Molecular approaches to improve biocontrol of soil-borne fungal pathogens

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Abstract: The highly toxic and climatically relevant soil fumigant methyl bromide was used to suppress soil-borne pathogens, which cause high yield losses world-wide. To develop environmentally friendly alternatives in plant protection, two target pathogens with a broad host range and high economic importance were studied: *Verticillium dahliae* Kleb. and *Rhizoctonia solani* Kühn. Strategies to select the most efficient antagonists were developed on the basis of hierarchical systems combining microbiological, molecular and phytopathological methods. To control Verticillium wilt, the biocontrol agent (BCA) *Serratia plymuthica* HRO-C48 was selected. The strain shows high antifungal and plant growth promoting activity and posses no risk for the environment and human health. RhizoStar® is registered and produced by E-nema, Raisdorf, Germany. For a biocontrol of *Rhizoctonia* diseases another strategy was developed: a combination of fungal and bacterial antagonists has been applied. While the fungi are used for direct disease protection and parasitism of sclerotia and hyphae, endophytic bacteria are applied to enhance plant growth and protect against the pathogen from outside and inside the rhizosphere. The mode of action of BCAs and of interaction with microbial community was studied using molecular and microscopic techniques (FISH in combination with Confocal Laser Scanning Microscopy). Results of these investigations lead to new strategies in application and formulation of BCAs.

Key words: *Verticillium, Rhizoctonia,* antagonist, BCA, mode of action

Introduction

Soil-borne pathogens cause plant diseases associated with high yield losses world-wide. In this research two target pathogens were studied. *Verticillium dahliae* Kleb. causes Verticillium wilt on a broad host range throughout the world and is associated with high yield losses. Since the banning of the soil fumigant methyl bromide, there are no chemical controls available to suppress the pathogen. *Rhizoctonia solani* Kühn also causes many diseases on a broad host range. Fungicides are available for some crops but there are no biocontrol options to suppress the pathogen on horticultural crops or for organic farming in general. Before biocontrol strategies can be developed it is necessary to study and understand the molecular ecology of the microbial populations in the crop environment especially those with antagonistic potential against the pathogens.

The use of polyphasic approaches combining novel cultivation-independent and more traditional techniques to study microbial communities led to a significantly better understanding of structure and function of plant-associated microbial communities in the last decade. Furthermore, molecular approaches were used to understand the mode of action of Biological Control Agents (BCA) and to improve their performance under greenhouse and field conditions. As examples, the involvement of molecular techniques in two different biocontrol strategies will be presented.

Results and discussion

Molecular methods in biocontrol studies

The use of polyphasic approaches combining novel cultivation-independent and more traditional techniques to study microbial communities led to a significantly better understanding of structure and function of plant-associated microbial communities in the last decade. An overview about these applications is shown in Fig. 1.

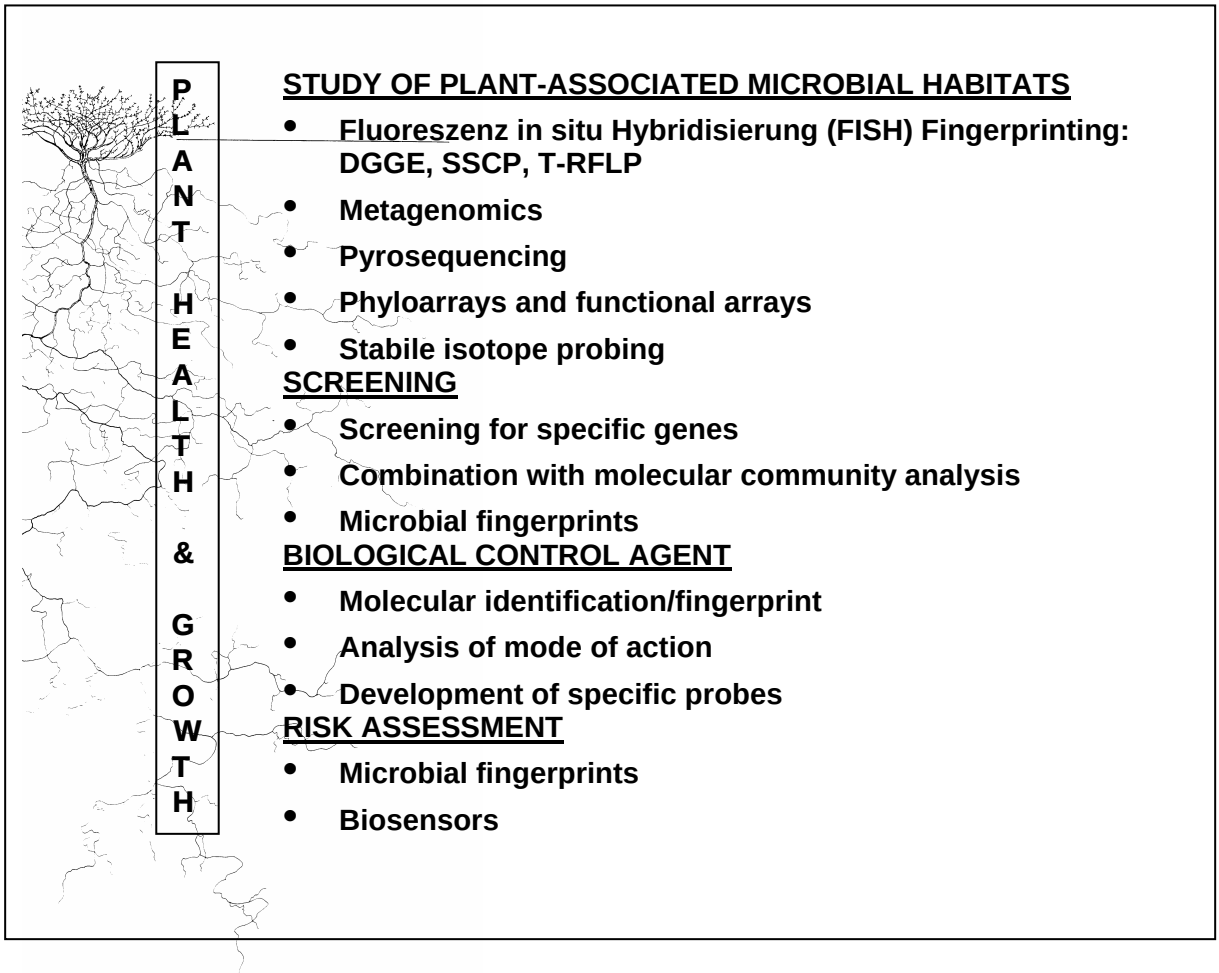

Fig. 1 Molecular techniques in biocontrol research
DGGE = Denaturing Gradient Gel Electrophoresis, SSCP = Single Strand Conformation Polymorphism, T-RFLP = Terminal restriction fragment length polymorphism

The involvement of these techniques is demonstrated in the following two examples.

A biocontrol strategy to suppress Verticillium diseases

To develop a management strategy for *V. dahliae* beneficial or antagonistic micro-organisms, which can suppress the soil-borne pathogen in the rhizosphere and endosphere, were screened. *Serratia plymuthica* strain HRO-C48, originally isolated from the rhizosphere of oilseed rape, was selected as biocontrol agent (BCA) by a hierarchical screening strategy according to plant growth promoting and antagonistic properties. The strain was evaluated in a variety of greenhouse and field trials (Berg et al. 1999, Kurze et al. 2001).

Although *Serratia* treatments resulted in plant protection and growth promotion, sometimes inconsistent effects especially under field conditions were observed. Therefore, the mode of action was intensively studied. Interestingly, the interaction with the plant as well as with the fungus was regulated in a quorum-sensing dependent manner (Lui et al. 2006, Müller et al. 2008). To analyze the influence of AHL-mediated communication on biocontrol activity in detail, the AHL-degrading lactonase AiiA was heterologously expressed in the strain, resulting in abolished AHL production. The comparative analysis of the wild type, the quorum quenched strain and, additionally, a miniTn5 mutant, in which an AHL synthase has been inactivated, led to the identification of several AHL-regulated phenotypes. In the pathosystem *V. dahliae*-oilseed rape the essential role of AHL-mediated signaling for disease suppression was demonstrated. In vitro, the regulatory function of AHLs in the synthesis of the plant growth hormone indole-3-acetic acid (IAA) is shown for the first time. In addition to IAA production swimming motility was found to be negatively AHL-regulated. On the other hand, production of extracellular proteolytic and chitinolytic enzymes is shown to be positively AHL-regulated. HRO-C48 emits a broad spectrum of volatile organic compounds (VOCs), which are involved in antifungal activity and, interestingly, whose relative abundances are influenced by quorum sensing. This study shows that quorum sensing is crucial for biocontrol activity of *S. plymuthica* HRO-C48. For efficient applications it is necessary to establish a high number (the required quorum) on/in the plant.

Another important step was to formulate the BCA, and to develop a specific application method for each crop (Müller and Berg 2007). The product development was carried out in cooperation with the Strawberry farm in Rövershagen, E-nema GmbH (Raisdorf), and Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Hohenlieth, all in Germany).

A biocontrol strategy to suppress Rhizoctonia diseases

A screening strategy was developed to assess the potential of plant-associated bacteria to control diseases caused by *Rhizoctonia solani* Kühn (Faltin et al. 2004). About 434 already characterized antagonistic bacterial strains isolated from diverse plant species and microenvironments were evaluated for biocontrol and plant growth promotion by a hierarchical combination of assays. Analyzing *in vitro* antagonism towards different *Rhizoctonia* isolates resulted in a selection of 20 potential biocontrol agents. The strains were characterized by their antagonistic mechanisms *in vitro* as well as their production of the plant growth hormone indole-3-acetic acid. The plant growth promoting effect by antagonistic bacteria was determined using a microtiter plate assay on the basis of lettuce seedlings. Lettuce and sugar beet as host plants were included in the biocontrol experiments in which the antagonistic effect of seventeen bacterial isolates could be confirmed *in vivo*. Sequencing of the 16S rDNA gene was used to identify the antagonistic isolates. Molecular fingerprints of isolates obtained by BOX-PCR were compared to avoid further investigation with genetically very similar strains and to obtain unique molecular fingerprints for quality control and patent licensing. According to our strategy an assessment scheme was developed and four interesting biological control agents *Pseudomonas trivialis* B3, *P. fluorescens* B1, *S. plymuthica* B4 and *Serratia ordorifera* B6 were found. While *S. plymuthica* B4 was the best candidate to biologically control *Rhizoctonia* in lettuce, *P. trivialis* B3 was the best candidate to suppress the pathogen in sugar beet. Interestingly, although only a small proportion of endophytic bacteria was included, with exception of B1 and B6, the two other strains were isolated from the endorhiza of potato.

To develop a biocontrol strategy, different potato-associated ecto- and endophytically living bacterial strains selected by the screening scheme were evaluated against *R. solani* in potato and in lettuce (Scherwinski et al. 2008). The aim of this study was not only to assess

the biocontrol efficacy of three bacterial antagonists against *R. solani* introduced into different naturally *Rhizoctonia*-infested lettuce fields, also to analyse their impact on the indigenous plant-associated bacteria and fungi. Lettuce seedlings were inoculated with bacterial suspensions of two endophytic strains, *S. plymuthica* B4 and *P. trivialis* B3, and with the rhizobacterium *P. fluorescens* L13-6-12 7 d before and 5 d after planting in the field. Similar statistically significant biocontrol effects were observed for all applied bacterial antagonists compared to the un-inoculated control. Single-strand conformation polymorphism (SSCP) analysis of 16S rDNA or ITS1 fragments revealed a highly diverse rhizosphere and a less diverse endophytic micro-flora of lettuce. Representatives of several bacterial (α-, β- and γ-proteobacteria, firmicutes, bacteriodetes), fungal (ascomycetes, basidiomycetes) and protist (oomycetes) groups were present inside or on lettuce plants. Surprisingly, as lettuce belongs to raw-eaten vegetables, species of the genera like *Flavobacterium*, *Burkholderia*, *Staphylococcus*, *Cladosporium* and *Aspergillus*, which contain potentially human pathogenic strains, were identified. Analysis of the indigenous bacterial and endophytic fungal populations revealed only negligible, short-term effects due to the bacterial treatments, and that they were more influenced by field site, plant growth stage and microenvironment. Altogether, again *P. trivialis* B3 and S. plymuthcia B4 showed the best effect under all tested conditions. Both strains are interesting candidates to develop a commercial product. Interestingly, an application of Serratia plymuthica B4 together with *Trichoderma gamsii* resulted in enhanced biocontrol effects. Using molecular and microscopic techniques (FISH in combination with Confocal Laer Scanning Microscopy) it was shown, that the BCA colonized not only the rhizosphere also *Trichoderma* hyphae were colonised and used as vector.

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