Short Research Article

Preliminary Evaluation: Hydrocarbons, Cobalt, & Urea Enhance Bacteria Benefits to Plants.

ABSTRACT:

Aim: Develop a method to induce PGPB to biosynthesize nitrile compounds that may act as a reliable and repeatable means to increase seed germination in plant species. **Study Design:** The germination experiment was conducted in a completely randomized design with two replications and 40 pots (80 seedlings) per experimental unit, following a 1x1 factorial design for each culture, treated or untreated soil and 1 germination period for both conditions. The nitrile hydratase experiment was conducted in a completely randomized design with 3 replications and 3 soil samples per experimental unit, following a 1x1 factorial design for each cultivar, induced or non-induced soil and 1 cultivation period. **Place and Duration of Study:** Germination work was executed at G&A Innovative Solutions, LLC, GA., April - May 2017. Nitrile Hydratase Assay work was executed at Georgia State University, Applied Environmental Microbiology Dept., Atlanta, GA. November 2010-August 2011. **Methodology:** Rhodococcus and Bacillus species were induced with short chained-hydrocarbons, cobalt, and urea in a triphasic system for 3 d to potentially make nitrile compounds to benefit seed germination. Increased NHase activity has been previously correlated to production of these nitrile compounds and an increased ability to affect plant development. NHase activity was measured after bacteria were suspended in soil for 6-7 d. **Results:** The induction method sustained and increased NHase activity by 200 %, during suspension in soil. Induced Bacillus increased germination by 34 %, shoot & root length by 67 % & 10 %. **Conclusion:** Enhancing biosynthesis of nitriles in PGPB may enhance bacteria ability increase seed germination rates. Measuring NHase activity may indirectly measure efficacy of PGPB in soil. The results are preliminary and require additional studies to confirm results.

1. INTRODUCTION

Current agricultural practices must change in order to meet the demands of a growing global population (McGarth *et al.,* 2014). New planting and non-tillage practices coupled with climate change have seen an influx in emergence of resistant fungal pathogen and decrease in some seed germinations, many farmers are now searching for organic non-chemical alternatives to improve plant health and increase germination rates (Sfiligoj, 2018; Van den Bosch et al., 2018; Zulauf, 2018; Battaglin et al., 2011). *Pisum sativum* plants are particularly sensitive to fungal

infections during the early stages of germination, infected seeds display decreased germination rates (Wani and Aalum, 2018; Sharma and Singh, 2003). Chemical fertilizers do not improve germination, excess application of fertilizer harm seedlings and decrease germination rates (Yadav et al., 2010; Carter 1967).

New studies suggest PGPB such as Bacillus, Pseudomonas, Rhodococcus, and Azobacter are a cost effective, safe, and eco-friendly answer to micronutrient depletion without applying harmful toxins or excess chemical fertilizers (Lynch, 1990; Singh, 2013). PGPB increase phosphate solubilization, nitrogen fixation, and production of plant hormones to benefit plant growth (Shaikh *et al.,* 2016; Souza *et al.,* 2015; Dubeikovsky *et al.,* 1993; Lynch, 1990). PGPB also biosynthesize nitriles like HCN (hydrogen cyanide) or IAN (indole-acetonitrile) that may increase seed germination and inhibit growth of several fungal species (Rijavec and Lapanje, 2016; Michelsen & Stougaard, 2012; Oh et al., 2012; Kobayashi et al., 1995; Kerr, 1994; Smissman et al., 1964).

This study aims to develop a method to induce PGPB to biosynthesize natural nitrile compounds that provide reliable and repeatable means to increase seed germination in *Pisum sativus* plant species. The production of these compounds was measured indirectly by assessing NHase activity, an enzyme known to degrade these nitrile compounds into indole-3-acetic acid in bacteria and plants (Duca et al., 2014; Kobayashi et al., 1995). The study focused on Rhodococcus and Bacillus species, both contain inducible NHase enzymes (Mpofu et al., 2019; Singh et al., 2018; Zheng et al., 2008; Kim et al., 2000; Nagasawa et al., 1991; Nagasawa et al., 1988). Bacteria were induced with a short chained hydrocarbon, cobalt, and urea for 3 d. under high pressure low aeration conditions (Perry, 2011; Perry, 2016). NHase activity was assessed after initial induction and after 7 d of suspension in soil to measure prolonged activity.

2. MATERIALS AND METHODS

2.1 Hydrocarbon, Cobalt, and Urea Induction Method

Rhodococcus rhodochrous DAP 96253 (ATCC 55899) and *Bacillus licheniformis* (ATCC 12759) were obtained from American Type Culture Collection (ATCC) located in Vienna, VA. Both species were cultured on nutrient agar for 3 d, scrapped from agar, suspended in 15 ml of (1X) PBS buffer (0.8% NaCl, 0.02% KCl, 0.02M PO4, and pH 7.2), then transferred to a to a IL flask that contained $CoCl_2$ 0.201 (g/L), Urea 7.5 (g/L), Glucose 5 (g/L), Ethylene 15% (v/v), and 300 ml Minimal Media for 3 d at 30˚C with shaking at 120 rpm (Perry, 2016; Perry, 2011; Shadowen and Sciortino, 1989). Cells were harvested & re-suspended to 1.37 10^5 CFU/ml.

Previous studies showed the induction media increased nitrile hydratase (NHase), amidase, and potentially a monooxygenase like activity in *Rhodococcus*. Induction method may induce prolonged biosynthesis of nitrile compounds like indole-3-acetonitrile, acetonitrile, or cyanohydrin to inhibit growth of fungal plant pathogens, (Perry 2011; Kobayashi, *et al.,*1995).

2.2 Germination Study

Uncoated *Pisum sativus* seeds were purchased from Ferry Morse Co. and stored at 23 °C (40% RH) until potted. Germination period of 7-14 d and required soil pH 5.5- 7.0 (Elzebroek and Wind, 2008; Hartman et al., 1988). Two seeds were planted in each peat soil pot 1.3 in. deep. The seeds were planted in biodegradable peat fiber pots, 80 pots were filled with 50 ml of Ecoscraps® (natural + organic) potting mix; 40 control; 40 experimental pots, then 15 ml of water or 15 ml of liquid biofertilizer were added to pots. Open free-standing screened wire mesh greenhouse was exposed to typical outdoor conditions in April 2017, avg. temp. high =78 ºF, low =51 ºF, 15 h sunlight, 8 h darkness, and precipitation of 3.39 in. (Southwest GA Regional Station).

2.3 Prolonged NHase Activity in Non-Sterile Soil Conditions

NHase activity was induced in *Rhodococcus rhodochorus* cells using the method described previously in Section 2.2. Previous studies Rhodococcus cells were induced, washed, and then resuspended in a 35 ml of minimal media and low amounts of ethylene released from ripening fruit placed near the bacteria. After 6 d in the aqueous suspension NHase activity increased by 153% (Perry 2014). In this study Rhodococcus cells were induced, washed, and then resuspended in 35 ml of minimal media and mixed into 5 g of non-sterile peat soil. No exogenous ethylene/propylene was introduced to cells. NHase activity was assessed on 7^{th} d, test was duplicated and averaged.

2.4 NHase/Amidase Enzyme Assay

NHase activity was quantified using 1000 ppm of an acrylonitrile solution as substrate described in Perry, 2011. Ammonium concentrations were determined using a colorimetric assay (Fawcett et al., 1960). Absorbances of diluted samples were read using a spectrometer (Wallac 1420 Victor, multi well plate reader; Waltham, MA) for 10 sec at 620 nm. One unit of NHase is the conversion of 1 μM of AN per min per mg dry weight (units/mg cdw) of cells at 30 ˚C, pH 7.

3. **RESULTS/DISCUSSION**

A previous study compared the ability of Rhodococcus to grow on propylene/ethylene hydrocarbons for 3 d in the absence of another C-source. Rhodococcus cells cultured on (4g/L) glucose, (200mg/L) cobalt, and (7.5g/L) urea, final biomass was (77 mg ± 2 mg) ≤0.01% while cells cultured without cobalt and urea final biomass was (42 mg ± 15 mg) ≤0.01%. The prior growth on cobalt and urea increased biomass by 83% (Perry 2014). The data suggested cobalt and urea may play a role in improving the bacteria ability to metabolize the short-chained hydrocarbon

into a metabolic product the bacteria could use for growth. The previous data provided the rational to use cobalt and urea were used as inducers along with a short-chained hydrocarbon. Cobalt may also play a special role in inducing NHase (Mitra and Holtz, 2007; Kobayashi and Shimitzu, 1998). Urea may donate a cyanate to induce NHase activity (Stark et al., 1960). Induction increased NHase activity by 200 % after 7 d, see Table 1.

Parameter	**Non-Induced**	**Induced**		T-Test (Equal Variance)
Initial Activity (Day 0)				
Mean	1	170	p-Value[1]	***
			T-Stat > T-Crit.[1]	3.80 >2.13
Stdv.	± 1.26	± 70.50		
Final Activity (Day 7)				
Mean	0.3	436	p-Value[1]	***
			T-Stat > T-Crit.[1]	4.51 > 2.13
Stdv	± 1.17	± 183.87		

Table 1: [1]-Statistical analysis performed through T-test (comparing control and sample data); n.s. =non significant or *, **, *** =significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

The pre-induced Bacillus cells displayed an ability to increase seed germination by 34%. Shoot & root length increased by 67% & 10%, respectively, see Table 2. Seedlings grown with pre-induced Bacillus appeared healthier and more uniform than seedlings cultured in controlled conditions, Fig.1.

Parameter	Control	Pre-Induced Bacillus		T-Test (Equal Variance)
Early Germination Rate (%)				
Mean	70.20	94.00	p-Value[1]	***
			T-Stat > T-Crit.[1]	4.65 >1.75
Stdv.	± 13.81	± 4.90		
Stem Length (cm)				
Mean	3.05	5.15	p-Value[1]	***
			T-Stat > T-Crit.[1]	10.57 >1.68
Stdv	± 0.66	± 0.56		
Root Length (cm)				
Mean	4.93	5.50	p-Value[1]	n.s.
			T-Stat > T-Crit.[1]	0.48 < 1.70
Stdv.	± 1.73	± 1.22		

Table 2: [1]-Statistical analysis performed through T-test (comparing control and sample data); n.s. =non significant or *, **, *** =significant at P ≤ 0.05, 0.01 and 0.001, respectively.

Figure 1: (A) 3 Seedlings from Control Group (B) 3 Seedlings from Pre-Induced Bacillus Group. Seedlings displayed varied appearance and root health.

4. CONCLUSION

Seed germination is a complex cascade of mechanisms controlled by plant hormones (such as gibberlins, abscisic acid, indole-3-acetic acid, auxins, & cytokinin) produced by plant & soil bacteria (Vishal and Kumar, 2018; Miransari & Smith, 2014). Unfortunately, in-vitro benefits are rarely achieved when studies are conducted in field (Helland, 2017). This study suggests PGPB may be able to me induced to perform in harsh real-world environments. Pre-Induced Rhodococcus may also provide an additional benefit. The induced bacteria and non-induced bacteria displayed a differential ability to control and/or inhibit the growth of some common soil fungi. Induced and non-induced were suspended on non-sterile potting soil from the same bag. However, after 7 d the soil containing the induced bacteria displayed growth of a common white mold on the surface and the soil containing the non-induced bacteria displayed growth of a gray fuzzy mold, See Figure 2.

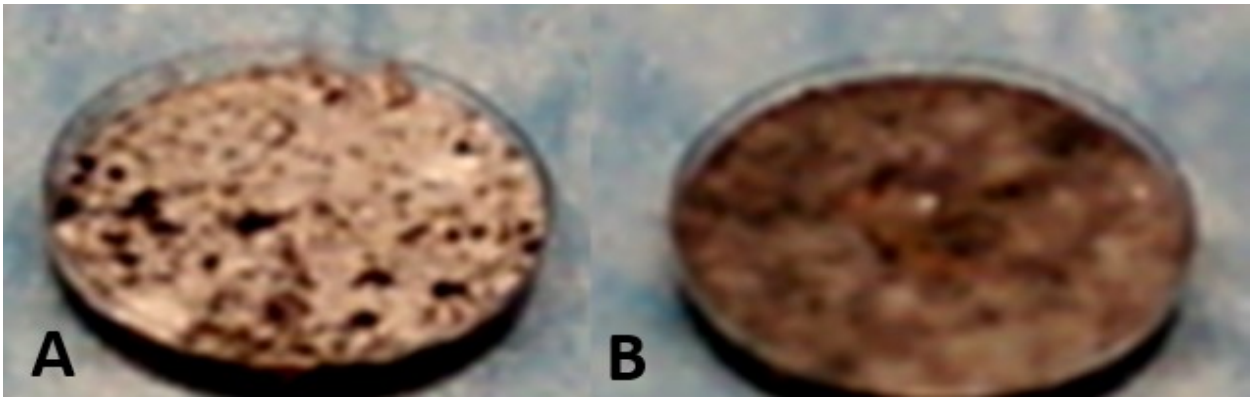

Figure 2: (A) Pre-Induced Rhodococcus (B) Non-Induced Rhodococcus. Induction may have enable bacteria to inhibit growth of certain soil fungal organisms.

This reliable performance may be related to nitrile compounds produced by bacteria after the induction method, (Perry, 2016). Measuring NHase activity may acts to ensure efficacy of cells before use in consumer products as biofertilizer and antifungal agents.

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