Streptomyces lividans inhibits the proliferation of the fungus *Verticillium dahliae* on seeds and roots of *Arabidopsis thaliana*

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Summary

Verticillium wilt, a vascular disease in more than 200 dicotyledonous plants, is due to the ascomycete fungus *Verticillium dahliae*. As documented by video-microscopy, the soil bacterium *Streptomyces lividans* strongly reduces the germination of *V. dahliae* conidia, and the subsequent growth of hyphae. Quantification by the use of DNA-intercalating dyes and Calcofluor-staining revealed that during prolonged co-cultivation, bacterial hyphae proliferate to a dense network, provoke a poor development of *V. dahliae* vegetative hyphae and lead to an enormous reduction of conidia and microsclerotia. Upon individual application to seeds of the model plant *Arabidopsis thaliana*, either the bacterial spores or the fungal conidia germinate at or within the mucilage, including its volcano-shaped structures. The extension of hyphae from each individual strain correlates with the reduction of the pectin-containing mucilage-layer. Proliferating hyphae then spread to roots of the emerging seedlings. Plants, which arise in the presence of *V. dahliae* within agar or soil, have damaged root cells, an atrophied stem and root, as well as poorly developed leaves with chlorosis symptoms. In contrast, *S. lividans* hyphae settle in bunches preferentially at the outer layer near tips and alongside roots. Resulting plants have a healthy appearance including an intact root system. *Arabidopsis thaliana* seeds, which are co-inoculated with *V. dahliae* and *S. lividans*, have preferentially proliferating bacterial hyphae within the mucilage, and at roots of the outgrowing seedlings. As a result, plants have considerably reduced disease symptoms. As spores of the beneficial *S. lividans* strain are obtainable in large quantity, its application is highly attractive.

Introduction

The genus *Verticillium*, belonging to a group of asco-mycete fungi, includes members that parasitize plants, insects and nematodes. Plant-pathogenic species, which exist worldwide, cause a vascular disease known as *Verticillium* wilt (Pegg, 1974; Pegg and Brady, 2002). Worldwide, the species *V. dahliae* and *V. albo-atrum* are responsible for the highest losses of crops.

The most intensely studied *V. dahliae* persists within soils for many years mainly as melanized resting structures, known as microsclerotia. Their germination leads to hyphae, which extend towards roots of host plants and colonize the root surface, frequently at or near root tips or root hairs. The proliferating fungal hyphae penetrate the epidermal root cells and reach cortical tissues. If an infection of the vascular tissue occurs, conidia arise ultimately within xylem vessels and move with the transpiration stream. Conidia often germinate to hyphae penetrating neighbouring vessels. The networks of hyphae (mycelia), clogging xylem-vessels, as well as fungal toxin-production, are implicated to impair the transport of water and minerals through the vascular system. As a result, gradual wilting, senescence, defo-liation and death of the plant occur. Within decaying plants, fungal budding leads ultimately to microsclerotia, which survive 10–15 years within soils (for reviews see Goicoechea, 2009; Klosterman *et al.*, 2009). *Verticillium dahliae* causes vascular wilt of over 200 dicotyledonous plant species including important field and horticultural crops, flowers and tree species in temperate and sub-tropical regions (see reviews Pegg, 1974; Pegg and Brady, 2002; Klosterman *et al.*, 2009). Due to the absence of specific and approved fungicides against *V. dahliae*, and the low-level resistance of the cultivars against this pathogen, crop losses are immense (Goicoechea, 2009).

Several ecotypes of the model plant *Arabidopsis thaliana*, belonging to the *Brassicaceae*, were found to be susceptible to *V. dahliae* and to *V. longisporum* (re-classified as *V. dahliae*, Pantou *et al.*, 2006) by using a root-dip inoculation technique (Veronese *et al.*, 2003; Eynck *et al.*, 2007). To date, studies on the interaction of *Verticillium* strains with seeds, including those of *A. thaliana*, are not available.

Like the seeds of *Brassicaceae*, resting structures of *Verticillium* species and spores of *Streptomyces* species persist within soils. Streptomycetes are abundant Gram-positive soil bacteria, which initiate their life cycle through the germination of spores. Subsequently, substrate hyphae proliferate to extended networks (mycelia). Upon depletion of nutrients, spores develop within aerial hyphae that survive harsh and varying conditions. Their vast repertoire of enzymes induces the turnover of chitin, cellulose, lignocellulose, xylans, pectins, lipids and proteins (reviews, Schrempf, 2007; Chater *et al*., 2010). Hundreds of different *Streptomyces* species together are the richest microbial source of thousands of low-molecular-weight chemically different compounds exhibiting antibacterial, antifungal, antiparasitic, cytostatic or other biological activities. As a result, efforts have focused to explore the chemical nature of these compounds, their mode of action and the genetics of their biosynthesis (reviews, Schrempf, 2007; Chater *et al*., 2010).

Within soils, streptomycetes present a large and varying proportion of the microbial community, and they are extremely important for bioconversions and humus formation (Kutzner, 1981). Although streptomycetes occur within these soils and their numbers increase within the rhizosphere, studies concerning their beneficial interactions with plants are still scarce (Schrey and Tarkka, 2008). Rhizobacteria have been isolated from the rhizosphere of strawberries. Those that suppressed *V. dahliae* in field trials included *Streptomyces* and *Pseudomonas* species (Berg *et al*., 2000). The role of certain beneficial Gram-negative rhizobacteria, mainly members of the genera *Rhizobium* and *Pseudomonas*, has been preferentially studied (see reviews, Emmert and Handelsman, 1999; Weller *et al*., 2002; Janssen, 2006; Mercado-Blanco and Bakker, 2007; Segura *et al*., 2009). Different root-associated *Pseudomonas* strains promote growth of various plants and several of them are endophytic colonizers. Some studies show that *Verticillium* wilt is suppressed by selected *Pseudomonas* strains (Mercado-Blanco *et al*., 2004; Humphris *et al*., 2005; Berg *et al*., 2006; Debode *et al*., 2007; Prieto and Mercado-Blanco, 2008).

Our investigations revealed that a selected *Streptomyces* species (*S. olivaceoviridis*) interacts with a filamentous fungus (*Aspergillus proliferans*), via a protein targeting chitin within the fungal cell wall (Siemieniewicz and Schrempf, 2007), and that chitinolytic enzymes contribute to the degradation of the fungal cell wall component chitin. Based on biochemical studies and genome sequences it has been concluded that the *Streptomyces* species have a large repertoire of proteins targeting chitin, degrading and utilizing this polysaccharide as carbon and nitrogen source through specific uptake systems (for review, Schrempf, 2007). Based on these findings we hypothesized a concerted interaction of a selected *Strep-*

tomyces strain with a fungal plant pathogen. In the frame of this report, we show in detail that a *Streptomyces* species inhibits the proliferation of *V. dahliae in vitro* and *in vivo* within the seed mucilage and on roots of *A. thaliana*, cultivated within agar or soil. As a result, wilt symptoms of the model plant are significantly reduced.

Results

S. lividans *inhibits* V. dahliae *during co-cultivation*

Like other streptomycetes, *S. lividans* had been isolated from soil, and has been used for many genetic studies (Kutzner, 1981; Schrempf, 2007). *Streptomyces lividans* spores or *V. dahliae* conidia, or both types together were seeded in liquid minimal medium in microtitre-plates. As scored by video-imaging, a large portion of the *Streptomyces* spores and a small number of the *V. dahliae* conidia germinated. These elongated within the first 10 h to small hyphae (Fig. 1A). *Streptomyces lividans* extended over a period of 15 h (Fig. 1B) or 20 h (Fig. 1C) to a denser network of hyphae, among which only few fungal hyphae proliferated. The fungal hyphae are rapidly identifiable by their larger width compared with those of *S. lividans*. Incubation for 37 h (Fig. 1D) led to additional growth of the *S. lividans* hyphae network, but the extension of the fungal hyphae barely progressed. The control cultures, which had been seeded either with *S. lividans* spores (Fig. 1E) or with conidia of *V. dahliae*, germinated well and proliferated rapidly to many hyphae (not shown). In the course of 37 h, *V. dahliae* built dense hyphae networks and many new conidia arose (Fig. 1F). As visualized by Dead/Life staining (see *Experimental procedures*), individual hyphae of both partners were distinguishable most easily during early stages of growth (Fig. 1G). Under these conditions (i.e. corresponding to Fig. 1C, see above), individual *S. lividans* hyphae were mostly alive (green fluorescence), rarely dead (red fluorescence) and attached closely to the fungal hyphae of *V. dahliae* (Fig. 1G). Fungal hyphae were barely stained with Dead/Life dyes. The fluorescence derived from *in situ* stained total DNA (Dead/Life dyes) within the hyphae of *S. lividans* grown alone for 44 h (Fig. 1H$_I$ block S, median value 14.2) was 3% higher than that of *S. lividans* cultivated in a mixture together with *V. dahliae* (Fig. 1H$_I$, block M, median value 13.76). Thus, the presence of *V. dahliae* did not significantly ($P = 0.254$) affect the quantity of *S. lividans*.

The fluorescent dye Calcofluor penetrates preferentially into the polysaccharides of fungal cell walls, rather than in the peptidoglycan of the *Streptomyces* bacterium (see Fig. 1I); hence, it is suitable to quantify the fungal hyphae. The total Calcofluor-derived fluorescence of *V. dahliae* hyphae grown alone for 44 h (relative median value 5.5 for 'V' in Fig. 1H$_{II}$) was 4.5 times higher than obtained from a mixed co-culture of *V. dahliae* hyphae raised together
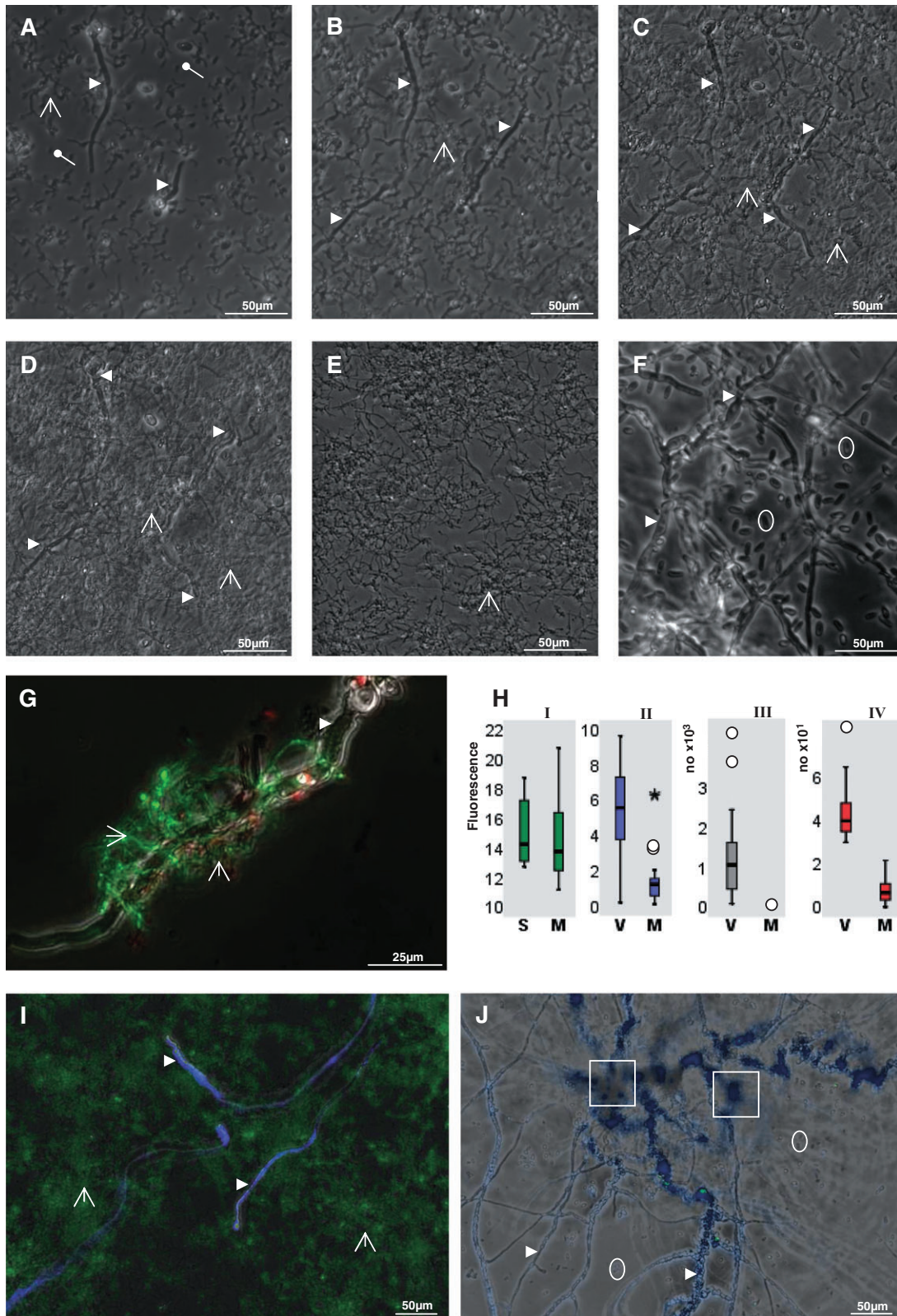
Fig. 1. Growth characteristics of *S. lividans* and *V. dahliae* within co-culture.

A–F. *Streptomyces lividans* spores and *V. dahliae* conidia were alone or co-incubated in minimal medium in microtitre plates. Images were recorded every 2 min over a period of 37 h. The images present incubation times for 10 h (A), 15 h (B), 20 h (C) and 37 h (D). Images of control cultures (E), which had been seeded with only either *S. lividans* spores or only with *V. dahliae* conidia (F), correspond to 37 h of incubation.

G. A co-culture comprising *S. lividans* and *V. dahliae* (corresponding to C) was grown for 20 h. Portions were treated with dyes (Dead/Life kit) and analysed by fluorescence-microscopy as outlined in *Experimental procedures*.

H. Evaluations by statistics.

H_I. *Streptomyces lividans* was grown alone (S) or in a mixture (M) together with *V. dahliae* as outlined above for 44 h. Portions were treated with the dyes (Live/Dead kit) as described in (C). The fluorescence values ($\times 10^{-7}$, *y*-axis) for *Streptomyces* hyphae within 18 wells of three independent experiments were determined (within photos each comprising an areas of 0.6 mm^2). The background fluorescence of each non-stained sample served as control and was set zero.

H_{II}. *Verticillium dahliae* was grown alone (V) or together (M) with *S. lividans* for 44 h. Aliquots were treated with Calcofluor as outlined in *Experimental procedures*. The fluorescence ($\times 10^{-7}$), which resulted from Calcofluor-binding to *V. dahliae* hyphae, was recorded (*y*-axis) within the measured area (0.6 mm^2) from three independent experiments. The background of unstained samples was set zero.

H_{III}. *Verticillium dahliae* was grown alone (V) or in combination with *S. lividans* (M) for 3 days as outlined below (see I) and photographed. Conidia were counted and evaluated (for details see *Experimental procedures*). The *y*-axis refers to calculated values per mm^2.

H_{IV}. The strains corresponded to those described, but these were cultivated for 7 days. The numbers of microsclerotia were counted and evaluated (for details see *Experimental procedures*). The *y*-axis refers to calculated values per mm^2.

Outliers (○) and the extreme (*) are marked.

I and J. *Verticillium dahliae* was grown alone (J) or together with *S. lividans* (I) for 4 days and inspected by microscopy. Samples were stained with SYTO9, which stains DNA (I), and with Calcofluor (I and J), which interacts with polysaccharides in fungal hyphae, leading to their blue fluorescence.

Spores (∿) or hyphae (⋀) of *S. lividans*, and hyphae (▷), conidia (○) and microsclerotia (□) of *V. dahliae* are marked.

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with *S. lividans* for 44 h (median value 1.2 for 'M' in Fig. 1H_{II}). The determined value ($P = 0.000$) underlines that the recorded differences are significant. The prolongation of the cultivation time (3 days) led to a very dense network of the *Streptomyces* hyphae (green fluorescence in Fig. 1I) and few residual fungal hyphae (Calcofluor-based blue fluorescence in Fig. 1I).

If *V. dahliae* grew alone, some conidia already arose after 37 h (Fig. 1F) and they increased considerably in number after 3 days of cultivation (Fig. 1J, median value: 106 per 0.1 mm^2, Fig. 1H_{III}, block V). After co-growth with *S. lividans* for 3 days, *V. dahliae* conidia were absent (median value: zero per 0.1 mm^2, Fig. 1H_{III}, block M). In contrast to conidia, microsclerotia started to arise after extended cultivation (3 days, Fig. 1J) and their number increased up to day 7 (median value: 40 per one mm^2, $P = 0.000$, Fig. 1H_{IV}, block V). The presence of *S. lividans* led to a significant reduction of the fungal microsclerotia (median value: seven per one mm^2, $P = 0.000$, Fig. 1H_{IV}, block M). Together, the results indicate that *S. lividans* significantly reduces the proliferation of vegetative fungal hyphae and inhibits the formation of conidia and microsclerotia.

V. dahliae *invades the seed mucilage of* A. thaliana

The *A. thaliana* seed coat differentiates during processes including growth, mucilage synthesis and secondary cell wall production. The mucilage is composed dominantly of pectins, whose key compounds are homogalacturonan and rhamnogalacturonan (Western *et al.*, 2000; Somerville *et al.*, 2004). Individual *A. thaliana* seeds were mixed with conidia of *V. dahliae*. During incubation (up to 48 h), images were taken by video (Fig. 2A–K) at 2 min inter-

vals. Alternatively, individual samples were analysed by microscopy (Fig. 2L–M). Compared with the control (Fig. 2A, no incubation), conidia germinated. In the course of 16 h (Fig. 2B) to 21 h (Fig. 2F), germ tubes extended mostly to branching hyphae, attaching frequently along the outer surface of the mucilage. After 24 h (Fig. 2C), 26 h (Fig. 2G), 31 h (Fig. 2H) or 32 h (Fig. 2D, and magnification in Fig. 2I), many branches initiated at the outer surface or within the mucilage.

Occasionally, germinating conidia were observed during the initial phase (14 h, Fig. 2J) within the mucilage, or germ tubes attached closely to a volcano-shaped structure – also known as columella. Upon further proliferation for 24 h (Fig. 2K) to 32 h (Fig. 2L, higher magnification), hyphae formed branches at or close to the volcano-shaped structures. The branches sometimes appeared to 'crawl' in the space between the columellae (Fig. 2K and L) and extended within the mucilage region. Due to extended proliferation for 48 h (Fig. 2E, video, Fig. 2M), seeds were completely covered by the fungal hyphae.

S. lividans *proliferates within the mucilage of* A. thaliana *seeds*

Spores of *S. lividans* were mixed with seeds of *A. thaliana* in minimal medium, and incubated for various time intervals. Under these conditions, the outer water-soluble mucilage-layer dislodges (Western *et al.*, 2000). After staining (Dead/Life), spores were found to be alive, and to adhere very well (Fig. 3A, and enlargements in Fig. 3B and C) to the water-insoluble mucilage-layer (hereafter referred as mucilage), and to occur along its cellulose radial fibres (stainable by Calcofluor, Fig. 3C), or close to the volcano-shaped structures (Fig. 3B and C). After
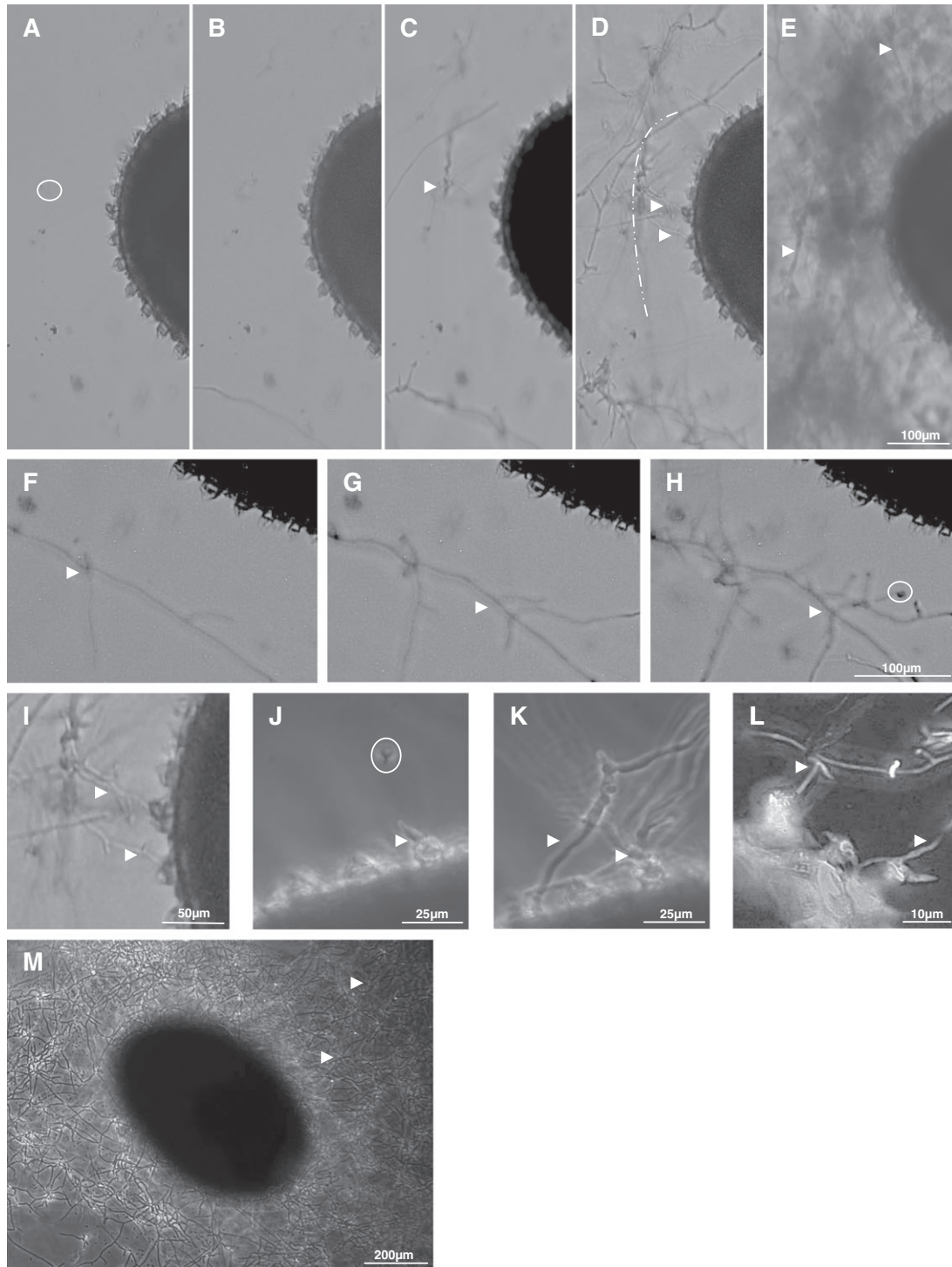
Fig. 2. Interaction of *V. dahliae* with *A. thaliana* seeds.

A–I. Seeds of *A. thaliana* and *V. dahliae* conidia were co-cultivated in microtitre plates in minimal medium as outlined in *Experimental procedures*. Video imaging (2 min intervals) was done over a period of 48 h. Photographs are presented prior incubation (A, control), after growth for 16 h (B), 24 h (C), 32 h (D) and 48 h (E). Areas corresponding to 21 h (F), 26 h (G), 31 h (H) or 32 h (I) are presented at a higher magnification. The border of the mucilage is marked (–···–) in D.

J–L. Samples were analysed by video imaging after incubation for various time-periods (J–K) or microscopy (L). An area containing a germinating spore within the mucilage (J, white arrow), and a germ tube at a volcano-shaped structure (J, white arrow) was analysed after 14 h. Continued proliferation for 24 h (K) or 32 h (L, higher magnification) led to branches between and close to volcano-shaped structures (K and L).

M. Dense networks of hyphae of *V. dahliae* (M) surrounding an *A. thaliana* seed (48 h of cultivation) were investigated by light microscopy. Hyphae (arrow) and conidia (○) of *V. dahliae* are marked.
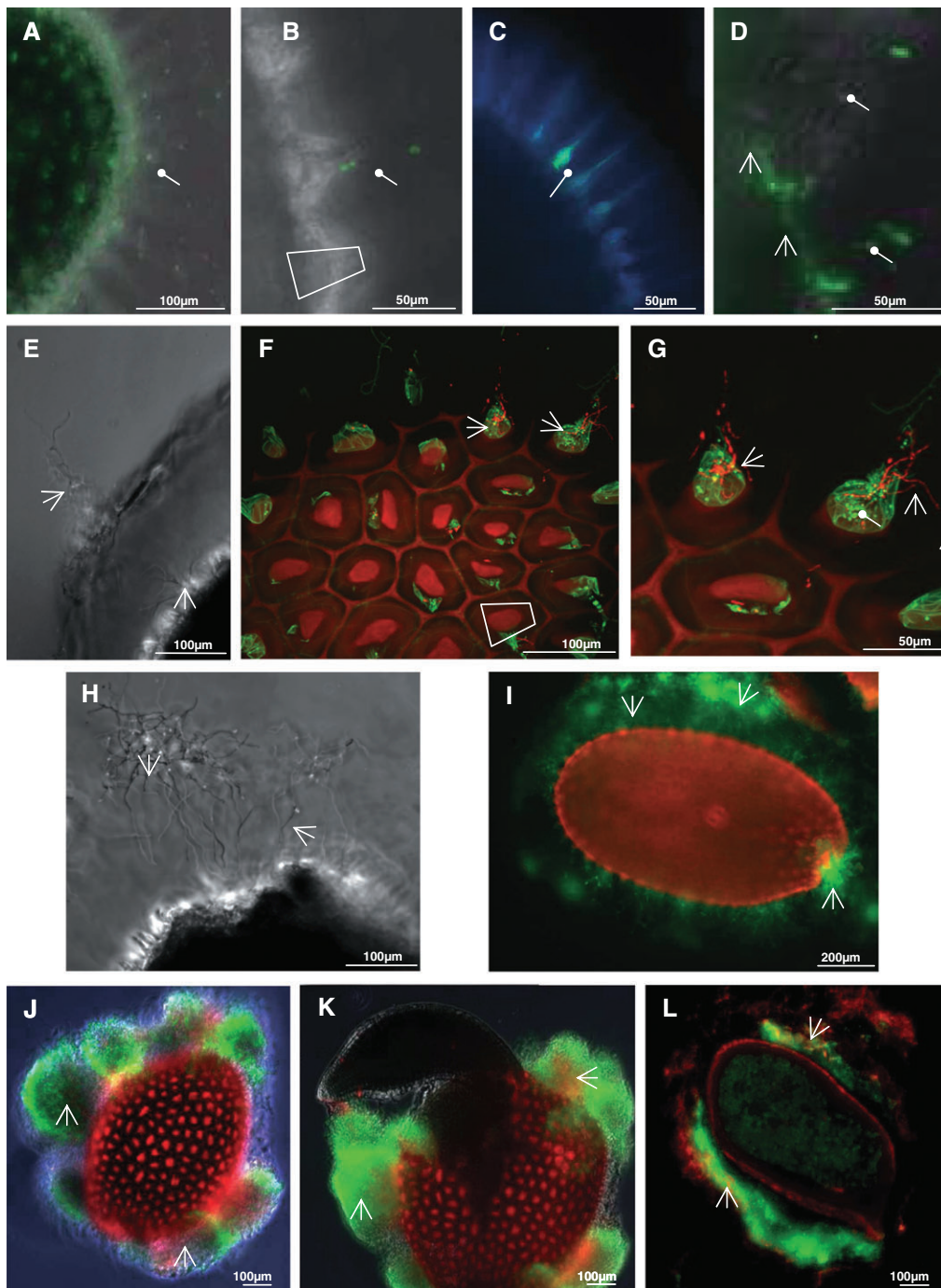
Fig. 3. Interaction of *S. lividans* with *A. thaliana* seeds. *Streptomyces lividans* spores were mixed with seeds of *A. thaliana* in minimal medium, incubated for various time-periods and analysed.

A–C. After 30 min, spores were detected within the mucilage (A) or close to volcano-shaped structures (B, higher magnification). Samples were exposed either to Dead/Life stain (A, B and D) or treated with Calcofluor and SYTO9 (C).

D–L. After incubation for 10 h (D) the *S. lividans* spores germinated, and subsequently, short tubes formed (22 h, E–G) within the mucilage, and volcano-shaped structures. Details are presented by enlarged views onto the volcano-shaped structures, being in close contact with *S. lividans* hyphae (F and G). Hyphae at the micropyle are shown after 24 h (H). Hyphae proliferated further, and extended within the whole mucilage (44 h, I). After 120 h of growth, huge bunches of hyphae aggregates surrounded the seed (J). An embryo emerged from the same seed 4 h later at the micropyle (K). A seed, which had been co-cultivated with *S. lividans* for 120 h, was embedded in agarose and sectioned, and inspected after staining (L) with dyes (Dead/Life).

Samples were inspected by phase contrast alone (E, H), fluorescence and phase contrast (A, B, D, J and K) or fluorescence (C, F, G, I and L) microscopy after staining with dyes (Dead/Life) using filters as described in *Experimental procedures*. Spores (⤵) and hyphae (ⵉ) of *S. lividans* or volcano shaped structures (▷) of *A. thaliana* are marked.
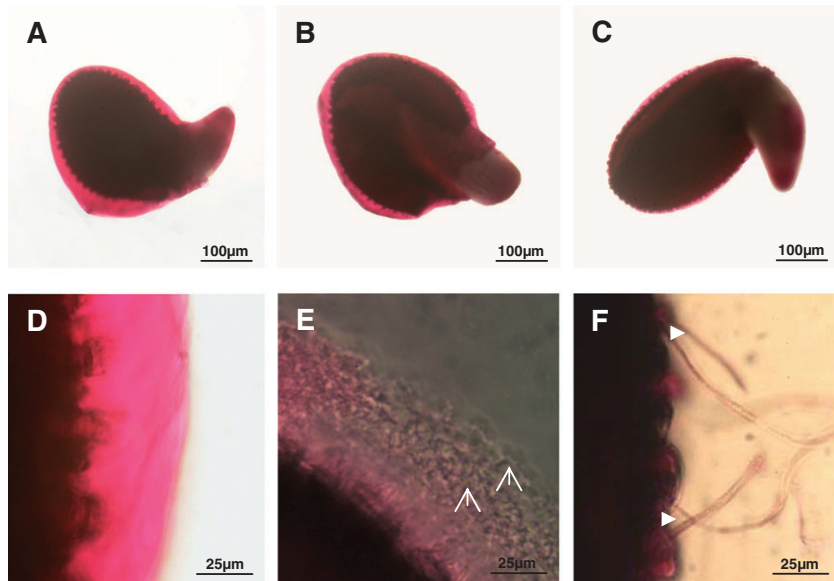
Fig. 4. Investigations of the seed mucilage during co-cultivation.
A–F. Seeds were placed onto agar plates alone (A and D), together with spores of *S. lividans* (B and E), conidia of *V. dahliae* (C and F) and incubated.
A–F. After 3 days, samples were stained with Ruthenium red (see *Experimental procedures*), and inspected by light microscopy at low (A–C) or at high magnification (D–F). Hyphae of *S. lividans* (⋀) or of *V. dahliae* (▷) are marked.

germination, living *S. lividans* hyphae (Fig. 3D and E) extended at the surface or within mucilage, and towards the columellae (Fig. 3E). By inspecting these structures from the top, hyphae were found closely attached. Short hyphae were mostly alive (green fluorescence), while a few extended ones had red fluorescence, indicating their decay (Fig. 3F and G). Upon further proliferation for 24–44 h (Fig. 3H and I), bunches of hyphae were mostly alive (Fig. 3I). Interestingly, the micropyle was covered rapidly (and often first) by the *Streptomyces* hyphae (Fig. 3H and I, right). After extended incubation (120 h, Fig. 3J), bunches of predominantly alive hyphae covered the seeds. The inspection of sectioned and dyed (Dead/Life) seeds revealed masses of hyphae outside but not within the seed (Fig. 3L). This observation correlated with the finding that the emerging embryo (Fig. 3K) was devoid of hyphae.

The seed mucilage is reduced by the microorganisms

Seeds were inoculated on agar plates (without extra carbon source) in the absence or the presence of *V. dahliae* or *S. lividans*. Alternatively, seeds were grown alone or together with both partners. During the first 23 h, hyphae of *S. lividans* or *V. dahliae* started to proliferate within the mucilage. After 3 days of co-cultivation, seeds were investigated with Ruthenium red (Fig. 4A–F), a dye that interacts with acidic polysaccharides (Western *et al.*, 2000). The mucilage of the *A. thaliana* seed had a gel-like, pink-violet stain and appeared intact (Fig. 4A and D,

controls). The incubation of *A. thaliana* seeds together with *S. lividans* (Fig. 4B and E) provoked a massive proliferation of *S. lividans* hyphae, which correlated with a reduction in staining (Fig. 4B and E). The growth of *V. dahliae* hyphae led to massive dismantling of the mucilage (Fig. 4C and F).

S. lividans adheres to roots of A. thaliana seedlings

Individual seeds of *A. thaliana* were incubated on agar plates together with *S. lividans*, for various time-intervals up to 21 days. Hyphae proliferated extensively within the seed mucilage over the course of 70 h (Fig. 5A, control). The roots of emerging seedlings carried hyphae preferentially at their tip (14 days, Fig. 5B). After further incubation (21 days), bunches of hyphae were sometimes observed alongside of roots (Fig. 5C) and at root hairs (Fig. 5D). The hyphae were mostly alive and well developed, as visualized by Dead/Life studies (Fig. 5A–C). Epidermal root-cells appeared intact. The analysis of stained root cross-sections showed that the *S. lividans* hyphae (Fig. 5E, left) were only found outside roots (Fig. 5E, right).

S. lividans reduces the proliferation of V. dahliae within the seed mucilage and at roots of A. thaliana

Individual seeds of *A. thaliana* were co-incubated on agar plates with *V. dahliae* in the presence of *S. lividans*. *Verticillium dahliae* conidia germinated at the seed muci-
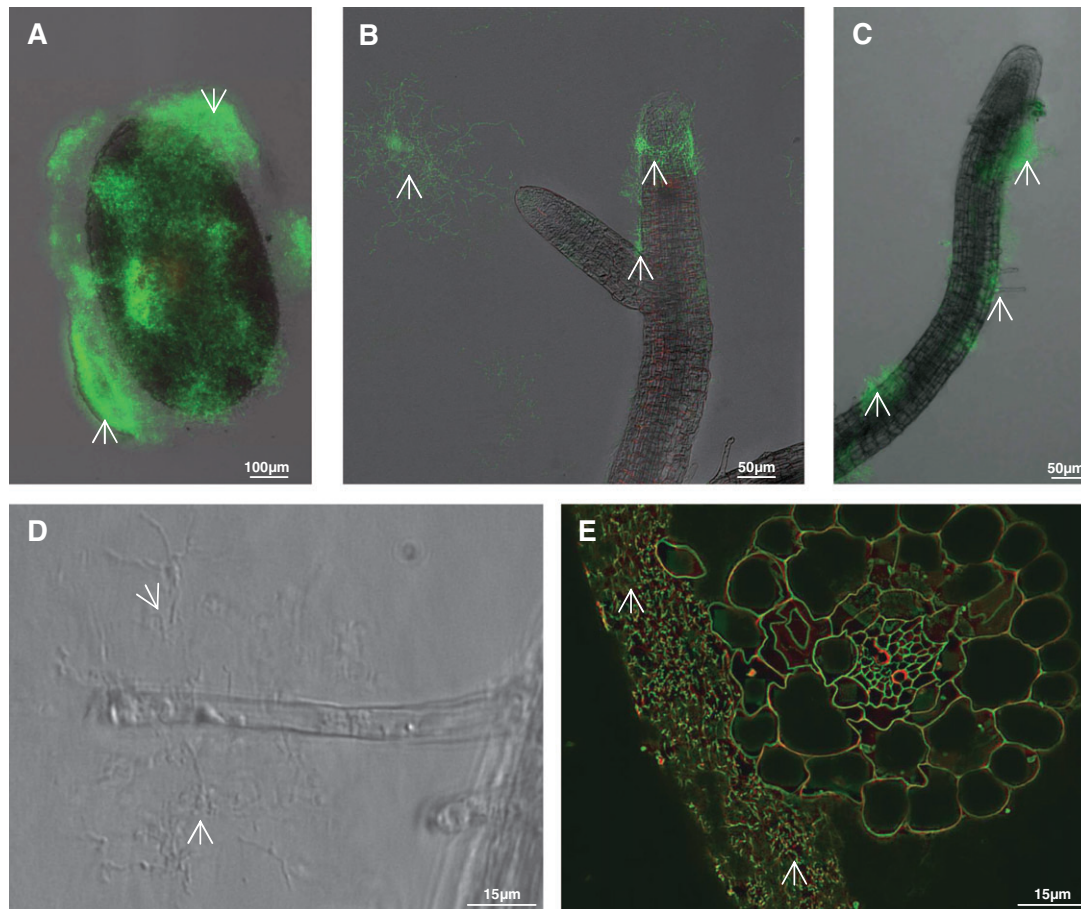
Fig. 5. Interaction of *S. lividans* with *A. thaliana* seedlings.
A–E. Portions comprising one *A. thaliana* seed and *S. lividans* spores on top of an agar-containing microtritre plate (see *Experimental procedures*), were inspected in the course of incubation after 70 h (A). Subsequently, roots (B and C) and root hairs (D) of emerging seedlings were inspected after 14 days (B), or after 21 days (C, D and E). Roots (E, right), to which *S. lividans* hyphae (E, left) were associated, were cross-sectioned.
Samples A–E (except D) were treated with dyes (Dead/Life), and inspected. Hyphae (arrow) of *S. lividans* are marked.

lage, and proliferated to a living hyphae network, which extended (control 70 h, Fig. 6A) additionally towards neighbouring regions of the seed. If the seeds were simultaneously exposed to *S. lividans* spores and *V. dahliae* conidia, large masses of *S. lividans* hyphae proliferated preferentially at and within the mucilage. However, only a few *V. dahliae* hyphae were detectable, and these were more frequent outside the dense *S. lividans* hyphae network (Fig. 6B). These results demonstrate that *S. lividans* considerably hinders the growth of *V. dahliae*.

After prolonged incubation of seeds (from Fig. 6A) for 20 days seedlings developed, and *V. dahliae* hyphae occurred randomly along the roots and its tips (Fig. 6C and D). Epidermal root cells often appeared disconnected, and were irregularly shaped (Fig. 6D), while root caps appeared mostly intact. Roots of developing seedlings, cultivated together with *V. dahliae* and *S. lividans*, were covered with a network of living bacterial hyphae at the tips and along their sides, and fungal hyphae were rare (Fig. 6E and F). The cells of the root epidermis had fewer defects. The corresponding cells of control plants, which had been raised in the presence of S. lividans alone, were intact (Fig. 5B). These data revealed that *S. lividans* suppressed *V. dahliae* growth on seeds and seedlings of *A. thaliana*.

As the natural habitat of the fungus is the rhizosphere, additional studies were done in soil. *Streptomyces lividans* spores within soil germinated and developed to a network of hyphae (44 h, Fig. 7A), a large portion of which attached closely to the mucilage of the *A. thaliana* seed. Hyphae were found to be only clearly detectable after staining within the complex soil structure. As outlined under the first chapter, the application of two different fluorescent dye-types (SYTO9 and Calcofluor) allows to distinguish the bacterial and fungal hpyhae. The *S. lividans* hyphae were stainable with the dye SYTO9 (Fig. 7A), but not with Calcofluor (Fig. 7D). Also, *V.*
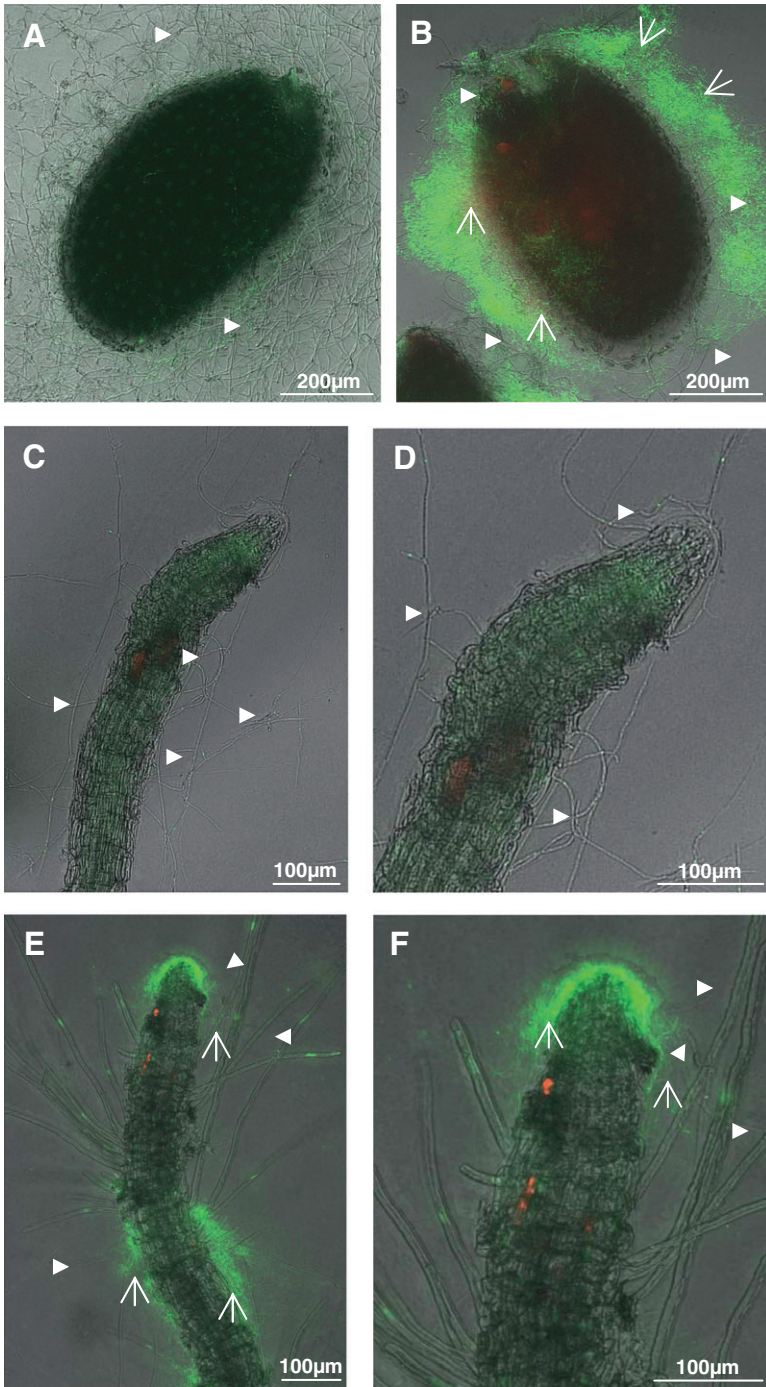
Fig. 6. Co-cultures of *S. lividans* and *V. dahliae* together with *A. thaliana*.
A, C–D. Portions containing one *A. thaliana* seed, and conidia of *V. dahliae* were placed onto agar plates, and incubated for 70 h (A) or for 20 days (C–D).
B, E–F. One *A. thaliana* seed was incubated together with *V. dahliae* conidia, and *S. lividans* spores (see above) and incubated for 70 h (B) or for 20 days (E–F).
Hyphae of *S. lividans* (⋏) or of *V. dahliae* (▷) are marked.

dahliae conidia proliferated well during 44 h to hyphae at and close to the seed mucilage within the soil. The fungal hyphae interacted with Calcofluor (Fig. 7E), but barely with SYTO9 (Fig. 7B). The simultaneous inoculation of *S. lividans* and *V. dahliae* led to an intensive growth of *S. lividans* at and next to the seed mucilage, which correlated with a lack of fungal development (Fig. 7C and F).

If Calcofluor was applied alone (Fig. 7D–F), or together with SYTO9 (Fig. 7A–C), it targeted also

cellulose fibres within the mucilage (for details see Fig. 3C).

S. lividans *reduces the* Verticillium-*induced disease symptoms*

Seeds were placed onto agar plates, and were incubated alone or together in different combinations with the microorganisms. In the absence of any microorganism (Fig. 8A,
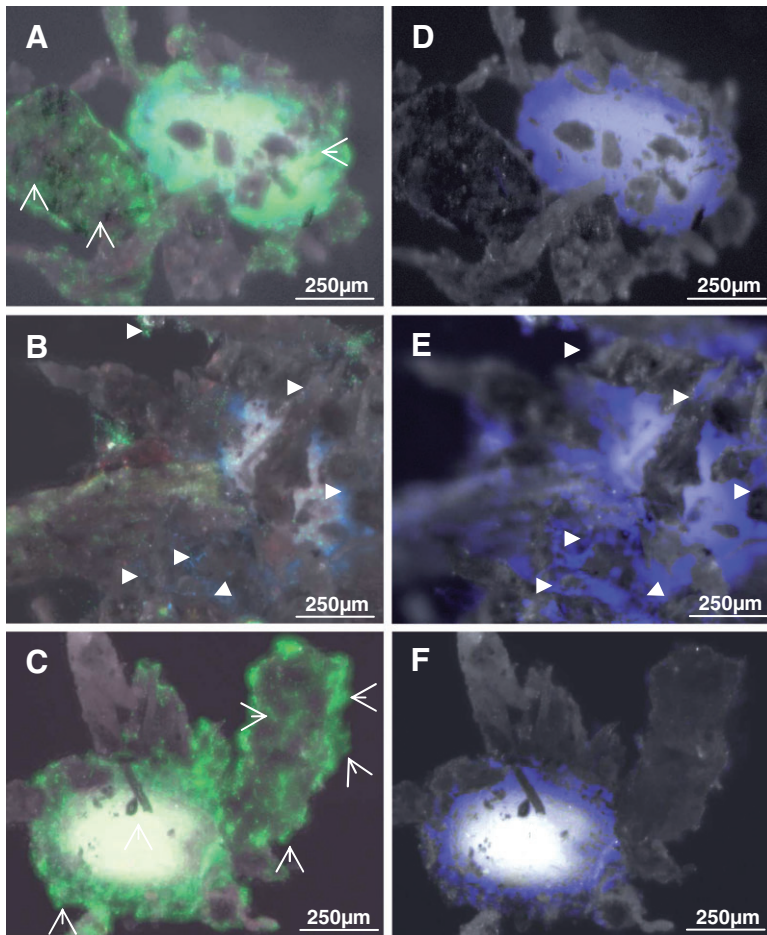
Fig. 7. Interactions within soil cultures. Within soil seeds of *A. thaliana* were inoculated for 40 h either with spores of *S. lividans* (A and D), with conidia of *V. dahliae* (B and E) or with spores of *S. lividans* together with conidia of *V. dahliae* (C and F). After incubation for 40 h, seeds including the attaching soil particles were stained with SYTO9 and Calcofluor (see *Experimental procedures*). Fluorescence corresponding to either Calcofluor (D–F) or both of them (A–C) were recorded. Hyphae of *S. lividans* (⋀) or of *V. dahliae* (▷) are marked.

controls), seedlings with green leaves, a short stem and long, barely branching roots were detected. If seeds had been incubated with spores of *S. lividans*, the resulting seedlings (Fig. 8B) resembled those of the control (Fig. 8A). In contrast, the presence of *V. dahliae* provoked the generation of seedlings with a varying degree of atrophy symptoms including chlorotic or barely developed leaves, and reduced or marginal stems and roots (Fig. 8C). The co-presence of *S. lividans* with *V. dahliae* led to seedlings with green leaves (Fig. 8D).

Furthermore, the surface of shoots and the length of roots were compared. The length of roots of *A. thaliana* seedlings (control) spanned a wide range, and the median was 12.9 mm (Fig. 8II, block A), and the shoot surface of these controls was highly variable with a median of 3.2 mm² (Fig. 8I, block A). All germinated seeds, which proliferated in the presence of *S. lividans*, had shoot surfaces comparable to those of the control (median 2.9 mm², *P* = 0.449, Fig. 8I, block B). The length of the roots stretched over a larger range (median 15.6 mm, *P* = 0.859, Fig. 8II, block B) than that of the control (Fig. 8I, block A).

Seedlings, which grew together with *V. dahliae*, had very short roots (median 4.9 mm, *P* = 0.000, Fig. 8C and

II, block C), and a reduced shoot surface (median 1.3 mm², *P* = 0.000, Fig. 8I, block C) compared with the control (Fig. 8II, block A). The co-inoculation of seeds with *V. dahliae* and *S. lividans* led to strong suppression of the fungus. The arising plant had a healthy appearance. Their mean root length was about 50% reduced (median 7.3 mm, *P* = 0.031, Fig. 8II and D) compared that of plants raised with *S. lividans*, and it increased about 43% compared with *V. dahliae* (*P* = 0.002, Fig. 8II, block C). The shoot surface in the presence of the bacterium and the fungus doubled in comparison to *V. dahliae* alone (median 2.1 mm², *P* = 0.006, Fig. 8I, block D), and corresponded to about 70% of the value for *S. lividans* (median 2.9 mm², *P* = 0.248, Fig. 8I, block B). Together, these data demonstrate that *S. lividans* suppresses the fungus-induced disease symptoms of the plant seedlings within agar.

The above outlined experiment was repeated by replacing the agar by soil. The shoots that arose from the *A. thaliana* control seeds had an overall healthy appearance with a well-developed stem and leaves (i.e. shoot surface; median of 2.4 mm², Fig. 8E and III, block E). Their roots had a median length value of 1.9 mm (Fig. 8IV, block E). Plants grown in presence of *V. dahliae* frequently had
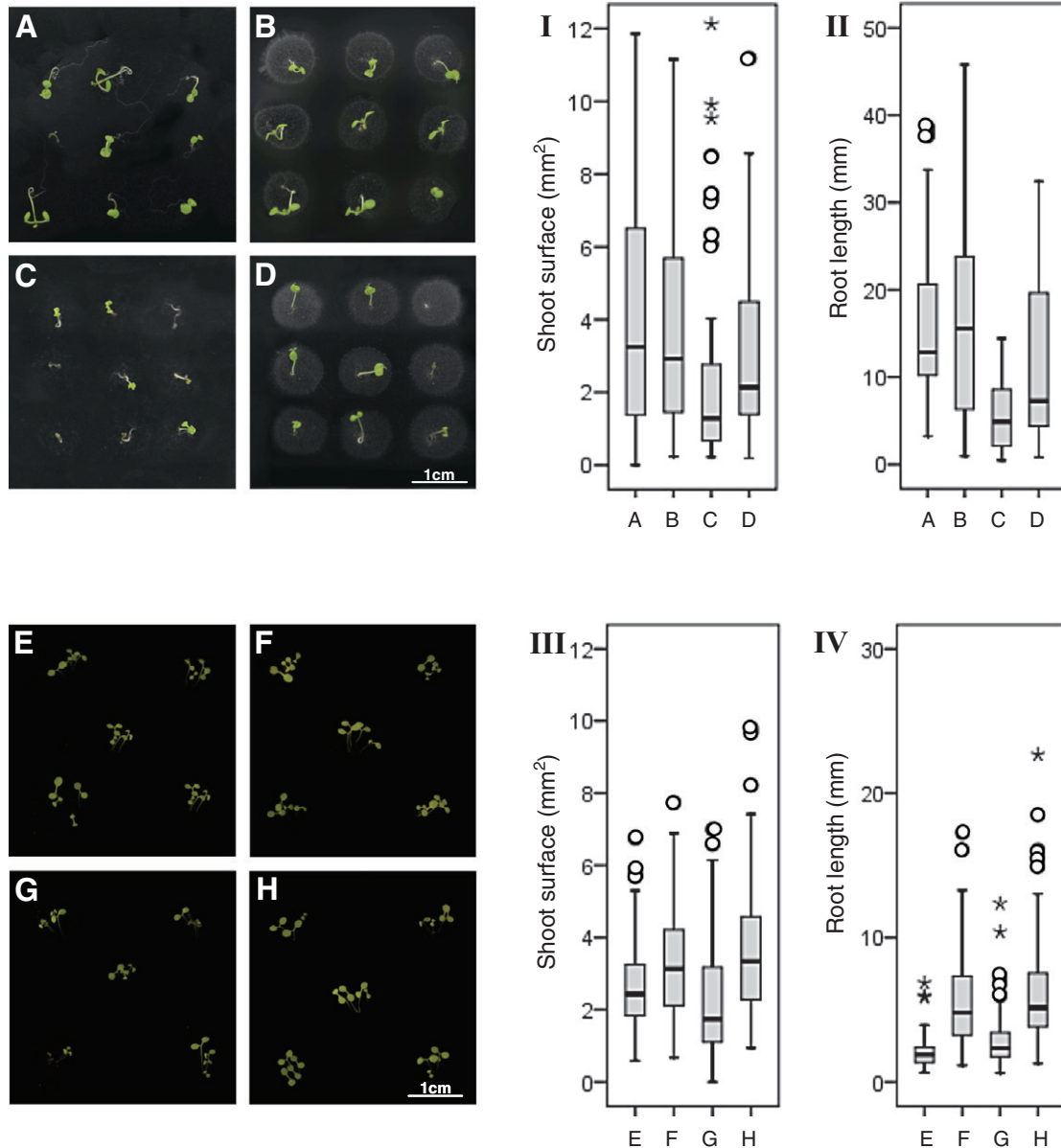
Fig. 8. *Arabidopsis thaliana* cultivated in different combinations with the microorganisms.

A–D. *Arabidopsis thaliana* seeds were placed at several distinct positions on top of an agar containing plate alone (A), together with spores of *S. lividans* (B), together with conidia of *V. dahliae* (C) or together with conidia of *V. dahliae* and spores of *S. lividans* (D) for 14 days. Plates were photographed from the top.

I–II. The shoot surface and the root length of 42–65 plants for each the combinations (A–D) were evaluated by statistics. Each black horizontal line indicates the determined median of the length.

E–H. *Arabidopsis thaliana* were seeded at several distinct positions within plates filled with soil alone (E), either with spores of *S. lividans* (F), with conidia of *V. dahliae* (G) or with conidia of *V. dahliae* together with spores of *S. lividans* (H) for 14 days. Plates were photographed from the top.

III–IV. The shoot surface and the root length of 106–130 plants for each the combinations within soil (E–H) were evaluated by statistics. Each black horizontal line indicates the determined median of the length. Outliers (○) and the extremes (*) are marked.

'dwarf' stems and leaves (Fig. 8G). The shoot surface was significantly reduced (median 1.74 mm², *P* = 0.001, Fig. 8III, block G), while their roots were about 20% longer (Fig. 8G and IV, block G, median 2.3 mm, *P* = 0.000). In the presence of *S. lividans* (Fig. 8F), the median length of roots extended about 2.4-fold (median 4.7 mm, *P* = 0.000, Fig. 8IV, block F), and correlated with a larger shoot surface (median 3.1 mm², *P* = 0.000, Fig. 8III, block F).

The co-inoculation of seeds with *V. dahliae* and *S. lividans* led to a strong suppression of the fungus within soil

(Fig. 7C and F). Hence, it was not surprising that healthy plants developed (Fig. 8H). Their root length (median of 5.1 mm, Fig. 8IV, block H) and the shoot surface (median 3.3 mm^2, Fig. 8III block H) differed little from those that had been grown with *S. lividans* (root length $P = 0.291$; shoot surface $P = 0.310$) and significantly from those cultivated alone with *V. dahliae* (root length $P = 0.000$; shoot surface $P = 0.000$).

The data showed that *S. lividans* suppresses the fungus within the soil even more effectively than in agar.

Discussion

We demonstrated, for the first time, that the spores of the soil bacterium *S. lividans* attach to the seed mucilage of *A. thaliana*. This interaction occurs efficiently within liquid medium, agar and soil (Figs 1, 3, 5 and 7). The mature seed coat cells of *A. thaliana* consist of a volcano-shaped secondary cell wall, covered by a ring of mucilage. Upon hydration, the outer radial cell wall breaks, the mucilage extrudes and forms a capsule around the seed. The mucilage comprises unbranched rhamnogalacturonan as the dominant pectin type, with homogalacturonan, arabinoxylan and arabinogalactan additionally present. Based on their physical properties, pectins within the mucilage will play roles in mechanical properties, molecular sieving, ion transport and adhesion (for review, Willats *et al.*, 2001). We found that *S. lividans* spores germinate at the outer surface, and within the mucilage. The subsequent proliferation to a dense hyphae network correlated with a thinner appearance of the mucilage that was apparent by a reduction of staining with Ruthenium red (Fig. 4). This dye is known to interact with acid polysaccharides, thus, it has also been used to trace pectins within the seed mucilage (Western *et al.*, 2000; Macquet *et al.*, 2007). To date, only a few authors have analysed pectinase activities from streptomycetes. Genome mining has led to the identification (Xiao *et al.*, 2008) of two pectate lyases from *Streptomyces coelicolor* A3(2), which is a very close relative of *S. lividans*. Therefore, we conclude that *S. lividans* provokes the recorded thinning or softening of the mucilage through enzymatic activity.

Interestingly, *S. lividans* spores, germ tubes and hyphae are also settling at or close to the volcano-shaped structures, and sometimes along the radial structures within the mucilage that are known to be comprised of cellulose fibres (Fig. 3C). However, the hyphae do not enter the interior of the seed. In frame of future studies, one should consider that oligosaccharides (the result of pectin-hydrolysis) function as signalling molecules in the course of defence responses (for review, Willats *et al.*, 2001).

The conidia of *Verticillium* are uniform compared with the complex microsclerotia. Conidia can be counted as individuals, and therefore serve as inoculums under laboratory conditions. In order to investigate the colonization process, researchers usually dip plant roots into a suspension of the *Verticillium* conidia and subsequently follow the proliferation. Researchers studied the *in situ* infection cycle of *V. dahliae* in lettuce cultivars (Vallad and Subbarao, 2008), the different interaction of *V. dahliae* and the variant *V. longisporum* with *Brassica napus* (oilseed rape) (Eynck *et al.*, 2007), and the colonization of a woody plant (olive) by *V. dahliae* (Prieto *et al.*, 2009). We demonstrated, for the first time, that *V. dahliae* conidia can be directly applied to *A. thaliana* seeds instead to roots to initiate the colonization process within liquid or agar containing medium or soil (Figs 2, 6 and 7). We discovered that *V. dahliae* germinates at or within the seed mucilage, or close to volcano-shaped structures. As a result, their hyphae proliferate significantly. This correlates with a reduction of the mucilage (Fig. 4). We anticipate that pectinolytic enzymes are important for this process (Marín-Rodríguez *et al.*, 2002). In the course of taxonomic studies, other authors have identified pectin-degrading activity within *V. dahliae* (Bidochka *et al.*, 1999). Previously, it has been proposed that seed mucilage may be important for seed-germination under water stress, and it is therefore dispensable under laboratory conditions (Penfield *et al.*, 2001). This view may have provoked other investigators to neglect a detailed study of microbial interactions with the seed mucilage.

Previous investigators analysed the interaction of pathogens with root caps including pectins and roots. The cellulose-cross-linking glycan network within root walls is embedded in a matrix comprising polysaccharides, proteoglycan and low-molecular-weight compounds. Pectin is highly abundant in this matrix, within the middle lamellae between primary cell walls where it assists in intercellular adhesion (for review see Willats *et al.*, 2001). It has been noted that the presence of pectin-degrading enzymes contributes to the virulence of certain pathogens. The inoculation of *Pisum sativum* (pea) roots with spores of *Nectria haematococca* led to the proliferation of fungal hyphae. Interestingly, the hyphae-containing root caps were dismantled into the surroundings. Thus, the root tips were mostly freed from the infecting fungus (Gunawardena *et al.*, 2005).

The co-inoculation of seeds with *S. lividans* and *V. dahliae*, respectively, led to a dominant network of *S. lividans* hyphae within the mucilage, and very few fungal hyphae. This result clearly demonstrates that this bacterium considerably suppresses fungal proliferation within agar or soil (Figs 6 and 7). In addition, the observation correlates with our finding that in a co-culture with *S. lividans*, *V. dahliae* proliferates poorly (Fig. 1). Our previous studies had revealed that *Streptomyces olivaceoviridis* inhibits the development of the filamentous fungus *A.*

proliferans during co-culturing, as it is targeted by a chitin-binding protein, and as chitinase-activity provokes the degradation of chitin within the fungal cell wall (Siemieniewicz and Schrempf, 2007). Chitinases are secreted by many streptomycetes including *S. lividans* (Vionis *et al.*, 1996; Schrempf, 2007). In addition, streptomycetes produce many secondary metabolites, some of which have antifungal activities. These include nikkomycin, which inhibits chitin synthase, or nystatin, which affects the permeability of the cell membrane, if ergosterol is present (for review, Schrempf, 2007). As demonstrated with plate-assays, culture filtrates of *S. lividans* contain antifungal activity against both *Verticilium* strains (data not presented). The determination of the chemical nature of the corresponding compound(s) awaits future investigations.

Interestingly, dense hyphae networks of *S. lividans* colonized dominantly at the *A. thaliana* root-tips, and bunches of hyphae were associated along the roots, and occasionally at root hairs. Therefore, components that facilitate adhesion to the plant cell wall will be important. One candidate is the protein AbpS, which has a high binding affinity to isolated plant cellulose, and is abundant among streptomycetes (Walter and Schrempf, 2003). CbpS could mediate an additional cooperative role, as this surface-anchored *Streptomyces* protein recognizes several polysaccharides including cellulose (Walter and Schrempf, 2008). It is interesting to note that *Trichoderma* spp. produce swollonin, an expansin-like protein with a cellulose-binding module. This assists *T. asperellum* to colonize root surfaces of cucumber seedlings (Brotman *et al.*, 2008).

Noticeably, *V. dahliae* germinates poorly in the presence of *S. lividans*, generates a highly reduced number of vegetative hyphae, and produces no conidia and considerably fewer micosclerotia. These originate from single or contiguous hyphae that swell and develop numerous septae. Lateral budding results in clusters of spherical cells, which become melanized (Griffiths, 1970). Mutants of *V. dahliae* carrying the inactivated gene *VMK*1 (encoding one Mitogen-activated protein kinase) exhibit reduced virulence, conidiation and microsclerotia formation (Rauyaree *et al.*, 2005). The presence of a functional hydrophobin gene *VDH1* provokes desiccation and cold tolerance of resting structures. The *VDH1* gene is transcribed in response to carbon limitation within developing microsclerotia, hyphae fusions and conidiophores (Klimes *et al.*, 2008). The morphological *Aspergillus flavus* transitions seem to depend on a quorum-sensing mechanism. Conidiation requires a lipoxygenase gene, which is likely involved in the formation of oxylipin (possibly linoleic acid or a related product) in dense cultures, while unknown factors stimulate sclerotium formation at low densities. Conidia arise at high population densities, in correlation with competition for nutrients. The niche of the robust sclerotium is to survive under harsh conditions (Horowitz Brown *et al.*, 2008).

Based on these findings, it can be speculated that streptomycetes have different tools that prevent either the formation of conidia and microsclerotia. Previous studies had revealed that the fungus *Talaromyces flavus* produces glucose oxidase. In the course of its catalytic activity, hydrogen peroxide is generated, which leads to destruction of microsclerotia (Stosz *et al.*, 1996). Various *Pseudomonas* species colonize the outer surface of *Verticillium* microsclerotia, which are less viable in the presence of bacterial biosurfactants and phenazines. The latter can act as reducing agent, resulting in the generation of superoxide radicals and hydrogen peroxide. Mutants of *P. cholororaphis*, which overproduce the phenazine PCA, were more effective than the wild-type in reducing germination and formation of microsclerotia (Debode *et al.*, 2007). It is interesting to note that the metamorphosis leading to sclerotia in *Sclerotium* and *Sclerotinia* is associated with oxidative stress, and the thiol redox state of the fungus (Patsoukis and Georgiou, 2008). As streptomycetes produce various enzymes, which govern the level of reactive oxygen species (Zou and Schrempf, 2000), one should reflect that these might play a role during co-cultivation with *V. dahliae*. In addition, cell wall-targeting proteins, degrading enzymes and low-molecular-weight antifungal metabolite(s) of streptomycetes (for review, Schrempf, 2007) need to be considered.

Our comparative studies showed that *S. lividans* suppressed *V. dahliae* within the established miniaturized agar and soil system. The process of microbial interaction can be more easily visualized within the transparent agar (Figs 5 and 6) than in the presence of adhering soil particles (Fig. 7). In the presence of *V. dahliae* disease symptoms of arising plants were already detectable after 14 days of cultivation either in soil or in agar. Within soil (Fig. 8III and IV), the seed inoculation with *S. lividans* alone or together with *V. dahliae* led to plants, which had a considerably extended root and shoot surface compared with the control. In contrast, the presence of *V. dahliae* alone correlated with a significant reduction of shoot surface compared with that of corresponding control plants. The data revealed that the presence *S. lividans* prevents the deleterious symptoms of *V. dahliae*. This effect occurs at a less pronounced level within the agar-cultures (Fig. 8I and II). As streptomycetes are soil bacteria, which utilize macromolecules within the soil as nutrients, this finding is not surprising. Additionally, we found that *S. lividans* stimulates plant growth better within soil than within agar. The newly established co-inoculation of seeds with *S. lividans* spores leads to a high protection level of the plant against the wilt pathogen.

The ratio of rhizosphere bacteria – including antagonists to *Verticillium species* – depends on the soil site and plant species. The rhizosphere of strawberries at one site comprised antagonistic species of *Streptomyces* and *Pseudomonas* (Berg *et al.*, 2000). Within the soil next to roots of oilseed rape, antagonistic *Serratia* strains were present. Among several hundred isolates (from differently planted fields), dominantly *Pseudomonas* species were found to inhibit *V. dahliae* (Berg *et al.*, 2006). In contrast to streptomycetes, *Pseudomonas* spp. have been studied extensively as they can promote enhanced plant growth, and some are endophytes (Mercado-Blanco and Bakker, 2007; Prieto and Mercado-Blanco, 2008). As a result, they suppress a range of pathogenic microbes. Hence, their use to biocontrol various diseases, including *Vertcillium* wilt, has been explored (Humphris *et al.*, 2005). For instance, the successful establishment of the endophyte *P. fluorescens* PICF7 within *Olea europaea* (olive) provokes *in planta* a reduced colonization by *V. dahliae* (Prieto *et al.*, 2009). In addition, other bacteria have potential as biocontrol agents against phytopatogens (De Mot, 2007). Biological control is a powerful alternative to chemicals. However, one of the difficulties in applying Gram-negative, non-sporulating bacteria such as pseudomonads is to make and preserve viable formulations. In contrast, the Gram-positive streptomycetes form robust spores, which withstand harsh conditions. Therefore, more intensified research on the example presented in this paper will be an excellent basis to reduce *Verticillium* wilt pathogens in agro-ecosystems.

Experimental procedures

Strains and seeds

The wild-type *Streptomyces lividans* 66 (in the text named *S. lividans*) has been described earlier (Siemieniewicz and Schrempf, 2007). To generate spores, the strains were grown on agar plates until sporulation occurred. Spores (2.5×10^9 ml^{-1}) were kept in 50% glycerol at -20°C. *Verticillium dahliae* (Eynck *et al.*, 2007) was kindly provided by Dr Braus-Stromeyer (University Göttingen, Germany). Each strain was grown on agar plates containing minimal-medium until conidia had formed. These were scraped off the plates with sterile water, filtered through cotton, concentrated (1.2×10^8 ml^{-1}) in 50% glycerol and stored at -20°C. *Arabidopsis thaliana* (ecotype Columbia) was grown in a glasshouse (kindly provided by Prof. Scheibe, the Department of Plant Physiology, University of Osnabrück, Germany) until seeds had developed. After harvesting, these were surface sterilized and kept in portions at 4°C for 3 months.

Growth in liquid culture

Aliquots of 400 µl (per well of microtitre plate per 24 wells) liquid minimal medium without extra carbon source (Siemieniewicz and Schrempf, 2007) were mixed with a suspension of 4 µl *S. lividans* spores (2.5×10^8 ml^{-1}) alone (control), or with 4 µl of a suspension of *V. dahliae* conidia (1×10^7 ml^{-1}). Alternatively, the *S. lividans* spores and conidia of *V. dahliae* were placed together with one seed (per well). In the course of cultivation at 25°C, the wells were inspected by microscopy.

For video recording, images were taken in 2 min intervals over varying time-periods (up to 48 h) with an inverted microscope (Zeiss Observer Z1). Using the software IPLab 4.04 in combination with the Microsoft Windows XP system, images were processed and combined.

Growth on agar-containing media

Aliquots (20 µl) containing one seed of *A. thaliana* and conidia (2×10^3) of *V. dahliae* and/or spores (5×10^4) of *S. lividans* were placed onto standard Petri-dishes or microtitre plates (24 wells). These had been filled with solified minimal medium (Hildebrandt *et al.*, 2006) without carbon source, and were grown for various periods. Incubation occurred at 25°C with alternating light (≈ 12 h) and dark cycles.

Growth in soil

A portion of 250 mg (about 1 ml volume) of autoclaved characterized soil (20% organic matter, and per litre: nitrogen 150 mg; 100 mg phosphate, 500 mg potassium, pH 6.2) and 180 µl of sterile water was filled into each well, and subsequently four sterilized *A. thaliana* seeds (in 20 µl water) were placed onto the top. The inner eight ones were used per microtitre plate (24 wells). Depending on the experiment, conidia (2×10^3) of *V. dahliae* and/or spores (5×10^4) of *S. lividans* were additionally placed together with the seed of each well. Three independent experiments comprised a total of 32 wells. The plates were incubated as described above (see experiments in agar under Results).

Staining procedures and microscopical visualization

To visualize the dead or life status of the bacterium *S. lividans* (Fernandez and Sanchez, 2002; Siemieniewicz and Schrempf, 2007), a mixture containing the DNA-intercalating dyes propidium iodide (diffusion into damaged hyphae) and SYTO9 (taken up by intact and dead hyphae) was applied according to the instructions of the supplier (Molecular probes or Invitrogen). The cultures were inspected under visible light at various magnifications. Subsequently, they were examined under reflected light using filter sets (for FITC: excitation: HQ 470/40, beam splitter: Q 495 LP, emission: HQ 525/50, for Texas red: HQ 562/40, Q 593 LP, HQ 624/40, from AHF Analysentechnik AG). Living bacterial hyphae exhibited green fluorescence, whereas dead ones appeared in red.

The cell wall including the volcano-shaped structures of *A. thaliana* seeds and the root cells were additionally noticed to interact strongly with the dye propidium iodide, leading to a high red fluorescence of the seed. Therefore, it was sometimes necessary, to reduce the red channel.

To stain the mucilage from *A. thaliana* seeds, which had been inoculated alone (control), or in the presence of *S. lividans* and/or *V. dahliae*, a suspension of Ruthenium red

(Fluka) was dropped onto each sample, which was subsequently washed as described earlier (Western *et al.*, 2000). Photographs were taken under visual light (Canon EOS 450D camera).

Preparation of plants and measurements of shoot-surface and root-length

Each soil containing well with seedlings was filled with 400 μl of sterile water, as prerequisite to pull them out carefully with forceps. Additional washing with water provoked the removal of most adhering debris. If cultivated in agar, plants could be gained easily without remains. Plants were flattened on glass covered with Whatman filter paper, and they were scanned (Epson Perfection 3170 PHOTO) at 2400dpi. The shoot surface (comprising the stem and leaves) and the main root and its side branches (each from hypocotyl to the tip) were measured by the use of IPLab 4.0.4.

Counting of conidia and microsclerotia

Portions of cultures of *V. dahliae* were photographed (see Fig. 1H$_{III}$ and H$_{IV}$). Conidia were counted within six areas (250×325 μm). The values were calculated per mm^2. The numbers of microsclerotia (three independent repetitions) within areas (each 0.6 mm^2) were counted. Data were statistically analysed.

Statistical analysis

Data from three identically prepared experiments were pooled and statistically assessed (for further details see Figs 1 and 8). Statistics were elaborated with the Mann–Whitney *U*-test implemented within programme from SPSS, version 17.0. A *P*-value of ≤ 0.05 was considered as significant.

Acknowledgements

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