BiOWiSH™ TECHNOLOGIES

Technical Report

Endophytic Potential Among BiOWiSH™ Bacterial Isolates

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Determination of Endophytic Potential Among BiOWiSH™ Bacterial Isolates

Introduction

An endophyte is a bacterium or fungus that lives within a plant cell without causing disease. Endophytes often confer benefits upon their host, such as improved nutrient acquisition, increased growth rate and increased resistance to abiotic stressors. Some produce enzymes, such as ACC Deaminase, which play a role in mitigating ethylene-based stress signaling pathways. Inhibition of the ethylene pathway allows the host plant to resist a variety of stressors, including hydric stress and excessive salt concentrations (Glick 2014). Bio-augmentation of plant seeds or of the rhizosphere itself with endophytic bacteria is one potential strategy for sustainably increasing agricultural yields.

Bacteria of the genus *Bacillus* have been well documented to form endophytic associations with a variety of vascular plants. Endophytic serotypes of *Bacillus subtilis* and *B. amyloliquefaciens* have been found in maize, where they produce antifungal lipopeptides and play a role in host gene expression (Gond et al 2015). Endophytic *Bacillus* have also been found in medicinal herbs (Zhao et al 2015), cacao plants (Melnick et al 2008), and dune grasses (Bibi et al 2011). In the case of cacao plants, *Bacillus* are indicated as antifungal agents.

BiOWiSH™ Crop is a proprietary assemblage of bacteria in the genus *Bacillus* which are known to enhance plant growth and yields through nutrient mobilization in the rhizosphere, fixing atmospheric nitrogen, and inhibition of phytopathogens. During field trials, it has been observed that the application of BiOWiSH™ Crop can result in increased root mass in several crop species (Figure 1). This effect compares favorably with the known activity of endophytic bacteria, especially those producing ACC deaminase.

Figure 1 – Differences in root mass between untreated control (left and right) and BiOWiSH (center) treated plants. Please contact a distributor near you, email agronomy@biowishtech.com, or visit our website biowishtech.com.

We endeavored to determine whether *Bacillus* isolates from BiOWiSH™ Crop are capable of crossing the cell wall of soybean root cells and occupying the cells as endophytes. Should they be found capable of this activity, additional research endeavors could be undertaken to determine their potential role in mitigation of ethylene signaling, regulation of host gene expression and cytokine elicitation. In order to determine whether these isolates are capable of crossing the cell membrane, an assay is required which differentiates between intracellular and extracellular bacteria.

Gentamycin is a broad-spectrum antibiotic which is unable to cross a cell membrane. Thus, treatment of eukaryotic tissues with gentamycin will result in the preferential killing of susceptible bacteria outside the cell, while leaving any bacteria inside the cell intact. Thus any bacterial growth obtained when treated eukaryotic cells are diluted and plated would putatively be comprised of endophytic bacteria protected from the gentamycin by the host's cell membrane. This is known as a gentamycin protection assay.

In the present study, we grew soybeans coated with BiOWiSH™ Crop in sterilized soil and subjected the root masses to a gentamycin protection assay to determine whether the constituent bacteria in this product are capable of forming endophytic associations with plant root cells.

Materials and Methods

Determining susceptibility of BiOWiSH™ bacterial isolates to gentamycin – In order for a gentamycin protection assay to work, the bacteria in question must be susceptible to the antibiotic. BiOWiSH™ Crop is composed of four distinct *Bacillus* species, referred to as BW283, BW284, BW285 and BW286. We used disks of gentamycin (Carolina Biological Supply) to determine whether these isolates were killed by gentamycin. A 100mL aliquot of a 24 hour TSB culture of each BiOWiSH™ isolate, respectively, was spread on a separate plate of Trypticase Soy Agar (TSA) using a sterile glass spreader. After the broth had absorbed into the cured agar, a gentamycin disc and a control disc were added to the plate. Plates were incubated for 48 hours at 37°C and examined for zones of inhibition around the gentamycin disc.

Growth of BiOWiSH™-Coated Soybeans – Commercially available soybeans (unsterilized) were coated with BiOWiSH™ Crop Liquid at 0.1% by mass and planted in sterilized soil (Helena Research Group) in a 48-cell planter disinfected with ethanol. Planters were placed beneath a 48" fluorescent light fixture (FloraGro) and watered daily with sterile DI water. Plants were allowed to grow for one month before root masses were harvested.

Collection of Root Masses – Soybean plants were severed just above the soil with flame-sterilized scissors. Root masses were placed inside bottles of sterile DI water and shaken vigorously for 5 minutes to remove soil particulates. Afterwards, root masses were transferred with flame-sterilized forceps to flasks of sterile Phosphate Buffered Saline (PBS, Cold Spring Harbor) and agitated for one hour on an orbital shaker set to 100 RPM. Control root systems were collected at this juncture for "pre-antibiotic shock" comparisons.

Gentamycin treatments – Rinsed root masses were individually added to 100mL of sterile PBS augmented with gentamycin (Carolina Biological Supply) at 100µL/mL and shaken at 100RPM for one hour. Treated root systems were transferred to flasks of sterile 0.1% peptone blank and incubated for 1 hour at 37°C to induce any extracellular endospores to germinate. Afterwards, gentamycin was added at 100µL/mL and flasks were incubated for an additional hour in order to kill any newly-germinated extracellular bacteria.

Dilution and plating of root masses – Antibiotic treated root masses were rinsed in sterile DI water and homogenized with a flame-sterilized mortar and pestle. The resultant tissue pulp was collected aseptically and subjected to serial dilution and plating using a standard APC assay (BiOWiSH™ SOP MLA3). Plates were incubated for 48 hours at 37°C before being examined for signs of bacterial growth.

Results

Gentamycin susceptibility assays – For all BiOWiSH™ isolates, robust clearing zones were observed around discs of gentamycin which were not present around untreated control discs (Figure 2). This indicates that BiOWiSH isolates are vulnerable to gentamycin and thus acceptable candidates for a gentamycin protection assay.

Figure 2 – Clearing zone in a lawn of BiOWiSH™ Bacillus isolate BW283 surrounding a disc of gentamycin.

Gentamycin protection assays – Results of plating assays on BiOWiSH™ treated soybean root systems, both pre- and post-gentamycin shock, are displayed in Figure 3. A tenfold reduction in microbial titer was observed subsequent to gentamycin treatment, suggesting that transient microbes on the root surface were killed by the antibiotic. Remaining bacterial isolates, which included morphologies identical to known BiOWiSH™ bacterial strains, are considered to have been within the plant cells and thus immune to the gentamycin treatment.

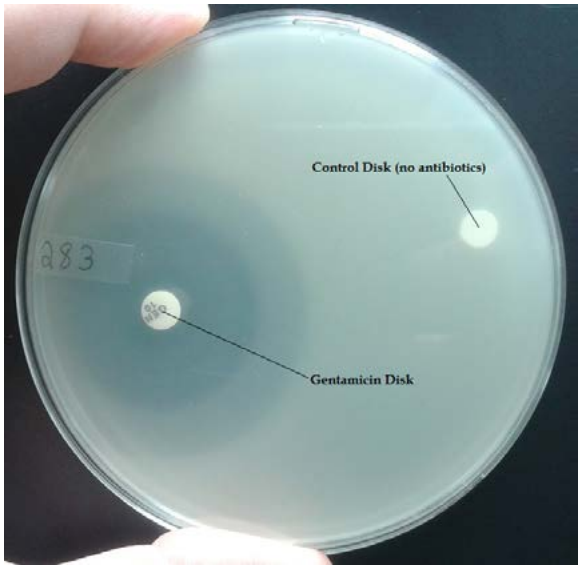
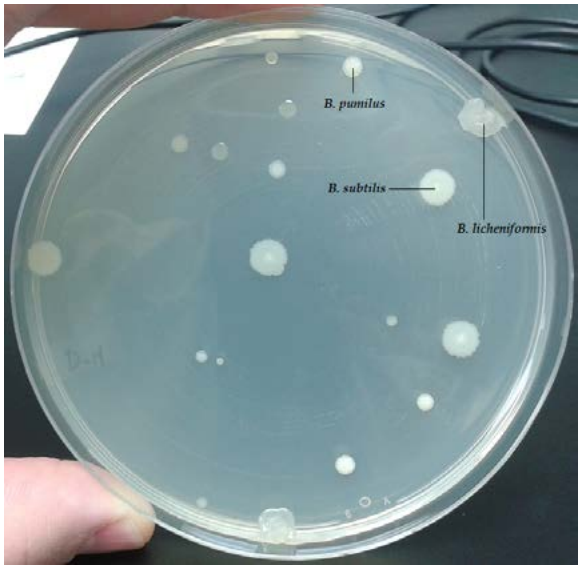


Figure 3 – Pre antibiotic treatment (left) and post treatment (right) soybean root system platings.

The isolates which survived the treatment (right) are considered to have been present within plant cell walls and thus immune to the gentamycin treatment.



Discussion

Endophytic bacteria, including several members of the genus *Bacillus*, confer many growth-promoting benefits upon their hosts and thus are an attractive means to sustainably increase crop yields worldwide. In the present study, we used a gentamycin protection assay to determine whether BiOWiSH™ *Bacillus* isolates were able to form endophytic associations with plant root cells.

Results of the gentamycin protection assay suggest that BiOWiSH™ *Bacillus* isolates are indeed able to form endophytic associations with soybean root cells. These results compare favorably with the published literature, in which endophytes of the genus *Bacillus* are well-documented. Confirmation of this finding using sterile plant tissue culture methodologies are needed to confirm this finding in a more quantitative manner, and are an area of future study. However, this study provides qualitative confirmation that BiOWiSH™ isolates are able to form endophytic associations with plant root cells. Additional research is necessary to determine whether BiOWiSH™ bacterial isolates are able to produce the enzyme ACC Deaminase, further elucidating their role in mitigating host stress signaling pathways.

Bacterial soil amendments such as BiOWiSH™ Crop represent an important of the sustainable agricultural practices which will become increasingly indispensable as agronomy professionals strive to meet the planet's growing need for increased crop yields.

Literature Cited

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