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STSM Topic: Interactions of grapevine plants with endophytic fungi and effects on stress tolerance of plants

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1. Background of the STSM

Fungal entomopathogens are important antagonists of arthropod pests and have attracted increased attention as biocontrol agents in integrated pest management programs. In addition to colonizing arthropods, evidence has accumulated that some entomopathogenic fungi like *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) can endophytically colonize a wide array of plant species. For a couple of crop plants it has been proven that endophytic *B. bassiana* can provide a systemic protection against damage by various insect pests (Akello et al. 2008; Gurulingappa et al. 2010; Quesada-Moraga et al. 2009) or might trigger induced systemic resistance mechanisms against plant pathogens (Griffin et al. 2005; Ownley et al. 2010; Ownley et al. 2008).

At the same time it is known that naturally occurring endophytic fungi and bacteria can play an important role in the expression of phenotypic characteristics such as increased stress tolerance of plants to abiotic factors (Davitt et al. 2010; Larimer et al. 2010; Parrent et al. 2010; Rodriguez et al. 2009). For a variety of monocots, some studies on these aspects recently provided important insights into mechanisms (Redman et al. 2011; Márquez et al. 2007; Malinowski and Belesky 2000). For grapevine, however, only limited information is available about the spectrum of mutualistic endophytic microorganisms and their significance. In this context, Gimenez et al. (2007) emphasized the currently missing knowledge about the influence of endophytic microorganisms on physiological processes regarding plant-pathogen interactions, which might represent a yet unknown and unused potential of plant endophytes for plant protection.

In my Ph. D. project I already investigated the antagonistic activity of endophytic *B. bassiana* against putative target pest insects and the protective potential against fungal grapevine pathogens. In addition to these investigations a microarray analysis was performed to work out the fundamental aspects of the interaction between grapevine and endophytic fungi. The results indicate an up-regulation of diverse defense genes in grapevine due to the endophytic establishment of *B. bassiana*.

2. Purpose of the STSM

During the proposed research stay, the infection and establishment mechanisms of endophytic *B. bassiana* in grapevine plants as well as its effects on the plant's stress response should be analyzed with specific techniques available in the host department.

GFP-Transformation and detection in planta

A key requirement in the study of the plant-fungus interaction is the ability to detect the endophytic fungus and to quantify the amount of the fungal biomass within the plant. A visual marker of fungal growth in plants is the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, which has the unique ability to emit green fluorescence under UV or blue light. The application of GFP as a marker in a fungus can be extremely useful in collecting information on the processes by which fungi invade and colonize the plants. Therefore, a commercially available *B. bassiana* strain should be transformed by *Agrobacterium tumefaciens*-mediated transformation using a plasmid containing the GFP-gen.

Stress tolerance and effect on physiological processes

Knowledge on the influence of endophytic fungi on the abiotic stress tolerance of plants is an important criterion for evaluating the potential of these fungi. Therefore, the impact of the endophyte *B. bassiana* on various plant physiological processes should be evaluated by measuring photosynthesis, chlorophyll levels, water usage and the response to drought stress.

3. Description of the work carried out during the STSM

GFP transformation

Agrobacterium tumefaciens-mediated transformation was used in order to create fluorescent conidia of *B. bassiana* strain ATCC 74040. The fungal transformation consists of three steps: (1) the induction of bacterial culture harboring the appropriate plasmid, (2) co-incubation of fresh fungal spores and the bacterium on solid support and (3) selection of transformants using a medium with suitable selection agents.

As preparation for the transformation the target plasmid pCAMB74 was isolated from competent cells of *E. coli* DH5α with QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's

instructions. The *Agrobacterium tumefaciens*-culture (AGL-1) was transformed by electroporation with approximately 1800 V for one pulse. *Agrobacterium tumefaciens* AGL-1 containing pCAMB74 was cultured on Luria Broth (LB) medium supplemented with 100 µg/ml kanamycin before inoculating 5 ml LB + kanamycin (100 µg/ml) by picking a single colony and incubating the culture on the shaker (200-210 rpm) at 23°C for 18-24 h.

Bacterial cells of this culture were harvested in a 400 µl aliquot and the LB medium was removed by centrifugation. For induction the cells were subsequently resuspended in 5 ml of *A. tumefaciens* induction medium (AIM) and grown for an additional 18–24 h to reach an optical density (OD 600) of 0.5–0.6. The co-incubation was achieved by mixing the culture with an equal volume of a conidial suspension of *Beauveria bassiana* (5 × 105 conidia ml−1) and plating 200 µl of the mixture onto nitrocellulose filters on modified solid AIM medium in the presence and absence of 200 µM acetosyringone (AS), respectively. To select the transformed fungus the filters were transferred to PDA plates supplemented with cefotaxime, 100 µg/ml carbenicillin in order to eliminate *A. tumefaciens* cells and 300 µg/ml hygromycin as the respective selective agent for fungal transformants.

Stress tolerance

Due to complications in culturing the postal delivered hardwood cuttings of grapevine at the host institution, it was not possible to perform the planned experiments with this plant species. We therefore selected two other plant species, which are known to be colonized by *B. bassiana* from the literature – tomato and corn. Two different treatments – pipetting the obtained spore suspension at the stem base and spraying the whole plant with the suspension – were chosen to inoculate both plant species with *B. bassiana*. The chlorophyll levels in the plant leaves were analyzed over a time period of 14 days after treatment (dat) for the tomato plants and 24 dat for the corn plants in endophytic and non-endophytic plants before and after exposure to drought stress. In addition to this, plant growth and development as well as water usage of inoculated and non-inoculated grapevine plants was measured with and without exposure to abiotic stress. All experiments were conducted under controlled conditions in the greenhouse.

4. Description of the main results obtained

GFP transformation

The transformation by electroporation of the *Agrobacterium tumefaciens* AGL-1 strain was successful. Because of an existing tolerance of the used *Beauveria bassiana* strain ATCC 74040 against hygromycin it was not possible to select the transformed colonies from untransformed ones with the hygromycin concentration used in other papers. Increased concentrations of hygromycin of up to 600 µl/ml were used in a plating test and a second transformation, but showed no positive

results either. A possibility to solve this problem would have been a selection of colonies under the microscope of the nearby University of Washington, but this step couldn't be carried out due to time constraints.

Stress tolerance

During the first time after inoculation the plants (tomato and corn) treated with *B. bassiana* showed a better performance regarding plant growth and chlorophyll levels of the leaves than the control plants. After exposure to the drought stress the differences in chlorophyll levels, plant height and wet weight (end of the experiments) were not as big as before and therefore not statistically significant. Due to the first indication of slight differences in performance, the experiments should be repeated with a greater number of plants and other plant species (e.g. grapevine).

Insights in the work and the methods of the company

In addition to my own experiments I got insights in the work of a company producing and selling their own products of endophytic fungi. Besides the coating of seeds with the product, the spore production in bigger scales and the formulation of the products I was involved in the conduction of different lab and greenhouse experiments to enhance further development of their own products.

5. Foreseen publications and further collaboration resulting from the STSM

The results obtained will not be directly included in articles, but are the basis for further investigations and experiments with grapevine and endophytic *B. bassiana* as part of my Ph.D. project. It is planned to establish the transformation system of filamentous fungi with the help of *Agrobacterium tumefaciens* at my home institution. Therefore I got an important contact to Jose Maciá-Vicente, former member of the staff of the host institution and now working at the University of Frankfurt.

6. Confirmation by the host institution of the successful execution of the STSM

I confirm the stay of Yvonne Rondot in our company Adaptive Symbiotic Technologies from 1st March to 19th April. Despite some complications and drawbacks with the experiments the fellow was highly motivated to carry out all experiments as planned and to learn the methods used in our lab. She integrated herself immediately into our team and was very active and truly independent. I am confident that Yvonne will apply the learned techniques in her further experiments and establish a successful transformation system at her home institute.

- Rusty Rodriguez

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