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The effect of growth-promoting methylobacteria on seedling development in *Ginkgo biloba* L.

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Summary

Microbes of the genus *Methylobacterium* are regularly associated with leaves and fruits of plants. In experimental tests, they promote the growth of germ-free liverworts and mosses, which are ancient land plants. In this study we analyzed the effect of *M. mesophilicum*, a bacterium that has been isolated from the organs of numerous plant species, including gymnosperms and angiosperms, on the development of sterile embryos of *Ginkgo biloba* L., a unique living fossil among the embryophyta. In addition, germ-free seeds of *Pinus sylvestris* were inoculated with the same strain of methylobacteria. In *G. biloba* seedlings that were raised in a 12 h dark/light regime, a promotion of root development was recorded in samples treated with the methylobacteria. A fresh mass increase of + 25 % occurred within 6 weeks of inoculation with bacteria, compared to the aseptic control. In contrast, shoot development of the same plants was not significantly affected by these bacteria. In *Pinus* seedlings, organ development was unaffected by the presence of methylobacteria. Our results document a differential sensitivity of the root system versus the shoot towards these ubiquitously distributed plant-associated bacteria. The data are discussed with reference to the isolated taxonomic position of *Ginkgo biloba*, one of the most primitive gymnosperms in the biosphere that is economically important as a medicinal plant.

Introduction

Nearly every botanical garden or arboretum in temperate and subtropical climate regions of the world has at least one *Ginkgo* (Maidenhair) tree. Due to veneration of this "sacred tree of the East", this unique organism, represented by only one extant species (*Ginkgo biloba* L. 1771), has been planted since ancient times in China and Japan in temple gardens near shrines and castles. The *Ginkgo* tree is the symbol of the Tokyo Metropolitan area; splendid individuals can be seen in the capital of Japan in many parks and along avenues. Moreover, in China, Korea and Japan grilled *Ginkgo* nuts are eaten; they are also used in porridges and soups or mixed with rice, stir-fried vegetables and mushrooms. Leaves of *Ginkgo* trees are used in Chinese medicine to treat bronchitis, asthma, stomach pain and other ailments. In Europe and the United States, *G. biloba* has also been recognized as a medicinal plant (HORI et al., 1997; ZHOU and ZHENG, 2003).

In a series of studies we have shown that gametophyte development in liverworts and mosses is enhanced by methylotrophic bacteria of the genus *Methylobacterium* (HORNSCHUH et al., 2002, 2006; KOOPMANN and KUTSCHERA, 2005). However, no such positive effect was measured when germ-free seedlings of higher plants (sunflower, maize) were treated with methylobacteria (KUTSCHERA, 2007). Bryophytes are, like *Ginkgo biloba*, organisms that display a phenotype reminiscent to that of ancient forms of life within the kingdom Plantae ("living fossil plants"). Therefore, we investigated whether the development of germ-free *Ginkgo*-seedlings is promoted by added methylobacteria. Taxonomists have classified the species *G. biloba* as a member of the Coniferopsida (HORI et al., 1997; SCHNEIDER et al., 2002; FROHLICH and CHASE, 2007). Hence, we have included a representative conifer species (*Pinus sylvestris* L.) in this experimental study for comparison.

Materials and methods

Seeds of *Ginkgo biloba* L. and *Pinus sylvestris* L. were purchased from a commercial supplier (Carmens Bonsai Garten, Memmingen, Germany). The seeds (in the case of *G. biloba* after the removal of the sarcotesta, see Fig. 1 A) were sterilized as follows. Batches of 5 to 20 pre-selected individuals were washed for 12 h in 300 ml of H_2O (aqua bidest). In the next step, the seeds were incubated for 20 min in a half concentrated sodium hypochlorite solution (NaOCl, available chlorine ca. 13 %) and thereafter washed five times with germ-free water. Half of the aseptic seeds were submerged for 24 h in a bacterial solution that contained 10^8 colony forming units per ml of methylobacteria (*M. mesophilicum*, ATCC 29983, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany); control seeds were treated with the same amount of distilled water. The seeds (± methylobacteria) were raised under aseptic conditions as follows.

Glass jars (750 ml), covered with a lid, were autoclaved (temperature 121 °C, pressure: 1.1 kg/cm^2) for 15 min and filled to a height of 5 cm with sterile vermiculite. Single seeds (*G. biloba*) or batches of 4 individuals (*P. sylvestris*) were planted under germ-free conditions. The *Ginkgo*- and *Pinus* seedlings were raised in a 12 h white light/dark cycle at 99.5 % relative humidity (25 ± 1 °C).

Six weeks after sowing, the sterile (or inoculated) plants were harvested. After careful excision, the length of the washed/dried root systems and the corresponding shoots were measured with a ruler. Thereafter, fresh masses of both organs were determined with a microbalance. In order to determine the extent to which the sterilized seeds and the resulting seedlings may nevertheless have been contaminated with bacteria the agar impression method of CORPE (1985) was used as described in detail in previous reports (KUTSCHERA, 2002; KUTSCHERA et al., 2002). All experiments were repeated at least four times with similar results.

Results

Fig. 1 A shows a representative *Ginkgo* seed, with the outer fleshy coating removed. After removal of the sarcotesta (the soft yellow-brown seed coat), the hard sclerotesta, which encloses the embryo, becomes visible. In all experiments, seeds without sarcotesta (i.e., embryos with attached endosperm) were used (Fig. 1 B).

In order to determine to what extent the smooth outer surface of the embryo is contaminated with spores of microorganisms non-sterile embryos were pressed for 1 day to the surface of petri plates that contained glycerol-peptone agar (Fig. 2 A). Seven days later, dense layers of bacteria and, occasionally, colonies of fungi had developed on the surface of the agar (Fig. 2 B). These results show that the embryo of *Ginkgo* seeds, which is in contact with the surrounding air via a terminal hole in the sarcotesta, is heavily contaminated by a

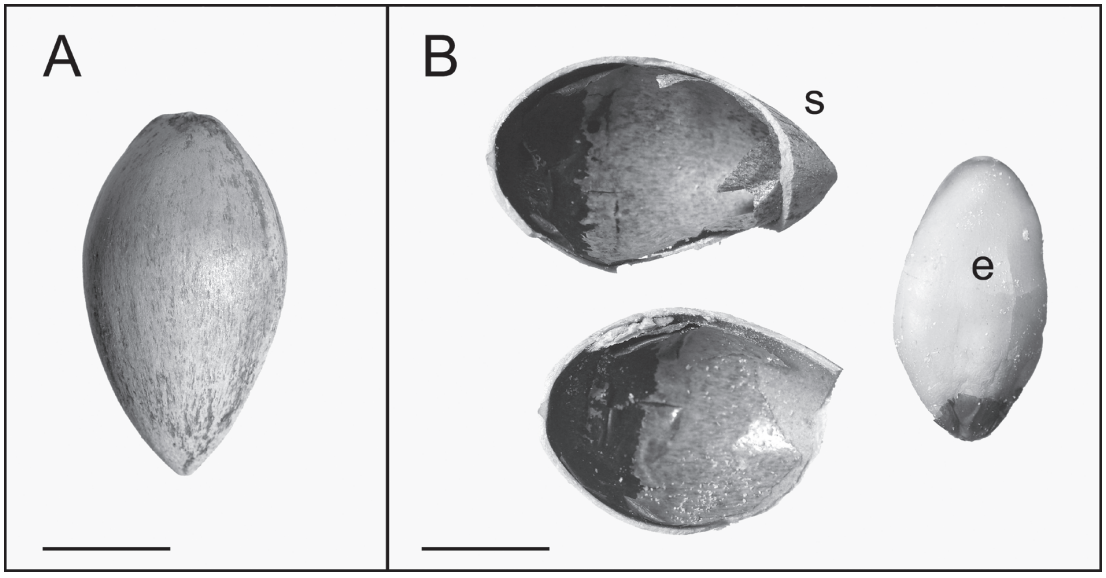

Fig. 1: Representative seed of *Ginkgo biloba* with sarcotesta removed to show the outer surface of the sclerotesta (A). The propagule is oriented with the micropyle downward. Broken sclerotesta, viewed from the inside (left) and isolated embryo with attached endosperm (right), oriented with the radicle downward (B). s = sclerotesta, e = embryo. Bar = 1cm.

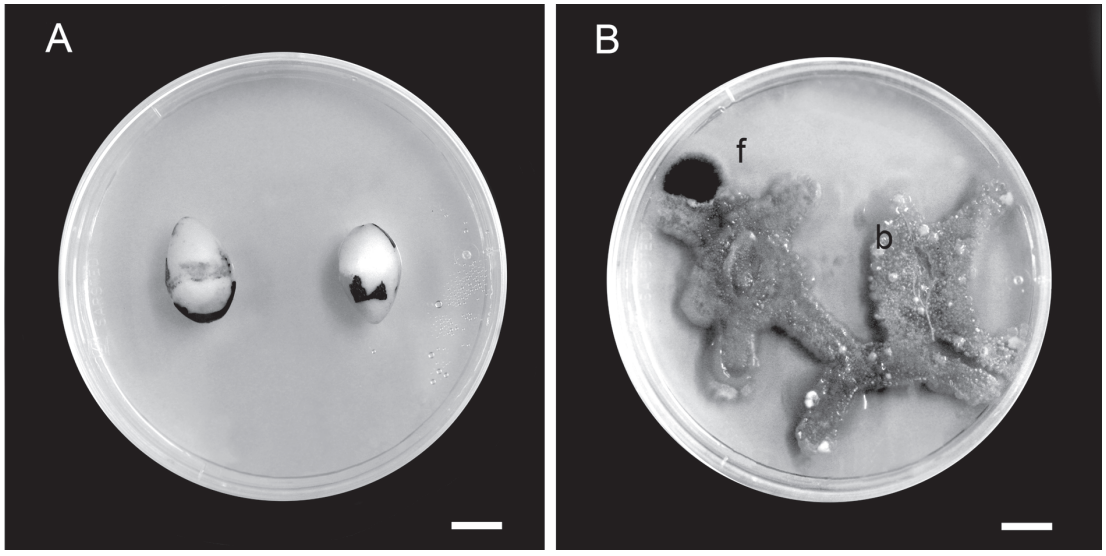

Fig. 2: Petri dish with two non-sterile embryos of *Ginkgo biloba* pressed onto the surface of sterile glycerol-peptone agar (A). After 7 days of incubation of the agar plate, bacteria (b) and fungi (f) developed around the places where the tissue was in contact with the agar (B). Bar = 1cm.

variety of microorganisms and their propagules. The corresponding experiment carried out with sterilized *Ginkgo* embryos, from which the sarcotesta had been removed, is shown in Fig. 3 A, B. It is apparent that the sterilization procedure employed here yielded aseptic propagules. The experiments shown in Fig. 2 and 3 were repeated with seeds of *Pinus sylvestris*. We obtained the same qualitative results as those depicted here for *Ginkgo biloba* (data not shown).

In a series of experiments with germ-free *Ginkgo* embryos (and *Pinus* seeds) we determined the quantitative effect of *Methylobacterium mesophilicum* on seedling mass. Sterile embryos were either inoculated for 24 h in germ-free water or in a suspension of *M. mesophilicum*. Thereafter, the seeds were planted into vermiculite and raised under aseptic conditions. A representative pair of *Ginkgo*-seedlings (± methylobacteria) is shown in Fig. 4 A, B. This photograph indicates that root development is promoted by the methylobacteria, but shoot growth is unaffected by these microbes.

The effect of *M. mesophilicum* on organ development was quantified by determining the fresh- and dry masses of sterile and inoculated seedlings (Fig. 5). On average, root fresh mass was 25 % higher in seedlings raised in the presence of methylobacteria compared with the germ-free controls. In the shoots of the same seedlings, no positive effect was measured. Hence, *M. mesophilicum* selectively promotes the growth of the root system but has no effect on the above-ground organs. To determine whether or not our seedlings were in fact sterile in the control and covered by methylobacteria in the inoculated samples, we used the agar-impression method to examine bacterial growth. The agar plate assays documented that our controls were germ-free and that added methylobacteria had colonized the entire

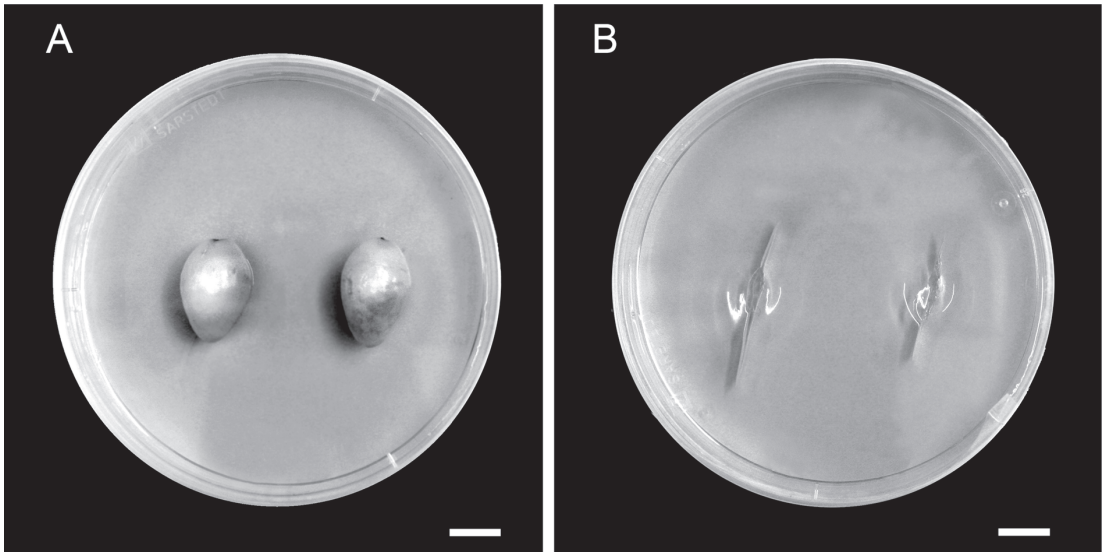

Fig. 3: Petri dish with two sterilized embryos of *Ginkgo biloba*, pressed onto the surface of glycerol-peptone agar (A). After 7 days of incubation of the plates the agar was still sterile, i.e., no microorganisms had developed in these germ-free samples (B). Bar = 1cm.

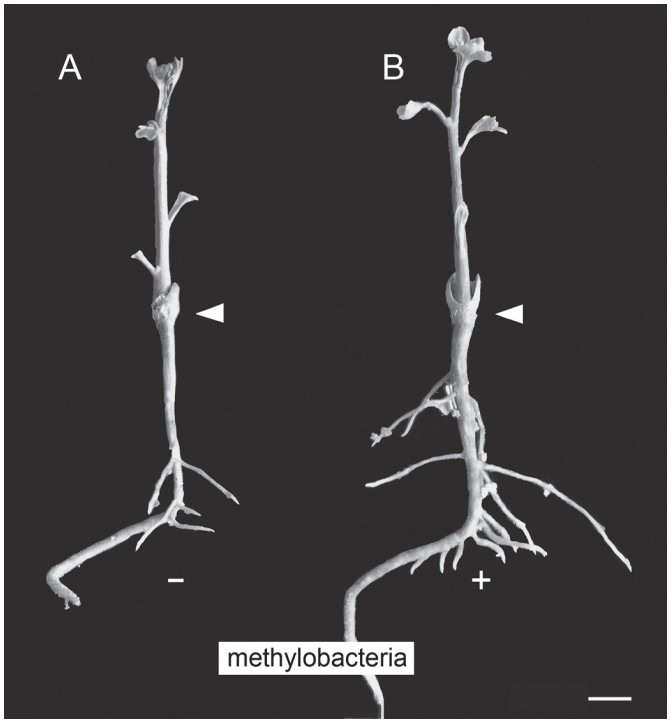

Fig. 4: Photographs of 6 week-old seedlings of *Ginkgo biloba* that were either raised in the absence (A) or presence (B) of methylobacteria. Sterile seeds were either soaked in germ-free water or a suspension of *Methylobacterium mesophilicum* and thereafter planted in sterile vermiculite. Note that the root system in the inoculated sample (B) is larger than that of the control (A). The onset of the root is indicated by arrowheads. Bar = 2cm.

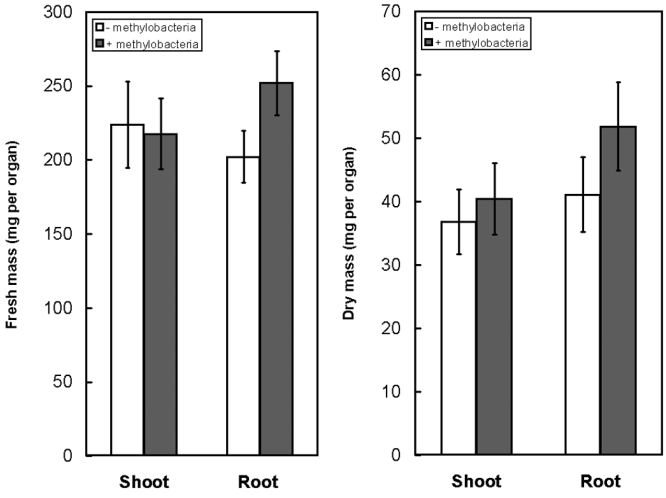

Fig. 5: Effect of methylobacteria on the development of root and shoot in *Ginkgo biloba*. Sterile embryos were either incubated in germ-free water or in a suspension of *Methylobacterium mesophilicum* and thereafter planted into moist sterile vermiculite. Fresh mass (A) and dry mass (B) (open and dark bars, respectively) was determined 42 days after start of the experiments (n = 15, ± s.e.m.).

Ginkgo seedling (results not shown).

The corresponding experiments with *Pinus* seeds yielded the following results. Average fresh mass of the shoots: sterile / inoculated (*M. mesophilicum)*: 35 ± 2 / 34 ± 3 mg (n = 25); fresh mass root system: 7.0 ± 0.5 / 7.3 ± 0.4 mg (n = 25). The measurements were carried out 42 days after inoculation. These data document that in seedlings of *Pinus sylvestris* methylobacteria exert no positive effect on organ development under the standard conditions employed here.

Discussion

In a series of studies it has been shown that plant-associated microbes of the genus *Methylobacterium*, which are probably distributed ubiquitously in nature, produce and secrete phytohormones such as auxins and cytokinines (HORNSCHUH et al., 2002, 2006; SCHAUER and KUTSCHERA, 2008). It is now definitively clear that methanol, emitted from the stomata of plants, is consumed by these epiphytes. Since the growth of axenically raised gametophytes of bryophytes, but not the shoots of sterile higher plants (sunflower, maize) is

promoted in response to the addition of methylobacteria, these microorganisms have been classified as phytosymbionts (KUTSCHERA, 2007). It should be noted that ABANDA-NKPWATT et al. (2006) analyzed the effect of the species *Methylobacterium extorquens* on the germination of surface-sterilized seeds of 10 species of angiosperms (wheat, barley, maize, carrot, pea, bean, tomato, strawberry, tobacco, and mustard). In the first six species of this list, no positive effect was measured, but in the last four a slight promotion of organ growth occurred. However, the authors did not distinguish between the roots and the above-ground phytosphere. Hence, it is not known whether these effects occur in the shoot or root of the corresponding seedlings.

In this study we report that in *G. biloba* methylobacteria exert a specific effect on root development, but the growth of organs above the surface of the earth are unaffected by these microbes. This finding has two implications: 1. The root system in this "living fossil plant" appears to be more sensitive to the addition of growth-promoting methylobacteria than the shoot, and 2. germination and subsequent seedling development can occur in the absence of any epiphytic microbes. SCHAUER and KUTSCHERA (2008) have shown that the roots of adult, field-grown sunflower plants are more heavily contaminated by methylobacteria than the leaves. This may be due to the fact that the zone surrounding the roots, the rhizosphere, provides more protection than the phyllosphere from light stress, high temperature, and desiccation. In addition, sources of mineral salts and organic substances are more abundant in the rhizosphere. Sugars, amino acids, organic acids and many other metabolites compose, together with small amounts of polysaccharides and proteins, the majority of the root exudates (BAIS et al., 2006). Since our *Ginkgo* seedlings (Fig. 4) were raised under moist, low-light conditions, the protective effect of the rhizosphere provided in the sun-exposed field is negligible in our experimental system. Hence, it is conceivable that the developing roots of our *Ginkgo* seedlings may be more densely populated by methylobacteria than the shoot; this may result in a promotion of root growth caused by phytohormones secreted by the epiphytic bacteria (KUTSCHERA, 2007). However, in seedlings of *Pinus sylvestris* that were treated the same as our *Ginkgo* samples no such differential effect of methylobacteria on root versus shoot development was measured. These data indicate that the root cells of *Ginkgo* seedlings may be more sensitive to the growth hormones secreted by methylobacteria than those of the shoot. However, direct proof in support of this hypothesis is currently lacking. At any rate, our data show that the "living fossil" *Ginkgo biloba*, which has extinct relatives that appeared in the Early Jurassic at a time when the first large-sized dinosaurs evolved (FROHLICH and CHASE, 2007), displays a behaviour that is analogous to that of mosses and liverworts (HORNSCHUH et al., 2002, 2006; KUTSCHERA and KOOPMANN, 2005). These ancient, morphologically primitive lower plants live in symbiosis with growth-promoting methylobacteria that consume waste products, such as methanol, that were released by the plant cells (KUTSCHERA, 2007). We suggest that the juvenile developing root system of *Ginkgo biloba* (Fig. 4) is likewise dependent on these ubiquitous, naturally occurring epiphytes of the genus *Methylobacterium*, but more experimental work is required to further elucidate this postulated plant root-microbe interaction.

HOLLAND (1997, a, b) postulated that higher plants obtain part of their phytohormones from attached methylobacteria and hence are dependent on these epiphytes. We have previously shown that shoot development in two representative higher plants (the angiosperms *Helianthus annuus* and *Zea maize*) is not promoted by added methylobacteria (KUTSCHERA et al., 2002; KUTSCHERA and KOOPMANN, 2005; KOOPMANN and KUTSCHERA, 2005). Here we document that two gymnosperms (*Ginkgo biloba, Pinus sylvestris*) can grow over a period of six weeks in the absence of any bacteria. Hence, these phylogenetically much older embryophytes are largely independent of bacterial phytohormones, although the root system of the "living fossil" *G. biloba* displays a behaviour that is similar to that of the most ancient land plants on Earth (liverworts).

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