Short Research Article

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

ABSTRACT:

Plant growth promoting bacteria (PGPB) are known to biosynthesize compounds that provide a wide range of benefits to plants. However, it is a challenge to replicate these benefits in real-word applications. The goal of this study was to develop a method to induce PGPB to biosynthesize natural nitrile compounds that may act as a reliable and repeatable means to increase seed germination in the 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 6-7 d period away from inducers to measure prolonged activity. The three inducers prolonged NHase activity in Rhodococcus by 200 % in the soil, compared to a 153% in a previous study using aqueous solutions. Induced Bacillus sp. increased seed germination by 34%. Enhancing biosynthesis of nitriles in PGPB may enhance bacteria ability increase seed germination rates and possibly regulating fungal growth in soil. Measuring prolonged nitrile production indirectly with NHase activity may be a legitimate means to measure efficacy of PGPB in the 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 the emergence of resistant fugal 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; Czyzewska, 1985).

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 ecofriendly 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 species of fungal pathogens (Rijavec and Lapanje, 2016; Michelsen & Stougaard, 2012; Oh et al., 2012; Kobayashi et al., 1995; Kerr 1994).

The goal of this study was to develop a method to induce PGPB to biosynthesize natural nitrile compounds that provide reliable and repeatable means to increase seed germination in the plant species *Pisum sativus*. The production of these compounds was measured indirectly by assessing NHase activity, an enzyme known to degrade these nitrile compounds into indole-3-acetcacidin 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 the American Type Culture Collection (ATCC) located in Vienna, VA. Both species were cultured on nutrient agar for 3 d, scrapped from agar, suspended in 15ml 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 rhodochrous*. 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 a., 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 the 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 the diluted samples were read using a spectrometer (Wallac 1420 Victor, multi well plate reader; Waltham, MA) for 10sec at 620nm. One unit of NHase is the conversion of 1 μM of AN per minute 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 play a key role in donating a cyanate to also induce NHase activity (Stark et al., 1960).

The induction method initially increased NHase activity 10 170 units of activity, but cells mixed with soil containing other microbes increased enzyme activity by 200 % after 7 d, see Table 1.

Pre-Induced Bacillus cells displayed an ability to increase seed germination by 34%, shoot & root length 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

4. CONCLUSION:

Seed germination is a complex cascade of mechanisms controlled by plant hormones (such as gibberlins, abscisic acid, indole-3-acetic acid, auxins, and cytokinin) produced by the plant and soil bacteria (Vishal and Kumar, 2018; Shaikh, 2016; Miransari and Smith, 2014). Unfortunately, in-vitro benefits are rarely achieved when studies are conducted in the field (Helland, 2017). This study suggests PGPB may be able to me induced to perform in harsh real-world environments. Pre-Induced Rhodococus cells even displayed a potential ability to control/inhibit the growth of some common soil fungi compared to non-induced cells, Fig. 2.

This reliable performance may be related to nitrile compounds produced by the 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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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 ≤ 0.05, 0.01 and 0.001, respectively.

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 $P \leq 0.05$, 0.01 and 0.001, respectively.

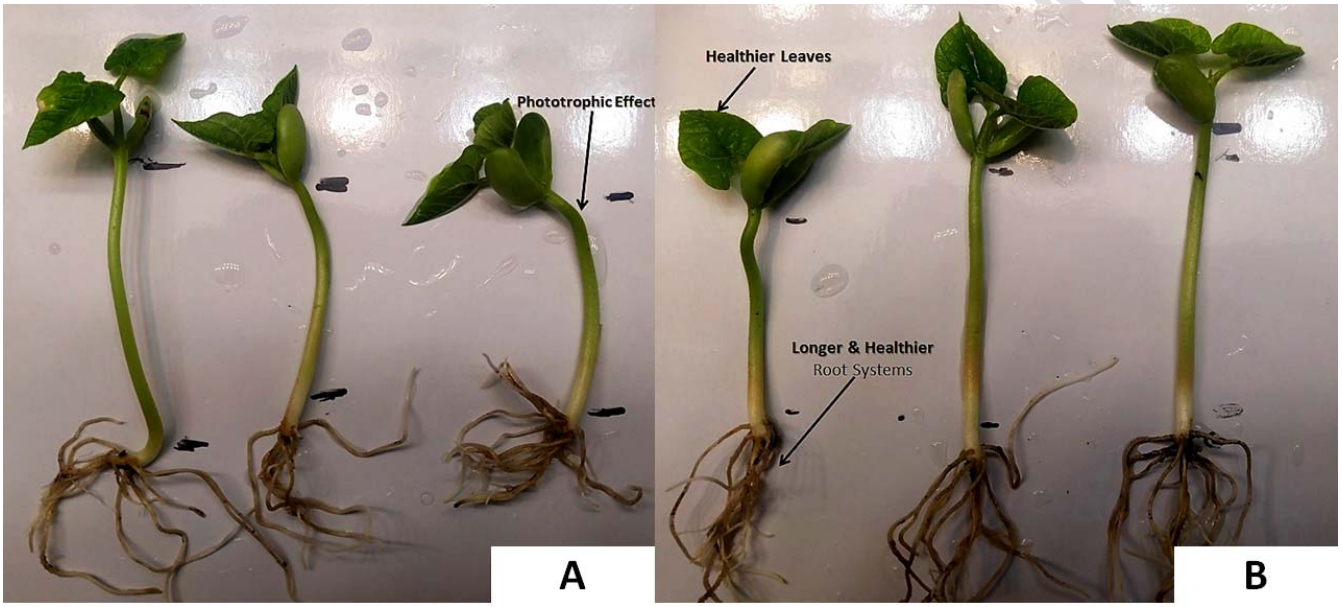

Figure 1: (A) 3 Seedlings from Control Group (B) 3 Seedlings from Pre-Induced Bacillus Group. Seedlings displayed varied appearance and root health.

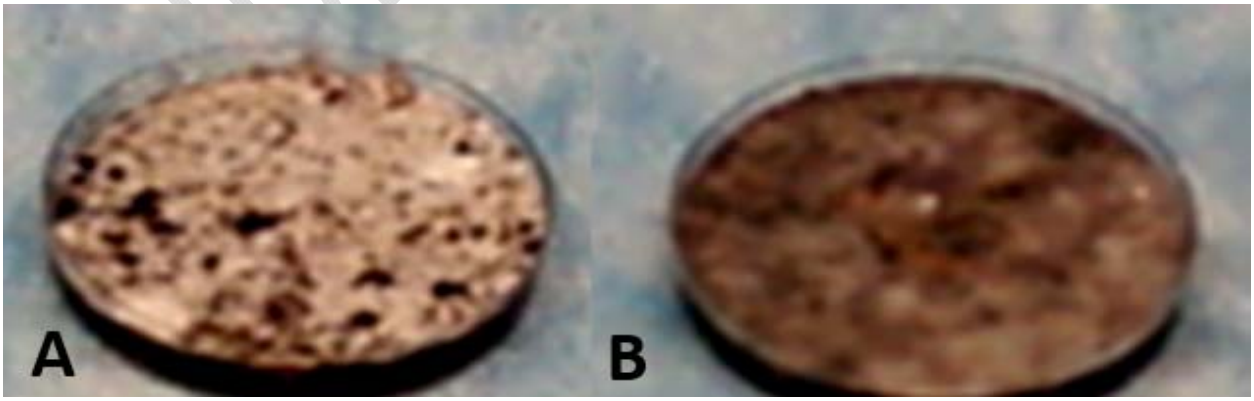

Figure 2: (A) Pre-Induced Rhodococcus (B) Non-Induced Rhodococcus. Induction may have enable bacteria to inhibit growth of certain soil fungal organisms.

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t-Test: Two-Sample Assuming Equal Variances

	Initial (3) Inducers	*Initial (1) Inducer*
Mean	156.3333333	1.56
Variance	4970.333333	1.5808
Observations	3	3
Pooled Variance	2485.957067	
Hypothesized Mean Difference	0	
df	4	
t Stat	3.801849786	
P(T<=t) one-tail	0.009536644	
t Critical one-tail	2.131846786	
P(T<=t) two-tail	0.019073287	
t Critical two-tail	2.776445105	

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