Application Techniques of Endophytes

Desiree Jakobs-Schönwandt, Matthias Döring and Anant Patel (eds.)

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Verantwortliche Chefredaktion
Prof. Dr.-Ing. Rolf Naumann
Fachhochschule Bielefeld
Fachbereich Ingenieurwissenschaften und Mathematik
Am Stadtholz 24
33609 Bielefeld
Telefon +49.521.106-7483
Telefax +49.521.106-7191
rolf.naumann@fh-bielefeld.de

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List of Participants

Abo-Elyousr, Kamal	Julius Kühn-Institut (JKI) Institut für biologischen Pflanzenschutz Heinrichstraße 243 64287 Darmstadt GERMANY kaaboelyousr@yahoo.com
Andersch, Wolfram	Bayer CropScience AG Alfred-Nobel-Straße 50 40789 Monheim GERMANY +49 2173 384306 wolfram.andersch@bayer.com
Bednarz, Hanna	Universität Bielefeld Centrum für Biotechnologie – CeBiTec Postfach 10 01 31 33501 Bielefeld GERMANY +49 521 106-4839 hanna@cebitec.uni-bielefeld.de
Bejarano, Ana	AIT Austrian Institute of Technology GmbH Health & Environment Department Bioresources Konrad-Lorenz-Straße 24 3430 Tulln AUSTRIA +43 (0) 6648251183 ana.bejarano.fl@ait.ac.at
Berninger, Teresa	AIT Austrian Institute of Technology GmbH Health & Environment Department Bioresources Konrad-Lorenz-Straße 24 3430 Tulln AUSTRIA +43(0) 50550-3650 teresa.berninger@ait.ac.at

Chebotar, Vladimir	All-Russia Research Institute for Agricultural Microbiology Podbelsky Chaussee 3 St.Petersburg, Pushkin 8 196608 RUSSIA vladchebotar@rambler.ru
Döring, Matthias	INOQ GmbH Solkau 2 29465 Schnega GEMANY +49 (0)58 42-98 16 72 doering@inoq.de
Garnier, Jérôme	INCOTEC Holding Westeinde 107 1601 BL Enkhuizen THE NETHERLANDS +31 228 358243 Jerome.Garnier@incotec.com
Garrido Jurado, Inmaculada	University of Cordoba Agricultural Entomology Department of Agricultural and Forestry Sciences Campus de Rabanales building C4 " Celestino Mutis" 14071 Cordoba, SPAIN +34 957218475 g72gajui@uco.es
Iasur-Kruh, Lilach	Newe Yaar research center Entomology department POBox 1021 Ramat Yishay ISRAEL +972-49539551 Iasur@agri.gov.il

Imani, Jafargholi	Institute for Phytopathology and Applied Zoology Justus Liebig University Heinrich-Buff-Ring 26-32 35392 Gießen GERMANY + 49 641/99-37571 jafargholi.imani@agrar.uni-giessen.de
Jakobs-Schönwandt, Desiree	University of Applied Sciences FH Bielefeld WG Fermentation and Formulation of Biologicals and Chemicals FB Ingenieurwissenschaften und Mathematik Wilhelm-Bertelsmann-Straße 10 33602 Bielefeld GERMANY +49 (0)521-1067220 desiree.jakobs@fh-bielefeld.de
Kleespies, Regina	Julius Kühn-Institut (JKI) Institut für biologischen Pflanzenschutz Heinrichstraße 243 64287 Darmstadt GERMANY +49 6151 407 226 regina.kleespies@jki.bund.de
Koch, Eckhard	Julius Kühn-Institut (JKI) Institut für biologischen Pflanzenschutz Heinrichstraße 243 64287 Darmstadt GERMANY +49 6151 407 227 Eckhard.Koch@jki.bund.de
Kron-Morelli, Roberto	Agrifutur srl Via Campagnole 8 25020 Alfianello (Brescia) ITALY +39 030 99 34 776 Roberto.Kron-Morelli@Agrifutur.com

Kukali, Edlira
Agriculture University of Tirana
Faculty of agriculture & Environmental
Department of Horticulture & Land designer
Architecture
ALBANIA
edlirakukali@yahoo.com

Lemke, Annika
University of Applied Sciences
FH Bielefeld
WG Fermentation and Formulation of
Biologicals and Chemicals
FB Ingenieurwissenschaften und Mathematik
Wilhelm-Bertelsmann-Straße 10
33602 Bielefeld
GERMANY
+49 (0)521-1067224
annika.lemke@gmx.de

Lohse, Rieke
University of Applied Sciences
FH Bielefeld
WG Fermentation and Formulation of
Biologicals and Chemicals
FB Ingenieurwissenschaften und Mathematik
Wilhelm-Bertelsmann-Straße 10
33602 Bielefeld
GERMANY
+49 (0)521-1067273
rieke.lohse@fh-bielefeld.de

Matecki, Christina
Julius Kühn-Institut (JKI)
Institut für biologischen Pflanzenschutz
Heinrichstraße 243
64287 Darmstadt
GERMANY
christina_matecki@yahoo.de

Niehaus, Karsten
Universität Bielefeld
Centrum für Biotechnologie – CeBiTec
Postfach 10 01 31
33501 Bielefeld
GERMANY
+49 521 106-5631
kniehaus@CeBiTec.Uni-Bielefeld.de

Öğüt, Mehmet	Selcuk University Cumra Vocational College 42500 Cumra Konya TURKEY +90 332 4475621 mogut@selcuk.edu.tr
Ooteman, Paul	INCOTEC Holding Westeinde 107 1601 BL Enkhuizen THE NETHERLANDS paul.Ooteman@incotec.com
Ozaktan, Hatice	Faculty of Agriculture Dep. of Plant Protection, University of Ege 35100 Bornova-Izmir TURKEY +90 2323111410 hatice.ozaktan@ege.edu.tr
Patel, Anant	University of Applied Sciences FH Bielefeld WG Fermentation and Formulation of Biologicals and Chemicals FB Ingenieurwissenschaften und Mathematik Wilhelm-Bertelsmann-Straße 10 33602 Bielefeld GERMANY +49 (0)521-1067318 anant.patel@fh-bielefeld.de
Rybakova, Daria	Institut für Umweltbiotechnologie Technische Universität Graz Rechbauerstraße 12 8010 Graz AUSTRIA +43 316 873 – 4316 daria.rybakova@tugraz.at

Schneider, Carolin

INOQ GmbH
Solkau 2
29465 Schnega
GEMANY
+49 (0) 58 42- 98 16 72
schneider@pflanzenkultur.de

Schoenebeck, Inka

Bactiva GmbH
Herongerstraße 2
47638 Straelen
GERMANY
+49 2834-703306
schoenebeck@bactiva.de

Schweiger, Rabea

Universität Bielefeld
Universitätsstraße 25
33615 Bielefeld
GERMANY
+49 521 106-5536
rabea.schweiger@uni-bielefeld.de

Spieth, Peter

University of Applied Sciences
FH Bielefeld
WG Fermentation and Formulation of Biologicals and Chemicals
FB Ingenieurwissenschaften und Mathematik
Wilhelm-Bertelsmann-Straße 10
33602 Bielefeld
GERMANY
+49 (0)521-1067358
peter.spieth@fh-bielefeld.de

Streminska, Marta

Wageningen UR Greenhouse Horticulture
Violierenweg 1
2665 MV Bleiswijk
THE NETHERLANDS
+31 317485752
marta.streminska@wur.nl

Van Vlaenderen, Johan	National University or Ireland Maynooth Callan Building co Kildare Maynooth IRELAND +353 (0)17086650 j.vanvlaenderen@gmail.com
Wijkamp, Ineke	Koppert Biological Systems Veilingweg 14 Postbus 155 2650 AD Berkel en Rodenrijs THE NETHERLANDS +31 (0)10 5140444 iwijkamp@koppert.nl

Welcome adress

On July 14th – 15th 2014, the workshop “Application Techniques for Endophytes” was held at the University of Applied Sciences Bielefeld. The university was founded in 1971 and has 6900 students and 190 professors and lecturers and 280 scientific and non-scientific staff. Apart from many individual projects, research is clustered in the research centres “Intelligent electronic and mechatronic systems”, “Intelligent materials: biomaterials and formulations, nanotechnology, plastics and composite materials”, “Intelligent production engineering” and “Intelligent energy distribution and generation”.

The participants were hosted by the working group “Fermentation and Formulation of Chemicals and Biologicals” (Fig. 1).

Fig. 1 Working group “Fermentation and Formulation of Chemicals and Biologicals”.

This working team investigates novel formulation materials, methods and technology that are needed to fill the gap between production of an active ingredient and its application. Here, they aim to understand the relationship between formulation components and their properties, e.g. stability or porosity of a capsule and the molecular structure and to transfer the results into practice. Furthermore, the beneficial interaction of the formulation material with the active ingredients are of interest.

Current research deals with: “Development of innovative formulation techniques for the establishment of the beneficial fungus *Beauveria bassiana* in crops as protection against insect pests”, “Biological Crop Protection with Innovative Formulations of Endophytic Entomopathogenic Fungi against Insect Herbivores”, “Biotechnology of endophytes”, “The EU project INBIOSOIL: Innovative biological products for soil pest control”, “Formulation of attractants based on CO_2”, “The project ATTRACT: Protection of crops from soil-borne insect pests with a novel attract and kill strategy”, “Formulation of antimicrobial plant extracts”, “Development of novel silica gels for the entrapment of a light-harvesting complex, the microalgae *Chlamydomonas reinhardtii* and cobalt nanoparticles” and “Co-immobilization of chemo- and biocatalysts for chemoenzymatic one-pot cascade processes”.

Foreword

Introduction of COST Action FA1103 and WG 3

Schneider, C.

Chair of COST Action FA1103

COST funds pan-European, bottom-up networks of scientists and researchers across all science and technology fields. These networks, called 'COST Actions', promote international coordination of nationally-funded research. COST does not fund research itself, but provides support for networking activities carried out within COST Actions. COST Actions are open to researchers from universities, public and private research institutions, as well as to NGOs, industry and SMEs.

COST Action FA1103 'Endophytes in biotechnology and agriculture' started in December 2011 and currently over 260 members from 57 countries are registered in one or more of the 4 Working Groups (www.endophytes.eu).

The scientific focus of Working Group 3 'Development of new microbial inocula' targets the development of inoculants (both bacteria and fungi) and inoculation technology. In addition, the impacts of the endophyte inoculants on population dynamics of pathogens, autochthonous MOs and communication in plants are investigated. Epigenetically inherited positive changes in plants after inoculation are taken into account and studied, as far this information leads to novel technologies.

In Working Group 3, different inoculation methods are investigated as the development of a suitable procedure for the production of bio-preparations by cultivation and stable formulation is a necessary precondition for the practical application and shelf life of endophytes.

Introduction

Application techniques of endophytes

Patel, A. V.

University of Applied Sciences Bielefeld, WG Fermentation and Formulation of Biologicals and Chemicals, Wilhelm-Bertelsmann-Str. 10, D-33602 Bielefeld, Germany,
anant.patel@fh-bielefeld.de

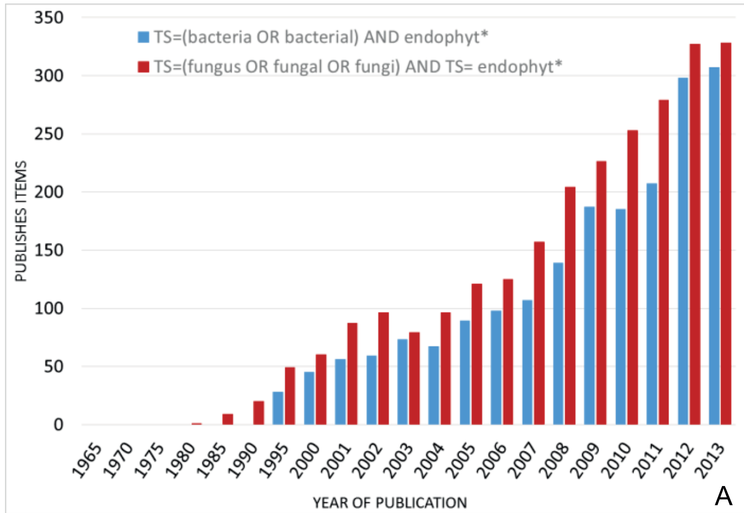
Endophytic microorganisms can have various beneficial effects, for example as plant growth and defense promoters by synthesizing phytohormones, by producing bio-surfactants, by fixing atmospheric nitrogen and CO_2 or by controlling plant diseases. However, the application of endophytes by seed coatings, sprays or granules and capsules still poses a range of scientific and technical challenges:
The formulation should increase establishment of the microorganism in soil and near or on the plant, support penetration and colonization of the host and reduce dosage and cost. Application techniques are closely linked to cultivation and formulation of the biologicals that ensure high shelf life and an in-depth understanding of the physicochemical and biological environment such as phyllosphere, soil, seed surface and rhizosphere.

In the case of mycorrhiza, advances have been made in the elucidation of the molecular plant-microbe interaction that needs to be converted into novel formulation and application techniques. In other cases such as endophytic entomopathogenic fungi, the mode of penetration and colonization is not

even known. Basic research studies still use only simple water-spores mixes instead of more sophisticated and effective application techniques.

This workshop will explore into application techniques fine-tuned to the delivery of endophytes to plants and will bring together scientists, technologists and representatives from companies from different parts of the industry. This talk will briefly introduce the participants to application techniques for endophytes with a literature survey, some key scientific and technical aspects and examples from our working group. At the end of the talk, questions from the participants that should be addressed during the workshop will be presented.

The number of publications on endophytes has risen dramatically in recent years as a literature research at the web of science revealed (Fig. 1A). However, much less publications can be found for application techniques for endophytes (Fig. 1B) thus reinforcing the timeliness of this workshop on application techniques for endophytes.



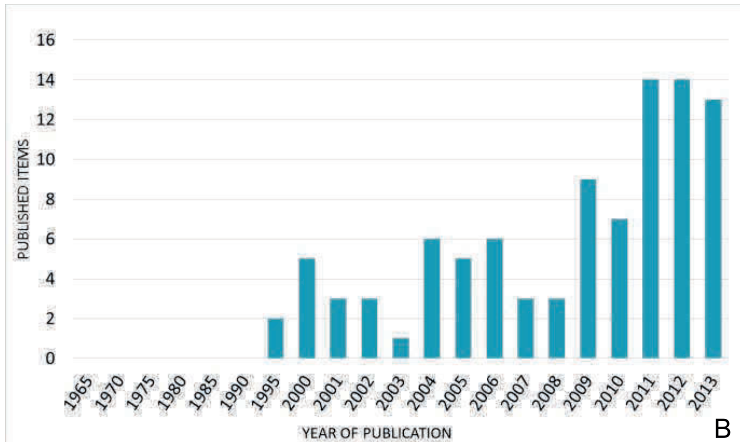


Fig. 1 A Published items of endophytes in recent years (Web of Science); **B** Application of endophytes: development in recent years (Web of Science, combination of search results containing formulation, seed treatment, spray and encapsulation).

Here, a brief survey on application techniques such as delivery by encapsulation, by seed coating and by sprays with examples from literature [e.g. 1-4] and our own work will be given. Focus is on application techniques for endophytes. In the following, we list the questions that are to be addressed within this workshop:

1. What are the special requirements for the application of endophytes?
2. Can we think of any methods for improvement of plant colonization after application?
3. Can we apply endophytes by inoculation of *in vitro* cultures (callus, meristem, protoplasts, and somatic embryos) to obtain seeds with endophytes inside?
4. Why do promising endophytes that work well under lab conditions fail when tested under more practical situations?
5. What is the actual increase in yield that may be expected from the application of endophytes (e.g. taking the application of rhizobia as a reference)? How is the interest of customers themselves to apply endophytes?
6. Can we register endophytes as biostimulants under the new biostimulant regulation 2016-2017 if they are co-applied within a co-formulation based on a consortium of microorganisms?
7. How to develop a mycorrhiza formulation for seed coatings?
8. What kind of novel seed treatments of oilseed rape in combination with endophytes application are promising?
9. How does application reflect on strain screening?
10. How can we fine-tune the cultivation of endophytes to increase the colonization of the plant?

Indirectly related to application techniques are the following questions:

1. The large amount of parameters that can be varied in the formulation process and may have an influence on the cell viability makes it difficult to identify the most critical factors, because it is simply impossible to try out every-thing! How can we identify critical parameters that affect cell viability during the drying step? (especially in the case of gram negative bacteria)?
2. There are also some technical problems with a seemingly simple method as plate counting: it is difficult to keep the formulation process sterile over many days (e.g. during air drying), which may result in a contamination. Also, the plate counting method may not detect the "viable but not culturable" bacteria. Alternatives?
3. Is the low recovery rate of entomopathogenic fungi out of plants a sign that entomopathogenic fungi are not natural endophytes even if we can observe them under this form in nature?
4. In the case of entomopathogenic fungi, what is the mode of action of plant colonization through roots or leaves?
5. How to produce bacterial inoculum in cheap substrates and to find the most effective formulation technique?
6. How much may a biocontrol agent cost in order to be able to compete with agrochemicals?

7. Which setups would be suitable for screening and identification of bioactive compounds from endophytes?

The results of a stimulating discussion round are presented in the conclusion on page 82.

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Presentations
Application via liquid root infection and seed coating

Development of Bacteria Formulations for Seed Coating

Bejarano, A. [1]; Mitter, B.[1] and Preininger, C.[1]

1. *Austrian Institute of Technology, Department of Health & Environment, Bioresources Unit, Austria.*

Inoculation of seeds is an efficient and convenient way of introducing effective plant-growth-promoting-bacteria (PGPB) to soil and consequently to the rhizosphere of plants. However, there are still limiting factors for commercial application of biological agents as biofertilizers which are mainly due to poor bacteria survival. Thus, developing formulations that provide high concentrations of microbial inoculant and high survival rates during storage constitute an important step in the development of effective inoculants.

So, the main aim of our work is to create "artificial spores" based on multilayer coatings [1] of good mechanical stability and selective permeability in order to develop environmentally friendly formulation technologies with increased product shelf life and efficient release and activation of encapsulated biomaterials.

To address this challenge, we are developing new surface coating solutions based on polyelectrolyte complexes for field application of plant growth promoting bacteria (PGPB), such as *Burkholderia phytofirmans* PsJN. *B. phytofirmans* PsJN

was chosen because it is a well characterized, prominent and efficient PGPB [2, 3].

The materials tested for seed coating of maize were non-toxic and biodegradable, cost-effective and readily available and include proteins like gelatin and polysaccharides like celluloses, alginate and xanthan. Depending on the kind of coating, the adhesive material concentration was 1% to 10% (w/v). Bacteria concentration used for encapsulation was 10^7 CFU/mL. Coating was carried out in a SATEC ML200 seed coater. Single and multilayer coatings were performed. Coating quality was defined by visual inspection while cell viability was determined by plating serial dilutions in Luria-Bertani agar. The effect of coating on seed germination was investigated in agar plates. Results compiled up to now showed favorable effects in germination, such as germination of maize treated with bacteria was enhanced by up to 60% compared with the untreated controls. Studies on release kinetics and stability testing are in progress.

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The Search for a Novel Seed Treatment for Oilseed Rape and *Brassica* vegetables

Rybakova, D.[1], Schmuck, M.[1] and Berg G.[1]

1. *Graz University of Technology, Environmental Biotechnology, Petersgasse 12, 8010 Graz, Austria.*

Abstract

Verticillium dahliae Kleb. and *Verticillium longisporum* are the cause of *Verticillium* wilt in a range of plants including *Brassica* spp. Currently, no fungicides are available for the control of *Verticillium*. The main objective of this project is to develop and optimize a seed treatment strategy against *Verticillium* wilt for oilseed rape (*Brassica napus L.*) and *Brassica* vegetables. The novel biological control will be based on the selected strains of *Serratia* and *Paenibacillus*. These biological control agents have already been evaluated under field conditions. The five strains of *Serratia* (*S. plymuthica* HRO-C48; *S. plymuthica* 3Re4-18; *S. plymuthica* 3RP8; *S. plymuthica* S13 and *S. proteamaculans* SP1-3-1) and five strains of *Paenibacillus* (*P. brasilensis* Mc2-9, *P. polymyxa* Sb3-1, *P. polymyxa* 302P5BS and *P. polymyxa* GnDWu39 and PB71) from the institute's strain collection were chosen for their antagonistic properties against *V. dahliae*, *Rhizoctonia solani* and *Fusarium culmorum* shown in preliminary tests. Our strategy for determining the most effective biocontrol agent involved biopriming of the surface-sterilized seeds of the oilseed rape and the cauliflower. The comparison of the colonization capacity of *Paenibacillus*

and *Serratia* strains showed no significant alterations within the same genus. The *Serratia* cells labelled with fluorescent markers were mostly observed on the upper parts of the roots using confocal scanning laser microscopy and were either present as clouds around the whole root system or they formed large micro-colonies in the root tissue. While *Serratia* treatment resulted in different levels of plant growth promotion (PGP), the opposite effect was found after *Paenibacillus* evaluation when plants were grown in a gnotobiotic system. With respect to the results, two bacteria were selected for further tests: *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1. The preliminary evaluation of the PGP effect of the selected strains did not significantly affect the biomass production when plants were grown in unsterilized soil making further tests for biocontrol effect under field conditions necessary.
The project being presented is a part of the overall BIOCOMES project (http://www.biocomes.eu/).

Key words: biocontrol, priming, plant growth promotion, PGP, BIOCOMES, Brassica, Serratia, Paenibacillus, Verticillium wilt.

Introduction

Verticillium spp. induce vascular wilting within a wide range of dicotyledonous plants, including economically important field crops such as oilseed rape (*Brassica napus L.*) and *Brassica* vegetables. *Verticillium* wilt is a significant soil-borne plant disease with no fungicidal cure available to date. Due to the genetically heterogeneous and polyphyletic character of *Verticillium* isolates, the fungus is amongst the most challenging

known diseases to control [5]. Given the current trend towards the environmentally friendly disease control of plants, biological control of the *Verticillium* wilt is especially desirable.

Serratia plymuthica HRO-C48 has been successfully used for controlling *Verticillium* wilt and other soil-borne fungi as a soil amendment in strawberry fields (RhizoStarR) [1, 6]. The application of the *S. plymuthica* HRO-C48 to the seeds of the oilseed rape via biopriming, pelleting or seed coating was shown to reduce the degree of *Verticillium* wilt in oilseed rape plants under field conditions [8. In the search for an optimal candidate for the seed treatment of *Brassica* plants, four further strains of *Serratia* (S*. plymuthica* 3Re4-18; *S. plymuthica* 3RP8; *S. plymuthica* S13 and *S. proteamaculans* SP1-3-1, Table 1) and five strains of Paenibacillus (*P. brasilensis* Mc2-9, *P. polymyxa* Sb3-1, *P. polymyxa* 302P5BS and *P. polymyxa* GnDWu39 and PB71, Tab. 1) from the institute's strain collection were selected for their antagonistic properties against fungal pathogens.

Material and methods

Biopriming of seeds for growth in germination pouches

The surface-sterilized seeds of the oilseed rape and the cauliflower were primed for 4 hours at room temperature with log10 6.0-7.0 CFUs/ml (*Paenibacillus*) or log10 8.4-9.5 CFUs/ml (*Serratia*) of each selected strain (Tab. 1) and the seedlings were grown aseptically in germination pouches for 14 days at 22°C under artificial 16/8 hours day/night conditions.

The initial selection strategy for determining the most effective biocontrol agent (BCA) included comparison of the three main parameters: (1) the ability of the BCA to induce plant growth promotion (PGP) in the oilseed rape and in the cauliflower seedlings determined by the seedling root and the shoot weight at the end of the incubation period; (2) the colonization ability of the BCA determined by the bacterial density on the roots at the end of the incubation period; (3) comparison of the colonization patterns of the different BCAs on the root using confocal laser scanning microscopy (CLSM) combined with fluorescent *in situ* hybridization and/or using microorganisms labelled with fluorescent markers.

Biopriming of seeds for growth in a soil system

For evaluation of PGP effect of BCA in the soil, the oilseed rape seeds were surface-sterilized and primed with log10 9.2 for *S. plymuthica* 3RP8 and log10 7.2 for *P. polymyxa* Sb3-1 for 4 hours. The seeds were sown in pots with a volume of 250.0 ml containing propagation compost (Einheitserdewerk, Uetersen, Germany) mixed with vermiculite (4:1, v/v). Plants were grown over a period of 14 days at 22°C under artificial 16/8 h day/night conditions. They were watered every second to fourth day. When the experiments were finished, the plants were weighed for the purpose of analyzing the effects of each BCA on biomass production.

Results and discussion

The inoculation of the seeds with the tested *Paenibacillus* strains resulted in the attachment of log10 2.8-4.1 CFUs per seed, where *P. polymyxa* PB71 showed the lowest abundance on the seed after priming (log10 2.6) and *P. polymyxa* GND-wu39 the highest (log10 4.4). The selected *Serratia* strains showed higher abundance of the bacteria on the seeds after priming than *Paenibacillus* strains, spanning from log10 5.8 for HRO-C48 to log10 6.5 for 3RP8 and for Re4-18. The abundances of the BCA on the roots of the 2-week old seedlings altered insignificantly within the same genus with log10 8.4-9.2 for *Serratia* and log10 7.7-8.5 for *Paenibacillus* strains. The evaluation of the colonization patterns of the *Serratia* strains labelled with fluorescent markers showed that *Serratia* cells were mostly observed on the upper parts of the roots. The *Serratia* cells were found either as clouds around the whole root system or in the form of large micro-colonies in the root tissue. Interestingly, the colonization patterns are similar to those observed for *Serratia* strains in the sugar beet rhizosphere [10].

Tab. 1 Selected bacterial isolates: identity, origin and properties.

Isolate	Closest database match	Environmental source	Antifungal activity*	Reference	PGP **
Paenibacillus					
Sb3-1	*P. polymyxa*	Agricultural Soil	+	Köberl *et al*. 2013	-
Mc2-9	*P. brasilensis*	*Matricaria chamomilla* Rhizosphere	+	Köberl *et al*. 2013	-
302P5BS	*P. polymyxa*	*Lobaria pulmonaria*	+	unpublished	-
GnDBl71	*P. polymyxa*	*Cucurbita pepo* Spermosphere	+	Fürnkranz *et al*. 2012	-
GnDWu39	*P. peoriae*	*Lobaria pulmonaria*	+	Fürnkranz *et al*. 2012	-
Serratia					
HRO-C48	*S. plymuthica*	Oilseed rape	+	Müller *et al*. 2008	+/-
3Re4-18	*S. plymuthica*	Potato endosphere	+	Berg *et al*. 2005; Zachow *et al*. 2010	+/-
3RP8	*S. plymuthica*	Potato rhizosphere	+	Berg *et al*. 2002	+
SP1-3-1	*S. proteamaculans*	Primula	+	Zachow *et al*. 2013	+/-
S13	*S. plymuthica*	*Lobaria pulmonaria*	+	Fürnkranz *et al*. 2012	+/-

* Antifungal activity was shown for different fungal pathogens such as Verticillium dahliae, Rhizoctonia solani and Fusarium culmorum among others. + Significant reduction of fungus growth as shown in dual plate assays.

** Positive PGP effect (+) was designated to the cases where a significant increase of the fresh weight of the roots of both cauliflower and oilseed rape seedlings after being primed with each BCA in a gnotobiotic system was observed. +/- designates cases where either no effect of the priming on the root weight or insignificant effect was observed. Negative PGP effect (–) was assigned to the cases where root weight was below the root weight of the unprimed seedlings (negative control).

The evaluation of the PGP effect of the selected BCAs showed that while *Serratia* treatment resulted in different levels of plant growth promotion, the opposite effect was found after *Paenibacillus* evaluation, with Sb3-1 being the least damaging for the plant. The priming of the *Brassica* seeds with the *S. plymuthica* 3RP8 strain resulted in increased root weights of the 2 week-old cauliflower seedlings in comparison to the unprimed negative control, while priming with other four *Serratia* strains did not result in a significant increase the weight of the roots. With respect to the results, the *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were chosen for further experiments. A deleterious effect of *Paenibacillus* on the *Arabidopsis thaliana* ecotype C24 was previously reported when plants were grown in a gnotobiotic system [9]. For that reason, we decided to evaluate the effect of the selected microorganisms under more natural conditions by sowing the seeds primed with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 in the unsterilized soil. Preliminary evaluation of the soil experiments showed that the average fresh weight of the plants primed with *S. plymuthica* 3RP8 was slightly higher than that of the unprimed seedlings and that plants primed with *P. polymyxa* Sb3-1 produced slightly less biomass than the control. However, these differences were insignificant. The results indicate that further testing of the selected strains for their biocontrol effects against *Verticillium* wilt in *Brassica* spp. in addition to further greenhouse experiments and field trials, are necessary

in order to fully evaluate the biocontrol effect of the selected strains.

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Application of Bacterial Endophytes to Cucumber Plants for Their Effects on Growth Promotion and Biocontrol

Ozaktan, H.[1], Gül, A.[2], Çakır, B.[2], Yolageldi, L.[1], Akköprü A.[3], Fakhraei, D[1]. and Akbaba, M.[1]

1. *University of Ege Faculty of Agriculture Department of Plant Protection, Izmir, Turkey, hatice.ozaktan@ege.edu.tr, mustafa.akbaba@ege.edu.tr, lalehan.yolageldi@ege.edu.tr*
2. *University of Ege Faculty of Agriculture Department of Horticulture, Izmir, Turkey, ayse.gul@ege.edu.tr, birsen.cakir@ege.edu.tr*
3. *University of Yüzüncü Yıl, Faculty of Agriculture Deptartment of Plant Protection,Van, Turkey, ahmetakkopru@gmail.com*

The aim of this study was to find within cucumber plants endophytic bacterial (EB) isolates that can provide significant biological control of *Fusarial* wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) and that can enhance plant growth under greenhouse conditions.

In this study, 104 EB strains were isolated from internal tissues of healthy cucumber plants which were grown in greenhouse and field in Turkey. These isolates were screened *in vitro* for their plant growth promoting traits and antagonistic activity against FOC [4]. The EB isolates exhibiting biocontrol and multiple plant growth promoting (PGP) traits were tested in growth chamber and greenhouse experiments [3]. The colonization and population dynamics of EB strains in the plant tissues for 75 days under growth chamber conditions were also monitored. EB inoculation took place two times; before sowing as seed coating and after transplanting as substrate drenching (30 ml per plant, 10^8–10^9 CFU ml^{-1}). For seed coat-

ing, bacterial inoculants were suspended with carboxyl methyl cellulose (CMC, 1.5%) to obtain the population density with 10^8–10^9 CFU ml^{-1}. Cucumber seeds (cv. Gordion) were treated with the bacterial suspensions in Erlenmeyer flasks by shaking for 30 min at 120 rpm, and the seeds were air-dried before sowing. It was determined that EB isolates colonized seed tissues at the rate of 10^6 CFU seed g^{-1} after bacterization. Cucumber plants with treated EB as seed coating and soil drenching were transplanted to peat inoculated with FOC spore suspension (10^5 spores ml^{-1}).

According to growth chamber test results, 38% of tested EB strains exhibited a disease reduction between 30 to 60 %, comparing to only FOC inoculated plants (Fig. 1).

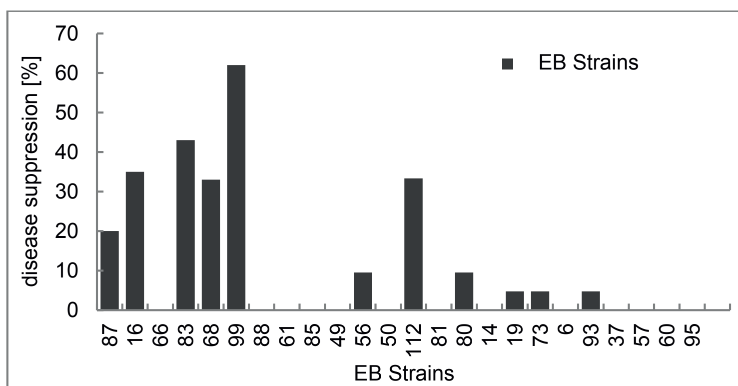

Fig 1. Effects of EB treatments to disease severity of FOC by pot tests.

Over 80 % of plants treated with EB by exhibited an increased dry weight concerning the root biomass by 5 to 160% compared to non-treated plants. On the other hand, 50% of tested

EB strains increased the dry weight of shoot biomass by 2 to 15%. Cucumber plants treated with EB as seed coating and soil drenching were transplanted to peat inoculated with FOC spore suspension (10^5 spores ml^{-1}). Then, the cucumber plants were transferred to perlite medium pots in greenhouse to monitor the disease development of FOC and fruit yield for 2 months after transplanting to a soilless system. EB treatments applied as seed coating and soil drenching inhibited the *Fusarial* wilt development at the rate of 49 to 52% compared to only FOC inoculated cucumber plants for two months growing period in a soilless growing system (Table 1).

Tab. 1 The effects of EB treatments on disease suppression caused by FOC ten weeks after beginning the greenhouse trials.

Treatments	Mean Disease Incidence [%]	Mean Disease Suppression [%]
Pathogen alone (FOC)	55 A*	---
BION +FOC	27 AB	51
99 + FOC	28 AB	49
83 + FOC	27 AB	52

*Statistical comparison within all seedlings was performed by Duncan's test (a<0.05). Different letters above the bars on the graphs indicate significantly different results

Moreover, EB treatments without being any disease pressure increased the total marketable fruit yield at the rate of 1 to 12 % compared to non-treated cucumber plants for 2 months growing period in soilless cultivation (Fig. 2).

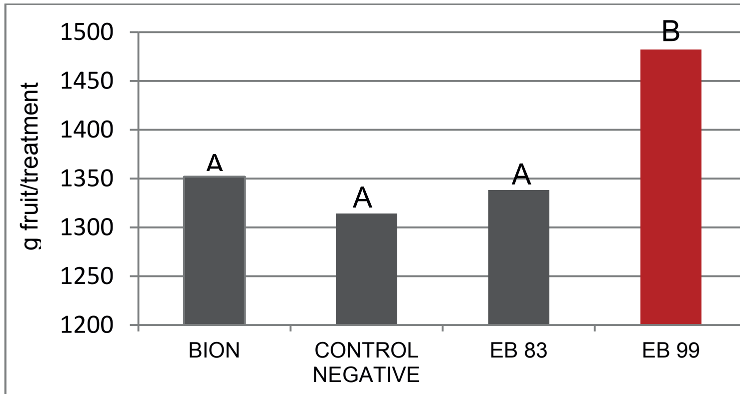

Fig. 2 The effect of EB treatments on total fruit yield (g/treatment) of cucumber plants ten weeks after beginning the greenhouse trials (*statistical comparison within all treatments was performed by Duncan's test ($a<0.05$). Different letters above the bars on the graphs indicate significantly different results

EB treatments to seeds and seedlings were monitored from sowing of seeds until 45 days after FOC inoculation in order to determine their colonization and population dynamics inside the cucumber plant tissues depending on the time, periodically. EB strains were marked as rifampicin resistant (200 µg/ml) in order to determine the endophytic populations in the plant tissues which were surface sterilized with Na-hypochlorite 1% for 5 min and then washed three times in distilled water. The last rinsing water was checked for surface contamination. The samples (roots and shoot parts of plants in sterile plastic bags) were then grounded with a stoemaker and homogenized in order to determine the microbial populations inside surfaced-sterilized roots, shoot, and leaves. The bacterial colonies were counted after 3 days of incubation at 24°C in rifampicin (200 µg/ml) supplemented Kings medium B.

Tested EB showed the ability to move within the stem 75 days after seed bacterization. We found that populations of bacterial endophytes ranged from $log10^5$ to $log10^7$ CFU/g-fresh weight in surface disinfested cucumber seed, in cucumber cotyledones from $log10^4$ CFU/g-fw to $log10^5$ CFU/g-fw and in cucumber roots from $log10^5$ to $log10^4$ CFU/g-fw in the growing season. Population of log^5 CFU of strain 83 introduced into plant stems increased after 45 to 75 days to log^6 CFU/g shoot tissue. Unfortunately, population density of EB strain 99 decreased and showed limited colonization after FOC inoculation to the pots. Population densities of the endophytic bacteria in roots were found to be about 10^4 to 10^5 CFU g^{-1} fresh root weight. Root colonization of EB decreased depending on the time whereas shoot colonization of EB was higher than root colonization 75 days after seed sowing.

Plant growth promotion mediated by endophytic bacteria may be exerted by several mechanisms, e.g. production of plant growth hormones, synthesis of siderophores or solubilisation of minerals such as phosphorous [1 - 2, 5]. In this study, it was proven that the tested EB strains are promising in respect to plant growth promotion and biocontrol parameters.

Furthermore, EB isolates demonstrated not only to significantly improve seed germination, seedling length and plant growth of cucumber but also, when used for seed/seedling treatment, to significantly reduce disease symptoms caused by vascular wilt pathogen f. sp. *cucumerinum* (FOC). All tested bacteria

were recovered from cucumber root, stem and leaf tissues after having been monitored for 75 days in growth chamber room conditions. When inoculated onto cucumber seeds and seedlings, both species colonized and produced plant-growth effects. So, the application of EB as seed coating and soil drenching was recorded as effective and practical techniques in terms of biological control, growth promotion of cucumber plants and colonization of bacteria inside the plant tissues.

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Inoculation of Entomopathogenic Fungi as Endophytes by Treatments of the Seeds and Rootlets of Sitka Spruce and Lodgepole Pine Seedlings

Van Vlaenderen, J.[1,2]; Meade, C.[2] and Griffin, C.[1]

1. *Behavioural Ecology and Biocontrol Laboratory, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, IRELAND, j.vanvlaenderen@gmail.com*
2. *Molecular Ecology Laboratory, Department of Biology,National University of Ireland, Maynooth, Co. Kildare, IRELAND*

Entomopathogenic fungi occur naturally as endophytes in different parts of mature coniferous trees including the seeds, needles and bark [1-3]. Furthermore, a wide variety of non-entomopathogenic fungi acts as endophytes and can be successfully inoculated by treatment of the seeds, the roots or by spraying the aerial parts of the plant. However, inoculation of entomopathogenic fungi as endophytes has been rare [4].The large pine weevil *Hylobius abietis* (Coleoptera : Curculionidae) is a major pest in coniferous forest especially targeting newly planted seedlings for reforestation and leading to up to 100% mortality in certain regions. Several entomopathogenic fungi such as *Beauveria bassiana* or *Metarhizium anisopliae* have shown potential to control this insect in lab and field trials [5]. The use of entomopathogenic fungi on the aerial parts of the plants poses the problem of the spores being washed away by rain or damaged by UV light. The difficulty of targeting adult weevils led us to develop new techniques including the inoculation of these fungi as endophytes. Thus, we tried to inoculate entomopathogenic fungi as endophytes in two commercially

important species of conifer, Sitka spruce and lodgepole pine, using a variety of techniques. Both spores and hyphae were used. Initial results showed that even if the recovery rate of endophytic entomopathogenic fungi is low, it is possible to inoculate an entomopathogenic fungus as an endophyte in coniferous seedlings.

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Endophytes for Commercial use in Plant Growth Promotion

Wijkamp, I[1]. and Ravensberg, W[1].

1. *Koppert Biological Systems, PO Box 155, Berkel en Rodenrijs, the Netherlands, WRavensberg@koppert.nl*

Interest in endophytes was generated recently, particularly by recognizing that entomopathogenic fungi can be used for other purposes than the traditional one. They may be used as antagonists of plant pathogens and for control of nematodes, as endophytes against insects, and as plant growth promoting agents. Microbial disease control agents can also affect herbivorous insects through triggering the plant's defense system. Endophytes have been hardly used in commercial applications. Broader use of existing products offers companies larger markets and a better return on investment. Secondly, endophytic use of these organisms and newly discovered endophytic fungi and bacteria eliminates important issues such as repeated and laborious applications, like spraying, and unfavourable environmental conditions needed for entomopathogenic fungi to be efficient, like a high relative humidity. Thirdly, they allow applications as seed treatment and/or early treatment in the propagation phase. In terms of regulatory aspects, exposure to these endophytic microorganisms will be much less than when applied via spraying. As a consequence, risks for applicator and workers are greatly reduced. This should allow easier and less costly registrations.

Preliminary results will be presented on research on plant endophytes for plant growth promotion at Koppert. The potential and advantages for the industry will be highlighted as well as the needs and challenges in research and development.

Molecular Uptake Mechanisms of Symbiotic Bacteria with Respect to Legume Plant Roots

Nounurai, P. [1]**; Densow, H.**[2]**; Bednarz, H.**[2] **and Niehaus, K.** [2]

1. *National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Thailand*
2. *Bielefeld University, Faculty of Biology, Department of Proteome and Metabolome Research, Germany, kniehaus@CeBiTec.Uni-Bielefeld.de*

The nitrogen-fixing root nodule is generally derived through a successful symbiotic interaction between legume plant and rhizobial bacterium. A nodule cell can shelter hundreds of Rhizobia which are thought to invade into the cell through an endocytosis-like process despite the existence of turgor pressure and inexpandable plant cell. We could demonstrate the endocytotic uptake of bacterial lipopolysaccharides (LPS) to plant cell cultures. Each invading Rhizobium is surrounded by peribacteroid membrane to form the symbiosome which results in higher acquisition of host membrane materials. In this study we show the involvement of Rab11F (a member of the large Rab11 family) which is highly expressed in nodules of *Medicago sativa* in the symbiosome membrane formation. Rab11F-labeled organelles accumulated FM4-64 and are sensitive to brefeldin A by forming aggregates after treatment with brefeldin A. By co-localization with *cis*-Golgi marker, GmMan1:mCherry, Rab11F-organelles formed tricolor organelles, whereby Rab11F was located to the opposite side of GmMan1:mCherry indicating that Rab11F-labeled organelles is localize on the trans-Golgi (TGN). In nodules, Rab11F is

localized on infection thread- and peribacteroid membranes. The symbiosome acquires Rab11F during the entry process and during differentiation. However, the symbiosome does not acquire Rab11F after cessation of division. In conclusion, legume plants seem to use secretion pathway from TGN, which are controlled by Rab11 to proliferate the symbiosome membrane. The importance and origin of endocytosis of bacteria to plant cells will be discussed.

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How to (Re)Integrate Endophyte Soil Potential in Field Crops?

Schneider, C.[1] and Mercy, L.[1]

1. *Inoq GmbH, Germany, schneider@inoq.de, mercy@inoq.de*

The political, social and economical context should favor the exploration of biotechnologies which (re)integrate the logic of the living concept in soil and support ecosystem service potentials in the next years. Among microorganisms which will play a key role for the “second green revolution” implementation, Arbuscular Mycorrhizal fungi (AMF) are pillars participating to sustain plant performances. AMF are known to improve host plant P acquisition, increase plant tolerance against biotic and abiotic stresses and serve also as a niche for many other endophytes. However, their efficient application in crop fields is challenging and several challenges contribute to a weak consideration of these endophytes under agricultural systems. Indeed, the mycorrhizal plant susceptibility is mainly limited due to (i) the loss of soil receptivity due to conventional fertilization regime and phosphorus soil content, (ii) the application methodologies, (iii) but is also linked to mycorrhizal inoculum formulation, quality and cost. Therefore, there is need to elaborate strategies which allow application of a cheap mycorrhizal formulation adapted to crop machineries, while improving plant colonization and performance under difficult conditions. Through several examples, we discuss some problems en-

countered and possibilities for crop management of mycorrhizal soil potentials.

Presentations
Application via encapsulation

Encapsulation of *Burkholderia phytofirmans* PsJN in Alginate Beads

Berninger, T.[1], Mitter, B. and Preininger, C.

1. *Austrian Institute of Technology GmbH, Department of Health and Environment, Unit Bioresources, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria, [1]teresa.berninger@ait.ac.at*

Abstract

With the aim of providing a protective formulation for the plant growth-promoting endophyte *Burkholderia phytofirmans* PsJN, the bacteria were immobilized in alginate. Viability was checked after lyophilization and rotavapor-drying and compared to the one of *B. subtilis* as a reference strain. While the viability of *B. subtilis* could be preserved throughout the drying process, *B. phytofirmans* PsJN suffered from a sharp decrease in viability. Addition of trehalose to the immobilization matrix was compatible with the desired physical bead properties, but proved ineffective for amelioration of desiccation stress of PsJN.

Key words: Burkholderia phytofirmans, encapsulation, alginate, endophytes

Introduction

During in-house research, the plant growth-promoting endophytic organism *Burkholderia phytofirmans* PsJN has been characterized [2] and studied regarding its effect on a

variety of crops such as maize [3] and its ability to colonize plants through different pathways. However, the beneficial effects of PsJN observed in green-house trials could not be replicated to the same extent in field trials. This may be due to a loss in cell viability of the bacteria during storage and transport. Furthermore, the competition by native soil microorganisms may prevent the introduced strains from establishing populations on site.

To tackle these issues, it is necessary to provide a protective formulation to the bacteria. Bearing in mind the needs of the agricultural industry, this formulation should ideally consist of cheap, readily available and biodegradable materials, should be cost-efficient in production and convenient in use.

One approach is the encapsulation of bacterial strains in alginate beads. This straightforward technique has been examined regarding its suitability for a range of plant growth-promoting organisms, such as *Azospirillum brasilense*, *P. fluorescens* and *B. polymyxa* (reviewed by [1]). Several parameters involved in the formulation procedure may be varied, the ones regarded as most critical being the culture conditions, composition of the immobilization matrix and the drying process.

The aim of our work is to evaluate a selected set of process parameters on the viability of PsJN immobilized in alginate beads. The late stationary growth phase was chosen as the

point of harvesting. For the protection of prokaryotic cells during desiccation, a range of substances is known, such as trehalose, glycerol and skim milk (reviewed by [4]. These three additives were checked regarding their compatiblity with encapsulation equipment and influence on bead properties. The most suitable substance – trehalose – was examined regarding its effect on PsJN viability after lyophilization or rotavapor-drying. For an increase in mechanical strength and improvement of structure of alginate beads, starch from rice was opted for due to its small-sized starch granules and resulting compatibility with production of small-sized alginate beads geared for application in seed coating.

Material and methods

Cultivation

Burkholderia phytofirmans PsJN::*gfp*2x and – as a robust reference strain – *Bacillus subtilis* 1A748 were grown at 28 °C on a shaker at 200 rpm in liquid LB medium amended with 25 µg/mL of the selective antibiotic kanamycin. Bacteria were harvested after 48 hrs in the late stationary phase by centrifugation at 4500 rpm and 4 °C for 10 min, the supernatant was discarded and the cell pellet was re-suspended in sterile 0.9 % NaCl. The bacterial suspensions were kept at 4° C for 12 – 48 hrs until encapsulation. The OD600 was measured by the plate reader Synergy MX (Biotek, Vermont, USA) and adjusted to 10^{10} CFU/mL.

Encapsulation

An immobilization matrix consisting of 1.8 % (w/v) low viscosity alginate (Büchi GmbH, Flawil, Switzerland) and 3 % (w/v) rice starch (Sigma Aldrich, St. Louis, MO, USA) was prepared in dH_2O. The solutions were mixed thoroughly with the bacterial suspensions in a ratio of 10:1 to reach a final concentration of 10^9 CFU/mL.

Beads were extruded into fast stirred 0.2 M $CaCl_2$ by help of the encapsulator B-390 (Büchi) through a nozzle of 200 µm diameter. The beads were allowed to harden thoroughly in the $CaCl_2$ solution for 30 min and were then rinsed and separated from the solution by a 3 times repeated cycle of washing with dH_2O and decantation.

To verify the maintenance of cell viability during encapsulation, 10 wet beads were kept for examination. The volume of a single bead with a diameter of 400 µm was calculated from the formula $V = 4*\pi*r^3/3$ to obtain the theoretical amount of CFU to be expected.

Drying

Alginate beads carrying *B. phytofirmans* or *B. subtilis* were dried by lyophilization in rectangular, conical aluminium trays (Rotilabo by Carl Roth GmbH, Karlsruhe, Germany) at a vacuum of 0.120 mbar, shelf temperature increasing stepwise from 0 °C – 25 °C, pre-freezing at -80 °C for 48 – 96 h or by vacuum-facilitated evaporation in Rotavapor (R-210 by Büchi)

at a vacuum of 32 mbar, water bath temperature 40 °C in a slowly rotating beaker for 3-4 h.

Determination of cell viability

Cell viability was measured by selective plate counting on LB agar plates with kanamycin (25 µg/mL). Dry beads were dissolved in sodium citrate (50 g/L) by shaking at 300 rpm. The solution was serially diluted with sterile 0.9 % NaCl and streaked on the previously prepared plates in duplicate. The same was done with the cell suspension used for encapsulation in order to confirm the cell density measured by spectrophotometry before. All plates were incubated at 28 °C for 2 – 4 days. Subsequently, colony counting was performed by help of the Stuart Colony Counter (Bibby Scientific Ltd., Staffordshire, UK). The identity of *B. phytofirmans* PsJN::*gfp*2x was verified by observing the fluorescent colonies under UV light (Biospectrum imaging system by UVP, Upland, CA, USA). The ability of PsJN to express its antibiotic resistance even after desiccation and rehydration stress was checked on LB plates without antibiotic.

Influence of protectants on physical bead properties and viability of PsJN

Three different matrices were prepared without bacteria, containing 1.8 % alginate and 1 % rice starch as well as the additive 1.5 % trehalose (Fluka, Buchs, Switzerland), 1.4 %

skim milk or 15 % glycerol (Sigma Aldrich). Beads were formed and dried as described above. They were examined by transmission type inverted microscope (Axiovert 200 M with camera AxioCam and software AxioVision Release 4.8 by Zeiss, Oberkochen, Germany). To examine the ability of trehalose to function as a protectant for PsJN, it was added to the growth medium in a concentration of 20 mM or to the encapsulation matrix in a concentration of 0.1 M. Beads were formed, dried and the cell viability measured as described above.

Results and discussion

Bacterial viability in alginate beads

While cells of *B. subtilis* only suffered from a minor loss in viability subsequent to drying, the decrease of viable PsJN cells of up to 10 orders of magnitude was dramatic regardless of the drying method applied (Fig. 1). Trehalose in medium or matrix did not improve cell survival of PsJN (results not shown).

The initial concentration of the PsJN suspension as determined by plate counting was much higher than the target value adjusted by OD600 measurement. In contrast, the one of the *B. subtilis* suspension was lower. This underlines the necessity of controlling the amount of CFU in the suspensions not only by spectrophotometry, but also by plate counting.

As observed by dissolution of wet alginate beads prior to drying, the encapsulation process itself was of no harm to PsJN cell viability as the expected amount of CFU was measured in wet beads. Furthermore, the amount of colonies counted on plates with and without kanamycin was identical, meaning that the low amount of CFU after drying is not due to an inability of PsJN to express antibiotic resistance.

Clearly, the drying process constitutes a critical step in formulation development especially for the less robust, Gram negative PsJN compared to Gram positive *B. subtilis*. For this category of plant growth-promoting endophytes it is crucial to closely monitor the drying conditions and design a very mild process. The second task is then to screen for efficient protective substances, which may enhance survival of bacteria during formulation and for extended periods thereafter.

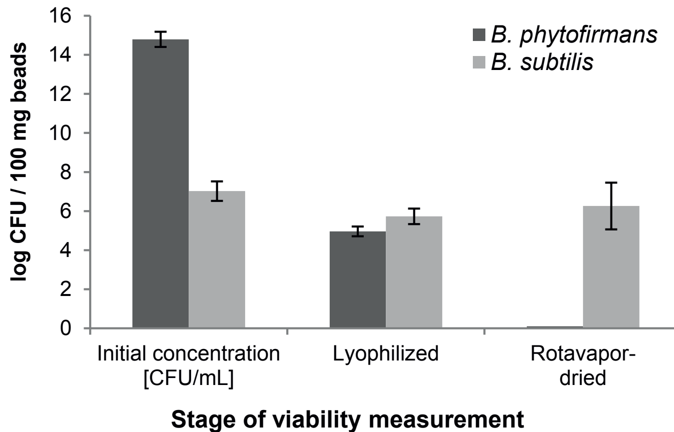

Fig. 1 Cell viability of *B. phytofirmans* PsJN decreases dramatically after drying, especially in case of rotavapor-drying. In contrast, *B. subtilis* only experiences a slight decrease in viability after drying.

Influence of additives on physical properties of beads

Not all additives proved suitable for homogenous bead production (Fig. 2). Skim milk resulted in foam formation on the surface of the fast stirred $CaCl_2$ bath, which gathered in the vortex, thereby impeding the formation of separate, gelled beads. This resulted in agglomeration and deformation of beads (Fig. 2C). While glycerol was compatible with the encapsulation process, it yielded sticky, agglomerated beads when dried by lyophilization (results not shown). Trehalose beads appeared homogenous both after lyophilization and rotavapor-drying, although a spherical shape was better maintained in the latter case (Fig. 2B). Rice starch improved structural stability compared to tests performed previously without it. Therefore, if aiming for spherical, homogenous

beads forming a free flowing powder, trehalose is the most suitable one among the tested additives and is best combined with drying by rotavapor and rice starch amendment. This test illustrates the necessity to not only evaluate additives regarding their effect on bacteria, but also on physical bead characteristics.

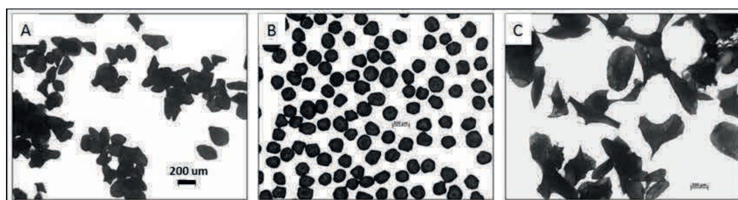

Fig. 2 Influence of additives and drying method on physical bead properties. Lyophilization of trehalose-amended beads results in some agglomeration and collapse of beads (A), rotavapor-drying of trehalose beads yields well separated, round-shaped particles (B), skim milk-amended beads are deformed and agglomerated after lyophilisation (C). Scale bar in (A) applies to all pictures.

Acknowledgements

The authors thank the Austrian Research Promotion Agency (FFG) for financial support of the FEMtech project “EndoCaps” (No. 839341).

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Presentations
Application via leaf treatment

Endophytically Colonized Plants by Entomopathogenic Fungi for *Spodoptera Littoralis* Control

Garrido-Jurado, I., Resquín-Romero, G., and Quesada-Moraga, E.

1. *Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba. Campus de Rabanales. Edificio C4 Celestino Mutis. 14071 Cordoba, Spain*

Isolates of *Beauveria* and *Metarhizium* (Ascomycota, Hypocreales) genera colonized tomato (*Lycopersicon esculentum* Mill), melon (*Cucumis melo* L*.,* hybrid F1- Galia) and alfalfa (*Medicago sativa*) plants by spraying application of fungal suspensions. The colonization of the tissues of the evaluated plants was determined by the fungus re-isolation of leaves, stem and roots. Two fungal strains, EAMb 09/01-Su and Bb04, showed an increasing colonization presenting from 4.0 to 24.3 % of colonization of the root tissues by 24 to 96h and 43.3 to 98.0 % of stem and leaves by 24 to 72h.

The potential of these isolates as mycoinsecticides for *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae) control was also evaluated. In the first step, larval mortality was determined after topical application of conidial suspension of the most virulent isolates, showing mortality percentage of 41.6% for EAMb 09/01-Su and 76.6% for EABb 01/33-Su. In the second step, the ingestion of alfalfa leaves endophytically colonized by larvae showed a significant larval mortality of 25.0% and 31.6%, respectively. No differences in leaf con-

sumption between treatments and controls were found without producing a repellent or a feeding deterrence effect. This study provides evidence for the ability of fungi to colonize internal tissues of tomato, melon and alfalfa, as well as to control *S. littoralis* larvae.

Key words: biological control, Metarhizium brunneum, Beauveria bassiana, endophyte, systemic protection.

Development of Spray Formulations for an Endophytic *Beauveria bassiana* Strain

Lohse, R.[1], Jakobs-Schönwandt, D.[1], Spieth, P.[1], Lemke, A.[1] and Patel, A.[1]

1. *University of Applied Sciences Bielefeld, Fermentation and Formulation of Biologicals and Chemicals, Wilhelm-Bertelsmann-Str. 10, D-33602 Bielefeld, Germany*

Abstract

Beauveria bassiana ATP-02 was raised in a 2 L stirred tank reactor to produce sprayable spores such as submerged conidiospores (SCS). In a mineral medium with 5 % molasses *B. bassiana* produced 0.24×10^{10} SCS/g sucrose in 216 h. Osmotic stress in form of 50 g/L NaCl added after 48 h increased the SCS yield to 1.68×10^{10} SCS/g sucrose. The SCS were formulated in a spray to support the endophytic colonization of oilseed rape plants. Moreover, the physiochemical properties and biocompatibility of different spray formulation additives like surfactants, humectants, sticker, nutrients and sunscreens were investigated. The resulting novel spray based on 0.1 % NIS3, 1 % molasses, 1 % titanium oxide and 10^6 spores/mL was applied to 6th secondary leaves. After 7 weeks, *B. bassiana* was re-isolated out of the 8th secondary leaves of 24 % of the plants whereas hyphae growth was observed in 100 % of mid rip cross-sections of these leaves by microscopy.

Key words: endophyte, Beauveria bassiana, submerged fermentation, spray formulation

Introduction

A promising biological plant protection strategy could be the use of the endophytic entomopathogenic fungus *Beauveria bassiana* isolate ATP-02. Before this endophyte can be applied as a commercial biocontrol agent, the fungus has to be mass-produced and formulated in such a fashion that it colonizes plants and protects them against insect pests from within similar to transgenic plants.

That is why three different formulation strategies for *B. bassiana* ATP-02, namely seed coating, encapsulation and spraying, were evaluated (Lohse, unpublished). The most suitable option was application of spores by spray. This reflected on the fermentation strategy where the focus was put on the production of sprayable spores such as submerged conidiospores (SCS) because of their higher shelf life than blastospores (BS) and their higher germination rate than aerial conidia (AC). Previous studies had shown that *B. bassiana* ATP-02 can be cultivated in a cost-effective fermentation process to high yields of BS [2] and is able to colonize sorghum [3], tomato, beans, cotton (Vidal, personal communication) and oilseed rape plants [2] via simple sprays under greenhouse conditions. Therefore, it can be assumed that this fungus shows great potential for a novel plant control measure in a variety of crops.

The objective of the present work was to increase SCS formation from *B. bassiana* by osmotic stress in different nutritive

situations and to systematically develop novel cost-effective spray formulations for these spores. The developed sprays should wet the hydrophobic leaf surface homogeneously, protect fungal propagules from harmful environmental effects like desiccation, rainfall and UV radiation, increase their persistence on the plant surface and enhance the endophytic colonization of oilseed rape plants. That is why physicochemical properties and biocompatibility of the single spray components were investigated.

Material and methods

Fermentation

B. bassiana isolate ATP-02, DSM 24665, was provided by Georg-August-University, Department of Crop Sciences/Agricultural Entomology, Göttingen and was isolated from the maize stem borer *Busseola fusca* at the Haramaya University, Ethiopia.

B. bassiana was grown in TKI medium with 5 % sugar beet molasses in a 2 L BIOSTAT® Bplus stirred tank reactor with a working volume of 1.5 L [2]. The basal salts were dissolved in 1200 mL ddH_2O and were autoclaved in the bioreactor. 300 mL of a carbon source stock solution (75 g molasses) were autoclaved separately and were inoculated with $5.0x10^4$ AC/mL. The fermentation was carried out at 25°C, 1 vvm, 600 rpm and pH 5.5 for 9 days. Concentration of SCS and BS was

determined with a Thoma counting cell chamber by light microscopy. At the end of fermentation, the culture broth was centrifuged for 10 min at 20,000 g, washed twice with ddH_2O and suspended with 0.9 % NaCl.

Development of a spray formulation

The suspensions of different non-ionic surfactants such as CP1-CP5 and NIS1-NIS3 in final concentrations of 0.01 %, 0.1 %, 1.0 % and 5.0 % (w/w) were dripped on a standardized leaf surface (sunflower wax) and the contact angles were determined. Different sunscreens like 5 % sugar beet molasses and 1 % titanium dioxide were autoclaved for 20 min at 121°C and afterwards, inoculated with 10^6 spores/mL. Besides, 0.9 % NaCl with 10^6 spores/mL served as control. Then 10 mL of each sample were placed on a 65 mm petri dish and were treated with UV-B radiation (UVM 57 Handheld UV Lamp 302 nm, UVP, UK) in an intensity of 100±5 µW/cm^2. After mixing of the suspensions, the viability of spores was checked by a colony forming unit (CFU) determination. Furthermore, suspensions of different humectants in sprayable concentrations were dripped on the wax surface; the contact angle and the volume of the drops were measured up to complete drying. To determine the biocompatibility, 50 mL of the water-based formulation additives and 0.9 % NaCl as a control were placed in 250 mL shake flasks and were inoculated with 10^6 spores/mL from a submerged cultivation. The cultures

were incubated at 25°C and 150 rpm for 48 h. Afterwards, the CFUs were determined.

Colonization assay

Spray formulations consisting of different compositions of a surfactant, a sunscreen, a humectant and a nutrient were autoclaved separately for 20 min at 121°C. After thorough mixing, the spore suspension (10^6 spores/mL) was added. The control did not contain fungal biomass. The sprays were brushed on the adaxial side of 6th secondary leaves from 7 weeks old oilseed rape plants. After 12 h darkness, the 12 h photoperiod was started. To increase the relative humidity to 95 %, the treated leaves were wrapped with plastic bags for 48 h. After 7 weeks the 8th secondary leaves were harvested (n=8). For microscopic detection of intercellular hyphae growth, cross-sections of the 8th secondary leaf mid rips were stained with 0.5 % rose bengal dissolved in 5 % aqueous ethanol for 15 sec and were washed with ddH_2O. For re-isolation experiments, the leaves were surface sterilized with 70 % ethanol for 2 min, 5 % sodium hypochlorite for 3 min and 70 % ethanol for 2 min, rinsed twice in sterile distilled water, and then placed on sterile tissue paper in a laminar airflow cabinet. Five leaf pieces with a diameter of 8 mm from each plant were randomly taken, placed separately on a modified *B. bassiana* selective medium and were incubated at 25°C for 4 weeks. DNA from fungal biomass was extracted and amplified by a *B. bassiana* specific two-step nested PCR [1].

Results and discussion

Fermentation

In a mineral medium with 5 % sugar beet molasses *B. bassiana* produced 0.24×10^{10} SCS/g sucrose in 216 h [2]. When 50 g/L NaCl added 48 h after inoculation, the SCS yield was increased to 1.68×10^{10} SCS/g sucrose at the end of fermentation. This is supported by a study which reported that in nature *B. bassiana* forms conidiophores from BS during the insect infection when the host's body is already exhausted, the pH value alters, the insect dehydrates and therefore salt concentrations rise [4].

Development of a spray formulation

Without any decrease of spore viability, 0.1 % NIS3 decreased the contact angle of water on a standardized leaf surface from 105° to 37° resulting in an increase of the wetted leaf area compared to the control based just on water (Fig. 1a). Furthermore, the use 1 % TiO_2 as a sunscreen increased the viability of spores after UV-B radiation (302 nm) for 120 min from 0 % to 77 % compared to a water-spore suspension (Fig. 1b). By the addition of 0.1 % gelatine Bloom 280 as a humectant the drying time of a droplet was increased from 23.8±2.1 min to 32.6±3.4 min. However, the droplets rolled off when the leaf was tilted. By adding 0.25 % xanthan as sticker the adhesion was increased up to a surface inclination of 90°. But the addition of 0.01 % NIS3 reduced the maximum tilt angle to 45° and the adhesion force decreased from 8.35 mN/m to 3.26

mN/m compared to the pure sticker. As a nutrient 1 % sugar beet molasses was added to the spray formulation due to the high yields obtained in fermentation.

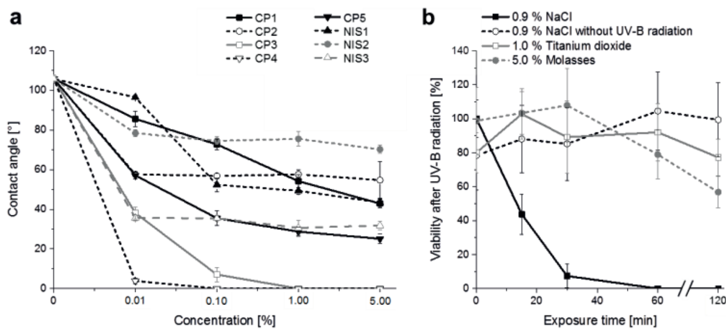

Fig. 1 (a) Influence of surfactant suspensions on the mean (±SE) contact angle on sunflower wax (n=10). **(b)** Influence of sunscreens on the mean (±Gaussian error) spore viability after UV-B radiation (n=3).

Colonization assay

The potential of the developed spray formulations to enhance the endophytic colonization of oilseed rape plants was investigated. Hyphae growth was not detected in the treatments without fungal biomass. Furthermore, hyphae were detected in 25 % of 8th secondary leaves of plants which were treated with a water-spore suspension. However, plants treated with a formulation based on 0.1 % NIS3, 0.1 % gelatine, 1 % molasses, 1 % TiO_2 and 10^6 TS/mL showed only 50 % colonized 8th secondary leaves (Fig. 2). Without gelatine intercellular hyphae growth was observed in 100 % of mid rip cross sections from 8th secondary leaves which indicated an inhibitory effect of gelatine. Besides the microscopic detection

of *B. bassiana*, the fungus was also detected via re-isolation and subsequent nested PCR in 24 % of the 8th secondary leaves of plants treated with this formulation. Compared to light microscopy, re-isolation tends to underestimate presence of *B. bassiana*.

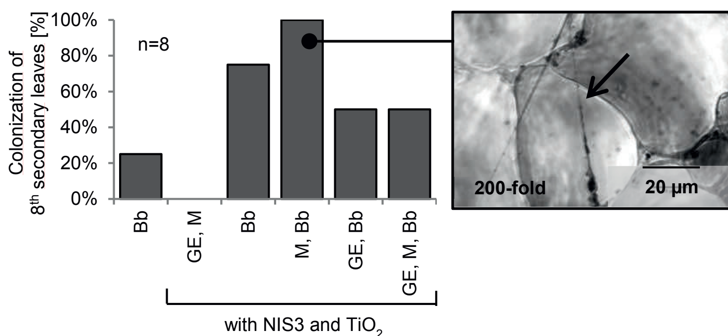

Fig. 2 Influence of different spray formulations (NIS3, TiO_2 titanium dioxide, GE gelatine Bloom 280, M sugar beet molasses, Bb 10^6 spores/mL) on the microscopically detected hyphae in the mid rip cross-sections of 8th secondary leaves (*picture*) in % of n=8.

To conclude, the endophytic *B. bassiana* was cultivated in a low-cost culture medium to high yields of sprayable SCS and a suitable spray formulation increased endophytic colonization of oilseed rape plants. To realize the full potential of this bio-control agent, more systematic research in further formulations, persistence of fungus and germination on leaves, penetration, colonization and efficacy in bioassays is needed.

Acknowledgements

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Detection of Entomopathogenic Fungi in Crop Plants by Immunofluorescence Techniques

Matecki, C.[1], Schäfer, J.[1], Ullrich, C.[1], Rabenstein[2], F., Kleespies, R. G.[1]

1. *Julius Kühn-Institut, Institut für Biologischen Pflanzenschutz, Heinrichstr. 243, 64287 Darmstadt, Germany regina.kleespies@jki.bund.de*
2. *Julius Kühn-Institut, Institut für Epidemiolologie und Pathogendiagnostik Quedlinburg, Germany*

Abstract

Leaves of three experimental plants, *Brassica napus* L., *Cucumis sativus* L. and *Vicia faba* L., were infiltrated with blastospore suspensions of *Beauveria bassiana* Naturalis, *Isaria fumosorosea* and *Lecanicillium muscarium*. Between 4 hpi and 27 dpi leaf samples were taken and submitted to various staining methods or PCR. Results on immunofluorescence localization of the applied fungi in leaf sections are reported. It is assumed that the most appropriate crop plants to harbour entomopathogenic fungi may be those which naturally live in a symbiosis with rhizobia or mycorrhizae.

Key words: Endophytes, entomopathogenic fungi, Beauveria bassiana Naturalis*, Isaria fumosorosea, Lecanicillium muscarium, Brassica napus L., Cucumis sativus L., Vicia faba L., immunofluorescence.*

Introduction

Entomopathogenic fungi are known to be efficient in biological protection of economically important plants against herbivorous insects [2, 4]. An actual aim is to establish entomopathogenic fungi directly in crop plants and finally in their seeds. However, apart from grasses and coniferous trees, the establishment and unequivocal detection of systemically growing endophytes in most dicots is still problematic. Therefore, investigations on leaf infiltration were conducted with three experimental plants, *Brassica napus* L., *Cucumis sativus* L., and *Vicia faba* L. Leaves were infiltrated with blastospore suspensions of *Beauveria bassiana* Naturalis [5], *Isaria fumosorosea* and *Lecanicillium muscarium*. Leaf samples were taken and submitted to various staining methods or PCR. Here, we report on our results on immunofluorescence localization of the applied fungi in leaf sections.

Material and methods

Leaves of our experimental plants, *B. napus*, *C. sativus* and *V. faba*, were infiltrated with blastospore suspensions (5×10^5 spores/ml) of *B. bassiana* Naturalis, *I. fumosorosea* and *L. muscarium* by means of a syringe. Prior to injection, the viability of the spores was checked by staining them with acridine orange. Between 4 hpi and 27 dpi leaf samples were taken and submitted to various staining methods. Ultrastructure of blastospores and conidiospores were compared by Transmis-

sion Electron Microscopy (TEM) as described by Kleespies (1993). The IgG fraction of polyclonal antibodies raised against *B. bassiana* in rabbit was used in combination with FITC-labelled goat anti rabbit secondary antibodies to detect the applied fungus in leaf sections by immunofluorescence localization [6, 7].

Results and discussion

B. bassiana hyphae were detected exclusively on the epidermal leaf surface of *B. napus*, in spite of infiltration (Figs. 1 H-K, 2 A-D). In contrast, in *V. faba*, *B. bassiana* and even more *I. fumosorosea* spores germinated within the mesophyll and hyphae could be detected growing alongside of the spongy and palisade parenchyma cell walls (Figs. 1 L, 2 E-G). Spores were equally injected into the intercellular space of all three plant species, but in particular in *R. napus*, the germination of spores must have been inhibited by defence mechanisms of the plant. After incubating sections of inoculated (with *B. bassiana*, 4 to 6 hpi) *R. napus* leaves in a solution of the peroxidase substrate DAB (diaminobenzidine), brown necrotic hyphae were detected within the stomata (Fig. 2 A), as well as necrotized chloroplasts generating H_2O_2 (signal for myrosinase activity, cleaving glucosinolates) within the guard cells (Fig. 2 H) (oxidative burst) [3]. We assume that *R. napus* leaf cells recognize the fungi as incompatible organisms and, being highly defensive, already inhibit the spore germination and

development of hyphae. In contrast, in the large stomata of *V. faba* no DAB reaction was observed. Interestingly, this different plant reaction towards non-pathogens obviously correlated with the ability of the Fabaceae to live in symbioses with rhizobia. We assume that the most appropriate crop plants to harbour entomopathogenic fungi may be those which naturally live in a symbiosis with rhizobia or mycorrhizae.

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Fig. 1 A. Production of submerged blastospores of *Beauveria bassiana* in Czapek medium. **B.** TEM section of a *Metarhizium anisopliae* blastospore and **C.** of a conidiospore, both with similar thick cell walls. **D.** Vitality test of *B. bassiana* blastospores with acridine orange: green = living, orange = dead. **E.** Injection of spores with a syringe into leaves by co-author Janina Schäfer. **F.** Completely infiltrated *Vicia faba* leaflet, tip of syringe at the leaf base (arrow-head). **G.** Guttation droplets at hydathodes (arrowhead) of a young *Brassica napus* leaf used for non-invasive application of the spores. **H.** Trypan blue staining of *B. bassiana* on *B. napus* leaf surface. **I.** Solophenyl flavine 7GFE (0.1%)/safranin (0.05%) staining of *B. bassiana* hyphae on leaf surface and **J.** by Blankophor (0.01%). **K.** *B. bassiana* (5 dpi) labeling on *B. napus* leaf sur-face with polyclonal IgG *B. bassiana* goat-anti-rabbit (143/4)/FITC-conjugated antibodies (1: 200 dilution each). **L.** *Isaria fumosorosea* (7 dpi) labeling on *V. faba* leaf surface with polyclonal IgG *I. fumosorosea* (144/4-2/1) goat-anti-rabbit/FITC-conjugated antibodies (1: 200 dilution each).

Fig. 2 A. Antibody-labeled *Beauveria bassiana* spores and hyphae on *Brassica napus* stoma. **B.** *B. napus* leaf cross section with antibody-labeled *B. bassiana* hyphae only on the lower epidermis (here on top). **C.** No labeled fungal hyphae in *B. napus* mesophyll or vascular bundles. **D.** No detection of hyphae in spongy parenchyma of inoculated *B. napus* leaf, stained with safranin/solophenyl flavine GFE. **E.** Detection of *B. bassiana* germinating spores in spongy parenchyma of *Vicia faba* leaf, stained as in **D**. **F.** Detection of *B. bassiana* hyphae by antibody-labeling **in** *V. faba* mesophyll and **on** the lower epidermis. **G.** Germinating *Isaria fumosorosea* spores on *V. faba* epidermis, labeled with *Isaria* antibodies (144/5). **H.** DAB staining of *B. bassiana* hyphae and guard cells of *B. napus* leaf upon H_2O_2 production as defense reaction. **I.** *B. bassiana* in *V. faba* mesophyll intercellular space, labeled with *Beauveria* antibodies. **J.** *I. fumosorosea* hyphae **in** *V. faba* mesophyll intercellular space detected with *Isaria* antibodies (144/4-2/1). **K.** *B. bassiana* hyphae **in** *V. faba* mesophyll intercellular space labeled with *Beauveria* antibodies. 1 dpi (**E**, **H**); 2 dpi (**F**, **I, K**); 5 dpi (**A - C**); 7 dpi (**D**, **G**, **J**).

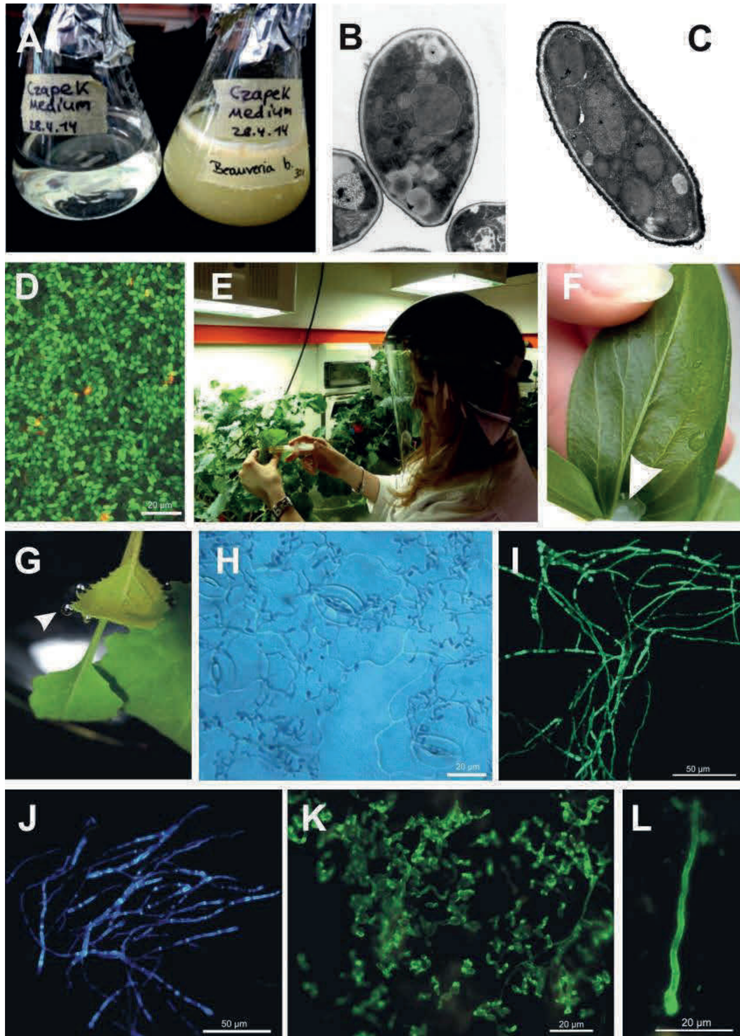

Fig. 1

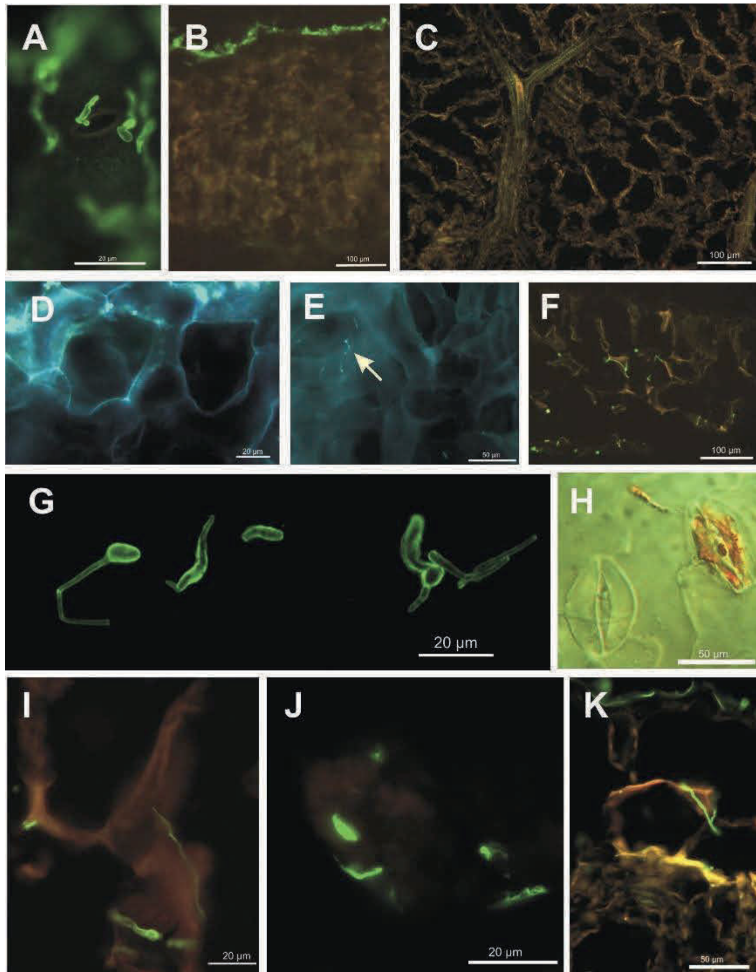

Fig. 2

Double Inoculation Leads into Impaired Colonization Pattern and Failure to Synergistically Increase Plant Growth in Wheat

Öğüt, M.[1] and Er, F.[1]

1. *Selçuk Üniversity, Cumra Vocational College, Çumra-Konya, Turkey*

There is a growing interest in microbial inoculation of plants with more than one microorganism to synergistically increase plant growth. However, there is little information regarding how colonization of one microorganism is altered by co-inoculation of another microorganism. The well-known *Azospirillum brasilense* Cd strain was genetically modified with a thermostable β-galactosidase coding gene to visualize the colonization pattern. *Bacillus sp.* #189 is a phosphate solubilizing microorganism. A rhizobox and a microcosm study were established in controlled conditions to study the effects of single and double inoculation in wheat (*Triticum aestivum* spp. Bezostaja). Colonization of *Azospirillum brasilense* Cd cells was concentrated at the base of the stem where the inoculation was performed in single *Azospirillum* inoculation treatment. However, co-inoculation with the *Bacillus* strain cleared off the *Azospirillum* colonization at the base of the stem. Total root length was reduced by 27 % in the *Azospirillum+Bacillus* treatment compared to the *Azospirillum* treatment. In terms of single inoculations, the *Azospirilllum* strain outcompeted the *Bacillus* strain in total root length and lumped root width. However, the two

strains produced similar fresh shoot weight. Consequently, double inoculation does not always lead into synergistically increased plant growth probably due to “enhanced” release of bacterial antibiotics, which may also negatively affect root growth at elevated concentrations.

Presentations
Open topics

An Endophytic Bacterium as a Possible Control Agent Against Yellows Disease in Grapevine

Iasur-Kruh, L.[1,2]; Rodoy, S.[1]; Mozes-Daube, N.[1]; Zahavi, T.[3]; Perlman, S.J.[5]; Sharon, R.[3,6]; Zchori-Fein, E.[1] and Naor, V.[3,4]

1. *Department of Entomology, Newe Ya'ar Research Center, ARO, Israel, einat@agri.gov.il*
2. *Faculty of Biology, Technion, Israel, Iasur@agri.gov.il*
3. *Golan Research Institute, Israel, vered_n@ohalo.ac.il*
4. *Ohallo College, Israel*
5. *Department of Biology, University of Victoria, Canada, stevep@uvic.ca*
6. *Northern R&D, Galilee Technology Center (MIGAL), Israel, rakefetsh@my.ohalo.ac.il*

Bacteria from the genus Phytoplasma pose a major threat to many agricultural crops including grapevine. The conventional application of chemical sprays for controlling phytoplasma and other phloem restricted pathogens is inefficient, and the role of endophytes in the induction of plant resistance against such pathogens has been suggested. We hypothesized that since phytoplasma are transferred by phloem feeding insects, the vectors, as well as the host plants, may serve as reservoirs of potentially beneficial bacteria. The current study is focused on the bacterial community composition of the phytoplasma's insect vector as a source for such potentially beneficial bacteria. Mass sequencing of 16S rRNA gene obtained from the insect vector showed dominance of *Sulcia* (77.95 % of sequences in the sample), *Wolbachia* (4.51 %) and a bacterium which belongs to Enterobacteriaceae (16.89 %), with the remaining 0.65 % of the sequences belonging to Actinobacteria,

Alpha- and Gamma-proteobacteria. Subsequently, different bacteria were isolated from the insect on artificial agar. One of these isolates belonged to the bacterial family Xanthomonadaceae, and its 16S rRNA sequence resembled one of the rare bacterial taxa found by the mass sequencing analysis. The isolate was introduced to healthy and phytoplasma-infected plants (Periwinkle and Chardonnay) and was detected in the grapevine phloem three weeks post inoculation. The presence of the isolate seems to affect the morphology of the infected plants but not the healthy ones. In some parameters, the effect of phytoplasma on plant morphology was markedly reduced. We further investigated this isolate and found it secreting substances that suppress Spiroplasma (a model pathogenic bacterium). The facts that the isolate inhabits the same location as phytoplasma and that it has the ability to secret antimicrobial substances makes it a good candidate for phytoplasma control in grapevine.

Presentations
Conclusions

Patel, A. and Döring, M.

Host and Chairman COST FA1103 Working Group 3

The working group 3 meeting of COST FA1103 was an excellent opportunity to present short reviews on various aspects of application technology of endophytes and practical experiences of companies involved in the manufacturing of microbial inoculants. Application of endophytes via root infection like seed coating and capsules and via sprays was considered. This workshop brought together 35 participants from 11 countries out which 25 % came from private companies. The final very active discussion round based on questions submitted by participants and presented in the introduction (page 16) led to the following conclusions:

With regard to the special requirements for the application of endophytes to and into plants, the participants agreed on the view that the endophyte needs to "show the right passport" which apart from the usual strain selection encompasses the choice of novel additives, and that some of these additives may be certain sugars and polysaccharides and substances with stimulation activity. Inspiration may be obtained from in-depth studies of pathogen infection mechanisms on molecular level and studies on the molecular dialogue of endophytes and plants.

Suitable methods for improvement of plant colonization after application may take into account that the plant growth may

need to be adjusted to the sometimes slow growth of the endophyte within the plant. This may also imply a search for cultivation methods that increase fungal growth within the plant. Furthermore, endophytes could be of interest that are transmitted vertically to the next generation.

Somewhat related to this is the idea that endophytes could be applied by inoculation of in *vitro cultures* so that seeds with endophytes inside, maybe even in the embryo, are obtained. This may be obtained by treating micropropagated plants. Another suggestion was to apply endophytes to embryogenic callus cultures by the particle-gun method. The participants also agreed that more studies on application of endophytes via flowers are needed. Preliminary data on poppy seem promising. The vertical transmission of bacterial endophytes was already proven in certain mosses, for examples.

The observation that promising endophytes that work well under lab conditions often fail when tested under more practical conditions may be attributed to a multitude of causes. Among these are the competition of the endophyte with soil organisms such as the microbial community, mycophagous nematodes and fungal gnats. Besides, the time of application and especially the sometimes low temperatures affect the growth and establishment of the endophyte. This calls for a screening of strains under “application conditions”. It was also felt that unlike lab conditions practical conditions exert a stress on plants and that plants need to divert some energy to stress

management instead of managing the endophyte. This may go hand in hand with an upregulation of defense systems and a change in metabolite production. The interesting conclusions here were to apply endophytes in the lab under stress conditions and that endophytes should be isolated from stressed plants instead of healthy ones. Other obvious conclusions were to adjust endophyte cultivation, e.g. production of spores and to adapt technical formulations to field conditions.

Another important issue was the actual increase in yield or other benefits that may be expected from the application of endophytes. Firstly, it was stated that the yield has to be better than of the untreated control. It was concluded that at least with the highly vigorous European seeds, a yield increase cannot always be expected. This partly explains high yield increase with endophytes from countries that still use inferior seeds. Also, it was suggested that uniform seeds which have no big variance in germination and growth should preferably be used.

Of special interest was the application of arbuscular mycorrhizal fungi. In this context the question of an adequate formulation for these fungi was briefly discussed. It was concluded that seed pellets may be better than a film coating. The big challenge seems to be a successful germination of spores and colonization of germinating roots. By the usage of a seed coating, the fungi may be able to compete against the soil (mycor-

rhizal) fungi and have an advantage especially in the early steps of root colonization.

The participants also commented that an increase of yield is not always the aim. Among other benefits could be saving time on the field, earlier emergence, saving cultivation time, earlier harvesting, higher product quality and decreasing fertilizer input. It was concluded that scientists should give more attention to these aspects so that more hard data become available.

In relation to these topics, it was asked if customers will be interested to apply endophytes themselves. The general answer was “all are interested but more information is needed”. More studies on customers, consumers and markets are needed for the companies. It was also mentioned that “everything that helps farmers is of interest” and that more customer-oriented “marketable” information needs to be communicated. It was advised that not only organic but also conventional farmers should be addressed. To stir and maintain the interest, one promising strategy is the early involvement of customers during product development.

Finally one of the key questions was if endophytes can be registered under the biostimulant regulation if they are co-applied within a coformulation based on a consortium of microorganisms. It was concluded that although the registration has become easier, similar to the procedure in the U.S.A.,

time to register still amounts to three years and costs between 100-500.000 €. The need for a bio stimulation regulation is urgent. With regard to registration aspects on endophytes with biocontrol activity, a statement from the German JKI that is involved in the registration process is available [1].

As an overall conclusion it is clear that many questions remain open to speculation and that much more systematic research into endophyte application is needed.

Reference

[1] **Schneider, C.; Leifert, C.; Feldmann, F. (Hrsg.)** (2013) Endophytes for plant protection: the registration process at a glance in Endophytes for plant protection : the state of the art ; Proceedings of the 5th International Symposium on Plant Protection and Plant Health in Europe, Berlin, Germany 26.-29. Mai 2013, Page(s):214-222, ISSN/ISBN: 978-3-941261-11-2

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