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Addendum

The growth of fungi and *Arabidopsis thaliana* is influenced by bacterial volatiles

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Dual culture systems, which only allowed volatiles to cross the boundary of a bipartite Petri dish, were used to investigate the effects of bacterial volatiles on the growth of 14 fungi and *A. thaliana*. The majority of tested combinations exhibited dramatic growth retardations of fungi and *A. thaliana*, indicating that volatiles can act as antibiotics. It therefore can be concluded that bacterial volatiles influence the growth conditions of organisms in a community and in a habitat.

Introduction

Bacteria as many other organisms produce a wide range of secondary metabolites. Many of these specialized compounds possess growth inhibitory effects to other organisms (antibiotics and antifungal metabolits (AFMs)) and are thereby beneficial for the producing organism, for example by preventing the invasion of other organisms into the ecological niches and/or eliminating potential competitors for nutrients. As a consequence the producing organisms strengthen their growth, reproduction and fitness, and establish and sustain their presence in a community. Antibiotics are a diverse group of chemical compounds, which derive from different biosynthetic pathways. Most of the presently known antibiotics of microbial origin are at room temperature either liquid or solid, and little is known about volatiles that could act as antibiotics. Volatiles are small molecules with molecular masses lower 300, with low polarity and low vapour pressure, altogether features which facilitate volatility. They occur in the biosphere over a range of concentrations and they are ideal infochemicals because they can act over a wide range of scales.[1] Due to these properties such compounds could have important influence on biological/ecological systems. Volatiles with growth inhibitory and sometimes even deleterious effects on organisms are widespread in the animal and plant kingdom.[2] The microbial world also synthesizes and emits volatile compounds.[3] For example, 120 different VOCs were identified in 26 *Streptomyces* species comprising alkanes, alkenes, alcohols, esters, ketones, sulfor containing compounds, terpenoids, which are emitted in different combinations from the different *Streptomyces* species.[4] In a marine *Streptomyces* a complex blend of volatile lactones and terpenoids (including geosmin), which exhibits antibiotic properties, has been observed, and *Myxococcus xanthus* emits 42 volatiles, two of them were identified as new natural products.[5,6] Volatiles of soil bacteria can influence the growth of fungi such as *Verticillium dahliae, Trichoderma viridae, Phanaerochaete magnoliae, Phytophthora cryptogea, Gaeumannomyces graminis* var. *tritici,* and *Sclerotinia sclerotiorum*.[1,7,8] A comprehensive overview of the compounds, which are emitted from bacteria was recently published.[9] In a previous survey we could show, that rhizobacterial isolates comprising *Serratia plymuthica, S. odorifera, Stenotrophomonas maltophilia, S. rhizophila, Pseudomonas fluorescens* and *P. trivialis* synthesize and emit complex blends of volatiles, which confer growth inhibitions to *R. solani*.[10] This work was extended such that the influence of the volatiles emitted from these bacteria were tested on fourteen soil- and human-born fungi and the model plant *Arabidopsis thaliana*.

Co-cultivation of Bacteria with Fungi and *Arabidopsis thaliana*

Overnight cultures of the bacteria were applied on one side of a bipartite Petri dish, on the other side of the dish a mycel plaque of *Aspergillus niger, Fusarium culmorum, F. solani, Microdochium bolleyi, Neurospora crassa, Paecilomyces carneus, Penicillium spec., P. waksmanii, Phoma betae, Phoma eupyrena, Rhizoctonia solani, Sclerotinia sclerotiorum, Trichoderma strictipile, Verticillium dahliae* or 10 *A. thaliana* plantlets were placed. This experimental design only allowed volatiles to move from one side to the other side of the Petri dish. Growth of the mycel was recorded from the 1st to the 7th or 9th day after inoculation. The bacteria possess different affects on the different fungi (Fig. 1A). For example, *Serratia plymuthica* inhibits the mycel development of *Penicillium waksmanii* (middle), while *Staphylococcus epidermidis* significantly supports mycel growth (right) compared to the set up without bacteria application (left). In summary, eleven fungi experience strong and very strong growth inhibitions. Growth retardation was often already visible after the 2nd day of inoculation, and in most cases these inhibitions accelerated after the 4th day

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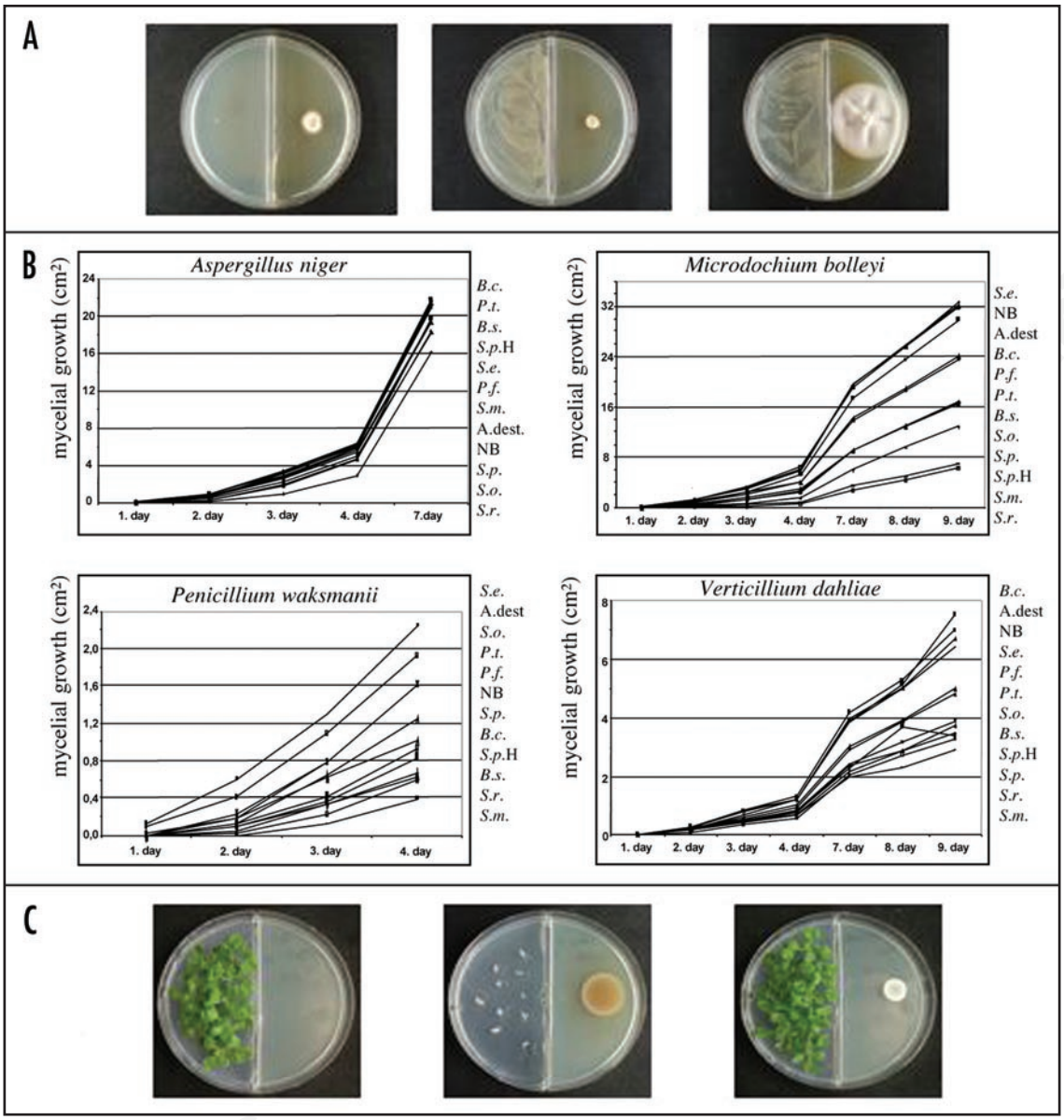

Figure 1. (A) Co-cultivation of bacteria with *Penicillium waksmanii* in a bipartite Petri dish. Left: control with nutrient broth (NB), middle: *Serratia plymuthica*, right: *Staphylococcus epidermidis*. (B) Development of mycelia after bacteria application. Growth curves of four fungi (*Aspergillus niger, Microdochium bolleyi, Penicillium waksmanii, Verticillium dahliae*) are shown. On the right ordinate the bacteria are arranged from low to high mycel inhibition, top to the bottom, respectively. *B.s. Bacillus subtilis* B2g, *B.c. Burkholderia cepacia* 1S18, *P.f. Pseudomonas fluorescens* L13-6-12, *P.t. Pseudomonas trivialis* 3Re2-7, *S.o. Serratia odorifera* 4Rx13, *S.p. Serratia plymuthica* 3Re4-18, *S.p.H Serratia plymuthica HRO C48*, *S.e. Staphylococcus epidermidis* 2P13-18, S.m. Stenotrophomonas maltophilia R3089, *S.r. Stenotrophomonas rhizophila* P69, Aqua dest., nutrient broth II. (C) Co-cultivation of bacteria with *Arabidopsis thaliana* in a bipartite Petri dish. Left: control with nutrient broth (NB), middle: *Pseudomonas trivialis*, right: *Staphylococcus epidermidis*.

(Fig. 1B). *A. niger* and similarly *F. culmorum* are only mariginally inhibited, while *F. solani* received little growth promotion. The highest growth promotion effect of 35% was recorded for *R. solani* when co-cultivated with *Burkholderia cepacia*.

The growth of *Fusarium* isolates and *A. niger* is not much restrained and therefore could be considered to be 'resistant' or 'mute' to the rhizobacterial volatiles. *M. bolleyi*, *P. carneus*, *P. betae* and *S. sclerotiorum* experience inhibitions of higher than 40% by all rhizobacteria. The other fungi reveal different and individual inhibition patterns, e.g., *V. dahliae*, *R. solani*, *P. spec*. and *N. crassa* experience in comparison to other tested fungi a moderate inhibition. *Serratia odorifera, S. plymuthica, Stenotrophomonas maltophilia, S. rhizophila* and *Pseudomonas fluorescens, P trivialis* volatiles inhibit the growth of *A. thaliana* significantly, while the volatiles of *S. epidermidis* possessed no effect (Fig. 1C).

Conclusions and Outlook

In summary, most of our experiments documented growth inhibitory effects rather than growth promotions indicating that the bacterial volatile organic compounds comprise the potential to act as antibiotics. The experiments show that pseudomonades are the weakest 'antifungal allrounders', best 'allrounders' are *Stenotrophomonas* spp. followed by *Serratia* spp. and *B. subtilis*. Highest fungal growth inhibitions are reached with *Stenotrophomonas* spp. The fungi which

are most sensitive to bacterial volatiles are *P. carneus*, *M. bolleyi*, *P. betae*, *S. sclerotiorum* and *T. strictipile*. Regarding the prominant phytopathogenic fungi, *Sclerotinia* experiences strongest inhibitions, followed by *Verticillium* and *Rhizoctonia*, while *Fusarium* is not inhibited.

Antifungal effects of anorganic volatiles has been demonstrated previously, e.g., ammonia (inorganic volatile) emitted by *Enterobacter cloacae* controls *Pythium spp*. growth, trimethylamine inhibits hyphal formation and extension of *Geotrichum candidum*, and hydrogen cyanide produced by pseudomonades control root rot of tobacco.[11-13] Organic compounds such as allyl alcohol inhibit germination of *S. sclerotiorum*, benzothiazole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-ethyl-1-hexanol and nonanal of pseudomonades inhibit mycel growth of *S. sclerotiorum*, or not further identified compounds of *B. subtilis* caused structural deformations of pathogenic fungi.[8,14,15] Our survey supports and extends the previous obervations that rhizobacteria produce biologically relevant organic volatile compounds. Beside pseudomonades and *B. subtilis* particularly potent volatiles are emitted by *Serratia odorifera, S. plymuthica*, *Stenotrophomonas maltophilia, S. rhizophila* and *Burkholderia cepacia*.

Bacterial volatiles not only have negative effects, but also promote growth, e.g., randomly isolated soil bacteria were found to stimulate fungal growth.[1] We showed that *B. cepacia* supports *R. solani* growth significantly. Allyl alcohol stimulates growth and enhances sclerotial colonization of *Trichoderma spp*.[14] 2,3-butanediol, acetoin (3-hydroxy-2-butanone), 3-methyl-1-butanol, 2-methyl-1-butanol and butane-1-methoxy-3-methyl of *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a promote growth and induce systemic acquired resistance in *A. thaliana*.[16-18] However, the latter observations are in contradiction to our findings, since the *B. subtilis* B2g isolate confered growth inhibition to almost all fungi and no significant effects were measured with *A. thaliana*. The reasons that may account for the different results could be (i) the use of different bacilli species or isolates, and/or (ii) variations of growth media and growth conditions. A plausible explaination is that the levels of synthesis and emission of the volatiles may differ in different isolates. Furthermore, as a consequence of the used growth media the composition the volatile formation may be altered as was previously shown.[1,8,19,20]

Finally the growth chambers and incubation systems have to be considered. In our surveys we used open systems and not sealed Petri dishes, since the open system reflects the natural situation more closely. It is conceivable that in experiments with volatiles often closed systems were choosen with the idea to maximize the propability to measure responses, but in the airtight chambers the composition of the air alters throughout the duration of the experiment and they influence the test organisms in a different way than in open or flow systems.

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