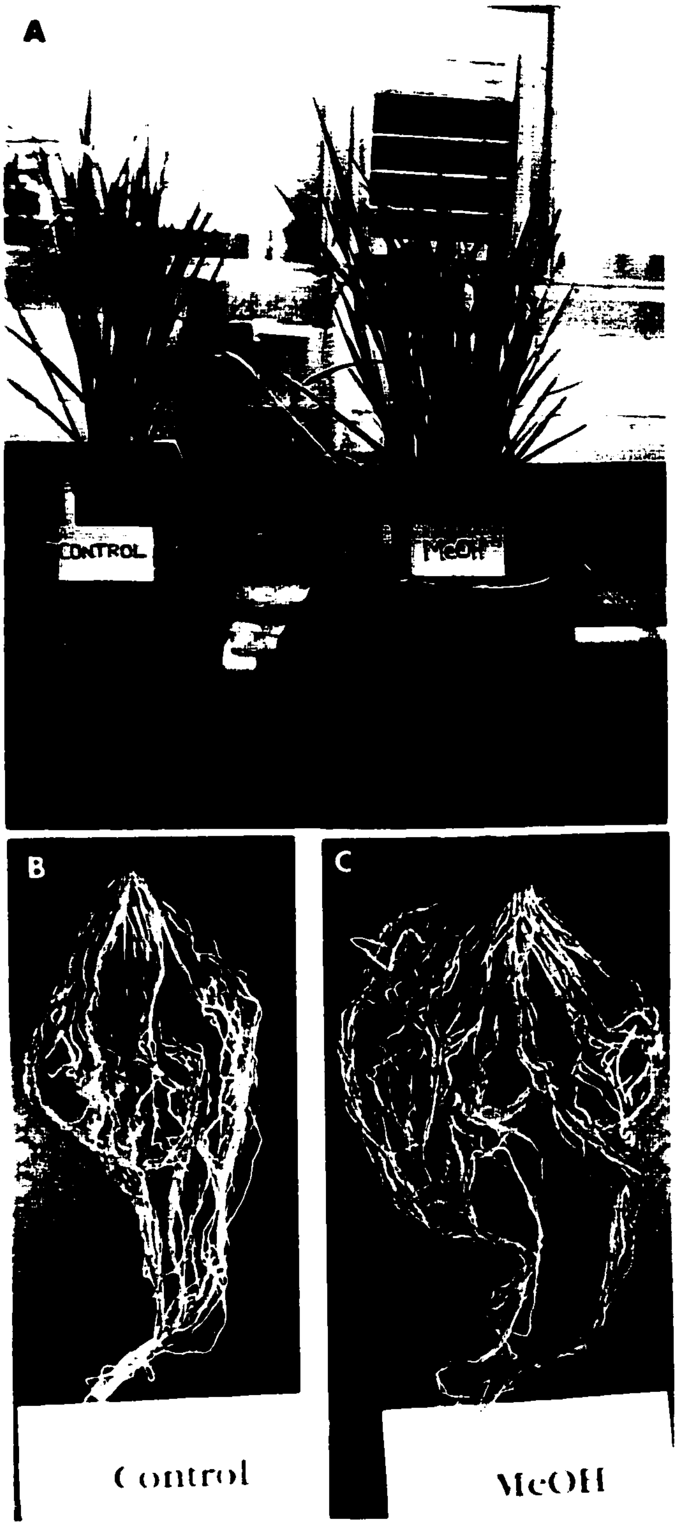

Figure 3.18 The growth of shoot vegetative and root structures of 17 week old elite semi-dwarf IR36 cultivar after two foliar-applied methanol treatements (MeOH) at week 4 and 11 of the growth cycle after seeding.

Table 3.14 The effects of foliar-applied methanol treatment on biomass accumulation in shoot and root structures (mean with SE) of greenhouse grown cv. IR36

Growth parameter	Control (Mean ± SE)	MeOH (Mean ± SE)	*t*-Test	df	*P* value
Shoot biomass	2.77 ± 0.11	3.04 ± 0.12*	1.762	28	0.0445
Root biomass	1.25 ± 0.06	1.44 ± 0.06*	2.262	28	0.0158

* Mean is significantly different from the control ($p \leq 0.05$)

** Mean is significantly different from the control ($p \leq 0.001$)

- p-values were based on unpaired one-tail comparison of means. The sample sizes were (n) = 15 in both control and in methanol treated groups. Shoot and root biomass were measured in g
- All treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l.. Treatment of plants was effected during week 4 and week 11 after seeding .

Table 3.15 Relative effect of foliar-applied methanol on shoot (vegetative) and root biomass (g) of cv. Pishori (Basmati 217) a tropical cultivars

Growth parameter	Control (Mean ± SE)	MeOH (Mean ± SE)	*t*-Test	df	*P* value
Shoot biomass	2.40 ± 0.09	2.40 ± 0.13	0.416	27	0.3403
Root biomass	1.52 ± 0.06	1.66 ± 0.10	1.118	27	0.1367

- p-values were based on unpaired one-tail comparison of means. The sample sizes (n) were = 14 (control) and 15 (methanol) in treatment groups. All methanol and control treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l.. The foliar-treatments were effected during week 4 and week 11 after seeding.

In all the rice cultivars, increases in accumulation of biomass in the root structures were positively correlated to increased development of root network in the treated plants. The biomass accumulation in root structures was significant in both Basmati 370 and IR36 cultivars (Table 3.14 & Table 3.16). Modest non significant increases in root biomass accumulation in treated plants were recorded in Pishori cultivar (Fig. 3.14). The response of plants to methanol treatments resulted in similar growth patterns, however there were variations among cultivars and thus the responses were not cultivar specific.

Table 3.16 The effects of foliar-applied methanol treatment on shoot and root biomass of greenhouse grown cv. Basmati 370.

Development indicator	Control (Mean ± SE)	MeOH (Mean ± SE)	*t*-Test	df	*P* value
Foliar biomass	1.95 ± 0.16	2.13 ± 0.08	0.993	23	0.1654
Root biomass	1.27 ± 0.10	1.56 ± 0.13*	1.888	23	0.0359

* Mean is significantly different from the control ($p \leq 0.05$)

- p-values were based on unpaired one-tail comparison of means. The sample sizes (n) were = 13 (control) and 12 (methanol) in treatment groups. The methanol and control treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l.. The treatments were effected during week 4 and week 11 after seeding.

A comparison of biomass productivity in treated plants showed that dry matter accumulation pattern in tropical cultivars shoot and root vegetative structures (Fig. 3.18) was similar to the observations made in temperate cultivars (Fig. 3.14). For instance methanol treatments resulted in 15.2%, 9.2% and 22.8% increase in biomass accumulation in the root system of IR36, Pishori and Basmati 370 tropical cultivars respectively (Fig. 3.19), relative to 17.9% and 17.5% in CR76 and A301 temperate cultivars. Thus in all the cultivars, the treated plants had higher root biomass mean values relative to the control. In the three tropical cultivars the partitioning of dry matter to the photosynthetic vegetative (shoot) structure and the root structure in the treated plants was at the ratio of 3 : 2.

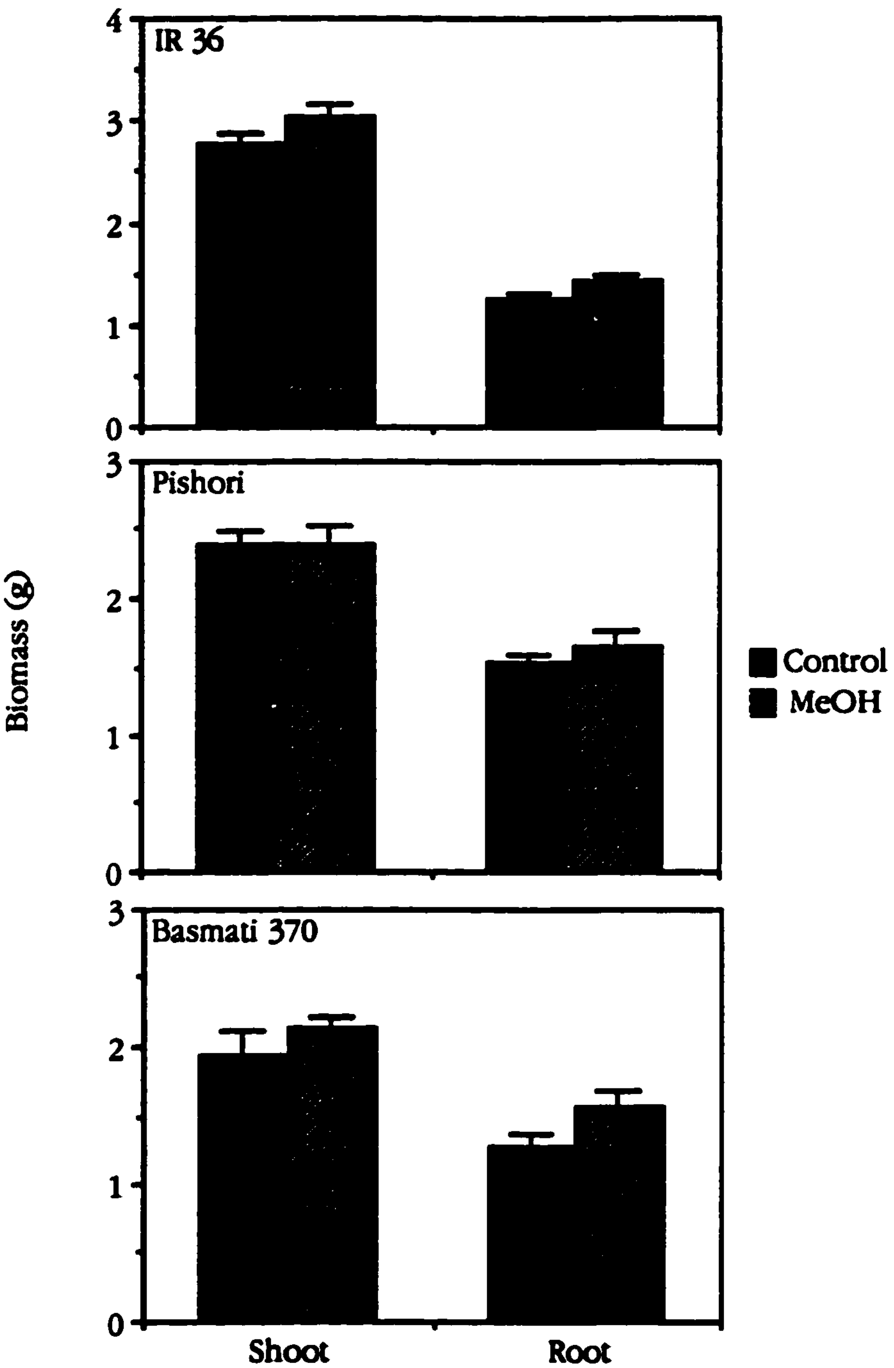

Figure 3.19 A comparison of the effects of foliar-applied methanol on shoot (vegetative) and root biomass productivity in three tropical cultivars: cv.IR36, cv. Pishori and cv. Basmati 370. Error bars indicate ± SE of means.

The plant biomass productivity in the tropical and temperate rice cultivars represented the cumulative effect of biotic and abiotic factors, that regulate the assimilation of carbon, one of the main substrates that limit plant productivity.

III) Reproductive characteristics

a) Flowering experiments

Early flowering and early grain ripening [change of filled grains (caryopsis) outer coat from green to brown] was observed in treated plants (Fig. 3.19 & Fig. 3.20). The onset of flowering (opening of the first flower bud) in treated plants occured on day 156 compared to day 157 in controls. The stimulation of early flowering was more evident on day 159. At this time 55% of the plants in the treated group had initiated flowers compared to 20% in the controls. At day 161, 82% of the treated plants had developed flowers, above the 75% mark point of successful flowering (Fig. 3.20). A relatively slow flowering rate was recorded in control group, on day 161 only 48% of the plants had developed flowers.

Further observations revealed that early flowering in treated plants in IR36 cultivar (Fig. 3.20) resulted in early maturity and ripening of the grains (Table 3.17). A significant early ripening ($F = 5.09$, $p = 0.032$) as indicated by 70% (reference mark point), change of green seed coat to brown (Fig. 3.18) was recorded in treated plants.

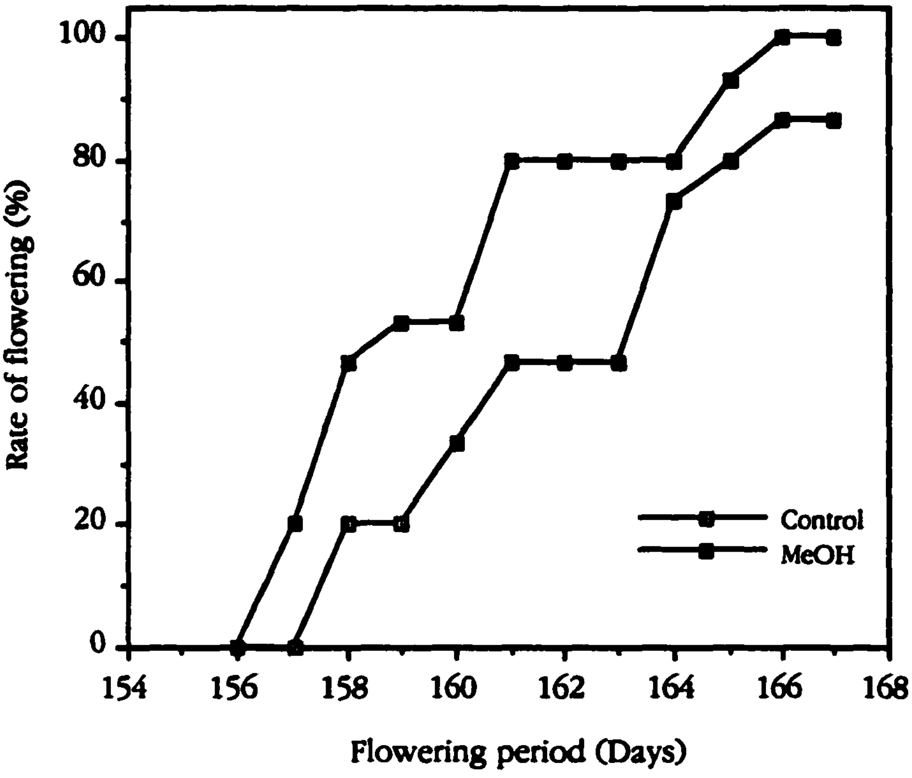

Figure 3.20 The effect of foliar-applied methanol on the rate of flowering of greenhouse-grown cv. IR36, a tropical cultivar.

Table 3.17 The effect of foliar methanol treatments on ripening of cv. IR36 grains from floral induction to 70% ripeness as a function of time in days (mean days ± SE) in greenhouse conditions.

Grain ripening condition	Control (Mean days ± SE)	MeOH (Mean days ± SE)	SS	F Ratio	df	Pro>F
70%	190.4 ± 0.88	187.6 ± 0.87*	58.8	5.09	(1,28)	0.032

* Mean is significantly different from the control ($p \leq 0.05$)

The N values are control = 15 and MeOH treated group = 15.

b)Yield measurements

An increase in the partitioning of biomass to reproductive structures (yield) measured as panicle number per plant and absolute grain weight produced per plant was recorded in plants treated with foliar-applied methanol. In IR36 cultivar, a significant 30.7% increase in the number of panicles per plant (t = 2.432, p = 0.011) was recorded in treated plants compared to the controls (Table 3.18). In addition, the absolute grain yield in g (a raw indicator of crop yield) in treated plants, increased significantly [(t = 3.353, p = 0. 0012 (in unpaired one tail Student's t-test)] by 38.1% to 2149.73 ± 145.34 mg/plant relative to 1557.27 ± 100.47 mg/plant in the controls.

Table 3.18 The effects of foliar-applied methanol treatment on development of fruiting panicles and absolute grain yield (mean with SE) in greenhouse-grown IR36 cultivar

Yield parameter	Control (Mean ± SE) n = 15	MeOH (Mean ± SE) n = 15	*t*-Test	df	*P* value
panicle number	1.53 ± 0.13	2.00 ± 0.14**	2.432	28	0.0108
Yield (grain wt)	1557.27 ± 100.47	2149.73 ± 145.34**	3.353	28	0.0012

** Mean is significantly different from the control ($p \leq 0.001$)

- p-values were based on unpaired one-tail t-test comparison of means. The absolute grain yields (dry weight at < 30% water content) were measured in mg. All treatments were supplemented with 0.2% w/v glycine/l and 0.5% w/v disodium D-L-glycerophoshate/l., at week 4 and week 11 after seeding .

In general, the growth stimulatory response of the plants to foliar-applied methanol treatments as indicated by measured vegetative growth parameters such as tiller number and shoot biomass productivity were not cultivar specific.

However there were variations among different cultivars in terms of the magnitude of response. Increase in yield as a result of methanol treatments appears to be partly due to the treatment induced increase in tiller number, a phenomenon that was observed in both temperate cultivars (Fig. 3.12 & Fig. 3.13) and tropical cultivars (Fig. 3.15, 3.16 & Fig. 3.17). In addition, measurements of grain yields (Table 3.18) showed a significant increase in harvest index in treated IR36 cultivar.

DISCUSSION

The aerial parts of green plants are colonized by numerous saprophytic microorganisms. The most prominent microflora, particularly on the actively growing young leaves, are the pink-pigmented facultative methylotrophic bacteria (PPFM) in the genus *Methylobacterium*. This group of microorganisms utilize methanol as their main source of carbon (Corpe and Basile 1982; Corpe and Rheem 1989).

The primary objective of this study was to evaluate the potential of Q4 and Q5 isolated *Methylobacterium* sp. strains as phylloplane microbial factors that could significantly increase growth, development of seedlings *in vitro* and absolute grain yield in greenhouse grown mature plants. In addition the experiments were also designed to determine if the observed growth stimulatory effects resulting from foliar-applied methanol treatment were partly mediated by some of the PPFM such as the isolated Q4 and Q5 strains.

The growth stimulatory effects of two isolated PPFM strains, designated Q4 and Q5 in the genus *Methylobacterium*, were reported and discussed in chapter one. The mechanisms which may account for the observed stimulatory effects in terms of vegetative growth and biomass productivity were hypothesized and supporting evidence was provided. The discussion in this section is focused on two main concepts.

a) the independent effects of methanol treatments on seedlings in axenic culture, relative to the independent stimulatory effects of Q4 or Q5 strain. The methanol-induced growth stimulatory effects in axenic cultures were demonstrated and reported (Fig. 3.3 to Fig. 3.6) in the *in vitro* experiments section (chapter 3). Possible mechanisms are suggested based on results and evidence derived from this study. Additional evidence is cited from research studies reported in recent years.

b) evidence suggesting that the growth and development stimulatory effects of the foliar-applied methanol is partly mediated by some *Methylobacterium* strains located on the leaf surface will be presented and discussed. The primary emphasis will be on the role of methylotrophic bacteria (Fig. 3.3 to Fig. 3.6), particularly the Q4 and Q5 designated PPFM strains.

Modulation of seedlings growth and development by foliar-applied methanol in axenic conditions

Methanol is one of the natural metabolic products, synthesized in small quantities during various plant cellular processes, such as demethylation of pectins in young tissues (Cossins *et al.*, 1964; MacDonald and Fall 1993). The plant-derived methanol is released to the atmosphere as a volatile organic compound (VOC) through the stomates. Even though plants tissues release methanol in small quantities, recent studies have shown that exogenously applied methanol treatments to C_3 plant foliage could increase growth and yield of some plants (Nonomura and Benson 1992; Rowe *et al.*, 1994; Li *et al.*, 1995).

In this study, preliminary greenhouse experiments (I) showed that the foliar-applied methanol significantly stimulated growth of seedlings. The treatment also supplemented the amount of methanol available to the phylloplane methylotrophs such as the PPFM . To test if the growth stimulatory effects were partly mediated by the PPFM, axenic (*in vitro*) studies conducted. The experiments showed that the application of methanol without PPFM (Q4 or Q5 strain) could stimulate growth of the seedlings significantly in some of the rice cultivars (Fig. 3.3 to Fig. 3.4). However treatment of seedlings with both methanol (MeOH) and Q4 or Q5 strains (Fig. 3.4 & Fig. 3.6) stimulated growth and development of the seedlings more than either treatment alone.

In addition, results from the *in vitro* experiments showed that MeOH can significantly stimulate shoot growth (length) in cv. CR76 and cv. A301 (Fig. 3.4 & Fig. 3.5), in the absence of the Q4 and the Q5 PPFM strains. Stimulation of leaf development over the controls was also observed in CR76 cultivar. Although this study does not provide information on mode of action or mechanism through which the foliar-applied methanol modulates plant growth and development, it unequivocally demonstrates that methanol applied on rice foliage stimulates biomass accumulation and development of vegetative structures. In some of the cases were the results where not significant (Fig. 3.3) relatively higher mean values in treated seedlings based on measured growth parameters were recorded.

The findings of studies reported in recent years suggest that there are several mechanism through which methanol treatments may stimulate/modulate plant growth and development. These mechanisms include inhibition of respiration, increase in photosynthesis rate in vegetative structures, increased metabolic rate, assimilation of methanol as a carbon source and increased turgor of leaves. Some of these reported mechanisms are suspect because some of the experiments were not carried out in axenic conditions but were conducted in the presence of the ubiquitous PPFM on aerial vegetative structures.

There are several mechanisms that may account for the reported growth stimulatory effect of the foliar-applied methanol observed in treated rice seedlings in axenic culture (Fig. 3.3 to Fig 3.6). First a study conducted by Cossins *et al.*, (1964) showed that plant tissues had the capacity of utilizing methanol in axenic conditions. It was further shown that the C^{14} in the radiolabelled methanol was incorporated in the structure of the biosynthesized amino acids in the plant tissue. In a separate study, Doman and Romanova (1962) showed that bean and barley plants tissues could metabolize radiolabelled methanol carbon. The carbon was later detected in sugars and in several amino acids such as α-alanine, serine, aspartic acid and leucine. Since most of the amino acids synthesized in plant tissues are integrated into plant cellular structures. Consequently if there is no other limiting factor, an increase in the amount of the available amino acids could enhance the growth of cellular structures which in turn translates to increased plant (rice seedlings) vegetative growth.

In addition to the assimilation of methanol carbon in the synthesis of amino acids, a study designed to evaluate the effects of methanol and ethanol on metabolic processes showed that methanol treatment induces local and systemic increase in metabolic rate in plant tissues (Rowe *et al.*, 1994). This finding is of interest since treatment of rice seedlings in both axenic and greenhouse conditions is highly likely to accelerate cellular metabolic rates. The methanol-induced increase in metabolic rate would in turn result in increased conversion of carbon dioxide in the photosynthetic carbon reduction (Calvin) cycle.

Based on these findings, it is reasonable to speculate that the methanol treatments in axenic conditions modulated the growth of the rice seedlings through stimulation of metabolic rate which leads to increased carbon conversion efficiency or through assimilation of methanol molecule as an alternate source of carbon and its final incorporation in cellular structures.

Superior stimulation of seedlings growth induced by combined Q4 or Q5 strain and foliar-methanol treatments *in vitro*

Results from *in vitro* experiments showed that combined treatment of seedlings with Q4 or Q5 strain and foliar-applied methanol resulted in superior growth stimulatory effects (Fig. 3.4 & Fig. 3.6) than either treatment alone. In addition, the observed positive shift in PPFM population count/cm^2 on methanol treated plants (Table 3. 11) and the significant increase in methylotrophic bacteria colonies count/cm^2 (population) in the treated greenhouse grown plants (Fig. 3.11) suggests that the reported growth stimulatory effect induced by methanol treatments is partly mediated by the methylotrophic bacteria located on the phylloplane in natural environment.

There are several mechanisms through which the Q4 and Q5 (PPFM) could stimulate seedlings growth. For instance, in chapter one, it was shown that both Q4 and Q5 stimulated callus proliferation (Table 1.1 & Fig. 1.10) when subcultured rice callus was inoculated with one of the strains. This finding was of great interest because the callus had been subcultured in a plantlet regeneration media. The inhibition of plantlet regeneration coupled with the stimulation of callus (cell) proliferation indicated that the Q4 and Q5 strains were synthesizing and secreting into the media biologically active compounds which have the properties of auxins and cytokinins. This is a unique property that is not found in all PPFM strains. It is possible that the synthesized compounds modulated growth by altering the auxin/cytokinin balance in the media. A low concentration of auxin/ and relatively high concentration of cytokinin ($A/C < 2$) in growth media are known to promote shoot proliferation (Skoog and Miller 1965).

It is therefore likely that, by being physically present on seedling leaves, the Q4 and the Q5 strains stimulated growth of the seedlings *in vitro* by modulating the concentrations of phytohormones within the rice seedlings apical meristems. It is noteworthy that is it not all the strains of the methylotrophic bacteria that have the capacity to stimulate seedling growth significantly (Fig. 1.6). Therefore the superior growth stimulatory effect observed in seedlings treated with both the foliar-applied methanol and Q4 or Q5 strain most likely represents the net effect resulting from, first alteration of phytohormone or secretion of bioactive phytohormone-like compounds by the Q4 and Q5 strains and secondly, the assimilation of methanol by seedlings or the methanol-induced increase in metabolic rate.

In addition to the processes described above, the Q4 and Q5 strain like all other *Methylobacterium* species, are aerobic. Thus during their life sustaining processes such as respiration, they release CO_2 which could result in elevated levels of CO_2 within the leaf microclimate. A study aimed at evaluating the effect of increased levels of CO_2 in ambient air, demonstrated that soybean plants grown under enriched CO_2 conditions, exhibited a significant increase in vegetative growth (Allen *et al.*, 1987). Elevated CO_2 concentrations are known to inhibit photorespiration and to increase photosynthesis and growth of C_3 plants as reported in *Chrysanthemum morifolium* (Mortensen and Moe 1983a).

Increased partitioning of biomass to vegetative and reproductive structures in mature plants. A factor of PPFM and MeOH ?

The phylloplane (aerial parts) of all green plants in natural environment are colonized by the pink-pigmented methylotrophic bacteria [(PPFM) Corpe and Basile 1982]. The *in vitro* experiments in this study showed that the isolated two PPFM strain designated Q4 and Q5 could significantly stimulate rice seedlings growth and development (Fig. 3.3 to Fig. 3.4) in axenic culture. Nevertheless it was further demonstrated that the combined treatment of seedlings with either one of the PFFM strain and foliar-applied methanol resulted in superior stimulation of growth (Fig. 3.4 & Fig. 3.6) than the methanol or the PPFM treatment alone. It is thus evident that all reported experiments involving the methanol treatments in nature should take into consideration the effects of the ubiquitous PPFM. Overlooking the role of PPFM is suspect since the net effect of methanol treatments as shown (Fig. 3.4 & Fig. 3.6) is partly mediated by these strains.

Carbon economy in relation to biomass productivity and yields

The carbon economy (budget) of a crop throughout a growing season is influenced by the balance between the carbon uptake (fixation) during photosynthesis and the carbon lost to respiration. Recent findings based on measurements of growth efficiency and respiration rate in rice plants have demonstrated that 60-70% of cellular respiration (energy generated) during booting (onset of development of floral bud) and flowering mainly supports growth of floral parts and early stages of seed development (Tanaka and Yamaguchi 1968)

The methanol treatments of greenhouse grown rice plants (naturally colonized by numerous strains of PPFM) resulted in significant increases in biomass and grain yield in temperate and tropical rice cultivars (Fig. 3.14, Fig. 3.19 & Table 3.18). Based on the findings of the *in vitro* experiments, it is reasonable to conclude that the observed effects were partly mediated by the PPFM. Nevertheless reports on experiments carried out in axenic culture (Cossins *et al.*, 1964) showed methanol, a simple organic molecule, is assimilated as a direct source of carbon by plant tissues and also stimulates metabolic (respiration) rate in plant tissues (Rowe *et al*, 1994).

Based on previously reported experiments, it is logical to conclude that the treatment of the greenhouse grown rice plants with the exogenously applied methanol increased the carbon available to photosynthetic tissues and, at the same time, stimulated both local and systemic of cellular metabolic rates. This could account for the recorded increases in the mean shoot (vegetative) and mean root dry weights in CR76, A301, IR36 and Basmati 370 cultivars. This process may also account for the observed increases in grain yield and in number of panicles per plant (Table 3.18) in the IR36 cultivar.

Treatment of rice plants at the onset of flowering would be more critical if increases in yields are to be realized. This is mainly because, in most of the cereal crops, the carbon assimilated prior to the flowering does not contribute directly to seed growth (Flinn 1977). Unlike carbon, the nitrogen assimilated before anthesis does contribute significantly to seed nitrogen requirement (Flinn 1977). Therefore the high metabolic rate associated with remobilization in vegetative tissues to support reproductive growth after anthesis are limited to the remobilization of nitrogen. In rice plants the respiration (metabolic) rates reach peak values just after flowering and then decrease until maturity (Yamaguchi 1978)

It is true that respiration represents the loss of carbon from the plant, in which case the straight forward assumption is that low rates of respiration would lead to more rapid crop growth and greater productivity due to positive carbon balance. But, it is also true that respiration is absolutely required for growth, maintenance and ion uptake (Gale 1974; Thorne 1982; Sinclair *et a.,l* 1987). Based on this reported findings, it is thus logical to suggest that methanol-induced rapid respiration (metabolic) rate in young rice plant tissues could trigger rapid growth, greater rates of nutrient uptake and effective maintenance, the net effect being increase in biomass and yield productivity.

Leaf development in relation to photosynthesis

The leaf is the principal organ developed by plants to harvest light energy. In this study treatment of seedlings with foliar-applied methanol, in the presence Q4 or Q5 strain in *in vitro* experiments (Fig. 3.4) and in the presence of naturally occuring PPFM in greenhouse experiments (Fig. 3.12, 3.13, 3.15 & Fig. 3.17), resulted in the significant stimulation of leaf development.

If all other factors that influence photosynthesis are held constant, an increase in number of leaves per plant is positively correlated to increased photosynthetic productivity, mainly due to increase in photosynthetic surface area. Therefore the increase in biomass productivity in treated plants may be partly due to the increase in number of leaves per plant. This could lead to increased fixation of carbon dioxide, among other effects.

In recently reported studies, Nonomura and Benson (1992a) showed that treatment of the tomato plant tissues with methanol increases turgidity of leaf cells. Turgid leaf cells (tissue) result is upright erect photosynthetically efficient leaves while flaccid cells result in droopy photosynthetically inefficient leaves (Tanaka *et al.*, 1969). The orientation and inclination of the leaf in space determines the surface area exposed to light, distribution of light on the leaf and the degree of penetration of the solar radiation through stand canopy. Therefore, the methanol treatment may have modulated leaf orientation indirectly and thus facilitated photosynthesis resulting in the observed increased biomass accumulation (Fig. 3.14 & Fig. 3.19) in vegetative structures of the tropical and temperate rice cultivars.

Further experiments need to be carried out to find long lasting mechanisms of modulating leaf orientation, since it has been shown that rice cultivars with erect leaves have higher photosynthetic rates (Tanaka *et al.*, 1969), lower light extinction coefficient at the crop canopy (Murata *et al.*, 1961; IRRI 1967) and relatively higher grain yields (Tsuoda *at a.,l* 1959).

The erect leaf plant architecture allows maximum crop photosynthesis due to reduced light interception by the uppermost leaves and the penetration of incident solar radiation to leaves at the lower levels. In rice, lower light extinction coefficient allows maximum crop growth rate (CGR) and maximum net assimilation rate (NAR). In addition, erect leaf architecture allows maximum leaf area index (LAI), primarily due to increased (dense) population of the plant in the field (Ashraf *et al.*, 1994), the overall effects include higher rate of photosynthesis, and increased net CO_2 uptake per unit leaf area.

Leaf chlorophyll content in relation to photosynthesis

In methanol treated tropical (Basmati 370, Pishori) and temperate (CR76, A301) rice cultivars (Fig. 3.9 and Fig. 3.10), significant increases in total chlorophyll and chlorophyll *a* in the flag leaf and other leaves were recorded. Even though increase in leaf chlorophyll content may not directly result in increase in photosynthetic rate, it translates into increased potential photosynthetic capacity.

The increase in leaf chlorophyll content (photosynthetic capacity) in rice plants may partly account for the observed stimulation of biomass accumulation in treated plants. In a separate study, Faver and Gerick (1993), reported an increase in photosynthesis in cotton plants that had been treated with foliar-applied methanol in greenhouse conditions.

Recently, additional supporting evidence was provided by Idso *et al.*, (1995) in experiments on quantitative effects of atmospheric CO_2 enrichment and foliar-applied methanol on net photosynthetic rate of sour orange trees (*Citrus aurantium*; Rutacea). In this study (Idso *et al.*, 1995), methanol treatments in ambient air increased leaf photosynthetic rate by 37%, while in a CO_2-enriched air, the treatments induced a significant 53% increase in photosynthetic rate.

Even though there in no compelling evidence to show that increased leaf chlorophyll content is positively correlated to stimulation of growth in treated plants. It is reasonable to speculate that the demonstrated increase in dry matter accumulation (Fig. 3.14 & Fig. 3.19) in treated plants may be partly due to modulation of photosynthetic rate.

Respiration and metabolic rate in relation to vegetative growth

Significant stimulation of shoot (vegetative) growth and leaf development were observed in Q4 or Q5 or methanol treated seedlings *in vitro* experiments (Fig. 3.4) and in greenhouse experiments (Fig. 3.12, 3.13, 3.15 & Fig. 3.17). The stimulation of cellular metabolic and respiration rates by methanol treatments could account for the stimulation of vegetative growth. Recent studies on crop respiration, growth efficiency and yield (Amthor 1989; Hansen *et al.*, 1994; Hemming *et al.*, 1995), showed that the rate of respiration (metabolic rate) is proportional to relative growth rates in plants.

In addition, one of the main physiological processes in plants reported to be modulated by foliar-methanol treatments is cellular respiration (Cossins *et al.*, 1964; Hemmings *et al.*, 1995). For instance, it is reported that metabolic heat rates in treated California Wonder bell pepper leaf sections stabilized at 35% level above the control (Hemming *et al.*, 1995). Furthermore in the same study (Hemming *et al*, 1995), localized treatment of a leaf on one branch of outdoors grown tomato induced a systemic increase in metabolic heat rate expressed throughout the entire plant continually for at least two weeks.

The relationship between respiratory rate, metabolic rate and specific growth rate has been clarified by a mathematical thermodynamic model proposed by Hansen *et al*, (1994).

$$R_{SG} = R_{CO_2} (\varepsilon/1-\varepsilon)$$

where - R_{SG} is the specific growth rate,

- R_{CO_2} is the specific rate of CO_2 production
- ε is the substrate carbon conversion efficiency.

The relationship (equation) shows that growth rate will increase as metabolic rate increases as measured by CO_2 production rate, if the efficiency of substrate carbon conversion (photosynthesis) is constant.

In young plant, tissues increase in metabolic rate is associated with synthesis of cellular components and cell structural molecules. Therefore in rice seedlings, methanol-induced increase in metabolic rate can be of benefit to plant growth and development. This process is of interest because it may partly account for the observed stimulation of growth of the two temperate rice cultivars, A301 and CR76 plus the three tropical cultivars Basmati 370, IR36 and Pishori. The process could equally account for the the observed increased partitioning of biomass to vegetative and reproductive structures in IR36 cultivar shown in Table 3.14.

Based on the thermodynamic model proposed by Hansen *et al*, (1994), the timing of final foliar-methanol treatments of rice plants should coincide with the flowering and grain filling period. This is mainly because the carbohydrates stored in grains are synthesized after anthesis (Yoshida 1972). Thus it is evident that the duration and the rate of post-anthesis photosynthesis and synthesis of cellular components would have significant effect on grain yields. This hypothesis is supported by the observations of Amthor (1989), which showed that flag leaf alone accounts for over 50% of the assimilate imported by seeds in many crops.

Biomass productivity as an indicator of potential yields

There exists a direct positive correlation between a cereal crop biomass and grain production (Slafer and Andrade 1991). The process of partitioning of biomass to vegetative and reproductive structures in cereals is governed by the rate of assimilation of carbon among other factors. Recent studies on grain yields in bread wheat (Slafer *et al.*, 1994) and rice (Ashraf *et al.*, 1994) showed that the grain yields are directly proportional to the total dry matter produced by the crop as dictated by harvest index (Akita 1989). In this study, increases in biomass productivity were recorded in the greenhouse experiments (Fig 3.14 & Fig. 3.19) involving methanol treatments in presence of naturally occuring PPFM and the *in vitro* experiments (Fig 3.3 & Fig. 3.5) involving both Q4 or Q5 strain inoculations and methanol treatments. It is therefore reasonable to hypothesize increase in biomass could translate to increased yield if the plants are grown to reproductive stage as shown in the IR36 cultivar experiments.

Harvest index (HI) which is a measure of the ratio of grain yield to total above the ground biomass is related to the total dry weight of rice plant. The harvest index for a given cultivated variety of a food crop is an indicator of the cultivar's capacity to partition assimilates between reproductive (grains) and vegetative structures. Typically tall varieties have low harvest index relative to the semi dwarf varieties, which have higher HI due to partitioning more of the assimilate into grains yields (Akita 1989)

Total biomass productivity (Y_{bio}) is both a direct measure of crop growth rate (CRG) in a given growing season and an indicator of potential yields. The grain yield (Y_{econ}) is a fraction of the Y_{bio}. Consequently the partitioning of biomass to the reproductive structures (grains) can be estimated using the formula:

[$Y_{econ} = Y_{bio} \times K_{econ}$]

where K_{econ} is the coefficient of the effectiveness of yield which is also refered to as the harvest index (Ashraf *et al.*, 1994)]. This equation shows that the grain yield can be increased by increasing total biomass production or increasing harvest index or both. Therefore, the increases in biomass productivity reported in the *in vitro* experiments following inoculation of Q4 or Q5 PPFM strains and also the reported stimulation of biomass accumulation in greenhouse experiments indicate that inoculations of the Q4 or the Q5 PPFM strains coupled with foliar methanol treatments represents a potential alternate technology that could have positive effects on rice production.

CONCLUSIONS AND FUTURE DIRECTIONS

The findings and information generated from the experiments carried out in this research have demonstrated that *Methylobacterium* bacterial strains naturally located on the phylloplane of cereal plants can stimulate seedlings growth *in vitro* and cell proliferation in tissue culture. The results showed that the inoculation of the isolated Q4 and Q5 strains of the *Methylobacterium* sp. results in stimulation of leaf development, shoot growth and biomass accumulation in vegetative structures of axenic rice seedlings grown *in vitro*. The basis or mechanisms for this stimulation have not been established. However indirect evidence suggests that the stimulatory effects may be mediated by microbial-derived bioactive compounds such as the nitrogen metabolism enhancing urease enzyme which is reported to be secreted by all PPFM (Polacco *et al.*, 1989). Furthermore, indirect evidence based on tissue culture and plantlet regeneration experiments suggests that the microbial-induced growth and cell proliferation is mediated by microbial derived cytokinin-like or auxin-like phytohormone analogs.

Secondly the relationship between the naturally occuring phylloplane PPFM and the stimulatory effects of methanol treatments in greenhouse conditions was revealed. The finding based on *in vitro* experiments showed that treatment of axenically grown seedlings with either Q4 or Q5 strain inoculant coupled with foliar-applied methanol treatment resulted in greater stimulation of growth than either treatment alone. The effects were demonstrated by the measured growth parameters, namely shoot length, leaf development and biomass productivity. The data indicates that the observed or reported stimulation of growth observed in greenhouse or field grown plants following foliar-application of methanol on green foliage is partly mediated by the naturally ubiquitous PPFM. Further investigation need to be carried out to establish the basis or mechanisms accounting for the stimulatory effects.

Foliar-treatment of greenhouse grown rice plants with methanol spray significantly stimulated leaf chlorophyll content and the partitioning of biomass to vegetative (shoot & root) and reproductive structures (panicle & grains) in tropical and temperate cultivars. Furthermore the data showed that the effects are not cultivar specific, although the degree of stimulation of growth varied among the cultivars. It is possible that the increased biomass productivity and yields in methanol treated cv. IR36 and other cultivars is partly due stimulatory effects PPFM inhabiting leaf surface, and partly due to assimilation of aqueous methanol into the carbon fixation pathway and/ or methanol-induced modulation of tissue respiration and metabolic rates.

Rice is the primary source of calories for over 65% of the total population. Future research should develop methods of inoculating rice plants in the field with the growth stimulating *Methylobacterium* strains such as the isolated Q4 and Q5. Site-specific technologies such as microbial seed coating need to be designed such that microbial treatments will directly increase grain yields or indirectly stimulate early tillering capacity, leaf chlorophyll content, biomass productivity and other growth parameters that are positively correlated to grain yield.

Based on the superior growth stimulatory effects observed in *in vitro* experiments as a result of Q4 or Q5 strains and foliar-applied methanol treatments, field tests need to be carried out to evaluate the potential of these treatments in increasing future food production through modulation of early tillering capacity, panicle development and grain yield.

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CHAPTER THREE

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