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RESEARCH PAPER

Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site

Daniel Abanda-Nkpwatt[1,*], Martina Müsch[2], Jochen Tschiersch[2], Mewes Boettner[3] and Wilfried Schwab[1,†]

[1] *Biomolecular Food Technology, Technical University of Munich, Lise-Meitner-Str. 34, D-85354 Freising, Germany*

[2] *National Research Center for Environment and Health, Institute of Radiation Protection, Ingolstädter Landstr. 1, D-85764 Neuherberg, Germany*

[3] *OrganoBalance GmbH, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany*

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Abstract

Four *Methylobacterium extorquens* strains were isolated from strawberry (*Fragaria*×*ananassa* cv. Elsanta) leaves, and one strain, called ME4, was tested for its ability to promote the growth of various plant seedlings. Seedling weight and shoot length of *Nicotiana tabacum*, *Lycopersicon esculentum*, *Sinapis alba*, and *Fragaria vesca* increased significantly in the presence of the pink-pigmented facultative methylotroph (PPFM), but the germination behaviour of seeds from six other plants was not affected. The cell-free supernatant of the bacterial culture stimulated germination, suggesting the production of a growth-promoting agent by the methylotroph. Methanol emitted from *N. tabacum* seedlings, as determined by proton-transfer-reaction mass spectrometry (PTR-MS), ranged from 0.4 to 0.7 ppbv (parts per billion by volume), while significantly lower levels (0.005 to 0.01 ppbv) of the volatile alcohol were measured when the seedlings were co-cultivated with *M. extorquens* ME4, demonstrating the consumption of the gaseous methanol by the bacteria. Additionally, by using cells of the methylotrophic yeast *Pichia pastoris* transformed with the pPICHS/GFP vector harbouring a methanol-sensitive promoter in combination with the green fluorescence protein (GFP) reporter gene, stomata were identified as the main source of the methanol emission on tobacco cotyledons. *Methylobacterium extorquens* strains can nourish themselves using the methanol released by the stomata and release an agent promoting the growth of the seedlings of some crop plants.

Key words: Methanol, *Methylobacterium extorquens*, *Nicotiana tabacum*, *Pichia pastoris*, PTR-MS, stomata.

Introduction

The living space on the leaf surface, known as the phyllosphere, shelters a great variety of organisms having beneficial, harmful, or neutral effects on the plant. The interactions between these micro-organisms and higher plants frequently affect the physiological activities of the plant (Yang *et al*., 2001; Holland *et al*., 2002). Pink-pigmented facultative methylotrophs (PPFMs) of the genus *Methylobacterium* are commonly found in association with plants and have been hypothesized potentially to dominate the phyllosphere bacterial population (Corpe and Rheem, 1989). PPFMs are aerobic, Gram-negative bacteria and, although they are able to grow on a wide range of multi-carbon substrates, they are characterized by the capability to grow on one-carbon compounds such as formate, formaldehyde, and methanol as their sole carbon and energy source. They are easily isolated on a methanol-based mineral medium. Recently published data suggest that the degree of the plant–*Methylobacterium* association varies from strong, or symbiotic (Jourand *et al*., 2004), to

* Present address: LAUS GmbH, Mandelring 47, D-67433 Neustadt/Weinstrasse, Germany.
† To whom correspondence should be addressed. E-mail: schwab@wzw.tum.de

Abbreviations: AOX, alcohol oxidase; CLSM, confocal laser-scanning microscope; GFP, green fluorescent protein; PPFM, pink-pigmented facultative methylotroph; PTR-MS, proton-transfer-reaction mass spectrometry; VOC, volatile organic compound.

loose, or epiphytic (Omer *et al*., 2004*a*), a range that also includes the intermediate endophytic association (Lacava *et al*., 2004). In the case of symbosis with nitrogen-fixing *Methylobacterium* strains, the benefit for the plant is self-evident, but this is not the case for the looser forms of association, although it has been suggested that the plant benefits from the production of plant hormones, such as cytokinins and auxins, by the epiphytic methylotrophs (Ivanova *et al*., 2000, 2001; Omer *et al*., 2004*b*). In addition, certain isolated *Methylobacterium* strains produce vitamin B_{12}, which has been suggested to stimulate plant development. Methylotrophic bacteria may also be associated with plant nitrogen metabolism through bacterial urease production (Holland and Polacco, 1992, 1994). However, the overall nature of their relationship with plants is as yet poorly understood, and the biological significance of these bacterial species is still unknown.

The consistent success of *Methylobacterium* strains in colonizing the phyllosphere is probably due to their ability to utilize methanol as a carbon and energy source, although the surfaces of plants are also covered with soluble carbohydrates, amino acids, organic and phenolic acids, terpenes, and alkaloids (Corpe and Rheem, 1989; Fiala *et al*., 1990). The considerable quantity of methanol emitted by plants is assumed to be a by-product of pectin metabolism during cell wall synthesis (McDonald and Fall, 1993; Nemecek-Marshall *et al*., 1995). Indirect experimental evidence has shown that most of the methanol is produced inside leaves and is emitted primarily through the stomata (Obendorf *et al*., 1990; Nemecek-Marshall *et al*., 1995).

Until recently, fluorometric assays and gas chromatographic methods have been widely utilized for the quantification of gaseous methanol (McDonald and Fall, 1993; Nemecek-Marshall *et al*., 1995; Hasunuma *et al*., 2004). However, since methanol has been recognized as an important constituent of the global atmosphere, methods allowing quicker and easier measurements of small amounts of the simplest alcohol have been developed (Nonomura and Benson, 1992). Proton-transfer-reaction mass spectrometry (PTR-MS) is a relatively new technique used to monitor real-time traces of methanol in the headspace of plants (Lindinger and Hansel, 1998). PTR-MS uses chemical ionization, in which the molecules of volatile organic compounds (VOCs) react with hydroxonium ions (H_3O^+) produced in an external ion source. VOCs with a proton affinity higher than that of water (166.5 kcal mol^{-1}) undergo a proton transfer reaction with H_3O^+. Primary and product ions are then detected by mass spectrometry in the usual way. The great advantage of this method is that fragmentation of the product ions is very much reduced, making the mass spectra much easier to interpret and more straightforward to quantify since the ion signal is linearly dependent on the concentration of the precursor molecule in the sample air (Hansel *et al*., 1995).

Recently, a biological sensor for the localization and quantification of sucrose on bean leaves was constructed by transforming *Erwinia herbicola* with a construct consisting of a sucrose-sensitive promoter and the gene (*gfp*) encoding the green fluorescent protein (GFP) (Miller *et al*., 2001). The mean fluorescence intensity of the transformed *E. herbicola* population increased with increasing concentrations of sucrose and therefore the spatial variations in sugar availability on the leaves could be demonstrated. Because of the ready availability of *Pichia pastoris* cells harbouring the highly inducible alcohol oxidase 1 (AOX1) promoter fused with *gfp*, it was only logical to use the transgenic methylotrophic yeast as a biological sensor to localize the methanol emission site on leaf surfaces (Boettner *et al*., 2002).

In order to elucidate the molecular interaction of PPFMs with plants, the ability of methylotrophic bacteria to promote seedling growth and germination of a number of important crop plants was tested, and the levels of methanol produced by the leaves and the consumption of methanol by the *Methylobacteria* were determined using PTR-MS. Finally, it was possible to localize the emission site of the alcohol from the leaf with a methanol-sensitive biosensor.

Materials and methods

Bacterial strains and culture conditions

Methylobacterium extorquens strains were isolated from strawberry (*Fragaria* × *ananassa* cv. Elsanta) leaves by imprinting on methanol agar plates. Single pure colonies were identified according to their 16S rDNA. For maintenance of *M. extorquens* strains, an ammonium mineral salts medium containing methanol (0.5 vol%) as the sole carbon source was used (Holland and Polacco, 1994). The yeast strains *P. pastoris* pPICHS/GFP and pPICHS were provided by Mewes Boettner (OrganoBalance GmbH, Berlin). Transformed cells containing pPICHS/GFP and cells carrying pPICHS were grown under non-inducing conditions on yeast peptone dextrose (YPD) medium supplemented with 2% D-glucose as the carbon source. For induction of protein expression on leaf surfaces, cells were harvested (20 min, 4 °C, 4000 *g*) and resuspended in 5 ml of fresh medium without a carbon source (Boettner *et al*., 2002).

Plant material

Tobacco seeds (*N. tabacum* var. Burley Jupiter) were purchased from Tabakanbau, Immenstaad, Germany. Bean (*Phaseolus vulgare*), strawberry (*Fragaria vesca*), mustard (*Sinapis alba*), barley (*Hordeum vulgare*), maize (*Zea mays*), pea (*Pisum sativum*), carrot (*Daucus carota*), tomato (*Lycopersicon esculentum*), and wheat (*Triticum aestivum* L.) seeds were obtained from a local market. *Fragaria*×*ananassa* cv. Elsanta plants were grown in fields belonging to the Institute of Botany II, University of Würzburg, Germany.

Effect of M. extorquens *on seedling growth*

Seeds were surface sterilized by soaking for 30 min in 50% sodium hypochlorite solution (NaOCl, available chlorine 12%) followed by

several washings with sterile, distilled water. Four filter papers wetted with either 4 ml of distilled water, 0.5% MeOH, 1% MeOH, or 0.1–0.0001% aqueous vitamin B_{12} solution were placed into Petri dishes and 1 ml of an *M. extorquens* culture medium (10^6 cfu ml^{-1}) was added. Seeds (10–75 depending on the species) were placed onto the moist filter papers with the help of a sterilized spatula. The Petri dishes were closed with parafilm and seeds were illuminated with ~7000 lx (Philips TLD 36W/840, Hamburg, Germany) for 16 h daily. After 7 d, seedling fresh weight and shoot length were determined. Germination experiments in which the seeds were not infected with *M. extorquens* ME4 served as controls. Additional control experiments were performed with the ammonia mineral salt medium instead of distilled water.

Fractionation of compounds produced by M. extorquens

Methylobacterium extorquens ME4 was grown in 100 ml of ammonia mineral salt medium with 1.0% MeOH for 7 d. The culture broth was centrifuged (4000 *g*, 25 min, 4 °C) and the supernatant extracted by solid phase extraction (40–63 μm; Chroprep RP18, Merck, Darmstadt, Germany). Compounds were eluted with 10 ml of MeOH, the solution was concentrated to dryness, and the residue was dissolved in 1 ml of sterile water. Aliquots (500 μl) of this solution were used for the germination experiments. Further fractionation of the extract was achieved by semi-preparative high-performance liquid chromatography (HPLC) separation using Eurochrom 2000 software and an HPLC system equipped with a Eurospher C-18 column (25 cm×4.0 mm id, particle size 5 μm) connected to a Maxistar pump and variable wavelength monitor (Knauer, Berlin, Germany). Separation was performed using a linear gradient proceeding from 95% water acidified with 0.05% formic acid and 5% acetonitrile to 100% acetonitrile in 30 min at a flow rate of 1 ml min^{-1}. Fractions of 3 ml fractions were collected, concentrated to dryness, dissolved in 1 ml of distilled water, and used for the germination experiments.

Consumption of gaseous methanol by M. extorquens

Methylobacteriom extorquens strains were streaked on agar medium while a small container containing 0.5 ml of methanol was placed on top of the agar. Plates were incubated at 37 °C.

Determination of methanol emission from tobacco leaves and consumption by M. extorquens *ME4*

Tobacco seeds (*N. tabacum* var. Burley Jupiter) were sterilized by soaking for 30 min in sodium hypochlorite solution (NaOCl, available chlorine 12%) followed by several washings with sterile distilled water. Seventy-five sterile tobacco seeds were placed into glass vessels with two opposite screw connections, each sealed with a septum (measurement of methanol by PTR-MS) or into Petri dishes (location of methanol on the leaf surface) on moistened filter papers. The seeds were then inoculated with 1 ml of a suspension of *M. extorquens* (measurement of methanol by PTR-MS) or *P. pastoris* pPICHS/GFP (location of methanol on the leaf surface) (10^6 cfu ml^{-1}). Seeds treated with sterile distilled water or a suspension of *P. pastoris* pPICHS were used as controls. The seeds were germinated at room temperature and 16 h daylight with an illuminance of 7 klux.

Proton-transfer-reaction mass spectrometer (PTR-MS)

The methanol emitted from growing tobacco seedlings was quantified by a PTR-MS built by Ionicon, Innsbruck, Austria. VOCs are protonated by H_3O^+ ions in a drift chamber and subsequently detected by a quadrupole mass spectrometer. Hollow needles were introduced into the two opposite screw connections of a glass vessel, one with a PTFE tube of ~50 cm length leading directly into the inlet of the PTR-MS, and the other one serving for pressure equalization. Five glasses were prepared with sterile tobacco seeds, while another five vessels contained seeds inoculated with *M. extorquens* ME4. The experiments were carried out at room temperature with 12 h light exposure, and measurements on each sample were taken twice daily for 1 week. The indoor room air was measured as the reference gas. The measurement programme was developed in preliminary experiments. For methanol (*m/z* 33; $[M+H]^+$), a measuring time of 2 s was chosen, and 50 ms was used for each of the other protonated masses (34, 37, 41, 42, 43, 45, 49, 50, 51, 55, 59, 61, 62, 63, 73, 75, 83, 85, and 95).

Laser scanning microscopy

Solutions or tobacco leaf surfaces inoculated with *P. pastoris* cells carrying pPICHS/GFP or pPICHS were viewed using a confocal laser-scanning microscope (CLSM) (model TCS SP2; Leica Microsyshoots, Germany). All images were generated with a CCD camera C5985 (Hamamatsu, Herrsching, Germany) and processed with Argus20/HPD-CP (version 1.5).

Results

Isolation of M. extorquens *strains*

Methylotrophs were isolated on selective media from the upper (adaxial) and lower surfaces (abaxial) of strawberry leaves and could be maintained with methanol as sole carbon substrate. On the abaxial surface, the micro-organisms colonized primarily the leaf veins, whereas on the adaxial surface they were more evenly distributed. Colonies isolated from strawberry leaves on methanol–agar media were pink, suggesting that PPFMs are ubiquitous in the *Fragaria* phyllosphere. The four strains isolated into pure culture, designated ME1–4, were characterized by partial 16S rDNA sequence analysis (424 up to 578 bp) and were most closely related phylogenetically to *M. extorquens*. The four strains were shown to share 99.9, 99.8, 100, and 99.9% sequence identity with GenBank entries AJ223453, Z23156, AF531770, and AF526936, respectively. Physiological tests by DSMZ (German collection of micro-organisms and cell cultures, Braunschweig, Germany) confirmed the classification (Table 1). Strain ME4 was chosen for the following experiments because it is the fastest growing strain. During propagation in liquid culture, the solution became turbid within 4–5 d.

Methylobacterium extorquens *can promote seedling growth*

To investigate the effect of methylobacteria on seed germination, surface-sterilized seeds of 10 different plant species were moistened with water, 0.5% MeOH, or 1.0% MeOH, and inoculated with the *M. extorquens* strain ME4 isolated from strawberry leaf. In the control experiments, the bacterial strain was omitted. The weight and shoot length of wheat, barley, maize, and carrot seedlings were not

Table 1. *Characteristics of the* M. extorquens *ME4 strain*

Property		Substrate utilization	
Shape	Rod	D-Glucose	Positive, but no pigment formation
Width	0.8–0.9 μm	D-Arabinose	Negative
Length	2.5–4.0 μm	Citrate	Negative
Gram reaction	Negative	D-Mannose	Negative
Lysis with 3% KOH	Positive	D-Fructose	Negative
Aminopeptidase	Positive	D-Trehalose	Negative
Oxidase	Positive	D-Sorbitol	Negative
Catalase	Positive	D-Inositol	Negative
Pigment	Pink	D-Mannitol	Negative
Alcohol dehydrogenase	Negative	D-Gluconate	Negative
Denitrification (24 h)	Negative	Malate	Positive
Urease	Positive	Ethanol	Positive
Indole production	Negative	Methanol	Positive
Hydrolysis of gelatin	Negative	Methylamine	Positive
Hydrolysis of esculin	Negative	Dimethylamine	Negative

significantly affected by the added MeOH in the absence of ME4, or by the presence of *M. extorquens* (Fig. 1A, B). A similar result was obtained for pea and bean seedlings (data not shown).

In contrast, the germination of the strawberry achenes was completely inhibited by 0.5% and 1.0% methanol but, in the presence of the bacterial strain, the seedlings developed similar lengths and weights to those germinated in water (Fig. 1C, D). It appears that the *M. extorquens* strain removes the cytotoxic methanol, thereby enabling normal germination. Methanolic solutions did not affect the germination rate of mustard, tomato, and tobacco seeds since almost all seeds (>95%) germinated, but the seedlings were heavier when inoculated with the bacterial strain (Fig. 2). The ammonia mineral culture medium used for the propagation of the methylotrophic strain did not enhance seed germination. The results clearly show that *M. extorquens* ME4 enhances biomass accumulation in mustard, tomato and tobacco seedlings.

Demonstration of the production of growth-promoting compounds by M. extorquens

The formation of vitamin B_{12} by methylotrophs and its growth-promoting effect on potato and tobacco plants has already been described (Kalyaeva *et al.*, 2001). In the present system, when tobacco seedlings developed in the presence of vitamin B_{12}, their weights and shoot lengths were not significantly different from those of the seedlings grown in sterile water (data not shown). However, when the cell-free supernatant of the *M. extorquens* ME4 culture broth was used in the germination experiments, considerable increases in seedling fresh weights and shoot lengths with respect to seeds germinated in ammonia mineral salt medium were observed. This observation is consistent with the promotion of seedling growth by compounds produced by the methylotrophs and excreted into the medium.

When the extract was separated further by preparative HPLC, only substances contained in two consecutive fractions stimulated the growth of tobacco seedlings in comparison with a control with water. Although the structure of the growth-promoting compounds could not be elucidated in detail due to insufficient quantities, it was possible to determine that the active ingredients that enhance the biomass of the tobacco seedling are semi-polar molecules eluting with 55–60% acetonitrile from the RP18 column.

Quantification of methanol emission and consumption by M. extorquens

After some preliminary experiments, tobacco seeds were chosen because the seedlings develop quickly and emit considerable amounts of methanol that are detectable by PTR-MS. Exactly 75 surface-sterilized tobacco seeds were placed on a moistened filter in an 80 ml glass vessel that was closed with a cap carrying two opposing threaded ports, each sealed with a septum (Fig. 3A). Similarly, a second vessel was prepared containing 75 surface-sterilized tobacco seeds inoculated with *M. extorquens* ME4. Needles were introduced into both of them, one connected directly to the inlet of the PTR-MS with a 50 cm long PTFE tube, while the other served for pressure balance. At the beginning of the measuring period, the seedlings were 7 d old with expanded cotyledons, but no true leaves (Fig. 2). The seedlings inoculated with PPFMs appeared to be better developed because they were bigger and had larger cotyledons.

On day 2 (9 d post-germination on filter paper moistened with sterile water), tobacco seedlings produced ~0.4–0.7 ppbv (parts per billion by volume) of methanol while room air contained ~0.01 ppbv. The methanol concentration in the headspace of tobacco seedlings inoculated with *M. extorquens* (0.005–0.01 ppbv) never exceeded the background level, i.e. the concentration in the room air. Typical data are shown in Fig. 3B for the measurements on day 4 (11 d post-germination). The methanol level in the vessels containing PPFMs was always lower than in indoor air.

One sterile and one inoculated sample were monitored for a longer period of ~20 min (Fig. 3B, Ia and IIa). Since the level of methanol remained constant during this period, it can be concluded that the vessels were tight. Furthermore, the methanol concentration in the headspace was not appreciably diluted by the air flowing in through the second needle. The glass vessel containing the abacterial sample used for the long-time test was also opened for 10 min and then closed again. When measured about half an hour later, the level of methanol had already again reached the level before the glass had been opened (Fig. 3B, IIb).

Throughout the duration of the measurement period (7 d), the average concentration (two measurements of each of

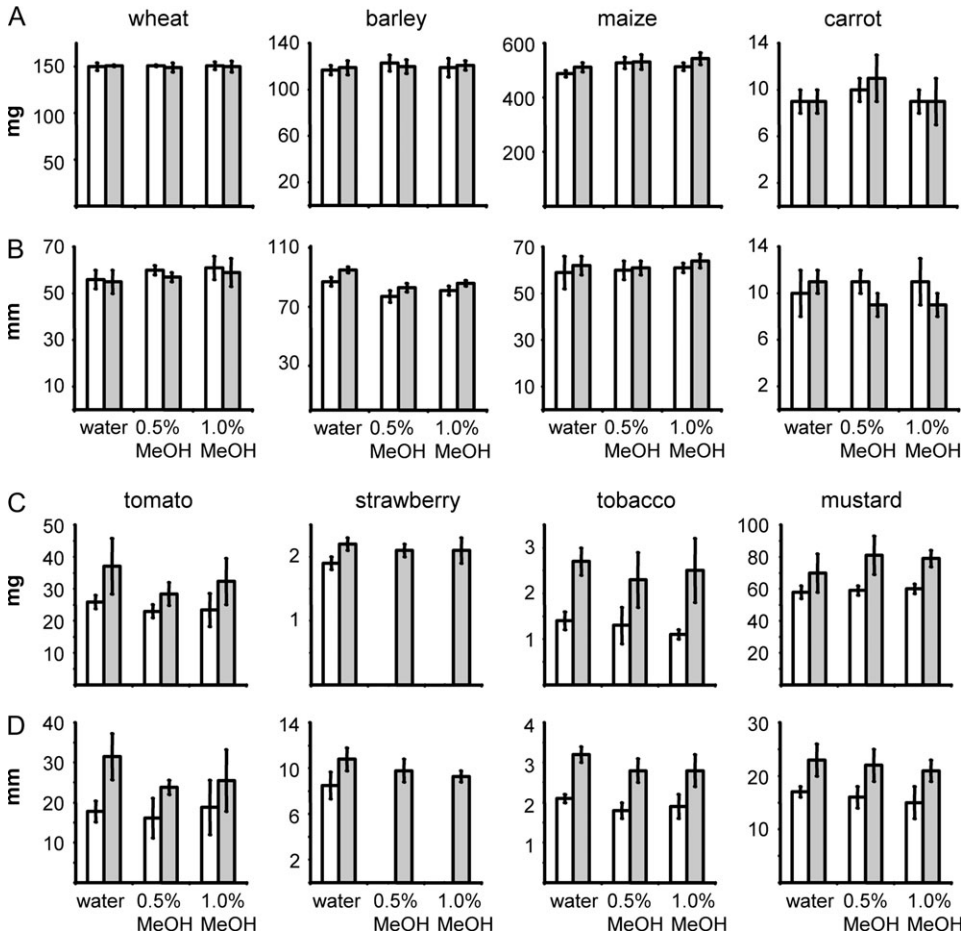

Fig. 1. Effect of *M. extorquens* strain ME4 (grey bars) on seedling fresh weight (A, C) and shoot length (B, D) of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*), maize (*Zea mays*), carrot (*Daucus carota*), tomato (*Lycopersicon esculentum*), strawberry (*Fragaria vesca*), tobacco (*Nicotiana tabacum*), and mustard (*Sinapis alba*) in comparison with untreated seeds (white bars) when cultivated in water, 0.5% methanol, or 1% methanol. Ten to 75 seeds, depending on the species, were used for the germination experiments. Error bars show the standard deviation of triplicate measurements.

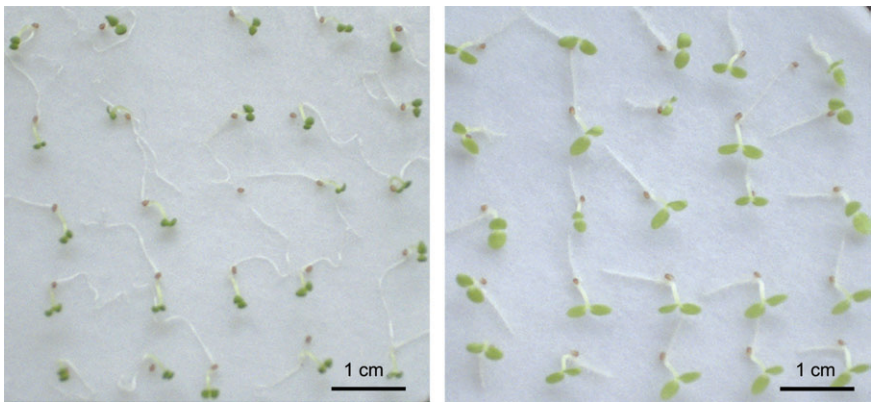

Fig. 2. Tobacco seedlings (7-d-old) germinated in sterile water (left) and after inoculation with *M. extorquens* ME4 isolated from strawberry leaf (right).

the five samples) of methanol in the headspace above the abacterial seedlings decreased from ~0.6 to 0.1 ppbv (Fig. 4). The presence of a small residual population which can be found under the seed coat and is difficult to remove by surface sterilization could explain the gradual decrease. A number of other substances showed a similar behaviour. One VOC determined at *m/z* 45 was emitted only by the plants inoculated with PPFM.

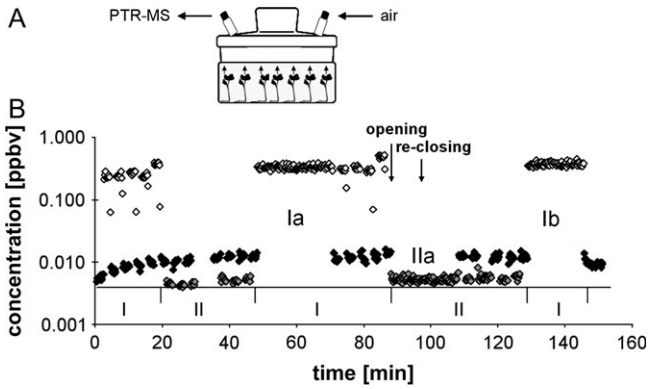

Fig. 3. Experimental set-up (A) and methanol concentration (B) determined by PTR-MS after 4 d (11 d post-germination) of measuring the methanol signal coming from sterile tobacco seedlings (white diamonds), room air (black diamonds), and tobacco seedlings inoculated with *M. extorquens* ME4 (grey diamonds). Room air and headspace of sterile seedlings (I) as well as room air and headspace of inoculated seeds (II) were alternately monitored (2 s measurements). Transition concentrations were excluded from the calculation of the mean values. Ia and IIa show long-term measurements, while Ib depicts the methanol level determined after opening and re-closing the vessel.

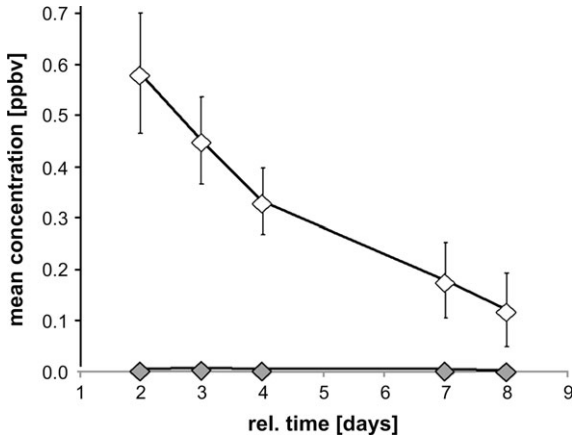

Fig. 4. Mean methanol concentration in the headspace of the sterile tobacco seedlings (white diamonds) and seedlings inoculated with *M. extorquens* ME4 (grey diamonds).

Localization of the methanol emission site on tobacco cotyledon

The well-characterized methylotrophic yeast *P. pastoris* transformed with pPICHS/GFP harbouring the AOX promoter that was fused to a reporter gene for GFP was used for the detection of methanol emitted by cotyledons (Boettner *et al*., 2002). In this strain, the *gfp* gene from the jellyfish *Aequorea victoriae* is expressed under the control of the promoter AOX, which is tightly regulated and induced by methanol. Accumulation of GFP fluorescence in cells of *P. pastoris* pPICHS/GFP was confirmed by a CLSM. *Pichia pastoris* pPICHS, containing the empty vector, served as control. Both strains were cultivated for 2 d in a methanol-containing medium, but GFP protein expression was only observed in *P. pastoris* pPICHS/GFP cells where it was homogenously distributed (Fig. 5A).

To determine the emission of methanol by cotyledons, *P. pastoris* pPICHS/GFP and pPICHS cells were added to tobacco seedlings and analysed by a CLSM 2 d after inoculation. Control experiments were performed with 1.0% methanol. Production of GFP was not observed in any experiments performed with the control strain (data not shown), but *P. pastoris* pPICHS/GFP cells colonizing the surface of the cotyledons of the tobacco seedlings produced the recombinant protein (Fig. 5B). GFP fluorescence was primarily detected in the close vicinity of stomata (Fig. 5C). However, the outlines of the single cells were not clearly visible. The individual cells probably overlap, they form biofilms, or GFP is liberated by spontaneous lysis. It is excluded that the yeast cells accumulate only in depressions in the stomata since GFP fluorescence is evenly distributed on the cotyledon surface, as shown when external methanol is added onto the cotyledons (Fig. 5D). The images clearly show that the levels of methanol emitted by young tobacco cotyledons are sufficient to induce the expression of GFP in the transgenic *P. pastoris* cells, providing direct evidence that methanol is emitted through the stomata.

Discussion

The ease with which PPFMs could be recovered from *F.×ananassa* leaves and purified into pure cultures as well as the detection of rDNA sequences coding for *M. extorquens* species suggests that methylotrophs are normal components of the strawberry leaf microflora in addition to other recently identified epiphytic bacteria (Krimm *et al*., 2005). The frequent reported isolations of PPFMs from plant material, in particular from leaf surfaces, and the demonstrated association with >70 plant species make them interesting as potential agents affecting plant growth (Corpe and Rheem, 1989; Holland and Polacco, 1994).

The present results show a growth-promoting effect of *M. extorquens* ME4 on seedlings of *L. esculentum*, *F. vesca*, *N. tabacum*, and *S. alba*, but a similar enhancement of the seedling weight and shoot length was not seen for the important crop plants *T. aestivum*, *Z. mays*, *H. vulgare*, *D. carota*, *P. vulgare*, and *P. sativum*, even after addition of methanol. *Methylobacterium extorquens* ME4 isolated from *F.×ananassa* leaves promotes the germination of different plant seeds and, therefore, exhibits no tight specificity for *Fragaria*, but the growth-promoting activity does not generally apply for all tested seeds. It has been demonstrated that colonization of tobacco explants with methylotrophic bacteria led to the stable association between the bacteria and its host plant that enhanced the latter's growth and capacity for regeneration and root formation (Kalyaeva *et al*., 2001). Colonization of

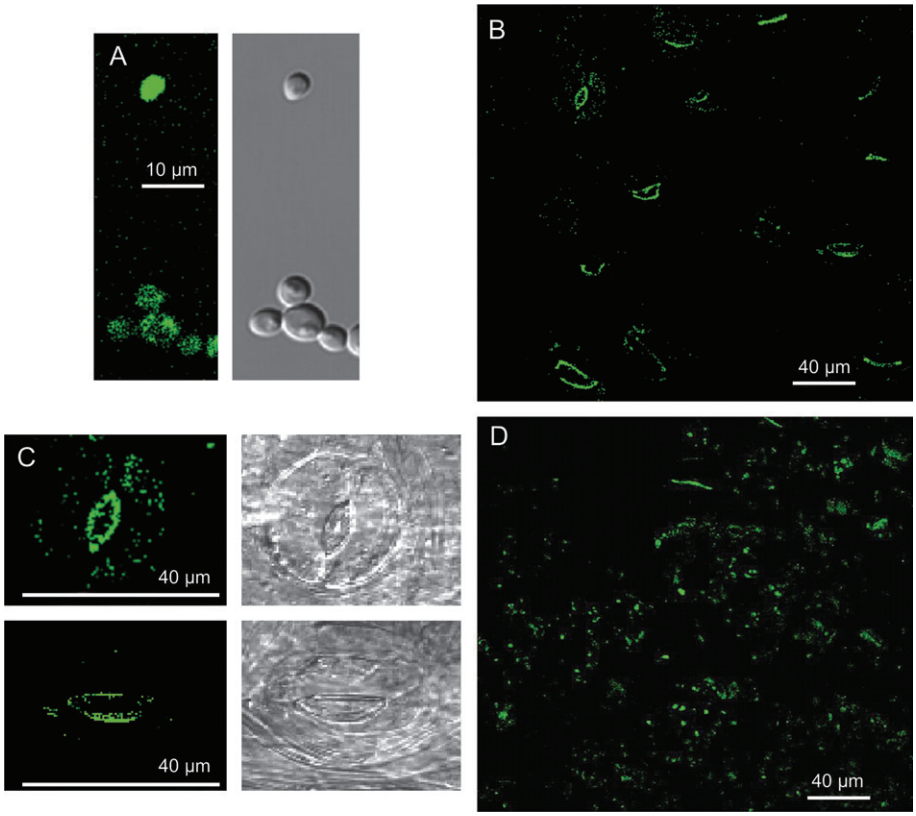

Fig. 5. (A) CLSM (left) and phase contrast image (right) of *P. pastoris* cells transformed with pPICHS/GFP expressing GFP in the presence of methanol. (B) CLSM image of the surface of a tobacco cotyledon 2 d after inoculation with *P. pastoris* pPICHS/GFP. (C) CLSM (left) and phase contrast image (right) showing the expression of GFP especially on stomata. (D). *Pichia pastoris* pPICHS/GFP cells are evenly distributed on the cotyledon as demonstrated by the expression of GFP after addition of external methanol onto the surface.

T. aestivum explants with *Methylobacterium* strains isolated from soil and rice also stimulated the formation of morphogenic calli and shoots and promoted development of the regenerated plants (Kalyaeva *et al*., 2003). However, the available data are too fragmentary to draw any general conclusion about specificity of the observed effects.

It is known that certain isolates of PPFMs produce phytohormones (auxins and cytokinins) and vitamin B_{12}, and interact with the plant nitrogen metabolism via the bacterial urease or through the induction of nitrogen-fixing root nodules, but the molecular basis for the growth-promoting activity is unknown (Jourand *et al*., 2004, Omer *et al*., 2004*b*; Lee *et al*., 2006). Vitamin B_{12} did not promote seed germination in the present experiments, but semi-polar substances (e.g. phytohormones) excreted by *M. extorquens* were shown to enhance the biomass of tobacco seedlings. Thus, urease activity positively tested for *M. extorquens* ME4 and the fixation of nitrogen probably do not account for the enhanced seedling growth.

Methylobacterium extorquens ME4 utilizes methanol but also D-glucose, ethanol, methylamine, and malate as carbon and energy sources (Table 1). Although levels of ~0.1, 0.3, and 1.1 mg m^{-2} D-glucose, D-fructose, and sucrose, respectively, have been determined on plant leaves (Fiala *et al*., 1990), methanol constitutes the primary substrate for the methylotrophic strain as demonstrated by PTR-MS analysis. The present data provide the first direct evidence that PPFMs predominantly utilize the gaseous methanol emitted by cotyledons. The emission rate of leaves depends on the leaf age (Nemecek-Marshall *et al*., 1995).

It has been shown that virtually all methanol emission from the leaf surface occurs through the stomata and that stomatal conductance correlates with methanol levels (Nemecek-Marshall *et al*., 1995). By means of a GFP-coupled bacterial biosensor system, it was directly demonstrated that stomata are the primary emission sites of methanol on the cotyledons. The variation in GFP fluorescence of biosensor cells on leaves suggested that large spatial variations in methanol availability occur on leaves, which is consistent with what has already been shown for sucrose (Miller *et al*., 2001).

Bacteria belonging to the genus *Methylobacterium* are ubiquitous in nature and colonize probably all land plants. They utilize the gaseous methanol that is emitted by the plants through the stomata as carbon and energy source and promote the growth of their host through the release of metabolites. Further experiments with other plant seedlings would be useful in order to obtain more general information on the symbiosis between plants and PPFMs.

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