Isolation of Mimosine-Degrading Endophytic Bacteria from the Invasive Plant: *Leucaena leucocephala*

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ABSTRACT

Leucaena leucocephala is an invasive plant in the state of Hawaii and other Pacific regions. It was listed as one of the 100 worst invasive alien species in the world (Lowe *et al*. 2000). One of the reasons for its invasiveness is its ability to produce a toxic chemical called mimosine. β-*N*-(3-hydroxy-4-pyridone)-α-aminopropionic acid, a non-protein forming amino acid that acts as a strong iron chelator which inhibits growth of some rhizobacteria (Fox and Borthakur, 2001). Mimosine is also an allelochemical, where it negatively affects the growth of surrounding plants (Xuan *et al*. 2006). However, there are currently three bacterial strains (TAL 1145, *Pseudomonas sp.* STM 905 and *Synergistes jonesii*) that are capable of degrading mimosine to its intermediate form 3-hydroxy-4-pyridone (HP) (Soedarjo *et al.* 1994, Awaya *et al*. 2005). Mimosine degrading capabilities has shown to be an advantageous trait as microorganism capable of doing so are able to use mimosine as a carbon/nitrogen source (Soedarjo *et al*. 1994). Bacterial endophytes have been recently recognized as major contributors to plant growth promotion, health, stress tolerance and preventing plant pathogens. Perhaps there are bacterial endophytes that are benefiting Leucaena and aiding it in its invasiveness. The main objectives of this study were to: 1) Isolate endophytic bacteria from the shoot tips of *Leucaena leucocephala* and identify them using 16S rRNA sequencing; and,- 2) determine if isolates were capable of degrading mimosine. After surface sterilization of shoot samples, eight total endophytic bacterial isolates (SH01-SH08) were cultured in 869 agar. Three of the eight isolates (SH05, SH07 and SH08) were able to degrade mimosine after monitoring their growth in AB minimal broth with 3 mM mimosine and 1 mM $FeCl_3$. Those three isolates are all members of the genus *Pseudomonas*. Considering that the young shoots of Leucaena contain the highest

concentration of mimosine, between 4-10% (Jones, 1979), this means isolates were able to tolerate that specialized niche and translocate from the soil up to the aerial parts of the plant.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………………………………………………..ii

ABSTRACT……………………………………………………………………………..iii

TABLE OF CONTENTS…………………………………………………...………...v

LIST OF TABLES………………………………………………………………………vi

LIST OF FIGURES……………………………………………………………………vii

CHAPTER 1.......………………….…...……………………………………….………......1

INTRODUCTION……………………………………………………………………...1

Leucaena leucocephala and its invasiveness……..……………………………………......1

Mimosine and allelopathy….……………………………………………………………...2

Endophytic bacteria………………………………………………………………….....3

Beneficial properties of bacterial endophytes.....………………………………………6

Mimosine degradation …………………………………………………………………...7

CHAPTER 2.......……………...………......................……………………………………10

MATERIALS AND METHODS..……………...……………..…………………………..…...10

Collection of plant tissue samples...10

Surface sterilization and culturing endophytes...10

16S rDNA extraction and sequencing..11

NCBI BLAST...12

Phylogenetic analysis..12

Growth of bacterial isolates in media containing mimosine and iron..............................13

CHAPTER 3..**14**

RESULTS..14

Characterization of endophytic bacteria...14

NCBI BLAST..15

Phylogenetic analysis..16

PCR Screen for mimosine degrading genes..17

Growth of Bacterial isolates in media containing mimosine and iron.............................21

CHAPTER 4..**22**

GENERAL DISCUSSION...22

LITERATURE CITED...**26**

LIST OF TABLES

Table 1. Primer sets…………………………………………………………………………....12

Table 2. Bacterial genes……………………………………………………………………….12

Table 3. NCBI Genbank 16S rRNA sequences used in Phylogenetic analysis………………….13

Table 4. Bacterial Isolate closest NCBI GenBank BLAST neighbor……………………………16

LIST OF FIGURES

Figure 1. Bacterial Isolates grown on 869 media containing 2mM mimosine…………………..15

Figure 2. Neighbor-joining tree of Bacterial isolates…………………………………………17

Figure 3. PCR of *MidD* genes…………………………………………………………18

Figure 4. PCR of *PydA* genes……………………………………………………….....18

Figure 5. Growth of bacterial isolates in media containing iron-mimosine complex…………....20

Figure 6. Mimosine concentration from bacterial isolates after 48 hours of incubation……......20

Figure 7. Growth of Bacterial isolates in 3mM mimosine and 1 mM $FeCl_3$…………………....21

CHAPTER 1

INTRODUCTION

Leucaena leucocephala and its Invasiveness

Hawai'i is among the most isolated island archipelagos in the world, which unfortunately makes it susceptible to exotic species invasions. Since the native biota is strongly endemic and vulnerable to disturbances, the chance for recovery is low (Vitousek 1990). For instance, *Leucaena leucocephala*, a member of the Mimosoideae family, is a leguminous tree/shrub that can grow rapidly in arid climates (Shelton and Brewbaker, 1994). It is originally from Central America and the Yucatan Peninsula of Mexico (Shelton and Brewbaker, 1998; Brewbaker *et al*. 1985), but has spread to Hawaii, Mariana Islands, southern Texas, southern Florida, Puerto Rico and the Virgin Islands. (Little and Skolmen, 1989). Leucaena was listed as one of the 100 worst invasive alien species in the world (Lowe *et al*. 2000). This plant was brought to tropical regions because of its agricultural uses such as cattle fodder, reforestation, biofuels, and mitigating soil erosion which makes it a 'conflict species' (Tuda et al. 2009; Elharith et al. 1980; Raghu et al. 2005, Olckers, 2011). One reason for its invasiveness is its high seed production. Each tree produces roughly 1,700 pods (Raghu *et al.* 2005), each pod containing approximately 20 seeds and there are 2-4 podding cycles per year (Raghu *et al*. 2005, Tuda *et al*. 2009). Seeds can be easily dispersed by rodents, birds or cattle into adjacent areas. *L. leucocephala* is also a fast grower, with sprouts capable of ~ 30 cm growth in a single month under optimal conditions (Kuo *et al*. 2005). Once sprouts reach 2-3 m; they can escape browsing which reduces herbivory on the species.

Mimosine and Allelopathy

One of the strongest defensive mechanisms for *L. leucocephala* is its ability to produce mimosine. β-*N*-(3-hydroxy-4-pyridone)-α-aminopropionic acid, a toxic, non-protein forming amino acid (Brewbaker and Hylin, 1965). Structurally, it is similar to dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxy-phenyl ring (Xuan *et al.* 2006).

In animals, ingestion can result in alopecia, growth retardation, cataracts and infertility (Crounse *et al*. 1962). Other symptoms include raw coronary bands, low thyroxin level and goiter (Hegarty, 1967). The presence of -OH and -O in the pyridine ring suppresses iron-containing enzymes, which is what makes mimosine toxic (Vickery *et al*. 1981). Leucaena has a relatively high protein content in leaves, about 26.14% and in seeds for about 33.44% (Von *et al.* 1985) making it an ideal food source for livestock. However, mimosine is most concentrated in the "growing tips" of Leucaena (4-10%) which prevents herbivory (Jones, 1979, Brewbaker and Hylin, 1965). Mimosine possesses antimitotic activity which impedes the cell cycle in the G1 phase and it also disrupts DNA synthesis by changing the deoxyribonucleotide metabolism, which prevents the formation of the DNA replication fork. (Gilbert *et al*. 1995; Tsvetkov *et al*. 1997). The free amino acid also acts as an antifungal agent (Tawata *et al*. 2008), and the growth of certain root nodule bacteria is inhibited by mimosine (Fox and Borthakur, 2001). Most importantly, mimosine acts as an iron chelator which allows it to block the biochemical activities of the cell through iron depletion (Katoh *et al*. 1992; Gilbert *et al*. 1995; Soedarjo and Borthakur 1998).

Mimosine can have adverse effects on other plants as an allelochemical. Allelopathy is the direct or indirect effects that a donor plant has towards other surrounding plants through excretion of a chemical compound (Rice 1984, Mishra et al. 2013), can be positive or negative

towards the target plants (Ishak and Sahid 2014). Leucaena begins its allelopathy by producing mimosine which is then leached out from the roots and plant tissues and directly absorbed by neighboring plants (Ishak and Sahid 2014). Mimosine has been shown to significantly contribute to weed reduction of *L. glauca* in paddy fields (Hong, 2004), and also been shown to inhibit seedlings of mung bean, lettuce, wheat and rice (Smith and Fowden, 1966, Ling *et al*. 1969, Xuan *et al*. 2006, Chou and Kuo, 1986). Because mimosine possesses strong allelopathic effects, it has potential commercial uses as a bioherbicide compound (Xuan 2006, Ishak and Sahid 2014).

Endophytic Bacteria

Legumes are agriculturally important plants because of their nitrogen-fixing capabilities. They can form root nodules in symbiosis with nitrogen-fixing bacteria to improve the plant's overall growth. Because of this importance, the rhizobia-legume symbiosis has been heavily studied (Wang *et al.* 2006, Mora *et al*. 2014). However, endophytic bacteria may contribute to plant growth and health as much, if not more than rhizobia bacteria in legumes (Dudeja et al. 2012). Endophytes are microorganisms residing in the plant tissue which cause no visible harm to the host plant (Hallmann et al. 1997, Wang et al. 2006). It is also defined as microorganisms that could be isolated from surface-sterilized plant organs (Hardoim *et al*. 2008) All plant species contain at least one or more endophytes (Strobel et al. 2004), but the presence of endophytes in all tissues is highly dependent on the soil microflora (Dudeja et al. 2012). It begins with endophytes originating from the soil as the plant is growing. Thus, bacterial communities within the endosphere of the host plant are likely dictated by stochastic events which are tied in with deterministic processes of colonization (Battin, *et al*. 2007). It can be said that the probability of endophytic colonization depends on the initial abundance, diversity, physiological status and

distribution of endophytes in the soil. Other factors such as plant genotype, growth stage, type of plant tissue and environmental soil conditions can also determine endophytic colonization. The initial steps required in endophytic colonization are similar to that of rhizobacteria and plant pathogens (Hallman *et al*, 1997). For example, well studied root-colonizing bacteria from the genera *Pseudomonas*, *Azospirillum* and *Bacillus* are often found as colonizers of the internal tissue of plants (Rosenblueth and Martinez-Romero, 2006, Hallman and Beng, 2006). However, it is assumed that endophytes are specialized members of these groups when accepted into plant tissue. There are several environmental and genetic factors that are presumed to have a role in allowing a specific bacterium to become endophytic (Hardoim *et al*. 2008). The endosphere region offers protection from the environment for outside bacteria that can colonize and establish *in planta* (Ryan et al. 2007). The intercellular spaces provide endophytes with a significant advantage over outside bacteria in the rhizosphere and phyllosphere because of stable pH levels, moisture, rich nutrients and lack of competition from large number of microorganisms (Backman and Sikora, 2008, Chebotar et al. 2015). Bacterial endophytes have been isolated from all plant compartments, including seeds (Posada and Vega, 2005, Ryan, et al. 2007.)

How bacterial endophytes colonize regions above ground such as seeds and vegetative plant parts is an active area of study. Endophytes are known to systematically spread throughout the plant and colonize the stems and leaves through use of the lumen of xylem vessels (Hardoim *et al*. 2008, Compant *et al*. 2005). But, it is unclear whether endophytes colonizing the roots or aerial plant tissues have different effects on the plant. It is now confirmed that plant growth promoting bacteria can migrate from one xylem compartment to another through the perforated plates (Compant *et al.* 2010) Endophytes can also travel through the plant by use of the plant transpiration stream. It is less likely for endophytes to travel through intercellular regions

because that would require cell wall degrading enzymes. It is important to note that only a few endophytes are able to colonize aerial vegetative plant parts (Hallman, 2001) because they have to pass multiple barriers as well as be physiologically capable to establish in different plant niches (Compant *et al*. 2010). Endosymbiont cultivable population densities can reach up to 10^3-10^4 CFU g^{-1} of fresh weight under normal conditions (Compant *et al*. 2010).

Our understanding of endophytic microbial communities is being accelerated through application of molecular techniques, e.g., 16S rRNA gene cloning, terminal restriction fragment polymorphisms (RFLP) and sequencing (Loh et al. 2013, Franks et al. 2006). Molecular advancements allow scientists to look into diverse microbial communities without necessarily having to culture them. The five endophytic taxa showing the most promise for colonization and an ability to persist were identified as *Cellulomonas, Clavibacter, Curtobacterium, Pseudomonas* and *Microbacterium* by 16S rRNA gene sequence, carbon source uptake analyses and other methods (Zinniel et al. 2002, Elvira-Recuenco & van Vuurde 2000). Recent studies conducted on bacterial endophytes in Leucaena (Nimnoi, P & Pongsilp, N. 2009; Maruya, J & Saeki, K. 2010) have successfully identified *Mesorhizobium loti*, *Ensifer (Sinorhizobium) meliloti, Rhizobium* sp. NGR 234, *R. tropici* and *Ensifer (Sinorhizobium) meliloti* 1021, all of which were isolated from Leucaena roots.

Our current knowledge of endophyte-host molecular interactions has developed over the past decade (Lugtenberg et al. 2002). For example, autofluorescent protein (AFP) methods are a key tool for studying the processes of microbe-plant interactions and biofilm formation (Ryan et al. 2007). Artificial inoculation of plants with bacterial endophyte isolates shows us how these bacteria colonize the endosphere and how they interact with the inoculated plants (Chebotar et al. 2014). There is also *in vivo* analysis of biological material for research on the molecular changes

of plant endospores and the spatial distribution of microorganisms over the course of the development of the plant. A common marker system uses green fluorescent protein (GFP), which allows the detection and counting of microorganisms *in situ* on plant surfaces and *in planta* (Gage et al. 1996, Tombolini et al. 1997, Tombolini & Jansson, 1998.) The host plant gene expression may be regulated by the type of bacteria endophytes.

Beneficial properties of Bacterial Endophytes

Bacterial endophytes have been shown to improve the overall health of their host plant. Plant wellness is aided by: improved nitrogen and phosphorus nutrition to host plant, regulation of osmotic pressure, modified root development, accelerated seedling emergence, promote plant recruitment under extreme climate conditions and synthesis of vitamins and siderophores (Rosenblueth & Martinez-Romero, 2006, Chebotar et al. 2015). Bacterial endophytes can reduce or prevent the deleterious effects of pathogenic microorganisms through a variety of mechanisms, including pathogenesis, production of various compounds and out-competing the pathogens within the endosphere (Whipps, 2001). A variety of bacterial endophytes that show superior antagonistic activities against plant pathogens has led to artificial inoculation (biocontrol activity) of plants with endophytic bacteria. For example, 9 out of 137 isolates of bacterial endophytes isolated from the tissues of stems, roots and nodules of soybean in vitro inhibited the growth of the pathogenic fungi *Macrophomina phaseolina, Fusarium udum, Rhizoctonia bataticola* and *Sclerotium rolfsii* (Senthikumar et al. 2009). Bacterial endophytes, particularly the ones from the genera *Pseudomonas, Burkholderia,* and *Bacillus* are known for producing secondary metabolites such as antibiotics, anticancer drugs, volatile organic compounds, and fungicidal, insecticidal and immunosuppressive agents (Chebotar et al. 2015).

Some key properties of endophytes are their ability to promote plant growth through signal inductions of hormones (López-López et al. 2010*)*. Ethylene is a phytohormone that controls plant growth and development, and has a central role in plant cellular metabolism (Ping and Boland, 2004). Ethylene is also tied in with the plant developmental cycle, disease resistance and microbe-plant interactions. It is a key regulator of the colonization of plant tissue by bacteria, which are able to control plant ethylene levels by two mechanisms: first by cleaving the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Glick *et al*. 2007) or by inhibiting ACC synthase or β-cystathionase, both enzymes are involved in the ethylene biosynthesis pathway (Sugawara, M, *et al*. 2007) Thus, bacterial endophytes with high locally induced ACC deaminase activities could be ideal plant-growth promoters because they can reduce plant stress by blocking ethylene production (Cheng *et al*. 2007).

Indole-3-acetic acid (IAA) is the main plant growth hormone with auxin activity (Davies, 1995). IAA is responsible for several physiological processes in plants such as root proliferation, cell division and shoot growth (Lambrecht *et al*. 2000, Davies, 1995). IAA production is known to be involved in processes of plant-growth promoting bacteria and symbiotic bacteria (Hunter, 1989; Lambrecht *et al*. 2000). One example is *Pseudomonas putida* GR12-2 produces IAA which stimulates the growth of the roots of canola seedlings (Patten and Glick, 2002).

Mimosine Degradation

Mimosine is antagonistic to a variety of plants and weeds. However, some bacterial strains (*Rhizobium sp.*TAL1145, *Pseudomonas sp.* STM 905 and *Synergistes jonesii*) can enzymatically break down mimosine into the intermediate: 3-hydroxy-4-pyridone (HP) and use it as a carbon/nitrogen source (Soedarjo *et al.* 1994, Awaya *et al*. 2005, Pandey & Dwivedi, 2006, Allison *et al*. 1992). There are specific genes (*mid*) that are required by the rhizobium strain TAL

1145 to degrade mimosine into HP (Borthakur *et al*. 2003). The *mid* genes are located within a 12.6-kb fragment of the TAL1145 chromosome. Two important steps are required to degrade mimosine. In the first step, the alanyl side chain is hydrolyzed, forming HP. The next step involves the expression of *pydA* and *pydB* genes respectively encoding for dioxygenase and hydrolase enzymes that further degrade mimosine into pyruvate, formate, and ammonia. The *pydA* gene is required for the cleavage reaction, opening up the aromatic ring structure of HP (Awaya *et al*. 2007). The *pydB* gene is used to catabolize the molecule into metabolically useful pyruvate (Awaya *et al*. 2007). The necessary proteins are located in the cytoplasm of TAL1145 (Soedarjo *et al*. 1994) Mimosine can be used as an exogenous siderophore by the rhizobial bacterium TAL1145, giving it advantageous characteristics over other nodulating *Rhizobium* (Soedarjo and Borthakur, 1998). The mechanism for degrading an allelochemical such as mimosine is through chemotaxis where bacteria use the allelochemical as a nutrient source. (Lugtenberg et al. 1999). It is believed that *L. leucocephala* originally evolved in regions with iron-deficient soils. Mimosine released from leucaena chelates to iron, forming Fe-mimosine complexes which are then taken up by Mid^{+} rhizosphere bacteria and converted to a form available to the plant (Khanna and Lavin 1993, Gilbert *et al*. 1995)

Microbes outside of the rhizosphere can degrade mimosine and other pyridine ring compounds (Allison et al. 1992, Watson et al. 1974). If there are endophytes that can degrade mimosine, this may help explain its plant growth promoting capabilities towards Leucaena. Overall, there is still very little research on endophytes within *L. leucocephala* in regions above the root system. Perhaps mimosine degrading bacteria inhabit the regions where mimosine is heavily concentrated such as the young shoots and stems. A better understanding of endophyte ecology and their molecular interactions can open up a near-future application of using

genetically engineered endophytes as a bio-control in some main crops. A promising potential for endophytes in agricultural uses is to increase crop yields, remove harmful chemicals, inhibit pathogens, and produce novel substances. The full effects and functions in plants are still not completely defined, thus this research field could contribute to economic and environmental impacts.

CHAPTER TWO

MATERIALS AND METHODS

Collection of Plant Tissue Samples

Fresh, young Leucaena shoot clippings and seed pods were aseptically bagged and collected from Pahoa-Kalapana Road (19°22'11.9"N 154°57'54.0"W) on June 23rd and July 15th 2016. Samples were immediately placed on ice for preservation and transported to the laboratory within 2 hours. Leucaena shoot samples were weighed and rinsed with tap water to remove particulates. Seeds were cleaned and placed in sterile falcon tubes for long-term storage.

Surface Sterilization and Culturing Endophytes

Fresh shoot samples (~1 g) were submerged in 5% sodium hypochlorite for 10 minutes, transferred into 70% ethanol for 2 minutes and then rinsed 3 times with sterile distilled water. A 100 ul aliquot of the third rinsate was plated on 869 minimal media petri dishes to check for contamination. Samples were done in duplicates. There was no sign of microbial growth on the control plates throughout the experiment.

Surface sterilized tissue samples were macerated in a sterile mortar with the addition of 5 mL of sterile water. Two 100 ul aliquots were plated on 869 media and placed in an incubator at 28℃ for 4 days. 869 minimal media was used for this experiment because it has been shown to have the best optimal growth for bacterial endophytes (Eevers et al. 2015). The media contained the following components per liter of deionized water: 0.035 g $CaCl_2.2H_2O$, 0.100 g Glucose D_+. 0.500 g NaCl, 1.0 g Tryptone, 0.500 g Yeast Extract, 15.0 g Agar. Successful endophytes were streak plated for single colonies and the isolates were stored in 40% glycerol at -80℃ for long term storage. The isolates were then grown in 869 liquid broth and in AB minimal media to detect mimosine degrading capabilities. AB minimal media contained the following components

per liter of deionized water: 1.5 g KH_2PO_4, 0.5 g NaH_2PO_4, 0.15 g $MgSO4 \cdot 7H_2O$, 0.075 g KCl, 0.005 g $CaCl_2$, 0.00125 g $FeSO_4 \cdot 7H_2O$ and 10 mL of 40 mg/mL mimosine stock solution (Soedarjo et al. 1994) for a final mimosine concentration of 3 mM.

16S rDNA Extraction and Sequencing

Bacterial DNA was extracted from overnight cultures of isolates using the MO BIO UltraClean Microbial DNA Isolation Kit. DNA quality and quantity was analyzed using a NanoDrop 1000 Spectrophotometer. The 16s, MidD and PydA primer sets (Table 1) were obtained from Dr. Jonathan D. Awaya. Polymerase Chain Reaction (PCR) was applied to detect mimosine degrading genes and for amplification of the 16S region for bacterial identification (Table 2). Promega GoTaq Green Master Mix was used for our PCR reactions. We followed the manufacturer's instructions (Part# 9PIM712) and standard protocol (Sambrook and Russell, 2001). The Eppendorf Mastercycler Pro with Vapo.protect thermocycler was set to the following cycling condition: 15 mins at 94ºC for 1 cycle; 1 min and 30 sec at 94ºC, 1 min at 52ºC, 1 min at 72ºCfor 30 cycles; 10 min at 10ºC for 1 cycle; and held at 4ºC. All 16S PCR products were verified for successful amplification by running gel electrophoresis using 1% agarose gel under 90 volts for 40 mins using Promega BenchTop 1kb ladder. PCR clean-up was performed using Promega Wizard SV Gel and PCR Clean-Up system. Clean PCR products were sent to the University of Hawaii at Hilo’s Genetics Core Lab for sequencing.

Table 1: Primer sets used in this study.

Gene	Primer Sequence	Annealing Temperature	Expected Product Size
16S	F 5' -AGA GTT TGA TCC TGG CTC AG-3' R 5' -GGG TTG CGC TCG TTG CGG G-3'	60ºC	1,000 bp
MidD	F 5' -CAT CTA CCC CTC CAT HCH AC-3' R 5'-CGT GCC GAT ATT CAG ACG GA-3'	60 ºC	800 bp
PydA	F 5' -ACG CCC TGA CTT GAT CAT CG-3' R 5' -ATC ACG GCC ATC CAG TTG AG-3'	55 ºC	800 bp

Table 2: Bacterial genes examined in this study.

Type	Gene	Description	Organism	Reference
House Keeping	*16S*	Small ribosomal subunit	Bacteria	--
Mimosine Degradation	*midD*	Primary step in degradation of mimosine to HP	*Rhizobium* TAL1145	Borthakur *et al.* 2003
	pydA	Primary step in degradation of HP to Formate and Pyruvate	*Rhizobium* TAL1145	Awaya *et al.* 2005

NCBI BLAST

Eight bacterial gene sequences were subjected to Basic Local Alignment Search Tool (BLAST) in order toidentify highly similar sequences reposited in GenBank at National Center for Biotechnology Information (NCBI). Max target sequence was set to 10.

Phylogenetic analysis

A neighbor-joining (NJ) tree (Saitou and Nei, 1987) was constructed for bacterial isolates SH05, SH07 and SH08 compared to several *Pseudomonas* species (Table 3). Sequences were

aligned using MAFFT alignment program with default settings. Five hundred bootstrap replications were used to evaluate support for tree topology.

Table 3. NCBI Genbank 16S rRNA sequences used in Phylogenetic analysis

NCBI GenBank Accession #	Description	Reference
NR074804	*Cellvibrio japonicus* (Outgroup)	Deboy *et al*. 2008
KY024584	*Pseudomonas aeruginosa*	Joseph *et al*. 2016
KX817232	*Pseudomonas fluorescens*	Zhu and Guo 2016
KY021743	*Pseudomonas mendocina*	Ding *et al*. 2016
KY021742	*Pseudomonas oleovorans*	Ding *et al.* 2016
LC191549	*Pseudomonas oryzihabitans*	Tani and Hamba 2016
KU550194	*Pseudomonas psychrotolerans*	NA*
KX817239	*Pseudomonas putida*	Zhu and Guo 2016
KU904408	*Pseudomonas stutzeri*	Wang *et al*. 2016

* Not Applicable

Growth of bacterial isolates in media containing mimosine and iron

Bacterial isolates were grown in 15 mL of AB minimal liquid culture containing 3mM mimosine and 1mM $FeCl_3$ in a shaker at 28ºC. Each growth rate treatment was done in triplicate. Optical density of bacteria was measured using a Genesys 20 spectrophotometer at 600 nm. 1 mL samples were removed every 8 hours for 48 until cultures reached stationary growth phase. Final mimosine content for bacterial cultures were observed by measuring absorbance of 1 mL samples at 535 nm.

CHAPTER THREE

RESULTS

Characterization of endophytic bacteria

A total of 8 isolates grew on the 869 media from the young shoot tissue of *Leucaena*. Control plates showed no growth from the rinsate which confirms that the isolates are endophytic. Initial growth of endophytes seemed to occur around 48 hours. All isolates seem to grow well at 28ºC. Isolate SHO1 was distinguishable from the others because of its orange-red color (Figure 5). It was also more difficult to form single colonies as it was a smoother, liquid form on 869 media agar. SH02 produced a yellow opaque color. SH03 had a creamy dull white color and smooth forming colonies. SH04 was bright yellow and creamy. SH05 was an opaque white color. SH06 was also bright yellow like SH04. SH07 and SH08 were very similar in morphology. Both had a translucent yellow color.

There was no successful bacterial growth from the seeds despite making adjustments to the culturing media, surface sterilization methods and incubation time.

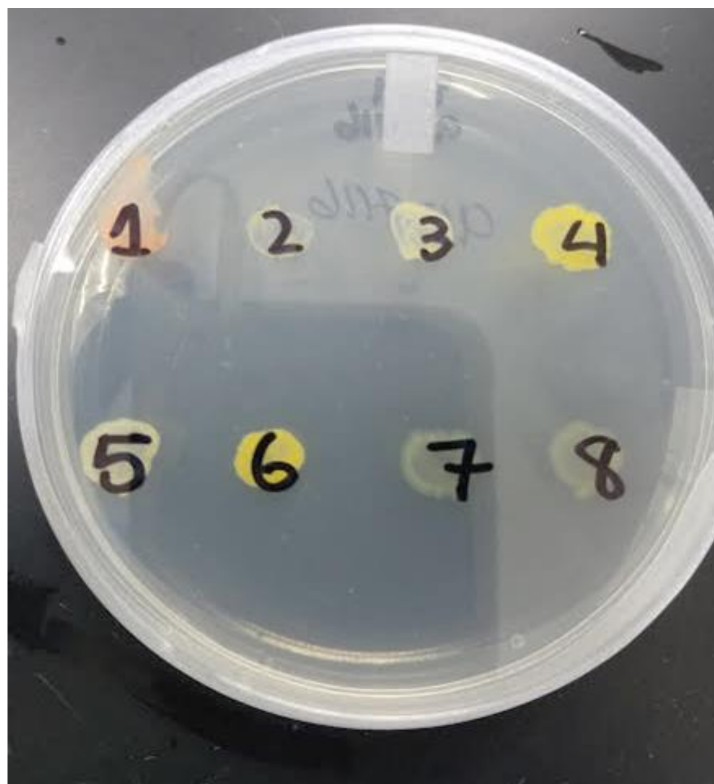

Figure 1. Bacterial Isolates (SHO1-SHO8) grown on 869 media containing 2mM mimosine.

NCBI BLAST

All 8 bacterial isolates had positive matches to 16S sequence BLAST results (Table 5). Isolate SH01 whose closest BLAST neighbor was *Rhodococcus kroppenstedtii* from phylum Actinobacteria, isolate SHO2 whose closest BLAST neighbor was *Sphingomonas paucimobilis* from the phylum Proteobacteria, isolate SH03 whose closest BLAST neighbor was *Microbacterium proteolyticum* from the phylum Actinobacteria, isolate SH04 whose closest BLAST neighbor was *Sphingomonas pseudosanguinis* from the phylum Proteobacteria, isolate SH05 whose closest BLAST neighbor was *Pseudomonas putida* from the phylum Proteobacteria, isolate SH06 whose closest BLAST neighbor was *Sphingomonas pseudosanguinis*, isolate SH07 whose closest BLAST neighbor was *Pseudomonas oryzihabitans* and isolate SH08 whose closest BLAST neighbor was *Pseudomonas oryzihabitans*.

Table 4. Bacterial Isolate closest NCBI GenBank BLAST neighbor and accession number.

Cultured Isolate ID	NCBI BLAST Closest Neighbor Description	Percent Identification	Closest Neighbor Accession #
SH01	*Rhodococcus kropspenstedtii*	98	JN873342
SHO2	*Sphingomonas paucimobilis*	97	NR104893
SHO3	*Microbacterium proteolyticum*	96	NR135869
SHO4	*Sphingomonas pseudosanguinis*	98	NR042578
SHO5	*Pseudomonas putida*	96	NR040860
SHO6	*Sphingomonas pseudosanguinis*	97	NR042578
SH07	*Pseudomonas oryzihabitans*	98	NR114041
SH08	*Pseudomonas oryzihabitans*	97	NR114041

Phylogenetic Analysis

The NJ tree showed strong bootstrap values for SH07 and SH08 being sister taxa along with being in the same clade with *Pseudomonas oryzihabitans*. Bootstrap values were moderate for the grouping of SH05 with *Pseudomonas putida*.

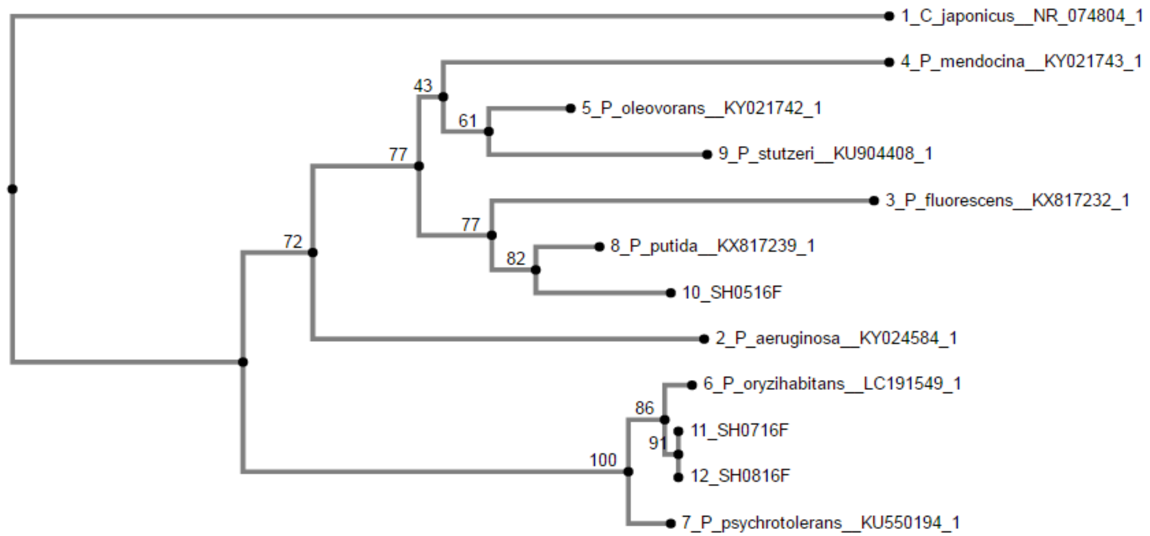

Figure 2. Neighbor-joining tree of Bacterial isolates: SH05, SH07 and SH08 compared to several *Pseudomonas* species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.The evolutionary distances were computed using the Jukes–Cantor method (Jukes and Cantor, 1969). *C. japonicus* was the outgroup.

PCR Screen for mimosine degrading genes

None of the 8 isolates showed any amplification of *midD* or *pydA* genes. Lane 1 served as a negative control and lane 2 was a positive control using DNA from the mimosine degrading bacteria: TAL1145. *MidD* and *pydA* primers were set to amplify ~800bp length fragments.

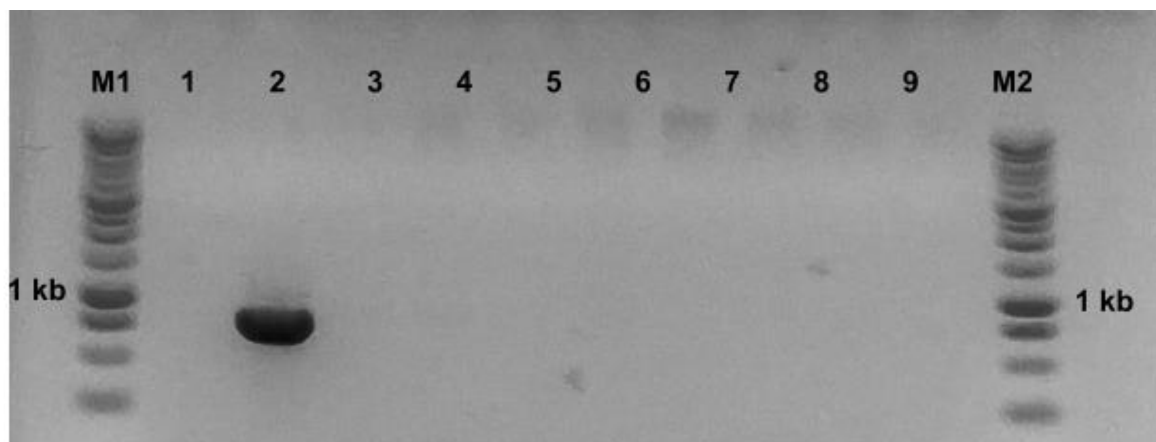

Figure 3. PCR of *MidD* genes. PCR analysis of DNA from all 8 isolates with primers used to amplify within *MidD*. M1 and M2 are 1.0 kb markers. Lane 1 is a *MidD* negative control. Lane 2 is a positive control using DNA from the rhizobium TAL1145. Lane 3-9 contains PCR products from isolates SH01-SH08 respectively.

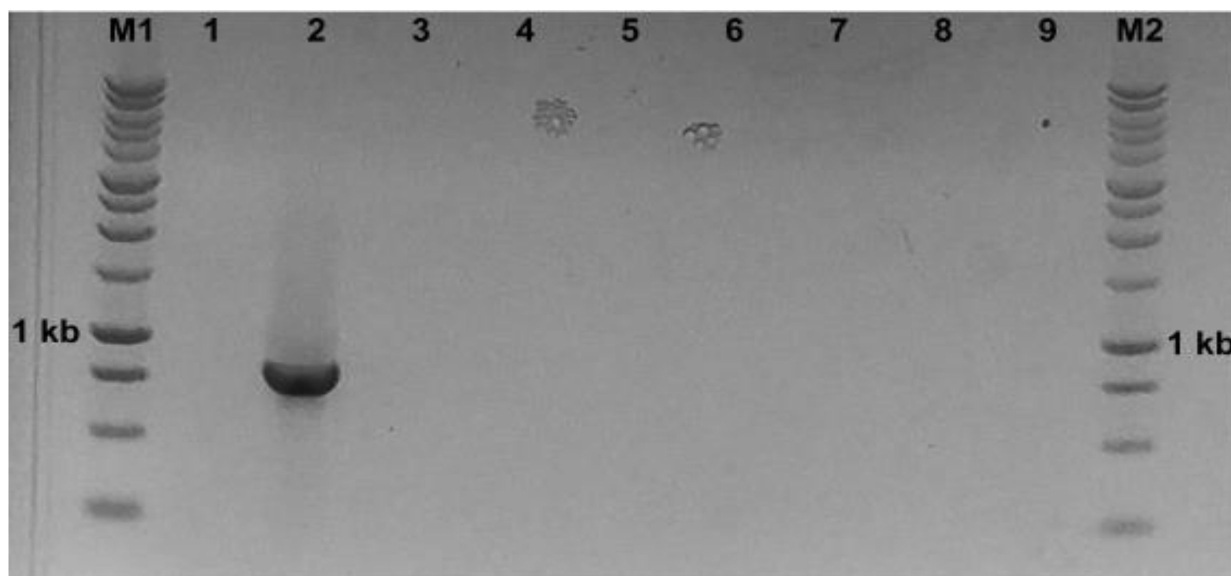

Figure 4. PCR of *PydA* genes. PCR analysis of DNA from all 8 isolates with primers used to amplify within *PydA*. M1 and M2 are 1.0 kb markers. Lane 1 is a *PydA* negative control. Lane 2 is a positive control using DNA from the rhizobium TAL1145. Lane 3-9 contains PCR products from isolates SH01-SH08 respectively.

Growth of Bacterial isolates in media containing mimosine and iron

Bacterial isolates SH05, SH07 and SH08 were able to grow in AB minimal media with 3mM mimosine and 1mM $FeCl_3$. Bacterial cultures for the three isolates seemed to grow exponentially around the 16 hour mark and reach the stationary phase around 32 hours. Isolate SH05 had a greater absorbance (~0.3) than SH07 (~0.24) and SH08 (~0.21), however, there is high variability due to minimal growth in the first replication. SH07 and SH08 seemed to have identical absorbance throughout the trial.

Mimosine content was measured at 56 hours. There was a difference in absorbance with isolates SH01, SH02, SH03, SH04 and SH06 having higher absorbance than SH05, SH07 and SH08. Normal absorbance for AB minimal media with 3 mM mimosine and 1 mM $FeCl_3$ was roughly 1.74. Isolates SH07 and SH08 had an absorbance below 1 which translates to mimosine degradation. Figure 11 shows a change in media color from dark red to a light yellow/orange color. This occurs when the mimosine-iron complex structure is cleaved.

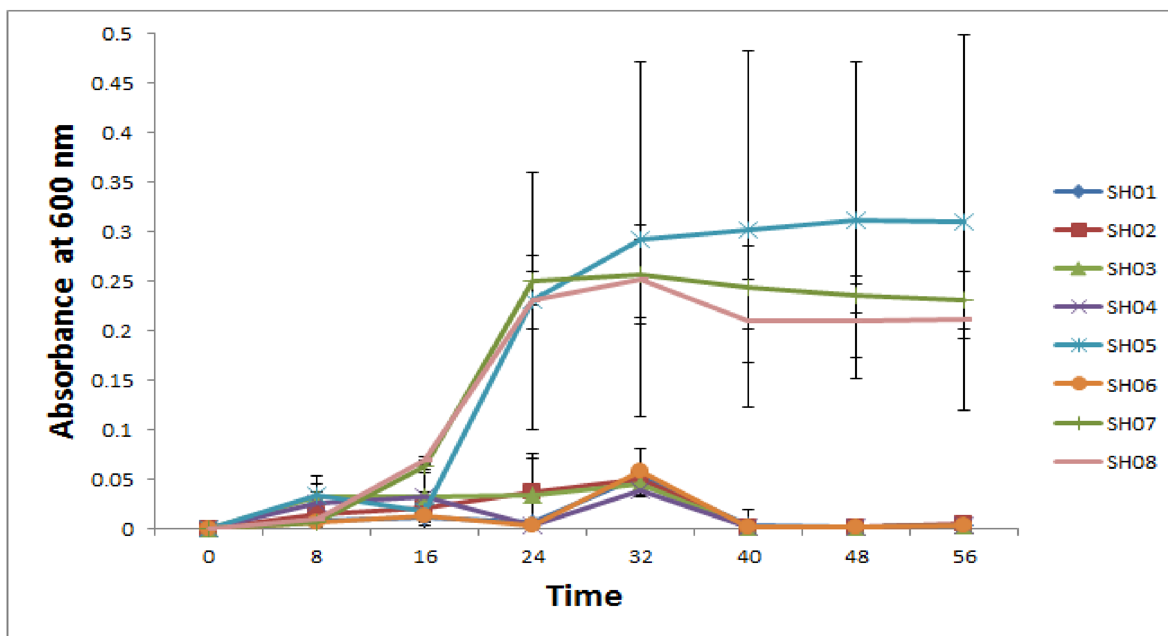

Figure 5. Growth of bacterial isolates in media containing iron-mimosine complex. Isolates were grown in AB minimal broth containing 3 mM mimosine and 1 mM $FeCl_3$. The data points in the growth curve are the means and standard deviations of three replicates.

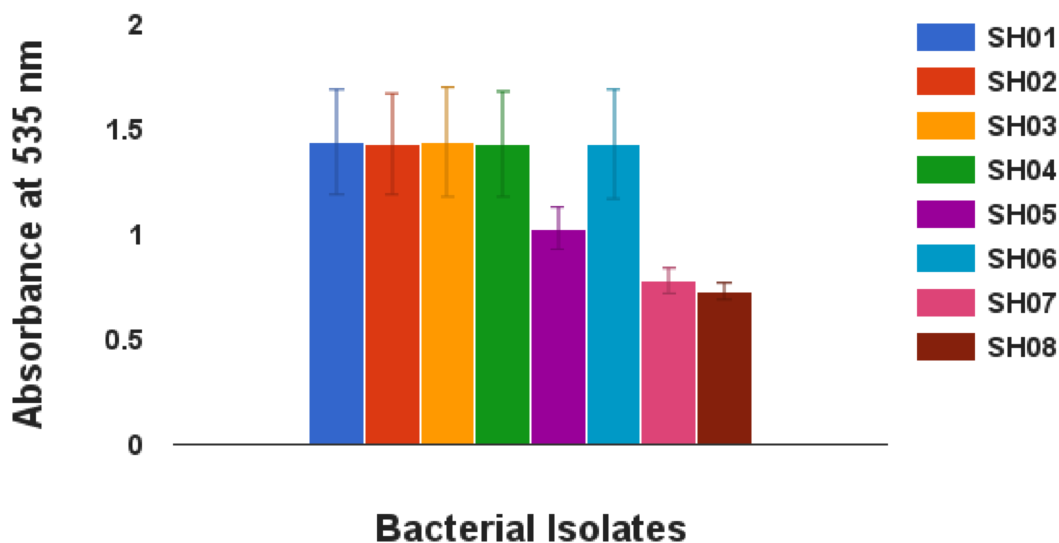

Figure 6. Mimosine concentration from bacterial isolates after 48 hours of incubation at 28°C. Initial concentration of mimosine was 3mM with 1mM $FeCl_3$, which had an absorbance of 1.74 at 535 nm.

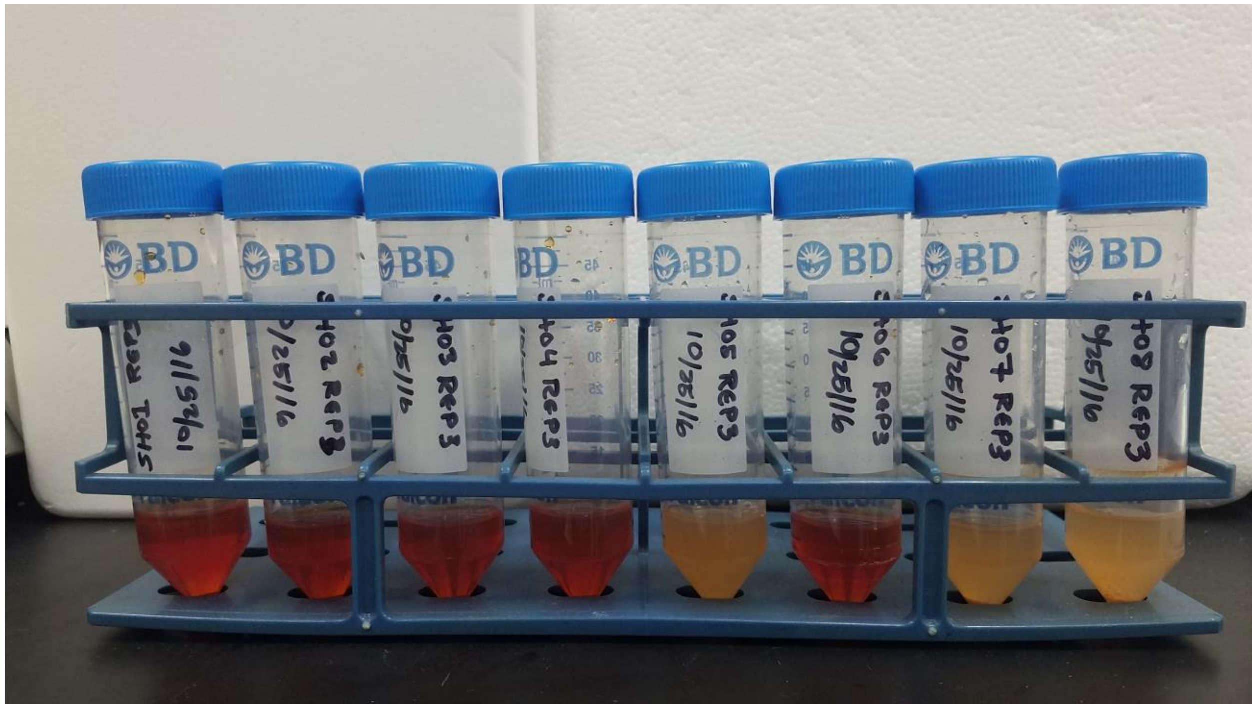

Figure 7. Growth of Bacterial isolates in 3mM Mimosine and 1 mM $FeCl_3$. Change in color of AB minimal media after 48 hour growth period.

CHAPTER FOUR

DISCUSSION

This study successfully demonstrates the isolation and identification of bacterial endophytes from the vegetative regions of *L. leucocephala*. There was no bacterial growth from the seeds despite making adjustments to culturing and surface sterilization methods. A total of 8 isolates were cultured from within the inner tissues of the growing tips of Leucaena sampled off Kalapana road. The sterile control plates confirm these isolates are endophytic and not surface dwelling bacteria. All 8 isolates have been identified and recognized as endophytes in previous studies. For instance, SH01 was closely related (98%) to *Rhodococcus kroppenstedtii*. The genus *Rhodococcus* is classified in the family *Nocardiaceae* (Stackebrandt *et al.* 1997). These microorganisms are shown to have a broad metabolic diversity, especially with hydrophobic compounds. This has piqued the interest of scientists to further study the biochemical and genetic characterization of their metabolic processes as they make ideal candidates for use in bioremediation (Guo-Zhen *et al.* 2012). *Rhodococcus kroppenstedtii* is Gram-positive, non-motile, non-spore forming, coccoid-like bacteria. Other member of the *Rhodococcus* genus have been isolated from the pharmaceutical plant *Artemeisia annua* L. (Zhao *et al*. 2012). Isolates SH02, SH04 and SH06 were closely related to the genus *Sphingomonas*. SH02 was more towards the species *paucimobilis,* whereas SH04 and SH06 were closer related to *pseudosanguinis*. Members of this genus are Gram-negative, yellow-pigmented, nonspore-forming and non-motile rods (Busse and Denner 1999). *Sphingomonas paucimobilis* showed it has plant-growth promoting abilities for a traditional Chinese medicinal plant; *Dendrobium officinale*. The growth of *D. officinale* seedlings, with *Sphingomonas paucimobilis* significantly increased stem growth by 8.6% (Yang *et al*. 2014). It was also shown that the strain ZJSH1 was

able to produce various phytohormones such as salicylic acid, IAA, Zeatin and abscisic acid (Yang *et al*. 2014). Isolate SH03 was closely related to *Microbacterium proteolyticum* which is Gram-positive, non-motile, aerobic and short rods (Alves *et al*. 2015). It was recently isolated from the roots of the salt-marsh plant *Halimione portulacoides* (Alves *et al*. 2015). Isolates SH05, SH07 and SH08 were closely identified in the genus *Pseudomonas*. SH05 is closely related to *Pseudomonas putida* as shown from the BLAST and NJ tree results. As mentioned earlier, there was a *Pseudomonas putida* strain (STM 905) that was able to degrade mimosine (Pandey, 2007). Isolates SH07 and SH08 might be the same species as sequence alignment. In the article from Kodama *et al.* 1985, *Pseudomonas oryzihabitans* are yellow-pigmented, oxidase-negative, Gram negative, rod shaped bacteria. They have been isolated from normal rice paddies and from soybean plants grown in soil treated with glyphosate herbicide (Kuklinsky-Sobral *et al*. 2004). Importantly, Belimov *et al*. 2001 observed that *P. oryzihabitans* isolates from *Pisum sativum* rhizoplane showed the capability to produce ACC deaminase. Furthermore, Kuklinsky-Sobral *et al.* 2004 discovered that endophytic isolates from soybean plants were able to produce auxin. These articles support that *P. oryzihabitans* has the potential for plant growth promotion.

Although isolates SH05, SH07 and SH08 showed the most growth in mimosine minimal media, the gel analysis showed that none of the bacterial isolates contained the *midD* and *pydA* genes required to degrade mimosine and HP previously identified in Rhizobium TAL 1145 (Fig. 4). This could indicate a different pathway is being used for mimosine degradation or that the primers were not able to properly anneal to the mimosine degrading genes. To our knowledge, this is the first attempt at isolating endophytic bacteria from *Leucaena leucocephala* above the root system. This is also a new discovery of bacteria that can degrade mimosine without being Mid^+. Discovering bacterial endophytes from regions above the roots and nodules unveils

another area of endophytic research that can help explain how these bacteria are benefitting the host plant. It seems that these endophytic isolates were successful in establishing themselves in a difficult niche and successfully translocating from the soil up to the growing tips of *L. leucocephala*.

Future work is needed to give us a better understanding of bacterial endophytes in Leucaena. Unresolved questions include how isolates SH05, SH07 and SH08 are able to degrade mimosine and which genes are induced from these isolate under high levels of mimosine? Investigating the biochemical pathways would be an interesting research topic because scientists can compare it with the currently known mimosine-degrading pathway from the rhizobium isolate, TAL 1145. Another research plan could be studying how these endophytes are benefiting the plant by detecting the presence of ACC deaminase gene. PCR can be used to assess if the isolates cultured in this study contain the ACC deaminase gene. These endophytes also hold potential for biocontrol activity to current plant pathogens. For example, *Acacia koa* is a native legume of Hawaii that is affected by a common fungal plant pathogen: *Fusarium oxysporum*. One can observe if *Fusarium* growth would be affected if one of the isolates was introduced to the culture. *Acacia koa* seedlings could also be inoculated with *Fusarium* and endophytic bacteria and observe if there is any difference in growth and symptoms.

A 16S metagenomics analysis within the endosphere of Leucaena is a good approach to observing the microbial community that may not be culturable. Little is known of the endophytic microbial communities within Leucaena and this could help us see which bacteria are most prevalent. The structure of bacterial communities is based on both cultivation-dependent and cultivation-independent techniques. Previous studies that have done this indicated that these communities can change over time, with endophytes showing a pattern of growth that correlates

with plant growth and development (Hardoim *et al.* 2008). In addition, observing endophytic communities is often simpler when compared to soil bacterial communities, encompassing hundreds of different bacterial types. 16S metagenomics could provide scientists with an idea of the bacterial community structure that resides in the tissues of Leucaena. Full genome sequencing of the mimosine degrading isolates will also provide good insight into which unique genes may be involved with mimosine degradation, and also which genes are involved with communication with host plant.

Hopefully this research along with future studies will open our eyes to how important these endophytes are to the overall success of Leucaena in its invasiveness and hopefully we can exploit them for native and other important flora.

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