Ethylene Induced Soil Microbes to Increase Seed Germination, Reduce Growth Time, and Improve Crop Yield in *Pisum sativum L*.

The main scope of this project was to identify a novel induction method to improve the effectiveness of biological fertilizers for organic farmers. To eliminate additional variables the plants were not treated with chemical or biological pesticides. The biological fertilizer used in this study was induced with co-factors and ethylene under specific conditions. Ethylene induced the soil to release acetonitrile, a component of indole-3-acetonitrile a precursor to the plant hormone indole-3-acetic acid (IAA). It is known that plant growth promoting bacteria can produce IAA and directly/ indirectly modify plant development and growth.

In this preliminary study, the ethylene induced biological fertilizer (**EIBF**) improved germination rate, enhanced quality, reduced growth time, and improved crop yield of *Pisum sativum L* (green peas) with a single application. Many biological fertilizer require two to three applications a year to see improved growth.Green peas grown in ethylene induced soil improved pea quantity by 200% per plant compared to control samples. **EIBF** increased the crop yield by over 57%. The average biological fertilizer only improves crop by 20-30%. The peas harvested from the plants grown in **EIBF** were 4 times larger peas collected from plants grown in the control soil. The improvement and continued study of **EBIF** may have a global applications, impacting farming techniques in poor developing countries or organic farms.

Ethylene Induced Soil Microbes to Increase Seed Germination, Reduce Growth Time, and Improve Crop Yield in *Pisum sativum L.*

Authors: Guenevere Perry
Contact: Guenevere Perry, PhD
Permanent: Research & Development Department
G&A Innovative Solutions, LLC
P.O. Box 5781
Albany, GA 31726
Cell: (678)-756-4832
Email:ideas2products@gaais.org

Tuesday, October 14, 2014

Keywords: Bio-fertilizers, Fertilizers, Plant Growth Promoting Bacteria, PGPR, Ethylene, Sustainable Agriculture, Agriculture, Farming, Crop Yield, Green Peas

INTRODUCTION:

1.1 Background

The US domestic farming industry is a $618 billion dollar business, with crop productivity dependent on the early planting techniques utilized by the farmer, including the inclusion of chemical fertilizers, (Johnson, 2014). Chemical or commercial fertilizers (CF) are effective tools to increase soluble nitrogen (N) and phosphorus (P) components in depleted soil, plants sequester the compounds to increase crop yields, quantity, and quality, (Al-Busaidi, 2013). Unfortunately, CFs are expensive and if used improperly can have negative effects on the surrounding ecosystem, by decreasing biological diversity, prompting run-offs and algae blooms, acidification of soil, and ozone pollution, (Aggani, 2013; Ribaudo et *al.,* 2011). High cost and adverse effects of CFs has forced large-scale farmers to consider alternative fertilizer treatments including organic fertilizers (i.e. manure or compost) and/or biological fertilizers. Organic fertilizers (OF) are all natural, environmentally friendly, naturally correct soil pH, and are cost effective, but OFs are messy, not readily available, and take longer time to provide visible benefits to crop yield and quality. BFs are typically cost effective, improve soil quality, and increase crop yields by 20-30%, but BFs are may require specialized storage, provide slower benefits compared CFs, and may require different equipment for application, (Aggani et *al.,* 2013; Banayo et *al.,* 2012; Ishfani, 2012; Ribaudo et *al.,* 2011). Biological fertilizers (BF) are defined as any fertilizer that contains plant growth promoting rhizobacteria (PGPR), nitrogen fixers, and/ or phosphate solubilizing microbes. PGPR are the soil bacteria that inhabit the area around/on the rhizosphere of several plant species. PGPR can biosynthesize and/or degrade phytohormones, such as indole-3-acetic acid (IAA), indole-3-acetonitrile (IAN), 1-aminocyclopropane-1 (ACC), cyanide, or ethylene. These bacteria are able to directly or

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

indirectly affect plant growth and/or development, (Arshad et al., 2007; DeBont and Albers, 1976; Miller et al., 1987; Vandeputte et al., 2005).

1.2 Enhancing Seed Germination

Most BFs focus on inducing PGPR or nitrogen fixers to hydrolyze N or P compounds into soluble by-products the plants to uptake. The scope of this project was to identify a novel and cost effective technique to enhance PGPR to biosynthesize phytohormones that stimulate plant growth and reduce the time it takes for biological fertilizers to improve crop yield and productivity within the first weeks of application.

A study conducted by Flematti et *al.,* 2011, suggest vegetation release increased concentrations of cyanohydrins during forest fires, the cyanohydrins undergo an abiotic reaction in water releasing cyanide into the soil to stimulate seed germination, (Downes et *al.,* 2013; Flematti et *al.,* 2011; Nelson et *al.,* 2012). A competing study conducted by Abeles, 2012, suggests vegetation release large portions of hydrocarbons such as ethylene during forest fires as a pollutant, (Abeles, 2012; Boubel et al., 1994; Brust, 2009).

This study focused on the idea that ethylene released by burning vegetation may induce cyanohydrin or nitrile production in plants and/or soil microbes to induce seed germination. The biological fertilizer used in this study was induced with co-factors and ethylene under specific conditions. Ethylene induced the soil to release acetonitrile, a component of indole-3-acetonitrile a precursor to the plant hormone indole-3-acetic acid (IAA). It is known that plant growth promoting bacteria can produce IAA and directly/ indirectly modify plant development and growth. The results from the study suggest ethylene induced biological fertilizer has increased biosynthesis of nitrile compounds that may enhance plant development.

MATERIALS AND METHODS:

2.1 Soil Collection

Based on a study conducted by Elsgaard, soil was collected from the base of fruit bearing plants *Pyrus communis, Solanum melongen*, and *Prunus persica,* the garden soil and store bought peat soil was mixed in a 1:1:1:1 ratio, (Elsgaard, 1998; Elsgaard & Anderson, 1998). Leaves, rocks, plant rooting systems, and nematodes/ insects were all subsequently removed from the soil samples. The control samples were mixed with 500 ml of dH_2O, ethylene induced bio-fertilizer **(EIBF)** samples were exposed to nitrogen, iron, and yeast lysate, and induced with ethylene similar to the conditions as stated in Perry, 2011 and Perry, 2014. Samples were stored for 4 d at 20˚C before gardening experiments.

2.2 Garden Preparation

Untilled land measuring 12x6 ft was cleaned and tilled for gardening, initial pH 6-8, and normal levels of N and P were present in the soil, (Mosser Lee, Soil Master Soil Testing Kit, Millstone, Wiscosin) .The garden as divided into three rows, the rows were separate by 3 ft on either side.

2.3 Planting

Pisum sativum L. seeds were used in this experiment, 23-25 seeds were sown in each row at a depth of 2 in. Seeds were covered with soil or a mixture of EIBF and soil, at a ratio of 1:3 (EIBF:Soil). Plants were watered, and analyzed bi-weekly for 8 wks. Experimental sample size > 50 seeds.

2.1 Data Analysis and Observations

Data analysis was used to understand the effect of EIBF on the early stages of plant development and root development in response to external stresses, (Harry et *al.* 1999). Four pea plants were harvested and analyzed biweekly analysis included stem length, root comparisons, node development, and leaf area per plant. Data analyzed using Microsoft Excel software. The pea plants were planted in late summer and conducted in an outdoor environment, *Pisum sativum L* (green pea plants) were harvested 1-3 weeks before complete maturity due to seasonal changes, (Frame, 2014).

2.4 GC-MS Head Space Analysis of Soil Samples

GC-MS analysis was performed by Millis Scientific, Inc. in Baltimore, MD. The soil samples were labeled as control or ethylene induced soil samples. There were four control samples mixed with dH_2O, and six induced soil samples exposed to cofactors and ethylene inducer. The tests were designed to identify the presence of ethylene, cyanide, cyanohydrin, and/or other nitrile compounds in soil samples. First 10g of each soil sample were aliquot into 40 ml vials equipped with septum. All containers were incubated at 50˚C for two hours, 2ml of 10M HCl was added to acidify soil for HCN assay. Samples were analyzed with a Waters/Micromass Quatro GC mass spectrometer interfaced to a ThermoElectron Trace gas chromatograph was utilized for the analysis. GC-MS conditions were as follows: Poraplot Q Plot column by Chrompack (10M 0.32 mm), carrier gas helium at (2.5 ml/min splitless), He; injection volume, 1 ml; injection temperature, 200°C; initial temperature, 50°C for 1 min, increasing by 10°C min^{-1} to a final temperature of 220°C, hold 1 min; ion source temperature, 150°C; and transfer line temperature 250°C. Mass-to-charge ratios (*m/z* values) from 10 to 400 were monitored using the scan mode.

For each of the samples, a set of target components was identified with the aid of AMDIS software. The components were identified using the NIST mass spectral library.

RESULTS & DISCUSSION

3.1 Ethylene Induced Acetonitrile Production in Soil Samples

Perry, 2013, hypothesized that ethylene induced the biosynthesize nitrile and/or cyanohydrin in PGPR. Soil samples induced with ethylene and cofactors were analyzed using GC-MS, the soil released acetonitrile a component of indole-3-acetonitrile a precursor to the plant hormone indole-3-acetic acid (IAA), in the headspace of induced soil samples. Control samples did not contain the nitrile compound, See Figure 1.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

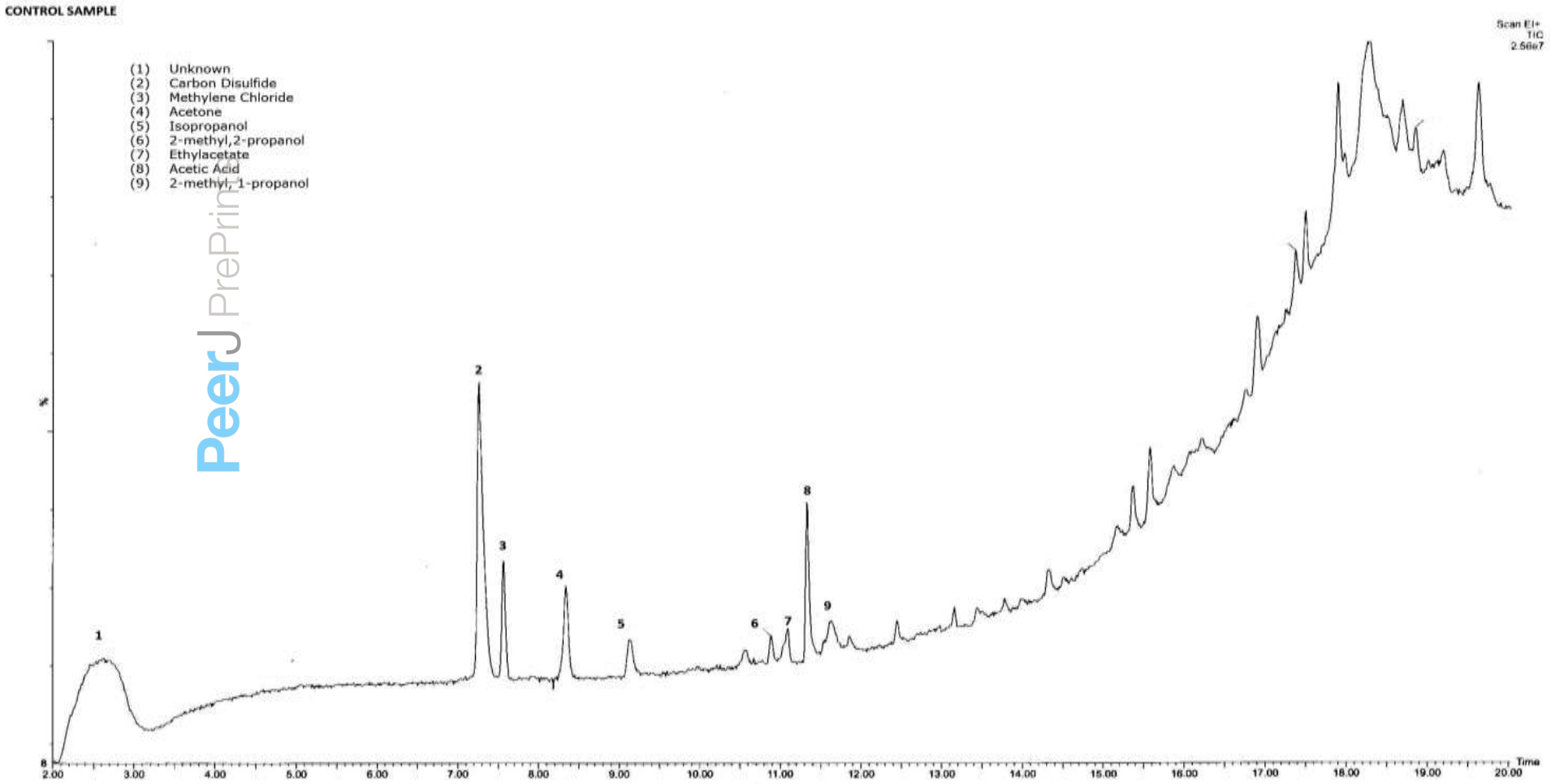

Figure 1: GC-MS Analysis, Composite. Control soil samples do not contain any nitrile compounds.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

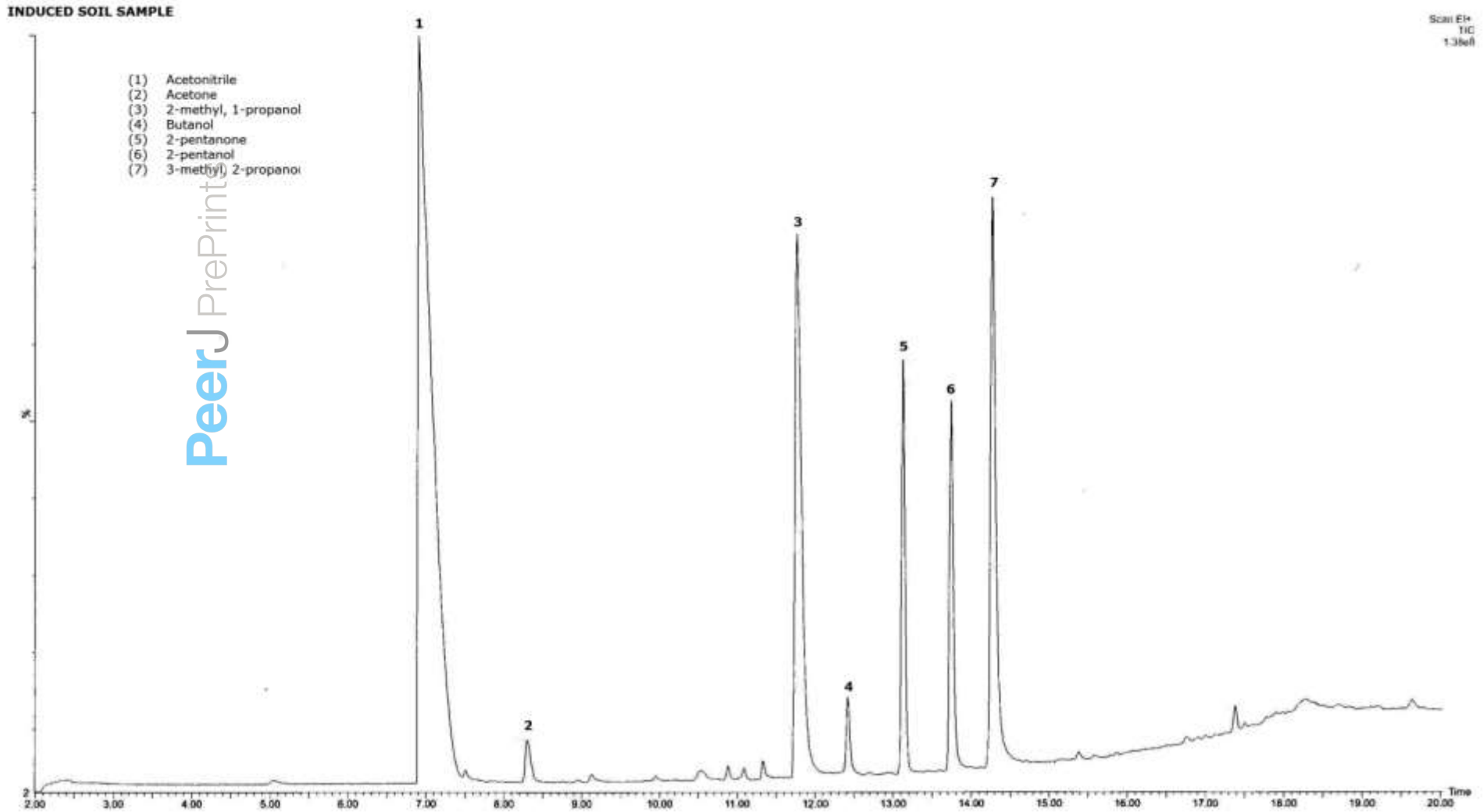

Figure 2: GC-MS Analysis, Composite. EIBF samples contained acetonitrile compounds.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

3.1 Comparison of Plant Growth and Development

During the early stages of plant development the pea plants grown in EIBF displayed increased stem length, increased leaf area, and increased nodule formation compared to control samples. As the pea plants developed over the 8 wk period the plants grown in the EIBF appeared to slow down in stem and leaf growth. The plants appeared to place more energy on the pea development and less energy on the plant development, see Figure 3 and Figure 4.

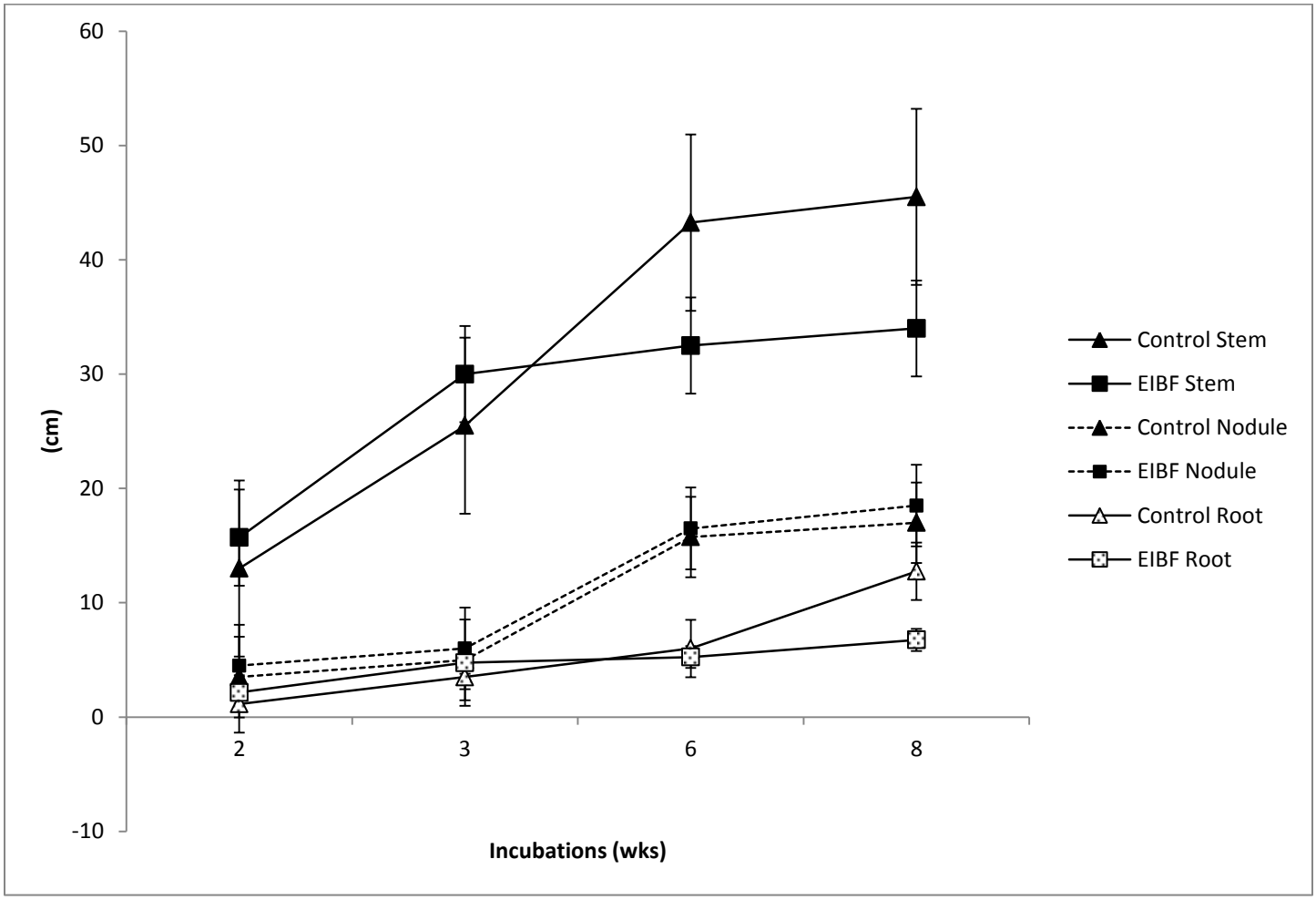

Figure 3: Comparing changes in stem length of pea plants over an 8wk period. Data includes the median length for 4-8 samples bi-weekly. Standard Error Bar represents the standard error of the mean value for each sample. Sample size > 50. Nodule formation calculated per plant. Stem Length & Root Length calculated in cm.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

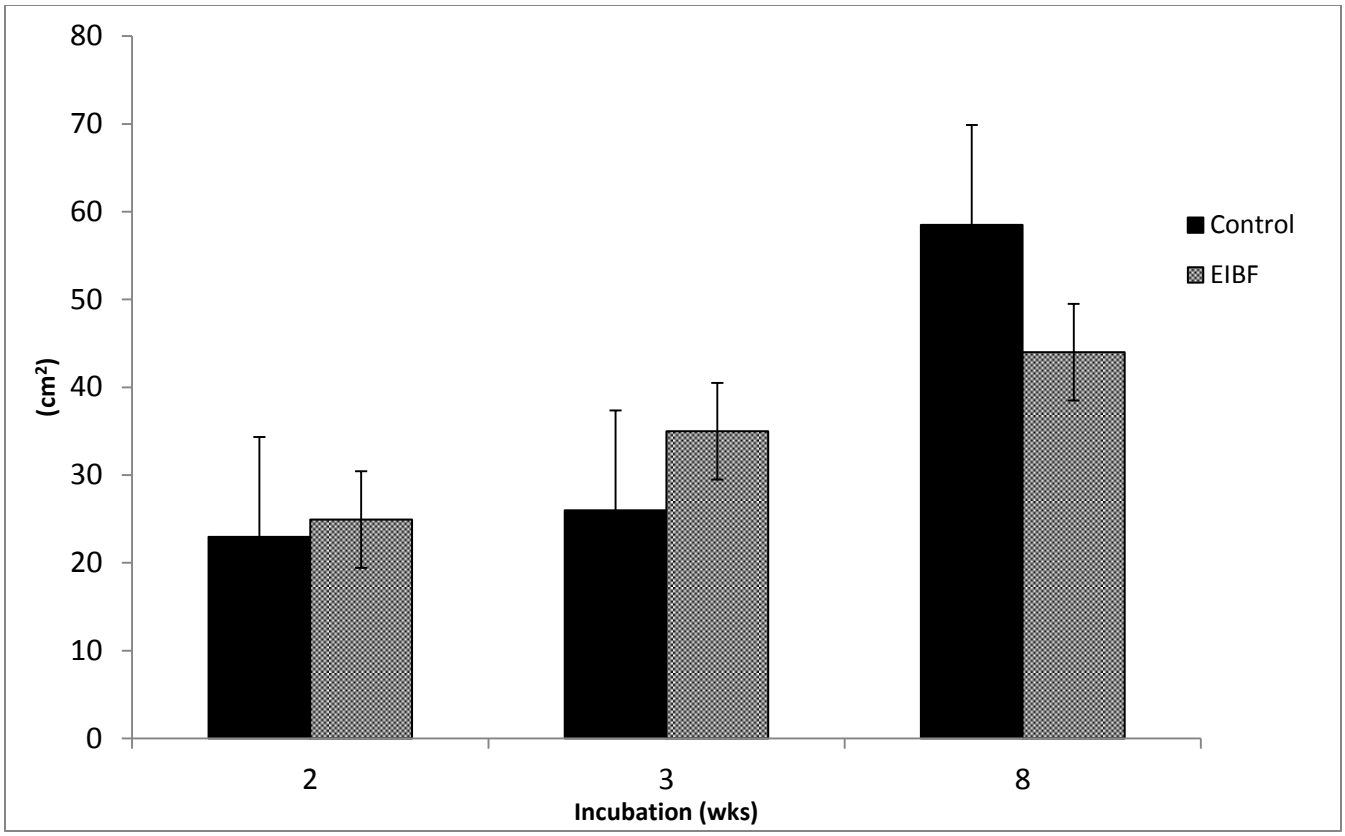

Figure 4: Comparing changes in leaf area of pea plants over an 8wk period. Data includes the median length(width) =A, for 4-8 samples per wks. Standard Error Bar represents the standard error of the mean value for each sample. Sample size > 50.

3.2 Visual Comparison of Plant Development

Pea plants were analyzed bi-weekly, four to eight plants were sacrificed to record phenotypic changes root, stem, and leaf area growth using a digital camera (Sony "Cyber Shot', 7.2 mega pixels, automatic flash). Images were stored and modified using an automatic correction tool on Microsoft Office Picture Manager Program to correct for variations in lighting and shade, see Figure 5, Figure 6, Figure 7, and Figure 8. Pea plants grown in EIBF displayed increased germination, smaller leaves, and smaller roots systems, but had increased crop yields. Pea plants grown in induced bio-fertilizer displayed increase in pea quantities by 200% per plant, the peas were 4 times larger than control samples.

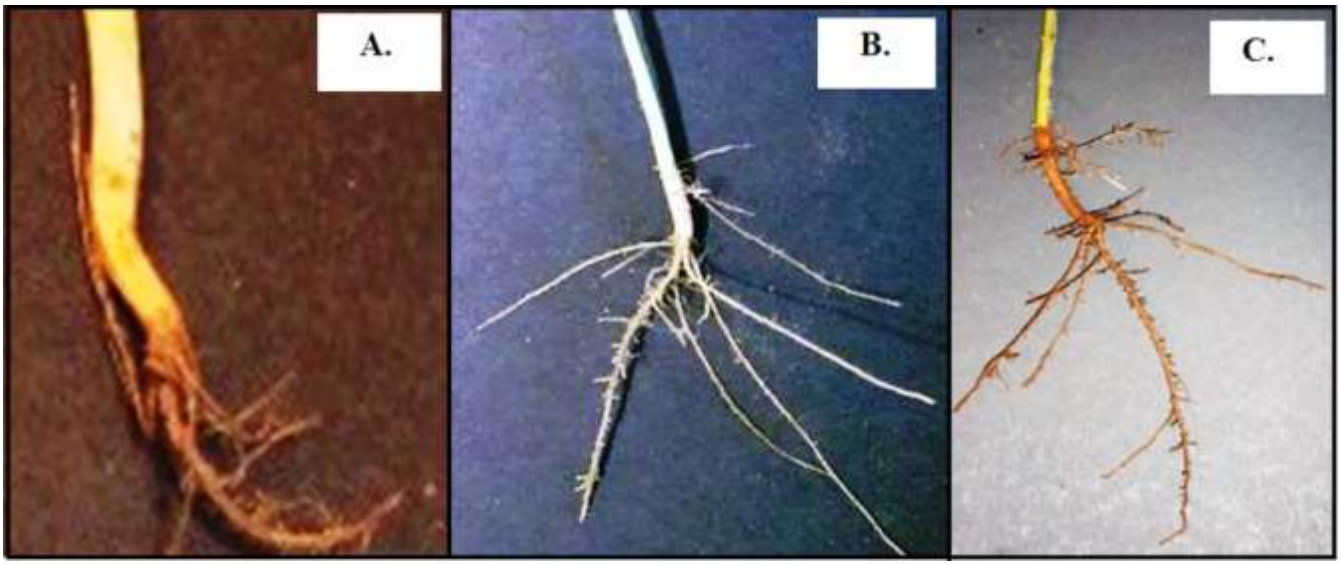

Figure 5: Comparison of Root Development A.) Roots of plant grown in control soil at 2 wks. B.) Roots of plant grown in control soil at 6 wks. C.) Roots of plant grown in control soil at 8 wks.

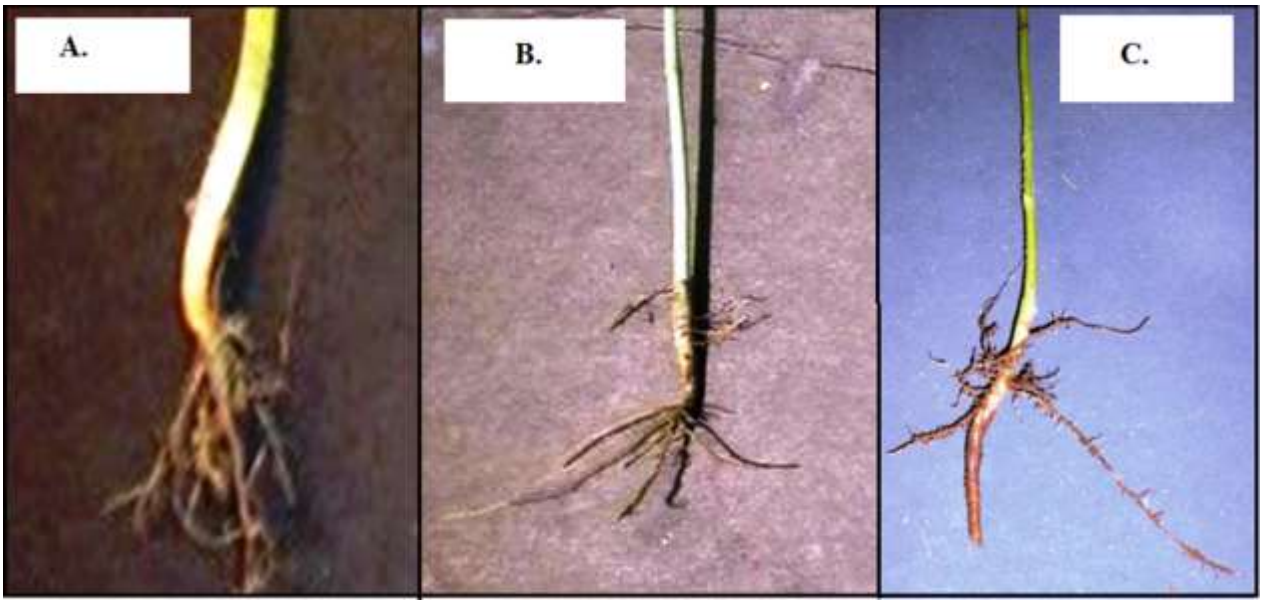

Figure 6: Comparison of Root Development A.) Roots of plant grown in EIBF at 2 wks. B.) Roots of plant grown in EIBF at 6 wks. C.) Roots of plant grown in EIBF at 8 wks.

Figure 7: Comparison of pea plant grown in control and induced soil. A.)Plant grown in ethylene induced soil for 8 wks. Pea plant contains increased number of larger pea, with reduced foliage. B.) Plant grown in control soil for 8 wks. Smaller peas present.

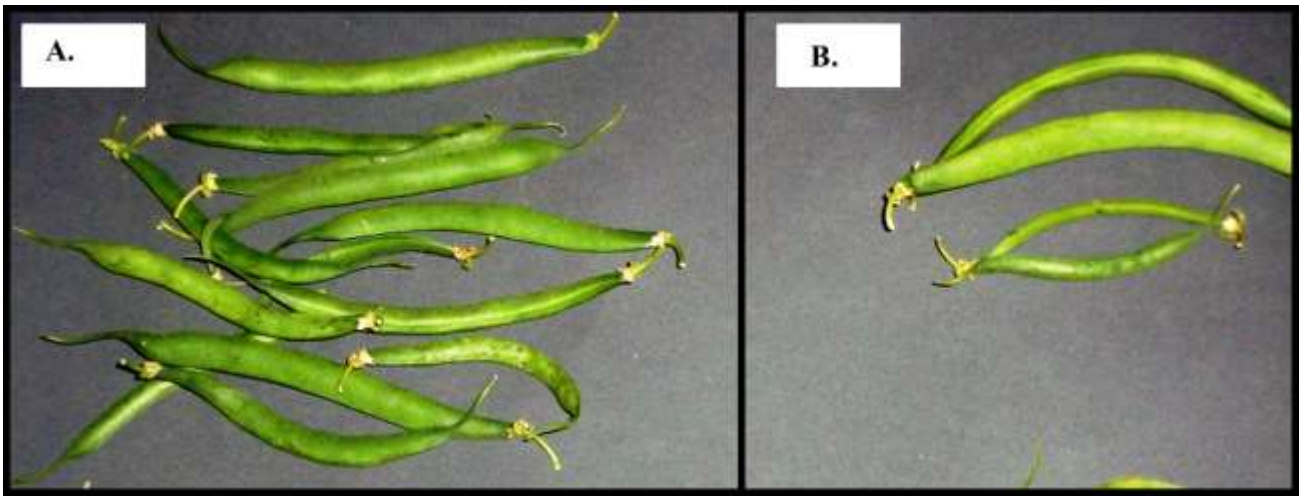

Figure 9: Comparison of pea products that exceed 4inches. A.)Peas collected from plant grown in ethylene induced soil for 8 wks, B.) Peas collected from plant grown in control soil for 8 wks, Pea plants grown in induced soil had 54% that measure 4-6inches in length, compared to peas harvested from plants grown in in control soil.

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

In conclusion, ethylene plays a significant role in the desired plant growth promoting effects of PGPR. The GC-MS analysis determined that soil samples exposed to the ethylene, nitrogen, and a heavy metal, released acetonitrile into the head space of test vials, acetonitrile is a component of indole-3-acetonitrile.

It is still unclear how ethylene affects the production of acetonitrile, but there are currently three known pathways that are used by both plants and microbes to biosynthesize IAN. Pathway (1): TRP→indole-3-acetaldoxime (IAM) → indole 3-acetonitirle (IAN)→ indole-3-acetic acid (IAA). Pathway (2): TRP→ IAM→ IAA. Pathway (3): TRP→ indole-3-pyruvic acid (IPA)→ indole-3-acetaldehyde (IAAId)→ IAA, (Gutierrez et al., 2009; Kobayashi et al., 1995; Schneider & Wightman, 1978; Sembdner et al., 165 1980; Vegan-Hernandez et al., 2002; Woong et al., 2003). Ethylene may affect a positive feedback system that promotes increased indoleacetaldoxime dehydratase (IAOx), nitrile hydratase (NHase), and/or nitrilase activity within the microbial cell, (Perry 2011). The nitrile is potentially released into the soil, where it undergoes an abiotic reaction releasing free cyanide into the soil to induce seed germination and plant growth. The non-induced soil samples did not contain the nitrile compounds, and displayed a reduction in plant growth and development. The shortened rooting system of green pea plants grown in the induced biological fertilizer may be linked to the fertilizers ability to maintain moisture. The plants did not need an expansive rooting system to obtain adequate water and nutrients for growth.

This study has global impacts and will be an important study for international organic farmers. This induction method can produce a bio-fertilizer than can improve soil quality within 4 weeks of application. If the induction method can improve PGPR ability to enhance plant growth similar to synthetic fertilizers, it may encourage more farmers to practice organic farming techniques. Additional research to understand the mechanism may lead to optimization techniques for specific plant traits such as longer roots or robust fibrous systems to combat high stress conditions such as dry climate areas, (Pace et al., 1999).

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

ACKNOWLEDGMENTS

Thank you Robert Perry for your support. Project Consultants, Ashlie Perry, PhD (Professor at Endicott College, Salem, MA) and Fengkun Du, PhD (Environmental Specialist at EBS, Mandeville, LA).

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

CITATIONS

Abeles, F., Morgan, P., and Salveit, M. Ethylene in plant biology, London: Academic Press (2nd eds.) (2012) **2:** 254

Aggani, S. Development of bio-fertilizers and its future perspective. (2013) Scholar Academic; Journal of Pharmacy **2**: 327-332

Al-Busaidi, K. Effects of organic and inorganic fertilizers addition on growth and yield of banana (Musa AAA cv. Malindi) on a saline and non-saline soil in Oman. (2013) Journal of Horticulture and Forestry **5**: 146-155

Arshad, M., Saleem, M., & Hussain, S. Perspectives of bacterial ACC deaminase in phytoremediation. (2007) Trends in Biotechnology (Review) **25**: 356-362

Banayo, N., Cruz, P., Aquilar, E., Badayos, R., and Haefele, S. Evaluation of biofertilizers in irrigated rice; effects on grain yield at different fertilizer rates. (2012) Agriculture **1:** 73-86

Boubel, R., Donald, L., Turner, D., and Stern, A. Fundamentals of Air Pollution, Academic Press (3rd eds.) (1994)

Brust, J. Green "air pollution" caused by ethylene. (2009) Weekly Crop Update, University of Delaware. agdev.anr.udel.edu/weeklycropupdate/?p=870

De Bont, J. & Albers, R. Microbial metabolism of ethylene. (1976) Antonie van Leeuwenhoek **42**:73-80

Downes, K., Light, M., Postz, M., Kohout, L., and Staden, J. Comparison of germination responses of Anigozanthos flavidus (Haemodoraceae), Gyrostemon racemiger and Gyrostemon ramulosus (Gyrostemonaceae) to smaoke water and smoke derived compounds Karrikinolide (KAR1) and glyceronitrile. (2013) Annals of Botany **111:** 489-497

Flematti, G., Merritt, D., Piggott, M., Trengrove, R., Smith, S., Dixon, K., and Ghisalberti, E. Burning vegetation produces cyanohydrins that liberate cyanide and stimulate seed germination. (2011) Nature Communications **2:** 1-6

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

Frame, J. *Pisum sativum* L. (2014) FAO Fiato Panis
http://www.fao.org/ag/agp/AGPC/doc/GBASE/Data/pf000493.HTM

Gutierrez, C., Matsui, G., Lincoln, D. & Lovell, C. Production of the Phytohormone Indole-3-Acetic Acid by Estuarine Species of the Genus *Vibrio*. (2009) Applied Environmental Microbiology **75**: 2253-2258

Isfahani, F. and Besharati , H. Effect of biofertilizers on yield and yield components of cucumber. (2012) **2**: 83-92

Johnson, R. The U.S. Trade Situation for Fruit and Vegetable Products. Congressional Research Service (2014) **7-5700**: 2-3

Kobayashi, M., Suzuki, T., Fujita, T., Masuda, M. & Shimizu, S. Occurrence of Enzymes Involved in Biosynthesis of Indole-3-Acetic Acid from Indole-3-Acetonitirle in Plant-Associated Bacteria, Agrobacterium and Rhizobium. (1995) Proc. Natl. Acad. Sci. USA **92**: 714-718

Nelson, D., Flematti, G., Ghisalbert, E., Dixon, K., and Smith, S. Regulation of seed germination and seedling growth by chemical signals from burning vegetation. (2012) Annu Rev Plant Biol **63:** 107-130

Miller, A., Walsh, C. & Cohen, J. (1987) Measurement of indole-3-acetic acid in peach fruits (*Prunus persica* L. Batsch vs Redhaven) during development. Plant Physiology **84**:491-494

Pace, P., Cralle, H., El-Halawany, S., Cothren, J., & Senseman, S. Drought-induced Changes in Shoot and Root Growth of Young Cotton Plants. 1999. The Journal of Cotton Science **3**:183-187

Perry, G. Pending Application Number: 14334392. Hydrocarbons and Heavy Metals Induce Bacteria to Modify Plant Development in Climacteric Fruit and Plants. Applied July 2014. Pending Patent.

Ribaudo, M., Delgado, J., Hansen, L., Livingston, M., Mosheim, R., and James Williamson. Nitrogen in agricultural systems: implications for conservation policy. (2011) USDA Economic Research Service *www.ers.usda.gov/publications/err127*

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014

Schneider, E. & Wightman, F. Phytohormones and Related Compounds: A Comprehensive Treatise, eds. Letham, D. S., Goodwin, P. B. & Higgins, T. J. V. (1978) (Elsevier, Amsterdam) **1**: 29-105

Sembdner, G., Gross, D., Liebisch, H. W. & Schneider, G. Hormonal Regulation of Development I: Molecular Aspects of Plant Hormones, ed. MacMillan, J. (1980) (Springer, Berlin) **9**: 281-289

Vandeputte, O., Oden, S., Mol, A., Vereecke, D., Goethals, K., Jaziri, M. & Prinsen, E. Infected plant tissues controlled by compounds specific to phytopathogen *Rhodococcus fascians* is biosynthesis of auxin by gram positive bacteria. (2005) Applied Environmental Microbiology **71**:1169-1177

Vega-Hernandez, M., Leon-Barrios, M., & Perez-Galdona, R. Indole-3-Acetic Acid Production from Indole-3-Acetonitrile in Bradyrhizobium. (2002) Soil Biology & Biochemistry **34**: 665-668

Woong, P., Kriechbaumer, V., Mueller, A., Piotrowski, M., Meeley, A. & Glawischnig, E. The Nitrilase ZmNIT2 Converts Indole-3-Acetonitrile to Indole-3-Acetic Acid. (2003) Plant Physiol **133**: 794-802

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.543v1 | CC-BY 4.0 Open Access | rec: 16 Oct 2014, publ: 16 Oct 2014