

In-vitro regeneration of sunflower plants: effects of a *Methylobacterium* strain on organ development

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(Received January 21, 2005)

Summary

Under natural conditions, plant surfaces are colonized by a variety of microorganisms. It has been postulated that non-pathogenic methylobacteria promote plant growth via the secretion of phytohormones. In order to study the putative role of these prokaryotic epiphytes during plant development we established a sterile regeneration system, using the sunflower (*Helianthus annuus* L.) as model organism. Apical hypocotyl segments, excised from 4-day-old etiolated seedlings, were placed into a shoot induction medium. Half of them were inoculated by immersing the hypocotyl segments in a bacterial suspension, containing a *Methylobacterium* strain that was isolated from a ligulate flower of a field-grown plant. After 14 days of cultivation in darkness (followed by 14 days in a light/dark-regime), regenerated shoots were excised from the hypocotyl segments. After 20 days on vitamin medium, juvenile plantlets were rooted on rooting medium. Regenerated sunflower plantlets started to flower after about 50 days of growth in glass jars. In the presence of the methylobacteria a promotion in the number of shoots and roots regenerated in-vitro was recorded, but no enhancement in growth occurred.

Introduction

The relationship between epiphytic microorganisms (bacteria, fungi and yeasts) and the epidermal cells in the above-ground part of land plants (embryophytes) is not completely understood. A number of reports indicate that microbes such as pink-pigmented facultatively methylotrophic bacteria (PPFMs) of the genus *Methylobacterium* are able to use metabolic waste products (methanol etc.) that are released by the plant cells during elongation growth (CORPE, 1985; CORPE and BASILE, 1982; HIRANO and UPPER, 2000). The degraded bacterial metabolites may be absorbed by the growing epidermal cells and improve the nutritional status of the developing plant. The epiphytic methylobacteria, on the other hand, may promote the rate of cell growth via the secretion of cytokinines and other phytohormones. This hypothesis was proposed by HOLLAND (1997), who speculated that microbes of the genus *Methylobacterium* may be interpreted as co-evolved participants in most (if not all) higher plants.

In a recent publication we have shown that germ-free seedlings of sunflower (*Helianthus annuus* L.) grow more rapidly than non-sterile controls, indicating that methylobacteria (components of the natural epiphytic flora) are not required for normal development in this representative higher plant (KUTSCHERA, 2002; KUTSCHERA et al., 2002). However, experiments with sterile protonemata of the moss *Funaria hygrometrica* revealed that methylobacteria promote the rate of cell growth in this lower plant (HORNSCHUH et al., 2002).

The aim of this study was to elucidate whether sterile plantlets of *Helianthus annuus*, regenerated from excised hypocotyl segments cultured in-vitro, grow more rapidly in the presence of methylobacteria. We used a selected strain of *Methylobacterium* that was shown in preliminary experiments to promote cell elongation in *Funaria* (HORNSCHUH et al., 2002).

Material and methods

To eliminate spores of fungi, achenes (seeds) of *Helianthus annuus* L. cv. Giganteus were soaked for 5 h in H₂O followed by immersing in hot water (51°C) for 10 min. Seeds were sterilized by a treatment with 6.5 % sodium hypochlorite solution for 30 min and thereafter washed in sterile distilled water. 40 seedlings were grown for 4 d (darkness) in 750 ml glass jars in sterile vermiculite moistened with a medium according to MURASHIGE and SKOOG (1962) (MSM). Hypocotyl segments with the apical meristem were excised in green

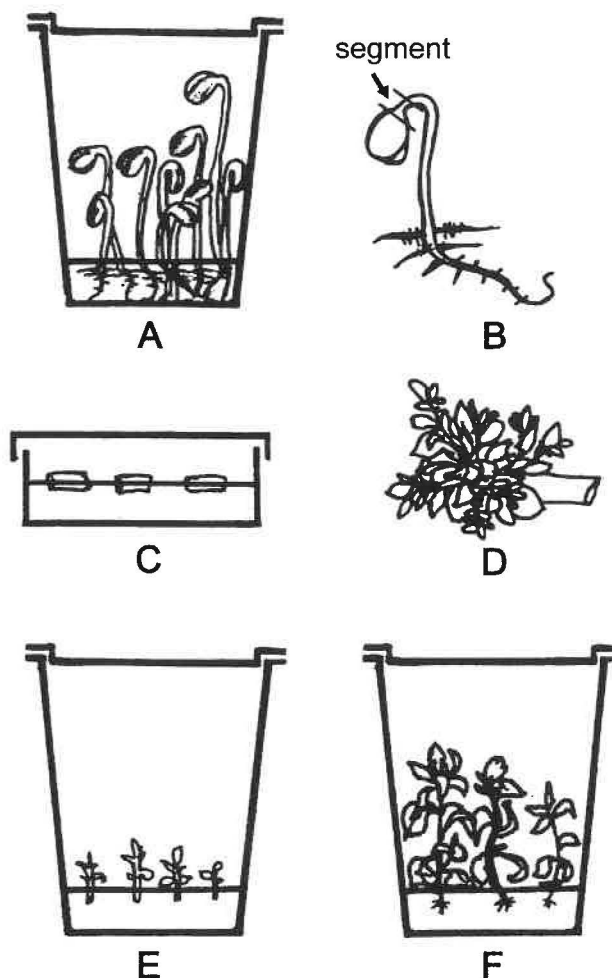


Fig. 1: Schematic representation of the in-vitro regeneration system used in this study. Segments were cut from etiolated seedlings that were raised under sterile conditions (A, B). The hypocotyl sections were incubated on shoot induction medium (C). After removal from the source tissue, individual shoots (D) were planted into vitamin medium (E) and thereafter rooted on a corresponding agar medium (F).

light and placed on MS-medium supplemented with sucrose (30 g/L), myo-inositol (100 mg/L), benzyl-aminopurine (1 mg/L), gibberellic acid (0.1 mg/L), KNO_3 (5 g/L), solidified with agar (0.8 Vol. %) (shoot induction medium SIM) (Fig. 1 A). Half of the explants were submerged in a bacterial solution, containing 10^4 colony-forming units of methylobacteria for 0.5 h (*Methylobacterium* sp., a strain isolated from a ligulate flower of a field-grown sunflower plant); control samples were treated with an equivalent amount of sterile water. For induction of shoots the sterile and inoculated hypocotyl segments were cultured for 14 days in darkness and for another 14 days in a continuous white light/darkness-regime (16 h / 8 h). Regenerated shoots were excised from the hypocotyl and placed for 20 d in glass jars that contained vitamin medium (VM), i. e., MS-medium supplemented with sucrose (30 g/L), gibberellic acid (200 mg/L), L-glutamine (439 mg/L), L-aspartic acid (133 mg/L), L-arginine (144 mg/L) and glycine (38 mg/L) solidified with agar (0.8 Vol. %). Thereafter, plantlets were rooted on rooting medium (RM), i. e., MS-medium that contained sucrose (10 g/L), myo-inositol (50 mg/L), naphthalene acetic acid (0.1 mg/L) and agar (0.8 g/L). All media were adjusted to pH 5.7. Sterile-filtered solutions of Benzylaminopurine and gibberellic acid were added after sterilization of the corresponding medium in an autoclave.

For scanning electron microscopy (SEM), leaves from plantlets on RM were fixed in 2 % glutaraldehyde in phosphate buffer, dehydrated, critical-point-dried, coated with platinum, and viewed in a SEM as described by KUTSCHERA et al. (1990).

The agar impression method was used for determination of bacterial contamination of plant organs (KUTSCHERA et al., 2002). Samples (shoots) were pressed firmly to the surface of glycerol/peptone-agar, a non-selective medium where many microorganisms grow and establish colonies (CORPE, 1985). After 24 h the samples were removed and incubated in darkness (27 °C). Two weeks later, the petri dishes were photographed. For selection of PPFMs from field-grown sunflower plants the medium described by COTE (1984) was used.

All experiments described here were repeated at least three times with similar results. Representative samples are depicted for documentation.

Results

To control morphogenesis in plants cultured in-vitro the media must be supplemented with various substances such as synthetic analogues of phytohormones, vitamins, and other special additives. Fig. 1 shows our sunflower in-vitro regeneration scheme as used in this investigation. Hypocotyl sections, excised from the hook of etiolated 4-day-old germ-free seedlings, were used because this tissue contains meristematic cells (KUTSCHERA, 2000; NEUMANN, 1995). The explants were cultivated in the presence or absence of a *Methylobacterium* strain that was isolated from an adult sunflower plant raised in the Botanical Garden of the University of Kassel (samples \pm methylobacteria).

The process of shoot induction is shown in Fig. 1 C and D. After 3 weeks of incubation on a corresponding medium, multiple shoots emerged on the explanted hypocotyl tissue adjacent to the apical meristem in both sterile and inoculated samples (Fig. 2 A, B). The number of shoots per explant was significantly enhanced in the presence of methylobacteria (8 ± 2 versus 5 ± 1 in controls, means \pm S.E., $n = 18$), indicating that the added microbes exerted a stimulatory effect on in-vitro regeneration in *Helianthus* (shoot induction in meristematic cells). These data document that the added microbes significantly promoted processes that lead to the induction of shoots. The number of roots per shoot was 7 ± 1 (control) and 10 ± 3

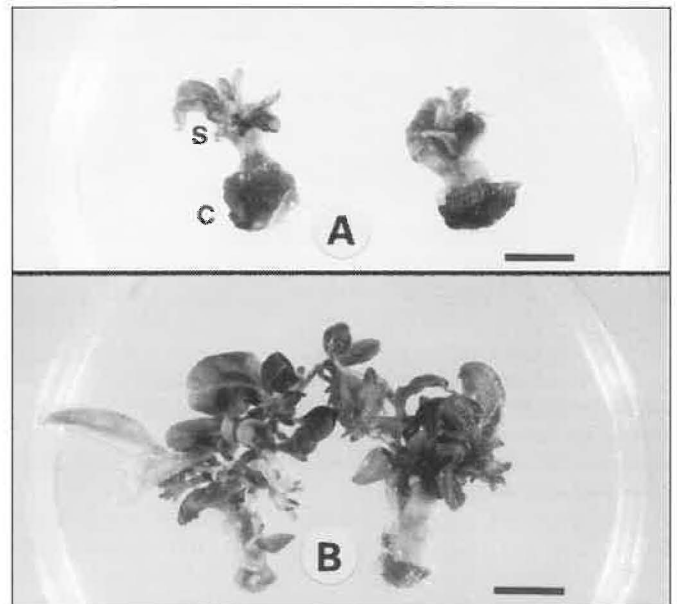


Fig. 2: Upon incubation on a shoot induction medium multiple shoots (S) emerged at the tissue close to the meristem in sterile (A) and inoculated (B) sunflower explants. More shoots and less callus tissue (C) occurred in explants treated with methylobacteria, resulting in a shift in fresh mass ratios. Bars = 1 cm.

(+ methylobacteria), respectively ($n = 100$). In the presence of methylobacteria rooting was very efficient (92 %). In control plantlets the percentage of rooted stems was significantly lower (61 %) ($n = 100$).

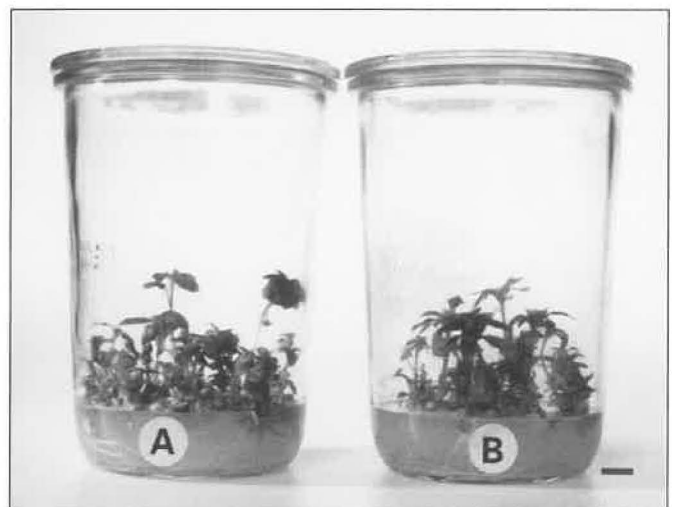


Fig. 3: Sterile (A) and inoculated (B) sunflower plantlets raised on rooting medium after 50 days of cultivation. No difference in stem length and organ development was observed. Both plantlets started to flower at about the same time after start of in-vitro cultivation. Bar = 1 cm.

However, the results shown in Fig. 3 A, B document that these positive effects do not cause an enhancement of growth. The lengths of regenerated shoots after a period of 20 days on vitamin medium was 22 ± 1 and 23 ± 1 mm for sterile and inoculated samples, respectively (means \pm S. E., $n = 100$).

Rooted plantlets (\pm methylobacteria) started to flower at approximately the same time (40 - 45 days after shoot regeneration) (Fig. 4). Average stem lengths of 30 ± 3 and 28 ± 4 mm were measured in sterile and inoculated plantlets, respectively (means \pm S. E., $n = 100$). These data show that the added methylobacteria exerted no stimulatory effect on elongation growth in the regenerated sunflower plantlets.



Fig. 4: Representative 50-day-old sunflower plantlet, regenerated in-vitro (sterile sample). A flower (F), green leaves (L) and yellow, senescing leaves (S) are visible. Bar = 1 cm.

In order to verify whether or not our regenerated control shoots were in fact sterile, the agar-impression method was used. In the controls, no bacterial contamination was detectable; in inoculated samples, however, numerous pink-pigmented colonies were visible on the agar plate (Fig. 5 A, B). This positive result indicates that the added methylobacteria established large colonies that invaded the entire cuticle of the epidermal cells.

In addition, mature leaves of 50-day-old sunflower plantlets were examined by scanning electron microscopy. In sterile samples, no bacteria were detected (Fig. 6 A). Clusters of methylobacteria were observed on epidermal cells and the stomata of inoculated explants (Fig. 6 B). Numerous methylobacteria were found in the cavity (pore) of some stomata where methanol is released. This gas can be used as a carbon-source by the prokaryotic epiphytes.

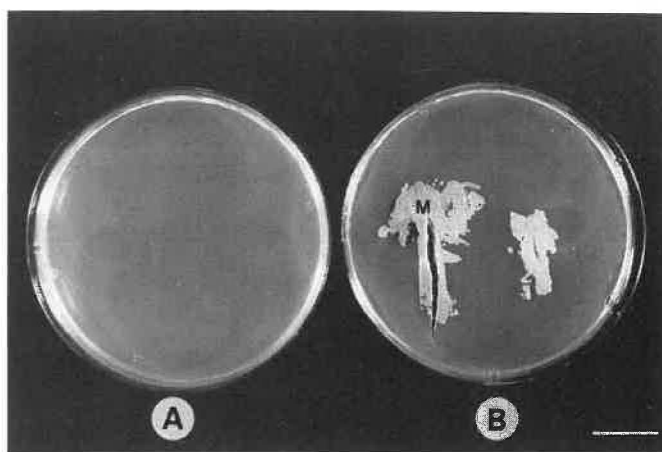


Fig. 5: For detection of bacterial contamination, 3-week-old explants with multiple shoots were pressed onto and removed from glycerol/peptone-agar plates. No colonies developed in the sterile control (A). Pink-pigmented colonies of methylobacteria (M) occurred all over the impressed surface of the inoculated explants (B). Bar = 2 cm.

Discussion

We introduce here an in-vitro system for routinely generating rooted plantlets of an important crop species of considerable economic relevance. It should be noted that methods for plant regeneration via somatic embryogenesis from etiolated sunflower cotyledons (FIORE et al., 1997) and from hypocotyl segments (PETERSON and EVERETT, 1995) have been described. In addition, MOHAMED et al. (2003) have established methods for the vegetative in-vitro regeneration of *Helianthus annuus* plantlets via shoot induction from the apical meristem.

The primary aim of this research project was to elucidate whether cytokinin-producing methylobacteria influence growth and organ development in sunflower plantlets raised under aseptic (germ-free) conditions.

Our selected *Methylobacterium* strain exerts a promotive effect on organogenesis. We suggest that cytokinins (and auxins as well as other unknown substances) are produced and released by these methylobacteria which leads to a stimulation of shoot- and root production. However, previous tests without cytokinin in the shoot induction medium (+ methylobacteria) resulted in no regeneration of shoots (data not shown). The positive effects recorded here were only transient and occurred about 3 weeks after the start of the experiment. Inoculated 5-week-old flowering *Helianthus annuus* (Fig. 4) developed stems that were not larger than those in sterile controls, i. e., epiphytic microorganisms exerted no persistent effect on plant growth in the sunflower.

HORNSCHUH et al. (2002) have shown that methylobacteria associated with protonemata of the moss *Funaria hygrometrica* enhance cell growth via the release of cytokinins. It is likely that the positive effect documented here for sunflower (Fig. 3 A, B) is likewise attributable to the action of these phytohormones, which were supplied by the bacterial epiphytes (*Methylobacterium* sp.).

Cytokinins have been defined as "substances which, in combination with auxin, stimulate cell division in plants and which interact with auxin in determining the direction which differentiation of cells takes" (ASHBY, 2000). In healthy (non-stressed) plants, cytokinins are synthesized primarily in the roots and thereafter transported into the shoot through the xylem. In a classical essay, HOLLAND (1997) proposed that cytokinins are not synthesized by plant cells de-novo,

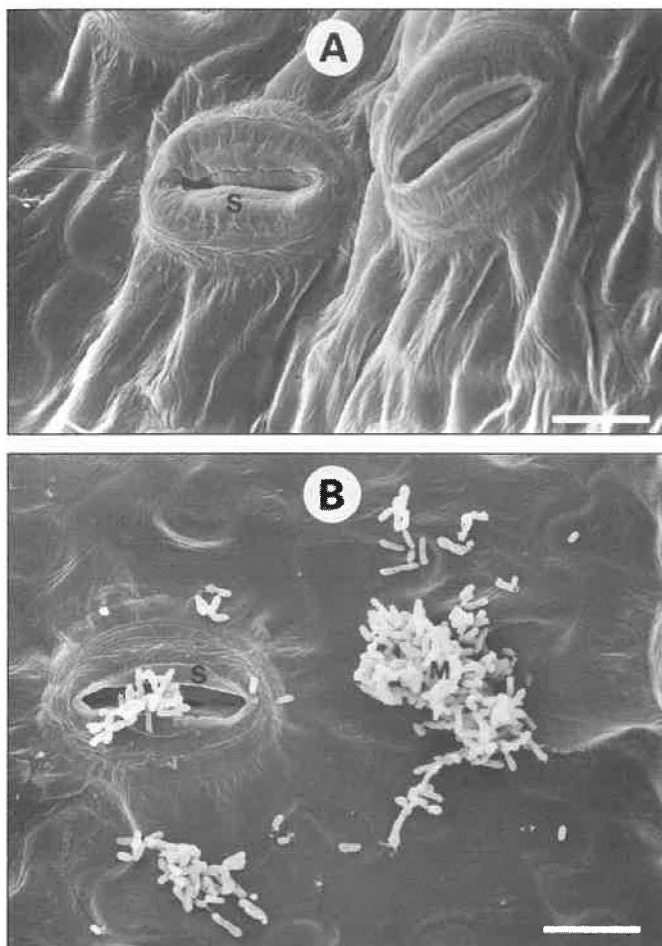


Fig. 6: Scanning electron micrographs of mature (green) leaves of 50-day-old sunflower plantlets. No bacteria were detected on the abaxial leaf surface of sterile samples (A). In explants that were treated with *Methylobacterium* sp. four weeks prior to preparation of the leaves (B) clusters of methylobacteria (M) were observed on epidermal cells and close to the stomata (S). Bars = 10 μ m.

but are derived from epiphytic methylobacteria. Three years later, ASHBY (2000) concluded that, in the absence of a documented molecular pathway in plants for de-novo cytokinin biosynthesis, microbes may be a fundamental component of this biochemical chain of reactions *in planta*. However, in the meantime sequences within the plant genome have been detected that correspond to some key enzymes of cytokinin biosynthesis (HORNSCHUH et al., 2002).

Our results provide independent support for the concept of *in planta* cytokinin production. In-vitro regeneration of adult sunflower plantlets occurs in the absence of methylobacteria (sterile samples that grow over a period of 20 days in the absence of benzylaminopurine and synthetic auxin). It is obvious that these germ-free control plantlets produce cytokinins de-novo, because without cytokinins no plant growth occurs (ASHBY, 2000). Our experiments provide

strong circumstantial evidence for the endogenous production of this essential phytohormone. However, more work is required to further elucidate the transient effect of methylobacteria on shoot and root regeneration in sunflower stem segments.

Acknowledgement

We thank Mr. H. Rühling (Abt. Zellbiologie, Universität Kassel) for technical help with the scanning electron microscopy.

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