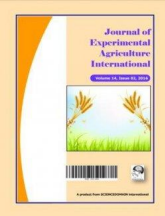

Journal of Experimental Agriculture International

36(3): 1-6, 2019; Article no.JEAI.49128
ISSN: 2457-0591
(Past name: American Journal of Experimental Agriculture, Past ISSN: 2231-0606)

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

Guenevere Perry[1*] and Don Spence[2]

[1]Department of Biology, Georgia State University, Atlanta, Georgia.
[2]Department of Natural Resources, Bethune Cookman-University, Daytona, Florida, USA.

Authors' contributions

This work was carried out in collaboration between both authors. Author GP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GP and DS managed the analyses of the study. Author GP managed the literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JEAI/2019/v36i330237
Editor(s):
(1) Dr. Mohammad Reza Naroui Rad, Department of Horticulture Crops Research, Sistan Agricultural and Natural Resources Research and Education Center, Iran.
Reviewers:
(1) Eduardo José Azevedo Corrêa, Empresa de Pesquisa Agropecuária de Minas Gerais, Brazil.
(2) Schirley Costalonga Department of Biological Science, Universidade Federal do Espírito Santo, Brazil.
Complete Peer review History: http://www.sdiarticle3.com/review-history/49128

Short Research Article

Received 03 March 2019
Accepted 16 May 2019
Published 22 May 2019

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

**Corresponding author: E-mail: guenperry@gmail.com;*

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.

Keywords: Germination; fertilizer; anti-fungal; nitrile hydratase; Rhodococcus; Bacillus.

1. INTRODUCTION

Current agricultural practices must change in order to meet the demands of a growing global population [1]. 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 [2,3,4,5]. *Pisum sativum* plants are particularly sensitive to fungal infections during the early stages of germination, infected seeds display decreased germination rates [6,7]. Chemical fertilizers do not improve germination, excess application of fertilizer harm seedlings and decrease germination rates [8,9].

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 [10,11]. PGPB increase phosphate solubilization, nitrogen fixation, and production of plant hormones to benefit plant growth [12,13,14]. PGPB also biosynthesize nitriles like HCN (hydrogen cyanide) or IAN (indole-acetonitrile) that may increase seed germination and inhibit growth of several fungal species [15,16,17,18,19,20].

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 [21]. The study focused on Rhodococcus and Bacillus species, both contain inducible NHase enzymes [22,23,24,25,26,27]. Bacteria were induced with a short-chained hydrocarbon, cobalt, and urea for 3 d. under high pressure low aeration conditions [28]. 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 [28,29,30]. 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, [28,18].

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 [31,32]. 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% [33]. 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 7th 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 [34]. 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 AND 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 (4 g/L) glucose, (200 mg/L) cobalt, and (7.5 g/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% [28]. 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 use as inducers along with a short-chained hydrocarbon. Cobalt may also play a special role in inducing NHase [35,36]. Urea may donate a cyanate to induce NHase activity [37]. Induction increased NHase activity by 200 % after 7 d, see Table 1.

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

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**

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

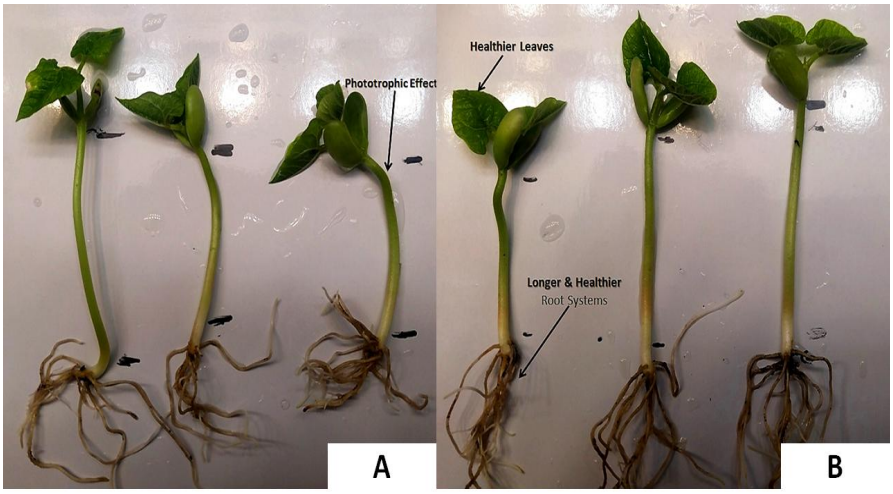

Fig. 1. (A) 3 Seedlings from Control Group (B) 3 Seedlings from Pre-Induced Bacillus Group Seedlings displayed varied appearance and root health

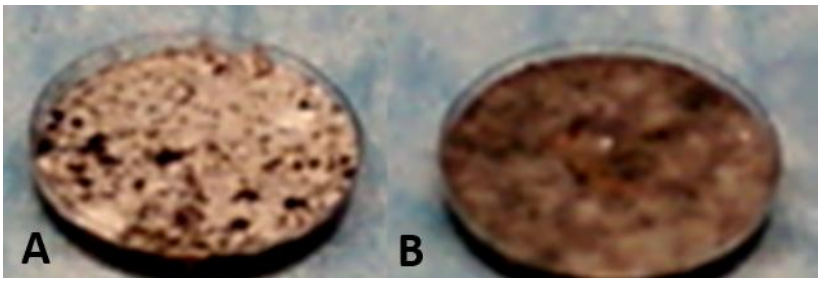

Fig. 2. (A) Pre-induced rhodococcus (B) Non-induced rhodococcus. Induction may have enable bacteria to inhibit growth of certain soil fungal organisms

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.

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 [38,39]. Unfortunately, in-vitro benefits are rarely achieved when studies are conducted in field [40]. This study suggests PGPB may be able to be 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 Fig. 2.

This reliable germination performance may be related to nitrile compounds produced by bacteria after the induction method [29]. Measuring NHase activity may ensure efficacy of cells before use in consumer products as biofertilizer and antifungal agents.

ACKNOWLEDGEMENTS

The authors appreciate Georgia State University and G & A Innovative Solutions for provision of laboratories that were suitable to accomplish this work. The assistance of laboratory

technician: Dian Williams is acknowledged and appreciated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. McGarth J, Spargo J, Penn C. Soil fertility and plant nutrition. Encyclopedia of Agriculture and Food Systems. 2014;5: 166-184.
2. Sfiligoj E. Fungicides in 2018: Looking at new pressures, including resistance. Crop Life; 2018. Available:https://www.croplife.com/crop-inputs/fungicides-2018-looking-new-pressures-including-resistance
3. Van den Bosch F, Lopez-Ruiz F, Oliver R, Paveley N, Helps J, van den Berg F. Identifying when it is financially beneficial to increase or decrease fungicide dose as resistance develops. Plant Pathol. 2018;67:549-560.
4. Zulauf C. Yield and price change from planting to harvest: How strong is the relationship? Department of Agricultural, Environmental and Development Economics. Ohio State University. 2018;8:215.
5. Battaglin W, Sandstrom M, Kuivila K, Kolpin D, Meyer M. Occurrence of azoxystrobin, propiconazole, and selected other fungicides in US streams, 2005-2006. Water, Air, and Soil Pollution. 2011;218 (1-4):307-322.
6. Wani I, Aalum K. Effect on seed germination and seedling vigor by seed bourne fungi of pea (*Pisum sativum L.*). International Journal of Advance Research and Engineering. 2018;7(4):186-191.
7. Sharma P, Singh SD. Effect of fungal metabolites on germination and seedling vigour of pea. Journal of Maharashtra Agricultural Universities (India); 2003.
8. Yadav J, Verma JP, Tiwari KN. Effect of plant growth promoting rhizobacteria on seed germination and plant growth chickpea (*Cicer arietinum L.*) under *in vitro* conditions. In Biological Forum. 2010;2(2): 15-18.
9. Carter OG. The effect of chemical fertilizers on seedling establishment. Australian Journal of Experimental Agriculture. 1967;7(25):174-180.
10. Lynch J. The rhizosphere. Wiley-Interscience, Chichester, UK; 1990.
11. Singh JS. Plant growth promoting rhizobacteria. Resonance. 2013;18(3):275-281.
12. Shaikh S, Sayyed R, Reddy M. Plant growth promoting rhizobacteria: An ecofriendly approach for sustainable agroecosystems. Plant, Soil, and Micro. 2016;181-201.
13. Souza R, Ambrosini A, Passaglia L. Plant growth promoting bacteria as inoculants in agricultural soils. Genetic & Molecular Biology. 2015;38(4):401-419.
14. Dubeikovosky A, Mordukhova E, Kochetkov V, Polikarpova F, Bovonim A. Growth promotion of blackcurrant softwood cuttings by recombinant strain *Pseudomonas fluorescens* BSP53a synthesizing an increased amount of indole-acetic acid. Soil Biology and Biochemistry. 1993;25(9):1277-1281.
15. Rijavec T, Lapanje A. Hydrogen cyanide in the rhizosphere: Not suppressing plant pathogens, but rather regulating availability of phosphate. Frontiers in Microbiology. 2016;1785.
16. Michelsen CF, Stougaard P. Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. Canadian Journal of Microbiology. 2012;58(4):381-390.
17. Oh S, Go GW, Mylonakis E, Kim Y. The bacteria signaling molecule indole attenuates the virulence of fungal pathogen *Candida albicans*. Journal of Applied Microbiology. 2012;113(3):622-628.
18. Kobayashi M, Suzuki T, Masuda M, Shimizu S. Occurrence of enzymes involved in biosynthesis of indole-3-acetic acid from indole-3-acetonitrile in plant associated bacteria, Agrobacterium and Rhizobium. Proc Natl Acad Sci USA. 1995;92(3):714-718.
19. Kerr JR. Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. Journal of Clinical Microbiology. 1994;32(2):525–527.
20. Smissman E, Beck S, Boots M. Growth inhibition of insects and a fungus by indole-3-acetonitrile. Science. 1961;17:462.
21. Duca D, Rose DR, Glick BR. Characterization of a nitrilase and a nitrile hydratase from *Pseudomonas* sp. strain

UW4 that converts indole-3-acetonitrile to indole-3-acetic acid. Appl. Environ. Microbiol. 2014;80(15):4640-4649.
22. Mpofu E, Vejarano F, Suzuki-Minakuchi C, Ohtsubo Y, Tsuda M, Chakraborty J, Nojiri H. Complete genome sequence of *Bacillus licheniformis* TAB7, a compost-deodorizing strain with potential for plant growth promotion. Microbiol Resour Announc. 2019;8(4):1659.
23. Singh R, Pandey D, Dhariwal S, Sood P, Chand D. Bioconversion of acrylonitrile using nitrile hydratase activity of Bacillus sp. APB-6. 3 Biotech. 2018;8:1-8.
24. Zheng YG, Chen J, Liu ZQ, Wu MH, Xing LY, Shen YC. Isolation, identification and characterization of *Bacillus subtilis* ZJB-063, a versatile nitrile-converting bacterium. Appl Microbiol Biotechnol. 2008;77:985–993.
25. Kim SH, Oriel P. Cloning and expression of the nitrile hydratase and amidase genes from Bacillus sp. BR449 into *Escherichia coli.* Enzyme and Microbial Technology. 2000;27(7):492-501.
26. Nagasawa T, Takeuchi K, Nardidei V, Mihara Y, Yamada H. Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1. Appl Microbiol Biotechnol. 1991;34:783–788.
27. Nagasawa T, Mathew CD, Mauger J, Yamada H. Nitrile hydratase-catalyzed production of nicotinamide from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. Appl Environ Microbiol. 1998;54:1766–1769.
28. Perry G. Enhancing the expression of enzymes used to degrade hydrocarbons and cyanohydrins in Rhodococcus sp. DAP 96253 by using inducers such as cobalt, urea, and propylene gas; also enhances the ability of the bacteria to delay the ripening of several fruit species; 2011.
29. Perry G. Hydrocarbons & heavy metals induce biocatalyst to modify development process in seeds, seedlings, and plants. Patent Application No.: US15/262,004; 2016.
30. Shadowen R, Sciortino C. Improved growth of *Campylobacter pylori* in biphasic system. Journal of Clinical Microbiology. 1989;27:1744-1747.
31. Elzebroek T, Wind K. Guide to cultivated plants. CAB International, Oxfordshire, UK; 2008.
32. Hartmann HT, Kofranek AM, Rubatzky VE, Flocker WJ. Plant science: Growth, development and utilization of cultivated plants. 2nd Ed. Prentice Hall Career and Technology, Englewood Cliffs, NJ; 1988.
33. Perry G. Ethylene induced soil microbes to increase seed germination, reduce growth time, and improve crop yield in *Pisum sativum L*. Peer J Preprints. 2014;2: e543v1.
34. Fawcett JK, Scott J. A rapid and precise method for the determination of urea. Journal of Clinical Pathology. 1960;13(2): 156-159.
35. Mitra S, Holz RC. Unraveling the catalytic mechanism of nitrile hydratases. Journal of Biological Chemistry. 2007;282(10):7397-7404.
36. Kobayashi M, Shimizu S. Metalloenzyme nitrile hydratase: Structure, regulation, and application to biotechnology. Nature Biotechnology. 1998;16(8):733.
37. Stark G, Stein W, Moore S. Reactions of the cyanate present in aqueous urea with amino acids and proteins. Journal of Biological Chemistry. 1960;235(11):3177-3181.
38. Vishal B, Kumar P. Review: Regulation of seed germination and abiotic stresses by gibberlins and abscisic acid. 2018;9:1-16.
39. Miransari M, Smith DL. Review: Plant hormones and seed germination. Environmental and Experimental Botany. 2014;99:110-121.
40. Helland IB. The effect of different plant growth-promoting bacteria on the root system of *Arabidopsis thaliana* WT and PP2A signaling mutants (Thesis, Univ. of Stavanger, Norway); 2017.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/49128