Spring 2017

Impact of *Burkholderia phytofirmans* strain PsJN on the *ex vitro* acclimatization and *in vitro* propagation of tissue cultured *Dionaea muscipula* (Venus fly trap)

Peter L. Riley
James Madison University

Impact of *Burkholderia phytofirmans* strain PsJN on the *Ex Vitro* Acclimatization and *In Vitro* Propagation of Tissue Cultured *Dionaea muscipula* (Venus Fly Trap)

Peter Lewis Riley

A thesis project submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Masters of Science

Department of Biology

May 2017

FACULTY COMMITTEE:

Committee Chair: Dr. Michael Renfroe

Committee Members/ Readers:

Dr. Steffen Morgan-Wurch

Dr. Jonathon Monroe

Acknowledgements

We would like to acknowledge The Institute of Advanced Studies in Danville for their generous donation of the bacteria organism and its GFP transformant for this research. We would also like to acknowledge Dr. Jerzy Nowak whose expert and kindly advice were monumental in the completion of this research. Specifically we want to thank him for his suggestion to explore maltose as an alternative sugar source for our media.

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Abstract

Dionaea muscipula, Venus fly trap, is an endangered plant that is propagated primarily through tissue culture. Tissue cultured plants must be acclimatized to their new environments when removed from their test tubes and the ensuing stress can result in plant mortality. A plant growth promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN, has been utilized in biotic hardening of tissue cultured plants and in the amelioration of stresses, particularly drought stress in potted plants. We investigated whether this bacterium could improve either propagation or acclimatization of tissue cultured Venus fly traps. Standardized inoculations were performed by adjusting cell density of inoculant by spectrophotometry. In *ex vitro* acclimatization, inoculation with the bacteria significantly improved survival but only in nonsterile soil media. Initial *in vitro* inoculations produced negative interactions with significant reduction in propagules and biomass in the plant and bacterial growth on the media. A modified plant tissue culture media using maltose instead of sucrose as a sugar source was created to address this problem. Inoculated plants grown in the maltose media were not significantly different than the mock inoculated controls in terms of biomass, traps produced and propagules produced. Additionally the bacteria did not visibly grow on media supplemented with maltose rather than sucrose as a sugar source. Plant infection was confirmed through fluorescence microscopy. Colony density estimates for both rhizosphere and endosphere populations of the bacteria in tissue cultured plants were generated using plate counting. These findings indicate that *Burkholderia phytofirmans* strain PsJN may be useful in reduction of mortality during acclimatization of *Dionaea muscipula*, but only in nonsterile growth situations or when drought stress is expected. The use of alternative tissue culture media using maltose when propagating biotized fly traps *in vitro* is an improvement over sucrose containing media. Further work must be done to determine whether mortality reduction by strain PsJN is the result of stress reduction by the endophyte or competitive inhibition of an unknown microbial agonist.

I. Introduction

PGPR

Plant growth promoting rhizobacteria (PGPR) are a polyphyletic category of plant mutualistic microbes whose presence in the rhizosphere or within the tissues of plants enhances growth and/or stress resistance through a number of mechanisms (Ahmad, Ahmad et al. 2008). This phylogenetically diverse group commonly includes genera such as *Pseudomonas*, *Enterobacter*, *Bacillus*, *Burkholderia*, *Azospirillum*, *Variovorax*, *Serratia*, and *Azotobacter*. Rather than being unified by phylogenetic relationship, it is their relationship with their plant hosts which typifies these organisms, and this common selective pressure along with horizontal transmission of symbiotic genes has resulted in convergent evolution of a suite of analogous growth promoting traits (Hontzeas et al. 2005, Ahmad, Ahmad, and Khan 2008). Chief among these are the induction of systemic resistance against pathogens (Alstrom 1991), the production of siderophores (Mahmoud and Abd-Alla 2001), the solubilization of minerals such as phosphorous and potassium (Raupach and Kloepper 2000, Han and Lee 2005), the fixing of nitrogen (Hurek et al. 2002), the degradation of ethylene through the production of ACC deaminase (Nadeem et. al. 2010), the production of growth stimulating phytohormones like auxin and gibberellin (Mitter et al. 2013b), and generation of protective bacterial growths (Bais et al. 2004). PGPR are characterized by their capacity to catabolize plant root exudates such as phenols and organic acids thereby thriving within the relatively carbon rich rhizosphere (Ryan and Delhaize 2001). Experiments have demonstrated that PGPR can

stimulate growth and enhance yields within agricultural settings, experimental greenhouses, and even within *in vitro* plant tissue culture systems (Lazarovitz and Nowak 1997). In the field PGPR promise a sustainable alternative to purely traditional methods of fertilization, irrigation, and pathogen management (Najafi et al. 2012). In the lab PGPR provide a means to enhance the speed and reliability of plant tissue culture through improved plant regeneration and acclimatization (Nowak 1998).

The Rhizosphere

The rhizosphere is defined as the soil region under direct chemical influence of plant root exudates (Bais et al. 2006). A plethora of plant-bacteria, plant-fungus, and plant-nematode interactions occur here ranging from the mutualistic association between arbuscular mycorrhizal (AM) fungus and AM plants to the parasitic relationship between plant species and their specialist root nematodes (Burgers 2012). The populations of soil bacteria in particular increase within 50 μm of the root surface or rhizoplane, a phenomenon noted early in the history of soil science and denoted as the 'rhizosphere effect' (Hiltner 1904, Foster et al. 1988). Cell densities in this region far exceed those in the bulk soil due to the carbon influx provided by root exudates and can reach densities of 10^{12} cells/g of soil (Foster et al. 1988). The community characteristics of these rhizosphere microorganisms (collectively referred to as the soil microbiota) determine more than individual plant fitness. For example, during the invasion of symbiotically disruptive plants such as garlic mustard, allelochemicals secreted by the non-native species affects AM fungal symbiosis and in so doing can destabilize entire native plant

communities (Rogers and Anderson 2001).

Considering the importance of the soil microbiota, it comes as no surprise that plants transfer up to 40% of their photosynthate to the rhizosphere in the form of a chemically diverse and energy rich root exudate (Burdman et al. 2000). This exudation occurs largely at the actively growing tip of the root and explains the great abundance of soil microbiota in this region, as root exudates are high in carbon whilst the soil is generally carbon limited (Odell et al., 2008, Burger 2012). In addition to its physical role as lubrication and protection for the growing root tip, exudate sugars, proteins and organic acids may act as a selective force on the rhizosphere and root microbiome (Grayson et al. 1998, Sharma et al. 2005). Plants must incur a net benefit from their rhizosphere microbiota in exchange for these resources, otherwise they would be outcompeted by more selfish variants. Likewise, plant beneficial microbes must incur a benefit from their plant association, and this is explained by access to the relatively high carbon resources of the rhizosphere and, in the case of endophytes, the relatively protected interior of plants themselves (Gray and Smith 2005). Although their plant growth promoting capacities are of primary importance to agriculturalists, plants may also select their microbial symbionts based on pathogen antagonism (Lugtenberg et al. 1999). This can be witnessed in a recent survey of fungal endophytes of the medical plant *Cannabis sativa*. The chemically mediated antagonism between these endophytic fungi and common fungal pathogen of *C. sativa, Botrytis cinerea*, was verified *in vitro* by culturing the two organisms together and observing restriction in growth and mortality of the fungus (Kusari et al. 2009).

Plant beneficial microbes have been known for a long time, but only recently has

our knowledge expanded beyond the well-studied and rigidly obligate mutualisms of nodulating diazotrophs and mychorrhizae (AM and EM). In addition to these obligate and relatively ancient symbioses, there are now many documented facultative symbioses which can display dramatic positive effects on plant growth and stress/pathogen resistance in particular (Kloepper and Schroth 1978). These facultative symbionts were not discovered until recently for a number of reasons. One is the 'great plate count anomaly' which obscured the vast abundance of microbial and fungal life from the view of laboratory scientists up until the emergence of high throughput sequencing technologies (Leigh 2006). Even now, the vast majority of plant symbionts (and microbial life in general) remain unculturable. This pattern of uncultivatability holds true for obligate endophytes, microorganisms which spend their entire life cycles within plant tissue (Koskimäki et al. 2010). Due to the relaxed selection resulting from their metabolite rich environment, these species have incurred gene loss which prevents their survival beyond the supportive interior of their respective plant hosts and impedes their growth in artificial media (Agnati et al. 2009).

Ecology

The biotic portion of the soil is a complex polyculture of microbes (fungi, bacteria and archaea) (Burgers 2012). These organisms are in constant relationship both with one another and macroscopic organisms (notably the plant rhizoplane). Microbial relationships typified by syntrophy where niche space is expanded and community

productivity enhanced by organisms adapting to utilize the waste products of their co-resident community members (Bashan and Holguin 1997, Shrestha 2011).

Focusing specifically on the microbial residents of the soil which interact strongly with vascular plants, we can initially divide them into two groups: facultative and obligate symbionts. The term 'symbiont' is preferred over 'mutualist' and 'parasite' since it has been continuously demonstrated that such inflexible designations are applicable only to specific relationships in specific phenological, biological and environmental contexts (Stone et al. 2004). For example, interactions between a bacterium and a fungus triggered a change from mutualistic to parasitic lifestyle in the fungal partner (Dewey et al. 1999) and plant pathogens can convert to endophytic mutualists with minimal deleterious mutation of pathogenic genes (Newton et al. 2010). Of course, when considering obligate symbionts such as mycorrhizae and nodulating rhizobacteria, relationships become more defined and it is thus safer to refer to such organisms as mutualistic. However, since this thesis focuses on the facultative microbiota the use of these static ecological labels will be limited to specific ecological and genotypic contexts.

Facultative symbionts are highly diverse when contrasted with mycorrhizae, especially in the case of AM fungi where the diversity is largely encompassed by the class Glomeromycota, but similar to the nodulating Rhizobia, a diverse paraphyletic group not typified by any low order taxa (Burgers 2012). In fact, facultative plant symbiotic bacteria include a wide diversity of largely gram negative bacteria ranging from *Bacillus* to *Pseudomonas, Burkholderia* and *Azobacter* species. Another difference is that obligate symbionts are generally more stable in terms of ecological relationships

than their facultative counterparts. For example, while inoculation of legumes with their relevant rhizobial strain increases yields in most ecological contexts, application of symbiotic bacteria and fungi tend to enhance yields mostly when they would otherwise be limited by abiotic stress or pathogen infection (Yang et al. 2009, Lathi et al. 2009).

Mechanism of Growth Promotion

Most growth promoting mechanisms can be grouped into a four categories: pathogen resistance, stress resistance, nutrient acquisition, and hormonal stimulation. Pathogen resistance is enhanced in inoculated plants primarily through the induction of systemic resistance, the nontarget specific immune response of plants (Bordiec et al. 2011). Simple competitive exclusion can explain much of this observed antagonism between symbiont and pathogen also (Thomashow et al. 1996). Nutrient exclusion can occur through simple competition for limited soil resources but can also occur when siderophores (iron cation chelating molecules produced by many PGPR) bind the iron in the soil resulting in iron deficiency in competing microorganisms (Mahmoud and Abd-Alla 2001). Finally, bacterial growths produced by some PGPR can physically exclude pathogens from entry into their plant hosts. This can be observed in the antagonistic relationship between *Bacillus subtilis* and *Pseudomonas syringae* when the former secretes a bacterial growth which deters the latter from entry into Arabidopsis roots (Bais, Fall and Vivanco 2004).

Induction of stress resistance in plants is one of the most promising aspects of PGPR, especially considering the likely effects of climate change on agriculture, namely

increasing aridity and incidence of temperature extremes (Kurukulasuriya and Rosenthal 2013). PGPR ameliorate abiotic stresses in their hosts through a number of pathways. One is their ability to downregulate ethylene production. Ethylene is a volatile plant hormone which signals stress and, in sufficient quantities, causes adverse reaction in plants including early senescence (Gepstein and Glick 2013). Through the synthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase, an enzyme which degrades the biosynthetic precursors to this plant stress hormone, PGPR can actively reduce the physiological pool of ethylene and, in so doing, dampen plant stress response (Hardoim et al. 2008). The centrality of this enzyme to plant growth promotion has been verified through gene knock-out experiments wherein deficient mutants lost their ability to promote plant growth under stress conditions (Sun et al. 2009).

PGPR can aid in nutrient acquisition through mechanisms which mirror those employed by their more obligate counterparts, the mycorrhizae and rhizobia. Like mycorrhizae, PGPR can increase phosphorus availability to plants, but unlike these fungi, the rhizobacteria do not translocate phosphorus directly to roots via hyphae. Instead they act to solubilize it in the soil through the secretion of low molecular weight organic acids so that it can be later absorbed by plant roots (Malboobi et al. 2009). As previously stated, many PGPR also produce siderophores which not only can exclude iron from pathogens, but can make it available to plants (Colombo et al. 2014). Finally some, but not all PGPR can fix inorganic nitrogen (Hurek et al. 2002). Although these PGPR diazotrophs do not form root nodules, they can raise the effective nitrogen levels in the rhizosphere and thus nitrogen availability for plants. Finally carbon acquisition can be enhanced in biotized (inoculated with PGPR) plants through improvement in

photosynthetic efficiency measured in terms of both quantum yield and stomatal conductance (Ryu et al. 2003).

Phytohormones are perhaps the least expected of these means of growth enhancement, for, as their name implies, they are typically considered purely as plant signals. However, bacteria also commonly synthesize auxins (specifically IAA or indole-3-acetic acid), the phytohormone responsible for cell expansion, root growth, and lateral root initiation, among a great many other things (Barka et al. 2006). The effect of this bacterial auxin on plant growth has been demonstrated in many species including *Brassica juncea* and *Zea mays* where production of IAA by PGPR correlated with increased yields (Arshad 2002, Naveed et al. 2014). One reason for this auxin synthesis is the expansion of the rhizoplane and root mass. More rhizoplane area means more area for bacterial colonization, and more root mass means potentially more exudate to supply these bacteria with nutrients. Promotion of lateral roots may also benefit bacterial colonization, as lateral root emergence opens cracks in the root epidermis, cracks which provide bacteria with entry into the plant apoplast (Gutierrez-Luna et al. 2010). The auxin producing PGPR *Burkholderia phytofirmans*, which has been shown to colonize the plant interior from the rhizosphere and has been verified as an auxin producer, may employ this strategy (Asghar 2002). In addition to auxin, some PGPR have been documented to mobilize cytokinin in the shoot by enhancing the uptake of artificial cytokinin from tissue culture media or promoting *de novo* synthesis of this and other phytohormones (Lazarovits and Nowak 1997, Kurepin et al. 2015).

Applications

Applications of PGPR can be divided into two areas, agricultural and biotechnological. Agricultural applications are vital, for PGPR may provide possible answers for some of the major problems facing agriculture in the 21st century (Bhattacharyya and Jha 2012). These problems include increasing aridity and climatic extremes due to anthropogenic climate change, decreasing freshwater reserves for irrigation, the buildup of resistant pathogens, and the increasing cost of inorganic fertilizers, particularly phosphorus (Tilman et. al. 2002). In field studies, the amendment of reduced inorganic fertilization with a PGPR biofertilizer resulted in greater sunflower yields (measured in seed size and overall oil content) than traditional chemical fertilizers or organic methods alone (Akbari et al. 2011). Inoculation of field grown lettuce with *Bacillus* spp. resulted in greater seed yield (Arkhipova et al. 2005), and treatment of wheat with *Azospirillum* spp. in a long term study resulted in a 30% yield increase (Okon and Labandera-González 1994). Such results are promising because they offer a clear path toward a reduction in wasteful and ecologically harmful inorganic fertilization (Orhan et al. 2006). However, great care must be taken in selecting such amendments, for PGPR are less predictable in the field when interacting with the native soil microbiota, native plants, and highly site specific edaphic factors (sediment size, C:N ratio, trace elements, etc.) (Kloepper et al. 1989). In many experiments PGPR have significantly reduced the requirements for inorganic fertilizers and enhanced the yield of crops exposed to osmotic or temperature stress. Yet in a few studies effects have been negligible or even negative (Kloepper 1978). One response to such variability is the

application of consortia of bacterial species rather than a single bacterial species. Such bacterial communities tend to produce more predictable field results, such as in wheat where plant pathogens were more reliably repressed by a polyculture of Pseudomonad species than by a monotypic inoculant (Thomashow and Weller 1990). This co-inoculation strategy can also be effective when used in conjunction with Rhizobia and was shown to increase nodulation and consequently nitrogen availability for mung bean and soybean (Mahmoud and Abd-Alla 2001, Bai et al. 2002b). When applying PGPR or any plant beneficial microorganisms, growers must consider the labile nature of ecological relationships, their potential for unpredictable cross-effects and cultivar-specific effects.

Cell inoculants of plants in field testing with PGPR can be suspended in a variety of media which are called carriers, the simplest of which is an isotonic suspension of either isotonic saline or phosphate buffered saline (Nowak and A'Hearn 2002). In agricultural settings peat based inoculants are common, but manure, biowaste and even biochar carriers have been employed with various degrees of success (Rivera-Cruz 2008, Yardin et al. 2000, Hale et al. 2015). Carriers can influence microbial colonization success by modifying rhizosphere soil properties such as water holding capacity and cation exchange capacity (Elsa et al. 1992). Additionally flour, talc, and gum based carriers have been demonstrated to lengthen the survival of bacterial cells in inoculum relative to controls, particularly with regards to Pseudomonads (Ali et al. 2001, Linda 2014).

Within the laboratory environment, PGPR have found promising applications in plant tissue culture, though currently there has been far less research done in this area

than in agricultural applications. Plant tissue culture is the process of propagating plants through a sterile nutrient medium, often stimulated by the action of physiologically relevant concentrations of phytohormone. This methodology is an essential part of plant biotechnology and a necessary means of propagation for some species with long reproductive cycles or expensive *in vitro* growth requirements such as Venus fly trap, carnations, orchids and other epiphytes. Biotization is defined as the method of inducing stress resistance in tissue cultured plants by means of microbial inoculation (Nowak 1998). Biotized plant tissue cultures have been shown to benefit from microbial presence both in terms of photosynthetic function and biomass (Elmeskaoui et al. 1995). Root biomass of tissue cultured plants in particular is enhanced, possibly due to PGPR synthesis of auxin, which allows for greater nutrient uptake by the plant from the tissue culture media (Pillay and Nowak 1997). Even calli, undifferentiated masses of totipotent plant cells, can benefit from treatment with PGPR as demonstrated in rice tissue culture where increases in final yields of treated plants were documented (Senthilkumar et al. 2008). Additionally, PGPR benefit cultures by down regulating ethylene, a gaseous stress hormone which can build up in closed vials, and by improving the acclimatization process possibly via the same mechanism (Nowak and Shulaev 2003). The acclimatization process, the most lengthy and costly in many tissue culture procedures, presents plants adapted to the moist interior of a test tube with an artificial drought stress due to the relative aridity of the *ex vitro* environment, a stress which can be reduced by PGPR via the same mechanisms used to dampen actual drought stress (Frommel et al. 1991, Chandra et al. 2010). Although more predictable than field applications due to the controlled nature of *in vitro* systems, PGPR can still vary greatly in their response based

on cultivar. This has been observed in the varied response of specific tomato and potato cultivars to the inclusion of the PGPR *Burkholderia phytofirmans* (Pillay and Nowak 1997, Bensalim et al. 1998).

Venus Flytrap

Dionaea muscipula Ellis, Venus fly trap, is an endangered plant restricted to specific microhabitats within the Carolina Coastal Plain of North America (Schnell 2000). This rare plant is threatened in the wild by illegal harvesting (Luken 2005). Tissue culture procedures are utilized for the mass production of *D. muscipula* due to the challenges of traditional cultivation of this species, but prolonged acclimatization increases the time and cost of such propagation procedures (D'Amato 1998). Therefore, any reduction in acclimatization times and cost can aid in *D. muscipula*'s recovery in the wild by allowing more cost efficient transplant of plants back into their native habitat and by reducing the market value of illegally harvested plants.

Acclimatization in tissue culture refers to the process of gradually altering the phenotype of tissue cultured plants to withstand the environment outside the relatively humid vessels in which they are originally grown (Chandra et al. 2010). Since tissue cultured plants regularly develop heterotrophically in an environment ranging anywhere from 80-95% humidity, they may develop an aberrant phenotype ill-suited to the relatively arid *ex vitro* environment (Chen 2004). This aberrant phenotype may be characterized by hyperhydricity, hypolignification, low photosynthetic capacity in leaves due to improper cell differentiation, reduced cuticle development, ethylene

overproduction and poor stomatal regulation (Preece and Sutter 1991, Ziv et al. 1995, Fila et al. 1998, Hazarika et al. 2003). Hyperhydricity, the increased water content of plant tissues resulting from growth within a high humidity environment, is of particular concern for tissue culture acclimatization as such plants can rapidly dessicate when exposed to normal humidity levels (Apóstolo and Llorente 2000).

Burkholderia phytofirmans strain PsJN is a nondiazotrophic PGPR originally isolated from the surface-sterilized root of a *Glomus vesiculiferum*-infected onion plant (Frommel et al. 1991). The genome of the organism has been fully sequenced (Weilharter 2011), and its genetic architecture is characterized by its large size relative to other bacterial endosymbionts. Also of interest is the presence of phytohormone synthesis genes, ACC deaminase synthesis genes, efflux pumps, a missing pillus for the T-3 secretion system (T-3SS), and a highly redundant quorum sensing system involving multiple molecular signals (Moran 2003). The missing T-3SS pillus and phytohormone and ACC deaminase production may indicate directed selection for plant mutualism (Weilharter 2011). Stabilizing selection for competence in the bulk soil, on the other hand, is implied by high numbers of efflux pumps and redundancy in quorum sensing strategies (Weilharter 2011). The modern genetic studies of *B. phytofirmans* thus supports its previous characterization as a mutualistic endophyte which has retained its competitiveness in the bulk soil environment (Sessitsch 2005).

Empirical study in both lab and field environments demonstrates *B. phytofirman's* colonization of diverse host plants in both sterile (Conn et al. 1997, Nowak 1998, Barka et al. 2006, Fernandez et al. 2012) and non-sterile (Mehnaz et al. 2010, Ambrosini et al. 2012, Kim et al. 2012) environments. Non-sterile inoculants, however, can reduce the

bacterium's ability to colonize its host. Attenuation of colonization is likely mediated by either competition or chemical mediated antagonism with resident microbiota resulting in reduced promotion effects on host plants and reduced microbial population densities within inoculants (Somasegaran 1985, Tittabutr et al. 2012). Unfortunately, all sterilization methods lead to physical and/or chemical changes to the soil substrate; though, gamma sterilization and autoclaving with recovery periods result in fewer alterations to media chemistry than do other methods such as traditional autoclaving and steam sterilization (Stotzky and Mortensen 1959, Ramsay and Bawden 1983, Berns et al. 2008). These alterations can result in phytotoxic byproducts including but not limited to free radical formation, ethylene, Mn^{+2}, Na^{+1} (autoclave only), and various Malliard reaction byproducts (sugar-amino acid compounds) (Rovira and Vendrell 1972, Benzing-Purdie 1986, Chen et al. 1991, Lotrario 1995). Autoclaving of potted soils can thus induce potential variables into an experiment despite resulting in greater controls for biotic factors. Although many papers have been published on similar plant-microbe systems using nonsterile media, these studies like ours are focused specifically on non-sterile systems (greenhouse, field agriculture, phytoremediation) (Ahmad 2008, Kim et al. 2012, Poupin et al. 2013, Naveed et al. 2014, Wang et al. 2015). For these reasons, when verifying stability of the plant-microbe interaction in a model industry system such as a greenhouse, we will use non-sterilized soil, but when addressing basic questions such as root colonization by *B. phytofirmans* or the utility of soil sterilization itself, we will employ autoclaved soil with a recovery period following the autoclaving process. A comparative study of media sterility is further justified due to the likelihood of synergistic interactions between multiple microorganisms in the soil environment

(Domenech et al. 2006, Sanon et al. 2009).

Biotization can be used to 'harden' tissue cultured plants to the *in vivo* environment and improve the subsequent acclimatization process both in terms of speed and mortality rates (Srivastava et al. 2002). Many of the mechanisms previously mentioned which might be utilized by PGPR to enhance plant growth under stress conditions are of particular use in promoting the growth of tissue culture plants either *ex vitro* or *in vivo* (Yan et al. 2010). Reduction of the ethylene biosynthetic precursor 1-aminocyclopropane-1-carboxylate and, consequently, ethylene levels *in planta* via bacterial production of the enzyme ACC deaminase may be central to the process of biotization in many plant-PGPR systems as indicated by knockout studies where the impairment of ACC deaminase expression results in loss of growth promotion by the strain (Onfroe-Lemus et al. 2009; Nascimento et al. 2014). Since ethylene is known to be produced in excess by tissue cultured plants and can accumulate in closed tissue culture vials resulting in plant stress, it is possible that its reduction would promote plant growth in tissue culture and post tissue culture (*ex vitro*) environments (Biddington 1992).

Acclimatization stresses in particular represent a time and economic bottleneck which encumbers many tissue culture procedures (Debergh 1988). Since the poaching of any endangered species is profit-motivated, a reduction in the cost of *Dionaea muscipula* production should reduce the environmental pressures from illegal harvest affecting this plant negatively in its remaining habitat. It is for this reason that I propose attempting to inoculate tissue cultured *Dionaea muscipula* plants *ex vitro* and *in vitro* in order to ascertain whether *B. phytofirmans* PsJN can significantly improve, in terms of growth parameters, either acclimatization or propagation. This experiment will evaluate the

hypothesis that inoculation with *B. phytofirmans*, will increase *in vitro* propagation and *ex vitro* acclimatization success in *Dionaea muscipula,* as measured by mortality and growth parameters, respectively. Either result would be significant for growers and for the conservation status of the plant.

Research Goals

In order to ascertain the validity of the hypothesis that *Burkholderia phytofirmans* strain PsJN can improve the acclimatization and propagation in *Dionaea muscipula*, we conducted a series of inoculations experiment both *in vitro* and *ex vitro*. We predicted that biotization with the bacteria would increase propagule, trap and fresh biomass production relative to controls in *in vitro* plants. We also predicted that plants inoculated with the bacteria would have reduced mortality during the acclimatization process. For *in vitro* inoculations we predicted most significant results at a moderate (10^6 CFU/mL) inoculant strength. Finally we made the prediction that *Burkholderia phytofirmans* would colonize plants endophytically and would therefore be observable in isolates from surface-sterilized, biotized plants.

II. Materials and Methods

Research Overview

In vitro experiments first tested the effect of inoculant cell density on propagation success by comparing three different density inoculants (10^4, 10^6, 10^8 CFU (colony forming units)/mL) with an unbiotized control. Inoculant density was chosen as an independent variable due to the fact that it has been shown to strongly affect growth promotion of plants inoculated with this strain of bacteria (Pillay and Nowak 1997). Since initial experiments utilizing standard Venus fly trap propagation media resulted in overgrowth by the bacteria, further experiments designed to test alternative media formulations were also conducted. Phytoautotrophic media, plant tissue culture media containing no sugar, proved insufficient to sustain Venus flytrap propagation in preliminary studies, but a medium replacing maltose with sucrose as the primary sugar source eliminated visible bacterial growth while resulting in comparable propagation rates and so was tested against the standard sucrose based medium for statistical analysis. Once maltose was identified as a viable alternative, the cell density experiment was repeated utilizing this medium. *In vitro* plant colonization was also tested. Endophytic colonization by the strain was first verified by inoculation and reisolation of a GFP tagged strain of the bacteria from surface sterilized plant segments. Due to loss of fluorescence by the strain and ensuing poor visibility, however, a plate counting method was adopted for further estimation and quantification of the level of colonization in both endosphere and rhizosphere. Additional verification of endophyte identity was provided

by 16s rRNA sequencing and bioinformatics analysis. *Ex vitro* experiments tested the effect of root inoculation on acclimatization success using a xanthan gum carrier in both a sterile and nonsterile system.

General Procedures

Burkholderia phytofirmans strain PsJN was provided by Scott Lowman of the Institute of Advanced Studies in Danville. The bacteria were stored in 50% gycerol at -80 C. Bacteria to be used in inoculation experiments were recovered from -80 C storage or from subcultures on Kings B media not more than 2 passages from cold storage (King et al. 1954). Bacteria were then subcultured into a 150 mL triple baffled flask containing Luria-Bertani (LB) broth. Bacteria were incubated in LB media for 12 hours on an orbital shaker at 150 rpm at 22 C and centrifuged at 3000 G for 10 min to produce cell pellets. Pellets were resuspended in 1 mL 10 mM phosphate buffered saline (PBS) at pH 6.5. A standard serial dilution method was employed to produce samples containing cell concentrations at different order of magnitudes. These dilutions were then tested for absorbance at 600 nm, and afterward colony counting was used to determine their approximate CFU (colony forming units)/mL. This calibration procedure was performed approximately once every three months during the research period in order to assure correlation between absorbance measures and inoculant CFU. Approximate measure of 10^9 CFU/mL during this time did not vary more than 0.1 ABS.

Bacterial inoculants were suspended after centrifugation in either a PBS or a xanthan gum carrier. Xanthan gum (NOW Real Food™ pure xanthan gum) was chosen

due to positive results in preliminary experiments and the advantages it provided over liquid baths, namely quick coating of roots without the chance for plant desiccation from atmospheric exposure. For PBS based inoculants, bacteria were simply diluted to the proper absorbance/CFU in 10 mM PBS. Xanthan gum hyrdogel was prepared by agitation in 10 mM PBS with a sterile whisk within a laminar flow hood until even consistency was achieved. The ratio of xanthan gum was optimized for adherence to root during preliminary experiments and a value of 0.022g/mL was used in all subsequent *ex vitro* experiments. For xanthan gum inoculants dilution in PBS was followed by the addition of 9 mL of xanthan gum hydrogel to 1 mL of inoculant. Final dilution in xanthan gum was accounted for in calculation so that the xanthan gum root dip had the desired cell concentration.

Venus flytraps were procured from Carolina Biological Supply (Burlington, NC) in order to assure axenic plants of similar genotype. These plants, along with all experimental tissue cultured plants, were grown in a closed tissue culture room kept at a stable 22 C and exposed to a 16:8 cycle with a photon flux of 30 μmol/m^2s lighting. *Ex vitro* plants were grown in a growth chamber at 24 C under a 16:8 cycle with an average photon flux of 89.9 μmol/m^2s. Higher temperature during *ex vitro* acclimatization was intended to more closely mimic temperatures in the native range of Venus fly trap. Plants were grown in plastic pots which were three inches in diameter and 200 mL in volume. Pots were manufactured by Kord Products of Toronto, Canada. Initial humidity above pots was increased through the application of a humidity lid created by cutting a 9 oz transparent plastic cup (SOLO^{TM}) to an approximate volume of 80 mL. The lid was affixed to the top of pots by pressing the cut edge into the exposed surface of the soil and

creating a sealed dome. Plants were acclimatized by punching a 0.5 cm diameter hole along the periphery of this humidity lid every two weeks to enhance airflow and lower humidity (Fig. 1). All media for *ex vitro* plants consisted of 50 mL 50% sphagnum peat, 50% pearlite (by volume), and plants were fertilized by applying 0.250 mL of half strength Hoaglands solution to the center of their rosette twice per week (Hoagland and Aronon 1938). Sphagnum peat was used to simulate the *D. muscipula'*s native environment of saturated, acidic bog soil and perlite was added to enhance drainage (Fagerberg and Allain 1991, Kruse et al. 2014). Hoaglands solution was diluted to one half strength to produce a closer approximation to the native low nitrogen soils carnivorous plants (Mattson 1980).

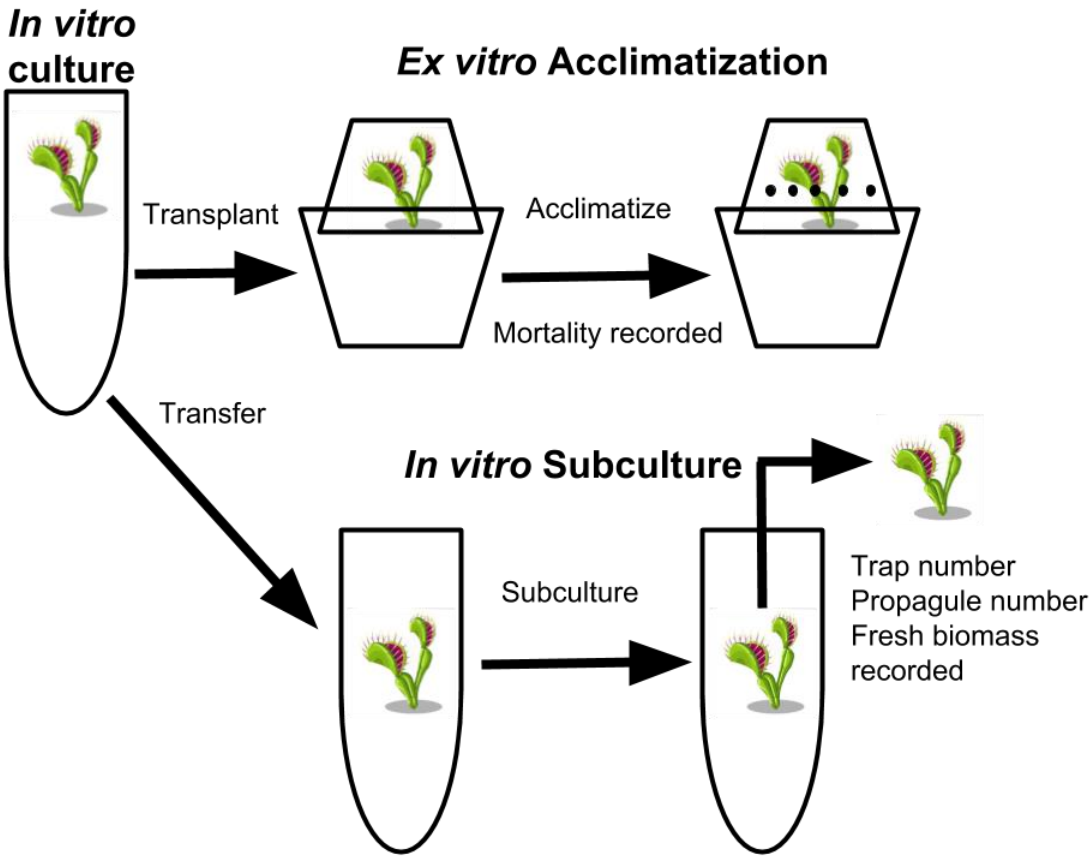

Figure 1: General overview of *ex vitro* and *in vitro* experiments along with timing of data collection.

All *in vitro* studies were completed in standard or modified Venus Flytrap Multiplication Media (VFM media) (Table 1) which is a variation of Murashige/Skoog nutritive media (Murashige and Skoog 1962). All media were adjusted to a pH value of 5.7 using NaOH before dissolving agar and sterilizing in autoclave at 121 C and 15 PSI for 15 min. All *in vitro* plants were grown under artificial lighting 16:8 hour daily cycle with 20 mL of sterile VFM or modified VFM media in 60 mL, 25 x 150 mm test tubes enclosed by parafilm.

Table 1: Components and their concentration in Venus fly trap media (VFM).

Component	Concentration
NH_4NO_3	0.4 g/L
KNO_3	0.48 g/L
$MgSO_4 \cdot 7H_2O$	0.37 g/L
$CaCl_2 \cdot 2H_2O$	0.44 g/L
$NaH_2PO_4 \cdot H_2O$	0.38 g/L
thiamine HCL	0.0004 g/L
myoinositol	0.1g/L
adenine sulfate	0.08 g/L
6-(γ,γ-Dimethylallylamino)purine	0.0002 g/L
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/L
$CuSO_4 \cdot 5H_2O$	0.025 mg/L
$CoCl_2 \cdot 6H_2O$	0.025 mg/L
$MnSO_4 \cdot H_2O$	16.9 mg/L
$ZnSO_4 \cdot 7H_2O$	10.6 mg/L
H_3BO_3	6.2 mg/L
KI	0.83 mg/L
$Na_2EDTA \cdot 2H_2O$	37.2 mg/L
$FeSO_4 \cdot 7H_2O$	27.8 mg/L
sucrose	30g/L
Bactoagar™	8 g/L

Ex vitro experiments

Two *ex vitro* acclimatization experiments were performed using axenic transplants from subcultures acquired from Carolina Biological Supply. Both followed the acclimatization procedure outlined in the general procedures section and used inoculation densities of approximately $1x10^8$ CFU as verified through spectrophotometry. This colony density was chosen for more extensive evaluation due to favorable mortality results in preliminary studies. All *ex vitro* experiments were concluded after three months of acclimatization. Throughout that time weekly assessments of mortality were conducted with dead plants classified as those with no visible live tissue nor indication of new meristematic growth (Fig. 1). Comparisons between mortality statistics between groups were done through a test of given proportions (R core team 2013). Control groups are considered those groups of plants given an inoculum without bacterial cells added (mock inoculum) while experimental groups are considered those inoculated with live PsJN cells.

The first *ex vitro* acclimatization experiment tested the effects of a xanthan gum based inoculant on mortality. Experimental plants were inoculated by dipping their roots fully in xanthum gum hydrogel containing *Burkholderia phytofirmans* strain PsJN and, after assuring a complete coating, transplanting directly into nonsterile media. Control plants were dipped in sterile xanthan gum instead.

The second *ex vitro* acclimatization experiment tested the additional effect of soil sterility on the xanthan gum carrier system. Sterile media was procured by autoclaving soil in biohazard disposable autoclave bags (Bel-Art Products^R) followed by a refractory

period of two weeks to reduce any phytotoxic byproducts of the autoclaving process. Inoculation of experimental and control plants was performed identically to the nonsterile experiment.

In Vitro Experiments

All *in vitro* experiments followed the procedures outlined in the General Procedures section unless otherwise noted. All tissue cultures were run for the maximum time allowable within the research period so as to ascertain long term effects when possible. Shorter term cultures were repeated to add additional support. Durations of tissue culture experiments were as follows: 226 days for assessment of various cell density in sucrose, 49 days for assessment of various cell density in maltose, and 140 days for comparison of maltose to sucrose at constant cell density. Experiments focused on the optimization of tissue culture media and inoculation density for the reduction of bacterial growth and enhancement of propagation and plant health in media. For all *in vitro* experiments, the following growth metrics were assessed at the close of the tissue culture period: trap number, fresh biomass, propagule number. Mature, countable traps were defined as those with visible ‘tines’, the interlocking protuberances along the distal edge of fly trap leaves. Fresh biomass was measured after washing plants of all visible bacterial growth and agar in distilled water followed by thorough blot drying on filter paper. Countable propagules were defined as individual plants capable of subculture due to presence of some root visible tissue at the plant base. This tissue was easily distinguished from shoot tissue during data collection due to the darkly pigmented nature

of Venus fly trap root tissue. In experiments where biomass accumulated in tubes, those tubes with biomass and/or necrosis were also counted. Tubes containing visible bacterial growth on the media and tubes with significant necrosis (>30% dead tissue) were recorded.

Three different media were assessed for their utility at promoting beneficial plant-microbe interaction *in vitro*: the standard VFM media using sucrose, an autotrophic media using no sugar and an alternative heterotrophic media using an equal mass of maltose in place of sucrose (J. Nowak, pers. comm.). Preliminary experiments showed negligible propagule production and high mortality in phytoautotrophic media so it was excluded from further study and analysis. Both the sucrose and maltose media were assessed for use in plant growth using three different concentrations of the bacteria ($1x10^8$, $1x10^6$, and $1x10^4$ CFU/mL) plus an uninoculated control. In sucrose experiments where bacterial over-growth impeded propagation, bacterial growth and necrosis were also assessed. In all experiments plants were immersed fully in PBS inoculant for 15 minutes followed by a thorough blotting with dry sterile filter paper before transfer to medium in test tubes.

For assessment of various cell density in sucrose, differences between groups were assessed by one-way ANOVA unless otherwise stated. Dunnet's Modified Tukey-Kramer Pairwise Multiple Comparison Test was used to assess significance of difference in trap production as these populations displayed unequal variance. A Moods Median Test was used to assess significance of difference in propagule production as these populations displayed nonnormal distribution. All other data were assessed for significance of difference via Tukey's Honest Significance Difference Test. For various

cell density in maltose media analysis was completed by assessing difference by one-way ANOVA and post hoc test for significance of difference utilizing Tukey's Honest Significance Difference Test. Maltose and sucrose media were compared directly using Student T Test.

Estimation of colony density in rhizosphere and endosphere was completed for plants grown on maltose and sucrose media without hormones. In both procedures a modified version of the surface sterilization procedure created by Compant for biotized *Vitus vinifera* was performed (Compant et al. 2005). To estimate endophytic colonization two plants of comparable size and appearance were selected from the maltose and sucrose groups based on similarity (general size of plant and number of petioles) and weighed. They were surface sterilized with 70% ethanol for 2 minutes, followed by a low strength bleach solution consisting of 0.0825 % NaClO and a 0.01% Tween 20 solution for 1 min. The low strength of the bleach solution is intended to eliminate surface bacteria while not penetrating tissues to kill endophytic populations. After surface sterilization, plants were washed three times in separate distilled water baths for one minute per bath. The samples were then ground in a sterile mortar and pestle with 1 mL of PBS. The resulting homogenate was washed with an additional 9 mL PBS into a 50 mL erlenmeyer flask and shaken for 1 hour at 200 rpm. Afterward the homogenates were serial diluted and plated on King's B media in triplicate. Aliquots of 0.1 mL from the final bath were also plated in triplicate to validate the success of the sterilization procedure. If any colonies grew on these plates after a week, the surface sterilization was considered ineffective and the results were discarded. Colonies were counted after three days incubation in the dark at 22 C. To calculate rhizosphere population, the procedure

was performed as above except without surface sterilization. The counts achieved from the former procedure were then subtracted from those achieved from the nonsterilized count in order to attain an approximate rhizospheric colony density. Results were averaged first among the three triplicates and then, after calculations, between the two samples to reach final estimates.

Verification of endophytic colonization was performed by inoculating plants with bacteria transformed via chromosomal insertion of GFP gene (Compant 2005). Plants inoculated with GFP transformed bacteria were subcultured in nonhormone maltose VFM for two weeks prior to bacterial reisolation. Surface sterilization was performed as described above and afterward plants were sectioned by sterile scalpel and the sections were plated on King's B media. After visible colonies emerged along the edges of plant sections, samples were taken and vortexed in 1 mL PBS. Aliquots of 0.1 mL were taken from this homogenate, placed on a glass slide and viewed under an Axioscope A1 (ZEISS Microscopy) to verify fluorescence of the emergent endophyte.

Bioinformatics

Due to the inconclusive results of microscopy, verification by 16s rRNA was also employed. Endophytes from *in vitro* inoculated fly traps grown in both in sucrose and maltose were harvested following the same surface sterilization procedure utilized for colonization estimation. Recovered endophytes were subcultured into 5 mL of tryptic soy broth (TSB) and incubated at 37 C on an orbital shaker set at 150 rpm. After twenty four hours incubation, bacterial DNA was extracted by centrifugation at 13,000 rpm for

one minute followed by 15 minutes boiling at 95 C. PCR was performed using the universal primers 27f (AGA GTT TGA TCM TGG CTC AG)and Univ1492R (CGG TTA CCT TGT TAC GAC TT) and 30 cycles at 95 C 1 min: 50 C 30 sec: 72 C for 1.5 minutes were used to complete the reaction. Presence of PCR product was verified using gel electrophoresis, and following verification, amplicons were sent to eurofins Genomics labs for sequencing. All sequences were analyzed using the DNA subway online tool including the BLAST and MUSCLE algorithm functions (Altschul 1990, Edgar 2004, DNA Learning Center 2011).

The hypothesis that a SigX ECF (extracytoplasmic function) transcription factor was present in PsJN was assessed by a geneBLAST of the PsJN genome using the NCBI database (Altschul 1990).

III. Results

Ex Vitro Experiments

Within nonsterile (unautoclaved) media, the effect of inoculation with *B. phytofirmans* strain PsJN on plant survival was significant (p=0.000750) with a mortality percentage of 47.2% total plants in the mock inoculated group and 11.1% in the inoculated group after an acclimatization period of three months (n=36) (Fig. 2). Within sterile (autoclaved) media, the effect of inoculation with *B. phytofirmans* strain PsJN was not significant with only a single mortality in both groups (n=28) (Fig. 2). When comparing between mock inoculated groups on sterile soil and nonsterile soil groups, autoclaving significantly reduces mortality (p value=0.000117). By contrast, autoclaving of potting media did not significantly reduce mortality in inoculated plants (p value=0.265).

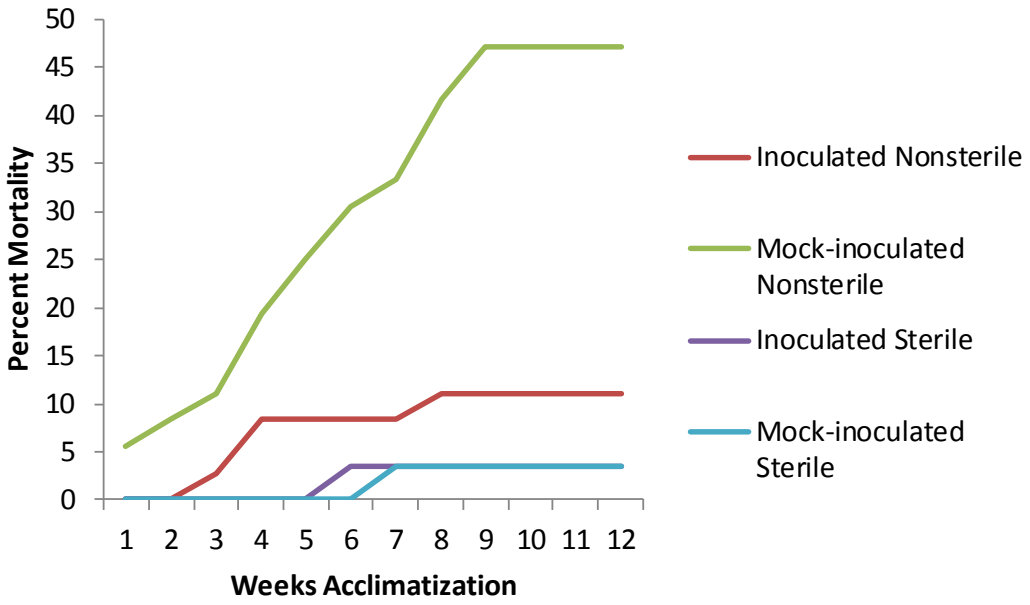

Figure 2: Percent mortality in four *ex vitro* treatments over 12 weeks of acclimatization. Inoculated plants inoculated with *B. phytofirmans* strain PsJN in xanthan gum carrier. Mock inoculated plants inoculated with xanthan gum only. Thirty six plants were acclimatized in sterile soil. Twenty eight plants were acclimatized in nonsterile soil.

In Vitro Experiments

After the tissue culture period, trap production was significantly different (greater than 95% confidence interval) between the control group which produced an average of 304 traps and the $1x10^8$, $1x10^6$ and $1x10^4$ CFU/mL inocula groups which produced 39, 112, and 73 traps respectively (Table 2). There also were significant differences between the $1x10^8$, $1x10^6$ and $1x10^4$ CFU/mL inocula groups. Axenic cultures in sucrose also produced significantly more (greater than 95% confidence interval) fresh biomass (average mass of 6.12 grams) than $1x10^8$, $1x10^6$ and $1x10^4$ CFU inocula groups (0.474, 0.884, and 0.676 grams respectively (Table 2). Median propagule production was

significantly different only between axenic culture and the $1x10^8$, $1x10^6$ and $1x10^4$ CFU/mL inocula groups (p values 0.0002, 0.0012 and 0.0002 respectively (Table 2)). The median propagule production in the axenic controls was higher with a median value of 139 propagules. Median production in the $1x10^8$, $1x10^6$ and $1x10^4$ CFU/mL inocula groups was 14, 35, and 34 respectively (Table 2). In all the inoculated tubes visible bacterial growth and necrosis of plant tissue was observed. In axenic tubes visible bacterial growth was only observed in contaminated tubes which were excluded from analysis.

Table 2: Average trap production, median propagule production, and average biomass for three different inocula cell densities and an axenic control cultured for 226 days in standard VFM media using sucrose as a sugar source. Visible bacterial growth and necrosis (greater than 30% dead tissue) are also listed as number of cultures where this was observed out of the total sample size. Asterisks indicate significant results ($p \leq 0.05$).

Inoculum Density	Average Traps	Median Propagules	Average Mass (g)	Bacterial growth (%)	Necrosis (%)	Sample Size
$1x10^8$	55.4±34	27±12	0.627±0.39	100	42.9	14
$1x10^6$	112±35	35±8.7	0.884±0.29	100	0	18
$1x10^4$	73±51	34±17	0.676±0.42	100	21.4	14
Axenic	304*±56	139*±24	6.12*±1.2	0	0	9

After 49 days of *in vitro* growth, no significant effect of inoculum CFU or control (cell free inocula) on trap production, fresh weight, or propagule production was observed in maltose media (Table 3). In the repeated experiment, these trends were comparable with no significant effects observed.

Table 3: Average trap production, average propagule production, and average biomass for three different inocula cell densities and an axenic control cultured for 49 days in Venus flytrap media using maltose as the sole sugar source. Table displays the results of two trials started at different times but continued for the same duration under identical starting and growth conditions.

Inoculum Density	Average Traps (Trial 1)	Average Traps (Trial 2)	Average propagules (Trial 1)	Average Propagules (Trial 2)	Average Mass (g) (Trial 1)	Average Mass (g) (Trial 2)	Sample Size (Trial 1)	Sample Size (Trial 2)
$1x10^8$	10.8±4.7	7.75±3.6	3.36±1.6	5.92±2.4	0.0951± 0.059	0.103± 0.042	11	12
$1x10^6$	9.27±3.3	5.75± 3.07	5.45±2.5	4.08±2.0	0.103± 0.045	0.0781± 0.031	11	12
$1x10^4$	8.00±2.8	7.92±3.6	5.00±3.2	5.58±2.7	0.113± 0.059	0.101± 0.025	10	12
Axenic	8.4±3.4	7.33±5.3	4.60±2.0	5.25±2.6	0.103± 0.053	0.110± 0.056	10	12

After 140 days *in* vitro growth, maltose grown biotized flytraps performed better than those cultured on sucrose in terms of biomass and propagule production, but not trap production (Table 4). Trap production was not significantly different between the two groups (p value=0.874). Fresh weight, however, was significantly different between the two treatments (p value = 0.0000277) with maltose media producing a greater wet biomass (mean=0.644 grams) than sucrose (0.2025 grams). Maltose cultures also produced significantly more propagules (p value=0.0006015) with maltose media producing an average of 20 propagules and sucrose media producing an average of 11 propagules per culture vessel. Bacterial growth production and necrotization of plant tissue was also recorded for this experiment. All sucrose cultures were recorded as having visible bacterial growth present while no visible bacterial growth was present in maltose cultures. Moreover 38.4% of maltose flytraps displayed significant necrotization

(greater than 30% of visible plant tissue). Apart from mortality from contamination there was no necrotization observe in maltose grown flytraps.

Table 4: Average trap production, average propagule production, and average biomass during culture on media containing two different sugar sources for 140 days. Visible bacterial growth and necrosis (greater than 30% dead tissue) are also listed as number of cultures where this was observed out of the total sample size. Asterisks indicate significant results (p value less than 0.005).

Sugar Source	Average Traps	Average Propagules	Average Mass (g)	Bacterial growth (%)	Necrosis (%)	Sample Size
Sucrose	24.9±18	11.0±7.4	0.201±0.15	100	46.2	26
Maltose	28.9±16	20.3*±10	0.644*± 0.41	0	8.33	24

Burkholderia phytofirmans strain:PsJN was verified as endophytic by reisolation from surface sterilized plant sections on KB agar. After endophytism was verified by reisolating GFP bacteria and visualization under fluroscope (Fig. 3), traditional plate counting was used to form an initial estimate of rhizosphere and endophyte populations on both sucrose and maltose. For biotized plants cultured on sucrose media the average rhizospheric colonization was $4.59x10^9$ CFU/g plant tissue and the average endophytic colonization was $7.04x10^8$ CFU/g plant tissue. For biotized plants cultured on maltose media colonization counts were lower. Average rhizosphere colonization of maltose cultured plants was $8.41x10^7$ CFU/g and the average endophytic colonization was.$3.32x10^7$ CFU/g.

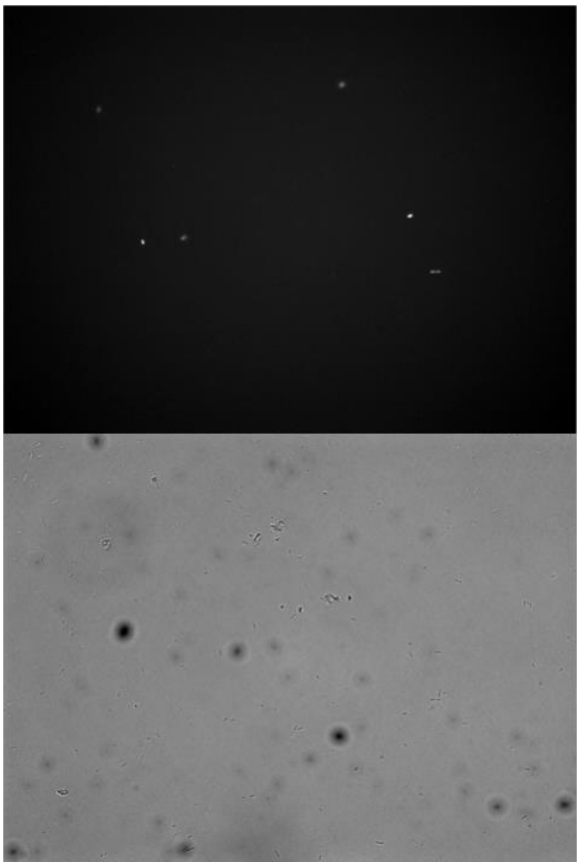

Figure 3: A fluorescent (top) and a brightfield (bottom) image of the same sample of reisolated endophytic bacteria diluted in phosphate saline buffer at 1000x magnification. Numerous bacteria in bright field image contrasted with few fluorescent signatures in fluorescent image demonstrates reduction in fluorescent phenotype through the infection/reisolation process.

Bioinformatics

Additional verification of endophytism was pursued using 16s rRNA amplification, sequencing and bioinformatic analysis. Twelve randomly selected endophytes isolated from maltose and sucrose cultured *in vitro* fly traps after sixteen days post-inoculation were assessed. BLAST was utilized to search the NCBI database for matching sequences after sequences were trimmed and unknown bases removed (Table

5). Percent identity and E-value for matching 16s rRNA sequences from *Burkholderia phytofirmans* strain PsJN listings from the NCBI database indicate phylogenetic identity of the isolated endophyte as *Burkholderia phytofirmans* strain PsJN(Table 5). The two highest percent identity samples are also the two high quality samples (less than 1% misreads) in the data set, further supporting strain identity as *Burkholderia phytofirmans* strain PsJN.

Table 5: Results of BLAST queries using endophyte isolated from *in vitro* propagated Venus fly trap 16 days post inoculation.

Isolate	BLAST Identity	E-value	% Identity	Sugar source
M1	*Burkholderia phytofirmans* strain PsJN	2e-124	87%	Maltose
M2	*Burkholderia phytofirmans* strain PsJN	3e-119	86%	Maltose
M3	*Burkholderia phytofirmans* strain PsJN	8e-55	84%	Maltose
M4	*Burkholderia phytofirmans* strain PsJN	3e-119	87%	Maltose
M5	*Burkholderia phytofirmans* strain PsJN	0.0	90%	Maltose
M6	*Burkholderia phytofirmans* strain PsJN	1e-90	84%	Maltose
S2	*Burkholderia phytofirmans* strain PsJN	0.0	90%	Sucrose
S4	*Burkholderia phytofirmans* strain PsJN	5e-141	86%	Sucrose
S5	*Burkholderia phytofirmans* strain PsJN	0.0	93%	Sucrose
S6	*Burkholderia phytofirmans* strain PsJN	0.0	95%	Sucrose

IV. Discussion

Ex vitro Experiments

Ex vitro mortality studies showed that both soil sterilization and inoculation increased survival, but inoculation did so only under nonsterile conditions. One possible explanation for this effect would be that inoculation primarily worked to reduce mortality by reducing biotic stress. Endophytes have been demonstrated to reduce such stresses by means of priming, an induction of plant defense pathways which results in future pathogen resistance (Yi et al. 2013). Another explanation of this difference has to do with the physical changes undergone by the media during the autoclave process. This unanticipated physical change caused the peat and pearlite constituents of the media to adhere in the autoclaved soil resulting in less run off of the peat in these pots. Run off could be visualized as discoloration in water draining from nonsterile but not sterile pots. Unfortunately, due to the fact that any plant being acclimatized (transferred from a high to a low humidity environment) is *ipso facto* under a drought stress, it becomes difficult to determine which portion of the drought stress is derived from acclimatization and which from peat loss. This increased peat/pearlite ratio could have resulted in better water holding capacity in the autoclaved media and increased drought stress in the nonsterile group. Conversely, since PSJN is well documented to enhance plant resistance to drought stresses, this might also explain both the higher general mortality in the nonsterile groups and the significantly lower mortality in the inoculated flytraps (Naveed et al. 2014, Wang 2015). These results indicate that PSJN would be most beneficial for

acclimatization in nonsterile propagation systems such as greenhouses. Additionally, PsJN may be a useful inoculant for enhancing survival when the grower anticipates a drought stress such as can occur in outdoor conditions and sunlit greenhouses. The results indicate that for small scale productions and controlled environments where soil can be sterilized and drought stress can be controlled, autoclaving soil is sufficient to reduce mortality to acceptable levels. In order to ascertain with more certainty whether PsJN's effect on mortality is purely the result of biotic stress reduction, future studies could make use of commensal microbes which have no growth promoting effect or ACC deaminase knockouts of PSJN with reduced growth promotion ability and less capacity for reduction of abiotic stress. If these microbes, which should still induce priming by means of microbe associated molecular patterns, cause a similar mortality reduction in nonsterile conditions then such a result would support this hypothesis. In order to eliminate the possibility of an increased drought stress as the cause, brands of peat which are less mobile during watering should be tested.

In vitro Experiments

Both sucrose and maltose formulations of Venus flytrap media (VFM) were tested with inoculants differing in CFU in order to determine cell density of inoculants could alter propagation success. However, since this methodology was investigated later in the research program, these tubes could not be cultured for the same length of time as earlier experiments. For this reason, any results from the maltose tubes should take this into account as longer term tissue cultures might magnify existing differences to significant

levels.

In sucrose VFM axenic cultures performed best in terms of propagation with significantly more propagules, traps and biomass than their inoculated counterparts. Although there were no significant differences between the inoculated groups in terms of morphometrics, more tubes in the $1x10^8$ CFU group had visible necrosis, twice the number as any other group. This may indicate some concentration dependence in the severity of the negative interaction in sucrose. In maltose, biotized plant tissue cultures were not significantly different from their axenic counterparts in plant biomass, trap production or propagule production but it must be considered that over a longer culture period, significant differences might appear. Since biotization did not improve propagation, it also must be considered that biotization of flytraps, poses no advantage in terms of propagation that justifies its application in conservation or industry. However, this study did not test the survival of prebiotized plants during acclimatization. Future research could make use of the biotized fly traps currently in culture at James Madison University to test whether such plants are hardier and have decreased mortality compared to axenic plants or *ex vitro* inoculated plants. Should this be the case, biotization may be warranted despite potential reduction in propagation rates.

When compared directly with sucrose, maltose VFM was superior across all propagation metrics except for trap production where there was no significant difference. This was likely due to the fact that production of small traps, which were none the less morphologically mature, continued even under stressful conditions. In addition to stimulating the generation of more biomass and propagules, maltose VFM resulted in a complete disappearance of visible bacterial growth from culture and a corresponding

reduction in necrosis. One hypothesis put forward as to why PSJN grew less upon the media when restricted to a maltose sugar source was the upregulation of extracytoplasmic function (ECF) factors in response to sucrose, a response documented in the related Proteobacterium *Pseudomonas aeruginosa* (Bouffartigues 2014). However a BLASTn conducted of the PSJN genome revealed no homologs for the SigX transcription factors indicated as responsible for the mucoid phenotype of *P. aeruginosa* in response to sucrose in this study.

Comparison of colony density on a per gram fresh weight basis showed relatively high colonization in both endosphere ($3.32x10^7$ CFU/gram) and rhizosphere ($8.41x10^7$ CFU/gram) in tissue cultured plants grown in maltose. Colonization densities were tenfold higher in sucrose VFM grown ($4.58x10^9$ CFU/gram rhizosphere colonization; $7.04x10^8$ CFU/gram endosphere colonization). This is higher than rates of colonization observed in *Vitis vinerea* by Compant et. al. (2005) where the effect of PSJN colonization was growth promoting. Despite the small sample size of these initial measurements, the orders of magnitude difference between colonization levels in sucrose and maltose cultured plants should be taken as an indication that over colonization is one possible reason for the negative interaction in sucrose VFM. We can be more confident based on the high colonization levels in all sampled plants that over colonization is a reason for the generally poor response of Venus fly trap to biotization. Such increased colonization rates are not necessarily caused by PsJN as colonization rates in endophyte symbiosis are the result of a balance between the plant immune response that limits colonization and the endophytes attenuation of that response (Iniguez et al. 2005).

Confirmation of endophytism by microscopy was complicated by loss of

fluorescence in the reisolated bacteria (Fig. 3). This phenotype in the transformed strain was verified as a known problem by correspondence with Dr. Jerzy Nowak (personal communication). To provide further support for endophytic colonization in this system, 16s rRNA sequencing was adopted as an alternative method of strain identification. Results of BLAST queries for sequences amplified from reisolated endophytes support the strain identity as *Burkholderia phytofirmans* strain PsJN (Table 5). Within the highest quality sequences with less than 1% missing base reads (S4 and S5), the highest percent identity among classified strains belongs to PsJN.

Considering the high bacterial colonization rates in our cultures, it might be suggested that future experiments either attempt inoculation with low CFU inoculants and/or amend tissue culture media with compounds such as salicylic acid which have been shown to heighten plant immune response to biotrophic interactions (Mauch-Mani and Métraux 1998). Future studies may also look to further improve the propagation of biotized traps by examining different sugar sources. We can conclude that *Burkholderia phytofirmans* can act as an endophyte in *Dionaea muscipula.* Although *in vitro* cultivation of fly traps biotized with *Burkholderia phytofirmans* strain PsJN is possible using maltose media this does not improve propagation rates. *Ex vitro* inoculation with this bacterium by contrast is a promising biotechnology for fly trap cultivation under nonsterile soil conditions and/or during drought stress where sterilizing all soil media is cost prohibitive.

Literature Cited

Agnati, L. F., Baluška, F., Barlow, P. W., and Guidolin, D. 2009. Mosaic, self-similarity logic, and biological attraction principles: three explanatory instruments in biology. Commun. Integr. Biol. 2, 552–563. doi: 10.4161/cib.2.6.9644

Ahmad, F., Ahmad, I., and Khan, M. S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163:173-181.

Akbari, P., Ghalavand, A., Sanavy, A.M., Agha Alikhani, M. and Kalkhoran, S. 2011. Comparison of different nutritional levels and the effect of plant growth promoting rhizobacteria (PGPR) on the grain yield and quality of sunflower. Australian Journal of Crop Science. *5*(12): 1570.

Ali, N. I., Siddiqui, I. A., Shaukat, S. S., and Zaki, M. J. 2001. Survival of *Pseudomonas aeruginosa* in various carriers for the inhibition of root rot-root knot disease complex of mungbean. Phytopathologia Mediterranea 40(2). 108-112.

Alstrom S. 1991. Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizosphere pseudomonads. J. Gen. Appl. Microbiol. 37:495–501

Altschul, S. 1990. Basic local alignment search tool. Journal of molecular biology. 215(3): 403-410.

Ambrosini, A., Beneduzi, A., Stefanski, T., Pinheiro, F. G., Vargas, L. K., and Passaglia, L. M. 2012. Screening of plant growth promoting rhizobacteria isolated from

sunflower (*Helianthus annuus* L.). Plant and Soil 356(1-2):245-264.

Apóstolo, N.M. and Llorente, B.E. 2000. Anatomy of normal and hyperhydric leaves and shoots of in vitro grown *Simmondsia chinesis* (Link) Schn. In Vitro Cellular and Developmental Biology-Plant 36(4):243-249.

Archana, M., Mathur A., Priyanka V. and Mahendra D. 2008. Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum* Sant. et Fernand. African Journal of Biotechnology 7(8): 1046-1053.

Arkhipova, T. N., Veselov, S. U., Melentiev, A. I., Martynenko, E. V., and Kudoyarova, G. R. 2005. Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. Plant and Soil 272(1-2): 201-209.

Asghar, H., Zahir, Z., Arshad, M., and Khaliq, A. 2002. Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in *Brassica juncea* L. Biology and Fertility of Soils 35(4): 231-237.

Bais HP, Fall R, Vivanco JM. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by bacterial growth formation and surfactin production. Plant Physiol. 134:307–19.

Bais, H., Weir T., Perry L., Gilroy S., and Vivanco J. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. 57: 233-266.

Barka, E. A., Nowak, J., and Clément, C. 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. Applied and Environmental Microbiology

72(11):7246-7252.

Barka, E. A., Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., and Clément, C. 2008. Endophytic colonization of Vitis vinifera L. by Burkholderia phytofirmans strain PsJN: from the rhizosphere to inflorescence tissues. FEMS Microbiology Ecology 63(1), 84-93.

Bensalim, S., Nowak, J., and Asiedu, S. 1998. A plant growth promoting rhizobacterium and temperature effects on performance of 18 clones of potato. American Journal of Potato Research 75(3): 145-152.

Benzing-Purdie, L.M., Cheshire, M.V., Williams, B.L., Sparling, G.P., Ratcliffe, C.I. and Ripmeester, J.A., 1986. Fate of [15N] glycine in peat as determined by carbon-13 and nitrogen-15 CP-MAS NMR spectroscopy. Journal of Agricultural and Food Chemistry 34(2):170-176.

Berns, A.E., Philipp, H., Narres, H.D., Burauel, P., Vereecken, H. and Tappe, W. 2008. Effect of gamma sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. European Journal of Soil Science 59(3):540-550.

Bhattacharyya, P. N. and Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology 28(4): 1327-1350.

Biddington, N.L., 1992. The influence of ethylene in plant tissue culture. Plant Growth Regulation 11(2): 173-187.

Bordiec, S., Paquis, S., Lacroix, H., Dhondt, S., Barka, E. A., Kauffmann, S., and Dorey, S. 2011. Comparative analysis of defence responses induced by the endophytic

plant growth-promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN and the non-host bacterium *Pseudomonas syringae* pv. pisi in grapevine cell suspensions. Journal of Experimental Botany 62(2): 595-603.

Bouffartigues, E., Duchesne, R., Bazire, A., Simon, M., Maillot, O., Dufour, A. and Chevalier, S. 2014. Sucrose favors *Pseudomonas aeruginosa* pellicle production through the extracytoplasmic function sigma factor SigX. FEMS Microbiology Letters *356*(2), 193-200.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., and Schulze-Lefert, P. 2013. Structure and functions of the bacterial microbiota of plants. Annual Review of Plant Biology 64: 807-838.

Burdman S., Okon Y., and Jurkevitch E. 2000. Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. Critical Reviews in Microbiology 26(2): 91-110.

Burgers, A. 2012. Soil Biology. New York, New York: Academic Press Inc. pp. 16-17, 40-42, 64-86.

Cassells, A.C. and Curry, R.F. 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell, Tissue and Organ Culture 64(2-3):145-157.

Chandra, S., Bandopadhyay, R., Kumar, V., and Chandra, R. 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnology Letters 32(9):1199-1205.

Chen, C. 2004. Humidity in plant tissue culture vessels. Biosystems Engineering 88(2):231-241.

Chen, Y., GamLiel, A., Stapleton, J.J. and Aviad, T. 1991. Chemical, physical, and microbial changes related to plant growth in disinfested soils. Soil Solarization 1:103-129.

Colombo, C., Palumbo, G., He, J. Z., Pinton, R., and Cesco, S. 2014. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. Journal of Soils and Sediments 14(3): 538-548.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., and Barka, E. A. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Applied and Environmental Microbiology 71(4): 1685-1693.

Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Barka, E.A. and Clément, C. 2008. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues. FEMS Microbiology Ecology 63(1):84-93.

Conn, K. L., Lazarovits, G., and Nowak, J. 1997. A gnotobiotic bioassay for studying interactions between potatoes and plant growth-promoting rhizobacteria. Canadian Journal of Microbiology 43(9):801-808.

DNA Learning Center. 2011. DNA Subway Tool. The iplant collaborative. Available at: http://dnasubway.iplantcollaborative.org/

D'Amato, P. 1998. The Savage Garden: Cultivating Carnivorous Plants. Berkeley, California: Ten Speed Press. pp. 915-916.

De-Bashan, L. E., Hernandez, J. P., and Bashan, Y. 2012. The potential contribution of plant growth-promoting bacteria to reduce environmental degradation–A

comprehensive evaluation. Applied Soil Ecology. 61: 171-189.

Debergh, P. 1988. Improving mass propagation of in vitro plantlets. Horticulture in High Technology Era pp. 45-57.

del Carmen Rivera-Cruz, M., Narcía, A.T., Ballona, G.C., Kohler, J., Caravaca, F. and Roldan, A. 2008. Poultry manure and banana waste are effective biofertilizer carriers for promoting plant growth and soil sustainability in banana crops. Soil Biology and Biochemistry. 40(12): 3092-3095.

Dewey, F. M., Wong Y. L., Seery R., Hollins T. W. and Gurr S. J. 1999. Bacteria associated with *Stagonospora nodorum* increase pathogenicity of the fungus. New Phytologist. 144(3): 489-497.

Domenech, J., Reddy, M.S., Kloepper, J.W., Ramos, B. and Gutierrez-Manero, J. 2006. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. BioControl. 51(2):245-258.

Edgar, R. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research 32(5): 1792-1797.

Elmeskaoui, A., Damont, J. P., Poulin, M. J., Piché, Y., and Desjardins, Y. 1995. A tripartite culture system for endomycorrhizal inoculation of micropropagated strawberry plantlets in vitro. Mycorrhiza. 5(5): 313-319.

Elsas, J. V., Trevors, J. T., Jain, D., Wolters, A. C., Heijnen, C. E., and Overbeek, L. S. 1992. Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. Biology and Fertility of Soils. 14(1): 14-22.

Fagerberg, W.R. and Allain, D. 1991. A quantitative study of tissue dynamics during closure in the traps of Venus's flytrap *Dionaea muscipula* Ellis. American Journal of Botany. 78(5):647-657.

Fernandez, O., Theocharis, A., Bordiec, S., Feil, R., Jacquens, L., Clément, C., Florence, F., and Barka, E. A. 2012. *Burkholderia phytofirmans* PsJN acclimates grapevine to cold by modulating carbohydrate metabolism. Molecular Plant-Microbe Interactions 25(4):496-504.

Fila, G., Ghashghaie, J., Hoarau, J., and Cornic, G. 1998. Photosynthesis, leaf conductance and water relations of in vitro cultured grapevine rootstock in relation to acclimatisation. Physiologia Plantarum. 102(3):411-418.

Foster, R. C. 1988. Microenvironments of soil microorganisms. Biology and Fertility of Soils. 6.3: 189-203.

Frommel, M. I., Nowak, J., and Lazarovits, G. 1991. Growth enhancement and developmental modifications of in vitro grown potato (*Solanum tuberosum* spp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiology. 96(3): 928-936.

Gepstein, S. and Glick, B. R. 2013. Strategies to ameliorate abiotic stress-induced plant senescence. Plant Molecular Biology. 82(6): 623-633.

Gray, E. J. and Smith D. L. 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biology and Biochemistry. 37(3): 395-412.

Grayston S.J., Wang S., Campbell C.D., Edwards A.C. 1988. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol. Biochem. 30:369-78.

Gutiérrez-Luna, F. M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H. R., and Macías-Rodríguez, L. 2010. Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis. 51(1): 75-83.

Hale, L., Luth, M., and Crowley, D. 2015. Biochar characteristics relate to its utility as an alternative soil inoculum carrier to peat and vermiculite. Soil Biology and Biochemistry. (81): 228-235.

Han H.S. and Lee K.D. 2005. Plant growth promoting rhizobacteria effect on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity. Res. J. Agric. Biol. Sci. 1(3): 210-215.

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology. 16(10): 463-471.

Hazarika, B. N. 2003. Acclimatization of tissue-cultured plants. Current Science-Bangalore. 85(12):1704-1712.

Hiltner, L.T. 1904. On recent experiences and problems in the field of soil bacteriology with particular regard to the founding and fallow. Work of the German Agricultural Society. 98: 59-78.

Hoagland, D.R. and D.I. Arnon. 1938. Growing plants without soil by the water-culture method. Circulation of California Agricultural Experimental Station. pp.16.

Hontzeas, N., Richardson, A. O., Belimov, A., Safronova, V., Abu-Omar, M. M., and Glick, B. R. 2005. Evidence for horizontal transfer of 1-aminocyclopropane-1-carboxylate deaminase genes. Applied and Environmental Microbiology. 71(11):

7556-7558.

Hurek, T., Handley, L. L., Reinhold-Hurek, B., and Piché, Y. 2002. Azoarcus grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Molecular Plant-Microbe Interactions. 15(3): 233-242.

Iniguez, A. L., Dong, Y., Carter, H. D., Ahmer, B. M., Stone, J. M., and Triplett, E. W. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. Molecular Plant-Microbe Interactions. 18(2): 169-178.

Kim, S., Lowman, S., Hou, G., Nowak, J., Flinn, B., and Mei, C. 2012. Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. Biotechnol. Biofuels. 5(37):1-10.

King, B. S., Ward M.K.W., and Roney D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301.

Kloepper, J. W., and Schroth, M. N. 1978. Plant growth-promoting rhizobacteria on radishes. In Proceedings of the 4th international conference on plant pathogenic bacteria. 1(2): 879-882).

Koskimäki, J., Hankala E. Suorsa M., and Nylund S. 2010. Mycobacteria are hidden endophytes in the shoots of rock plant [*Pogonatherum paniceum* (Lam.) Hack.](Poaceae). Environmental microbiology reports. 2.4: 619-624.

Kurepin, L.V., Park, J.M., Lazarovits, G. and Bernards, M.A. 2015. *Burkholderia phytofirmans*-induced shoot and root growth promotion is associated with endogenous changes in plant growth hormone levels. *Plant growth regulation*. *75*(1), pp.199-207.

Kurukulasuriya, P., and Rosenthal, S. 2013. Climate change and agriculture: A review of impacts and adaptations.

Kusari, P., Kusari, S., Spiteller, M., and Kayser, O. 2013. Endophytic Fungi Harbored in Cannabis Sativa L.: Diversity and Potential as Biocontrol Agents Against Host-Plant Specific Phytopathogens. Fungal Diversity. 60:137-151.

Latha, P., Anand, T., Ragupathi N., Prakasam V. and Samiyappan R. (2009). Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixtures of PGPR strains and Zimmu leaf extract against *Alternaria solani*. Biological Control 50.2: 85-93.

Lazarovits, G., and Nowak, J. 1997. Rhizobacteria for improvement of plant growth and establishment. HortScience. 32(2), 188-192.

Leigh, M.B. 2006. Methods for rhizoremediation research: Approaches to experimental design and microbial analysis. Phytoremediation Rhizoremediation. Springer Netherlands. 33-55.

Linda, A. 2014. Pseudomonad Fluorescent Preservation Using Tapioca and Rice Flour Carrier and the Addition of Glycerol Stabilizer. In Proceeding of International Conference On Research, Implementation And Education Of Mathematics And Sciences 2014. Yogyakarta State University. pp. 1-6.

Lotrario, J.B., Stuart, B.J., Lam, T., Arands, R.R., O'Connor, O.A. and Kosson, D.S. 1995. Effects of sterilization methods on the physical characteristics of soil: implications for sorption isotherm analyses. Bulletin of Environmental Contamination and Toxicology. 54(5):668-675.

Lugtenberg, B.J., Kravchenko, L.V. and Simons, M., 1999. Tomato seed and root

exudate sugars: composition, utilization by Pseudomonas biocontrol strains and role in rhizosphere colonization. Environmental Microbiology. 1(5), pp.439-446.

Luken, J.O. 2005. Characterizing Illegal Harvest of the Venus fly trap (*Dionaea muscipula* Ellis) at Lewis Ocean Bay Heritage Preserve, South Carolina, USA. Natural Areas Journal. 25(3):295-299.

Mahmoud, A. L. E., and Abd-Alla, M. H. 2001. Siderophore production by some microorganisms and their effect on *Bradyrhizobium*-Mung Bean symbiosis. Int. J. Agric. Biol. 3(2), 157-162.

Mattson Jr, W.J., 1980. Herbivory in relation to plant nitrogen content. Annual review of ecology and systematics. 11(1): 119-161.

Mauch-Mani, B. and Métraux, J. P. 1998. Salicylic acid and systemic acquired resistance to pathogen attack. Annals of Botany. 82(5): 535-540.

Mehnaz, S., Kowalik, T., Reynolds, B., and Lazarovits, G. 2010. Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions. Soil Biology and Biochemistry. 42(10):1848-1856.

Mitter B., Petric A., Shin M. W., and Chain P.S.G. 2013. Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. Frontiers in Plant Science. (4), p. 120

Malboobi M., Owlia P., Behbahani M., Sarokhani E., Moradi S., Yakhchali B., Deljou A., and Morabbi Heravi K. 2009. Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. World Journal of Microbiology and Biotechnology 25 (8): 1471–1477.

Moran, N. A. 2003. Tracing the evolution of gene loss in obligate bacterial symbionts. Current Opinion in Microbiology. 6(5):512-518.

Nadeem S.M., Zahir Z.A., and Naveed M. 2010. Microbial ACC-deaminase: prospects and applications for inducing salt tolerance in plants. Crit. Rev. Plant Sci. 29:360–393.

Najafi, A., Reza Arkadami, M., Rejali, F., and Sajedi, N. 2012. Response of winter barely to co-inoculation with *Azotobacter* and mycorrhiza fungi influenced by plant growth promoting rhizobacteria. Ann. Biol. Res. 3(8), 4002-4006.

Naveed, M., Qureshi, M. A., Zahir, Z. A., Hussain, M. B., Sessitsch, A., and Mitter, B. 2014. L-Tryptophan-dependent biosynthesis of indole-3-acetic acid (IAA) improves plant growth and colonization of maize by *Burkholderia phytofirmans* PsJN. Annals of Microbiology. 1-9.

Nascimento, F.X., Rossi, M.J., Soares, C.R., McConkey, B.J. and Glick, B.R. 2014. New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance. PLoS One 9(6):e99168.

Nowak, J. 1998. Benefits of in vitro "biotization" of plant tissue cultures with microbial inoculants. In Vitro Cellular and Developmental Biology-Plant. *34*(2), 122-130.

Nowak, J., Sharma V.K., and A'Hearn E. 2002. Endophyte enhancement of transplant performance in tomato, cucumber and sweet pepper. In XXVI International Horticultural Congress: Issues and Advances in Transplant Production and Stand Establishment Research. 631, pp. 253-263.

Nowak, J. and Shulaev, V. 2003. Priming for transplant stress resistance in in vitro propagation. In Vitro Cellular and Developmental Biology-Plant. 39(2): 107-124.

Newton, A.C., Fitt, B.D., Atkins, S.D., Walters, D.R., and Daniell, T.J. 2010. Pathogenesis, parasitism and mutualism in the trophic space of microbe–plant interactions. Trends in Microbiology. 18(8), 365-373.

Odell, R. E., DumLao, M. R., Samar, D., and Silk, W. K. 2008. Stage-dependent border cell and carbon flow from roots to rhizosphere. Am. J. Bot. 95, 441–446.

Okon, Y. and Labandera-Gonzalez, C. A. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry. 26(12), 1591-1601.

Onofre-Lemus, J., Hernández-Lucas, I., Girard, L. and Caballero-Mellado, J. 2009. ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. Applied and Environmental Microbiology. 75(20):6581-6590.

Orhan, E., Esitken, A., Ercisli, S., Turan, M., and Sahin, F. 2006. Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Scientia Horticulturae. 111(1), 38-43.

Perilli, S., Di Mambro, R., and Sabatini, S. 2012. Growth and development of the root apical meristem. Current Opinion in Plant Biology. 15(1), 17-23.

Pieterse, C., and Dicke, M. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology.Trends Plant Sci. 12, 564–569.

Pillay, V. and Nowak J. 1997. Inoculum density, temperature, and genotype effects on in vitro growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicon esculentum* L.) seedlings inoculated with a pseudomonad bacterium. Canadian Journal of Microbiology. 43.4: 354-361.

Poupin, M.J., Timmermann, T., Vega, A., Zuñiga, A. and González, B. 2013. Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS One 8(7).

Preece, J. and Sutter, E. 1991. Acclimatization of micropropagated plants to the greenhouse and field. Micropropagation 1:71-93

R Core Team. 2014. R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org/

Ramsay, A.J. and Bawden, A.D. 1983. Effects of sterilization and storage on respiration, nitrogen status and direct counts of soil bacteria using acridine orange. Soil Biology and Biochemistry. 15(3):263-268.

Raupach G.S. and Kloepper J.W. 2000. Biocontrol of cucumber diseases in the field by plant growth-promoting rhizobacteria with and without methyl bromide fumigation plant disease. Vol.84 (10), pp. 1073-1075.

Roberts, K. and Anderson, R. 2001. Effect of Garlic Mustard [*Alliaria petiolata* (Beib. Cavara and Grande)] Extracts on Plants and Arbuscular Mycorrhizal (AM) Fungi. The American Midland Naturalist 146(1):146-152.

Ryan P. and Delhaize E. 2001. Function and mechanism of organic anion exudation from plant roots. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:527–560.

Sanon, A., Andrianjaka, Z.N., Prin, Y., Bally, R., Thioulouse, J., Comte, G. and Duponnois, R. 2009. Rhizosphere microbiota interferes with plant-plant interactions. Plant and Soil 321(1-2):259-278.

Schnell, D., Catling, P., Folkerts, G., Frost, C., Gardner, R., et al. 2000. *Dionaea muscipula*. 2006. IUCN Red List of Threatened Species. IUCN 2006.

Senthilkumar, M., Madhaiyan, M., Sundaram, S.P., Sangeetha, H. and Kannaiyan, S., 2008. Induction of endophytic colonization in rice (*Oryza sativa* L.) tissue culture plants by *Azorhizobium caulinodans*. Biotechnology letters. 30(8): 1477-1487.

Sessitsch, A., Coenye, T., Sturz, A.V., Vandamme, P., Barka, E.A., Salles, J.F., Van Elsas, J.D., Faure, D., Reiter, B., Glick, B.R. and Wang-Pruski, G. 2005. *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. International Journal of Systematic and Evolutionary Microbiology 55(3):1187-1192.

Sharma S., Aneja M.K., Mayer J., Munch J.C., and Schloter M. 2005. Characterization of bacterial community structure in rhizosphere soil of grain legumes. Microbial Ecology 49:407-15.

Shrestha, M., Shrestha P., and Conrad, R. 2011. Bacterial and archaeal communities involved in the in situ degradation of 13C-13 labelled straw in the rice rhizosphere. Environmental microbiology reports. 3.5: 587-596.

Somasegaran, P. 1985. Inoculant production with diluted liquid cultures of *Rhizobium* spp. and autoclaved peat: evaluation of diluents, *Rhizobium* spp., peats, sterility requirements, storage, and plant effectiveness. Applied and Environmental Microbiology. 50(2):398-405.

Srivastava, P.S., Bharti, N., Pande, D. and Srivastava, S. 2002. Role of mycorrhiza in in vitro micropropagation of plants. *In* (Mukerji, K.G. ed.) Techniques in Mycorrhizal Studies. Springer, Netherlands. pp. 443-468.

Stone, J., Polishook, J., and White, J. 2004. Endophytic fungi. Biodiversity of fungi. Elsevier Academic Press, Burlington. pp. 241-270.

Stotzky, G. and Mortensen, J.L. 1959. Effect of gamma radiation on growth and metabolism of microorganisms in an organic soil. Soil Science Society of America Journal 23(2):125-127.

Sun, Y., Cheng, Z., and Glick, B. R. 2009. The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. FEMS Microbiology Letters, 296(1), 131-136.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. 2002. Agricultural sustainability and intensive production practices. Nature, 418(6898), 671-677.

Tittabutr, P., Teamthisong, K., Buranabanyat, B., Teaumroong, N., and Boonkerd, N. 2012. Gamma irradiation and autoclave sterilization peat and compost as the carrier for rhizobial inoculant production. Journal of Agricultural Science. 4(12):59.

Thomashow, L. S., Weller, D. M., and Hornby, D. 1990. Application of fluorescent pseudomonads to control root diseases of wheat and some mechanisms of disease suppression. *In* ed. Biological control of soil-borne plant pathogens. pp. 109-122. CAB International.

Thomashow, L. S., and Weller, D. M. 1996. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In Plant-microbe interactions. pp. 187-235. Springer US.

Wang, B. 2015. *Burkholderia phytofirmans* strain PsJN effects on drought resistance, physiological responses and growth of switchgrass.

Wang, B., Seiler, J.R. and Mei, C. 2015. *Burkholderia phytofirmans* strain PsJN advanced development and altered leaf level physiology of switchgrass. Biomass and Bioenergy 83:493-500.

Weilharter, A., Mitter, B., Shin, M. V., Chain, P. S., Nowak, J., and Sessitsch, A. 2011. Complete genome sequence of the plant growth-promoting endophyte *Burkholderia phytofirmans* strain PsJN. Journal of bacteriology. 193(13), 3383-3384.

Yan, J., Hao, W. and Shaodong, W. 2010. Application potential of microorganism fertilizer in agricultural production. Soybean Science and Technology, 5.

Yang, J., Kloepper J., and Ryu, C. M. 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science 14.1: 1-4.

Yardin, M.R., Kennedy, I.R. and Thies, J.E. 2000. Development of high quality carrier materials for field delivery of key microorganisms used as bio-fertilisers and bio-pesticides. Radiation Physics and Chemistry, 57(3), pp.565-568.

Yi, H. S., Yang, J. W., and Ryu, C. M. 2013. ISR meets SAR outside: additive action of the endophyte Bacillus pumilus INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper. Frontiers in plant science, 4, 122.

Ziv, M., 1995. In vitro acclimatization. *In* (Mukerji, K.G. ed.) Automation and environmental control in plant tissue culture. Springer, Netherlands. pp. 493-516.