Ethylene Induces Soil Microbes to Delay Fruit Ripening

The consumer demand for fresh fruits and vegetables increases every year, and farmers need a low cost novel method to reduce post-harvest loss and preserve the quality of fresh fruits and vegetables. This study identifies a method to induce soil bacteria to biosynthesize a nitrile compound that potentially enters the plants tissue and negatively affects climacteric ripening and delays the ripening process at 20-30°C. This study used soil rich with soil microbes, to delay the ripening of climacteric fruit. The soil was treated with nitrogen, a heavy metal, and ethylene gas.

Ethylene induced the soil to delay the ripening of organic bananas and peaches. A prototype transportation container maintained fruit fresh for up to 72 h at 20-30°C. The fruit retained color, firmness, texture, no bruising and minimal spotting. The soil also prevented fungal infection in all samples. GC-MS analysis suggests ethylene induced the soil microbes to release an acetonitrile compound into the gaseous environment. The nitrile is released in low concentrations, but mature plants (fruits) contain very low levels of indole-3-acetonitrile (IAN) or indole-3-acetic acid (IAA).

The nitrile may obstruct or modify the mature plants (fruit) late stages development process, thus delay the climacteric ripening process and retarding the physiological and phenotypic effects of fruit ripening. We believe this study may have strong applications for post-harvest biotechnology.
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1 INTRODUCTION:

A report generated by the USDA in 2011, states American consumption of fruits and vegetables has increased steadily since the 1980s, the demand for fresh fruits and vegetables has increased by 22% and 16.5%, respectively, (Johnson, 2014). Unfortunately nearly 20% of all fruits and vegetables never reach the targeted consumer sites due to spoilage and post-harvest loss (PHL), 19.6% of PHL is attributed to the effects of pre-mature or prolonged climacteric
ripening that lead to senescence, apoptosis, lesions, spotting, bruising, infection, and/or eventual spoilage that render the produce unsellable (Burg, 2004; Sakimin et al., 2012; Seymour et al., 2013). Abiotic and biotic signals from the surrounding environment trigger ethylene production within the cell, overtime ethylene re-enters the plant tissue and binds to ETR complexes (ethylene receptors) embedded in the surface membranes of plant/fruit cells. When ethylene binds to ETR complexes it initiates climacteric ripening and there is an immediate increase in cellular respiration (also referred to as “sweating”) and utilization of soluble sugars during glycolysis (sucrose→ glucose + fructose), (Theologis 2004; Trobacher 2009). The US domestic farming industry is a $618 billion dollar business; the agricultural and agronomy industry is searching for an innovate product that will reduce PHL, prevent spoilage, and pre-mature/prolonged ripening of fresh fruits and vegetables, (Barth et al., 2010; Blankenship, 2001; Johnson, 2014; Kader, 2005).

Previous literatures states that plant growth promoting bacteria (PGPB) biosynthesize and/or degrade phytohormones, such as indole-3-acetic acid (IAA), indole-3-acetonitrile (IAN), 1-aminocyclopropane-1 (ACC), cyanide, or ethylene, therefore directly or indirectly affecting plant growth and/or development, (Arshad et al., 2007; DeBont and Albers, 1976; Miller et al., 1987; Vandeputte et al., 2005). PGPB are the soil bacteria that inhabit the area around/on the rhizosphere of several plant species. Studies conducted by Pierce et al., 2011 & Perry, 2013, explore the idea that PGPB, specifically Rhodococcus rhodochrous strain DAP 96253, have the ability to delay the ripening of several species of climacteric fruits.

Pierce et al., 2011 and Pierce, et al., 2013 studies suggested Rhodococcus ability to delay ripening was enabled by culturing the cells on a specialized YEMEA media, induced with cobalt, urea, and/or asparagine, (Ganguly, 2005; Goodfellow & Anderson, 1977; Pierce et al., 2011;
Pierce at al., 2013). Pierce did not identify the mechanism or biochemical compound biosynthesized by Rhodococcus to delay ripening, the study did demonstrate that bacteria cultured on the specialized induced YEMEA media were able to reduce spotting and spoilage of climacteric fruit. Perry, 2013, study suggested that ethylene/propylene was induced biosynthesis of a nitrile compound that enabled Rhodococcus to delay fruit ripening. The study hypothesized that exposure to ethylene/propylene, cobalt, and an ammonium compound induced the bacteria to produce a nitrile or cyanohydrin compound that delayed the ripening process in several species of climacteric fruit. The study did not provide GC-MS or HPLC analysis of microbial samples to identify the nitrile compound biosynthesized by the bacteria, but enzymatic assays demonstrated that ethylene/propylene induced nitrile hydratase (NHase) (EC 4.2.1.84) and/or nitrilase like activity in Rhodococcus. Cells with high NHase and/or nitrilase like activity were able to maintain fruit firmness and reduce spotting, spoilage, and reduce color change, while cells with low NHase and/or nitrilase were unable to delay fruit ripening, (Perry, 2013).

However the current study expands upon this research by focusing on two project goals. The first goal is to determine if ethylene can induce top soil samples to produce a nitrile compound, such as IAN or acetonitrile compound. The second goal is to determine if ethylene can induce the soil to modify or deter plant development and/ or maturation in climacteric fruits and vegetables. This study used top soil collected around/on roots of young fruit bearing trees; the top soil was used because the soil contained high levels of PGPB and other microbes. The top soil samples were exposed to several inducers including ethylene. The results from the study suggest ethylene induced microbes found in the top soil to produce an acetonitrile compound that may have enabled the samples to delay the ripening process of organic fruit.

MATERIALS AND METHODS:
2.1 Soil Collection

Elsgaard demonstrated that a peat soil mixture, rich with soil bacteria, was able to effectively remove ethylene from a closed tri-phasic system environment, if the soil sample were gradually exposed to ethylene at increasing concentrations, (Elsgaard, 1998; Elsgaard & Anderson, 1998).

In this study, soil was initially collected from the rhizosphere area of two young fruit bearing plants, the top soil was rich with nutrients and soil microbes, leaves, rocks, root systems, and nematodes/ insects were removed from samples. The fruit bearing plants included flordahome pear tree (*Pyrus communis*) and black beauty eggplant tree (*Solanum melongen*), and one mature peach tree (*Prunus persica*) trees were grown in nursery in Albany, Ga. Soil was mixed in a 1:1:1 ratio.

The control samples were mixed with 500 ml of dH$_2$O, while the test samples were mixed with 500 ml of AP Mix (components included: Nitrogen, Phosphate, Iron, Yeast Lysate at pH 8.5), forming a slush-like substance, Perry 2014. The control soil mixture was aliquot into three 80 ml samples; the test sample was aliquot into six 80 ml samples. Each sample was stored in a 20˚C for 24 h before experiments.

2.2 Pre-Induced Exposure to Ethylene & Cyanide

Soil was induced with ethylene released from various species of over ripened fruit. The fruit and soil samples were stored in air tight containers for varied incubation periods at 20- 26˚C. Soil samples were harvested and stored at room temperature and fruit was discarded.

2.3 Delayed Fruit Ripening

Test samples were stored in air tight containers with organic bananas for 72 h, at 20-26˚C. The soil samples were in close proximity to the fruit, but were never allowed to directly contact
bananas. Bananas were purchased from Green Tomato Organic Market in Tallahassee, Fl. All bananas were certified organic, and were not sprayed with chemicals or pesticides, prior to experiments, unless otherwise noted. Fruit was imaged using a digital camera (Sony “Cyber Shot”, 7.2 mega pixels, automatic flash) to identify physical changes in the fruit.

2.4 Shipping and Storage at 20-30°C

Fruit samples were transported and stored in a box that contained the ethylene induced soil. The box was designed to prevent the soil from contacting the fruit. The box did allow the exchange of gaseous compounds released by the fruit and bacteria. Fruit was transported over 70 miles and stored with soil in closed box for 48 h to 72 h, temperatures ranged from 20-30°C. Control samples were stored at same temperature in closed brown paper bag.

2.5 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 induced soil samples. There were four control samples mixed with dH₂O, and six induced soil samples were mixed with the AP mixture and induced with ethylene released from ripening fruit. 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 Induced Soil Delays Ripening

Control bananas were stored in air tight containers with 65 ml of dH₂O at room temperature, for 72 h. Test bananas were stored in air tight containers with 65 ml of ethylene induced soil at room temperature, for 72 h. After four days soil samples were harvested and stored at -4°C, sample size > 3 hands (45 – 60 fingers of fruit).

Bananas labeled as control fruit were stored with water. There was heavy condensation in the containers, a sign of increased cellular respiration and climacteric ripening. The bananas were soft, decreased firmness, and change in texture. The skin was bruised and spotted. Peeled bananas showed visible bruising, in the form of a slimly yellow spotting and breakage, Figure 1 (A- Control bananas at 0 h. B- Control bananas at 72 h. Bananas are soft and bruise easily, image shows indent in fruit tissue. C- Control bananas peeled at 72 h.).

Test samples were stored with ethylene induced soil. There was minimal condensation, bananas firm, no change in hue, and no visible spotting. The peeled bananas were firm with no bruising or breakage, Figure 2 (A- Sample bananas at 0 h. B- Sample bananas at 72 h. Bananas are firm. C- Sample bananas peeled at 72 h. Tissue has no visible spotting.). The ethylene induced soil was able to delay the ripening process in climacteric bananas.
3.2 Ethylene Induced Soil vs. Non Induced Soil

The experiment contained three experimental conditions, including a control sample, induced sample, and non-induced sample. Fruit was purchased from an organic market in Tallahassee, FL and retailer verified fruit meet USDA qualifications for organic produce. Produce was not previously exposed to chemical fertilizers or pesticides.

Control samples were stored in air tight containers with 65 ml of dH₂O. Non-induced test samples were stored in air tight containers with soil mixed with 500 ml of AP mixture. Induced test samples were stored in air tight containers with soil mixed with 500 ml of AP mixture and induced with ethylene by exposing soil to ripening fruit. All samples were stored at room temperature for 72 h. After 72 h, bananas were imaged and the soil samples were harvested and stored at -4°C. The experiment was repeated four times with triplicate samples, total experimental size > 4 hands (50 – 65 fingers of fruit).

Control bananas were soft, bruised, and spotted. Peeled bananas were fragile and bruised, and fungal infection was present on some samples, Figure 3 (A- Control bananas at 0 h. B- Control bananas at 72 h. C- Control bananas peeled at 72 h.). Non-induced samples were soft and spotted, but bananas were firmer than control samples. The peeled non-induced samples were bruised and contain some fungal infections, Figure 4 (A- Non-induced sample at 0 h. B- Non-induced samples peeled at 72 h. C- Non-induced samples at 72 h.). Induced samples were firm and retained color and texture. Peeled induced samples did not display fungal infection or visible
bruising or spotting, Figure 5 (A- Induced samples at 0 h. B- Induced samples peeled at 72 h. C- Induced samples at 72 h.).

The results for the non-induced samples resembled images of bananas exposed to Rhodococcus cultured on YEMEA induced media reported by Pierce et al., 2011. The AP mix, that included nitrogen, phosphorous, a heavy metal, and yeast, enabled the soil bacteria to affect the ripening process of the climacteric fruit, but the exposure to ethylene enhanced the soils bacteria ability to effectively delay fruit ripening.

### 3.3 Shipping with Ethylene Induced Soil

Fruit was purchased from an organic market in Tallahassee, FL and transported for 70 miles; retailer verified fruit meet USDA qualifications for organic produce. Produce was not previously exposed to chemical fertilizers or pesticides.

Control fruit were stored in brown paper bags and stored in a storage room, temperatures ranged 20-30°C for 48 h to 72 h. Test samples were stored in transport container with 1 qt of ethylene induced soil bags in a storage room, temperature ranged 20-30°C for 72 h. The experiment was repeated twice with triplicate samples, total experimental size > 2 hands (35 – 40 fingers of fruit).

The transport container was made out of pre-treated wood and maintained an air tight seal throughout the experiment, Figure 6. Both control and sample fruit started to ripen after 24 h, Figure 7 (A- Control samples at 0hrs. B- Control samples at 24 h. C- Control samples at 48 h in brown paper bag. D- Induced samples at 0 h. E- Induced samples at 24 h transportation containers. F- Induced samples at 48 h transportation containers.). Bananas stored with ethylene induced soil were firmer than control samples and maintained texture after 48 h, Figure 8 (A- Control samples peeled at 48 h from brown paper bag. B- Induced sample peeled at 48 h from transportation container.). Peaches stored with ethylene induced were firmer than control
samples, and peaches had less visible spotting or bruising, Figure 9 (A- Control samples whole and cut at 48 h from brown paper bag. Control samples were soft and spotted. B- Induced samples whole and cut at 48 h from transportation container. Peaches were firm, yellow, and no visible bruising.).

The transport container that contained the induced soil slowed the ripening process in climacteric fruit. The gaseous compounds released by the soil slowed the physical effects of ripening in species of climacteric fruit.

3.4 Ethylene in Induced Soil Samples

GC-MS analysis identified the presence of aromatic or volatile compounds that may be released by soil samples to delay fruit ripening. It was hypothesized that the ethylene induced soil samples released a low concentration of a nitrile compound that entered plant tissue and delayed ripening.

Soil samples were heated to release gaseous or volatile compounds from the aqueous mixture. An integrated ion count showed ethylene concentrations differed in control and ethylene induced soil samples. Soil exposed to ripening fruit ethylene concentration was six time higher than control samples, Table 1. Ethylene cyanohydrin was not present in any samples and cyanide concentrations were low in all samples.

Based on the initial GC-MS data collected from the soil samples, the data suggest microbes within the soil samples were sequestering ethylene from the surrounding environment. Then the soil microbes were exposed to high levels of heat the ethylene was released into the vial headspace. Suggesting the microbes removed the ethylene to degrade or metabolize into a useful byproduct.
3.5 IAN/ACN in Induced Soil Samples

As stated in the previous study, it was hypothesized that ethylene induced soil bacteria to biosynthesize a nitrile compound such as indole-3-acetonitrile or a cyanohydrin, (Perry, 2013). The GC-MS analysis did not find detectable levels of ethylene cyanohydrin, and excluded the cyanohydrin as a potential nitrile produced by the induced soil samples. GC-MS analysis identified an acetonitrile compound, the methyl hydrogens are substituted with a 1H-indol-3-yl group.

Control samples did not contain nitrile compounds. Control samples contained moderate levels of carbon disulfide, methylene chloride, and acetone, Figure 10. Induced soil samples contained acetonitrile in all six samples. The head space analysis also contained acetone, 2-methyl-1-propanol (fermentation by-product), 2-pentanone, and 3-methyl-1-butanol, Figure 11.

Ethylene Induce Nitrile Production in PGPB

In conclusion the data ethylene released by plant cells may play a significant role in the plant growth promoting effects of PGPB. 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. The GC-MS analysis was designed to identify cyanide or cyanohydrin compounds, but reduction in heating levels of the injection column may results in identification
of an indole-3-acetonitrile released by microbes. The presence of the compound has a negative
effect on the climacteric ripening process in several species of fruit.

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) → IAN→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 positive, causing an overexpression or increased activity of indoleacetaldoxime
dehydratase (E.C. 4.99.1.6), Figure 12 (Conversion of TRP → IAN, the nitrile is released and
binds to plants tissue to potentially delay fruit ripening.). Non-induced soil samples showed a
significant reduction in the soils ability to delay fruit ripening. The direct mechanism used by the
bacteria to delay climacteric ripening is still unknown. Additional research to understand the
mechanism and enzymes used to imitate this process requires further investigation.

This study can have global impacts. This study could be a useful tool for organic farmers
in developing countries. The materials required for the project are cheap, and the final developed
product would be reusable. A product developed with induction method will improve with each
usage. Additional research should be conducted to study a broader scope of potential enzymes
that and biological compounds required by PGPB to delay the climacteric ripening process.

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CITATIONS


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Figure 1

A- Control bananas at 0 h. B- Control bananas at 72 h. Bananas are soft and bruise easily, image shows indent in fruit tissue. C- Control bananas peeled at 72 h.
Figure 2

A- Sample bananas at 0 h. B- Sample bananas at 72 h. Bananas are firm. C- Sample bananas peeled at 72 h. Tissue has no visible spotting.
Figure 3

A- Control bananas at 0 h. B- Control bananas at 72 h. C- Control bananas peeled at 72 h.
Figure 4

A- Non-induced sample at 0 h. B- Non-induced samples peeled at 72 h. C- Non-induced samples at 72 h.
Figure 5

A- Induced samples at 0 h. B- Induced samples peeled at 72 h. C- Induced samples at 72 h.
Figure 6

A- Control samples at 0 h. B- Control samples at 24 h. C- Control samples at 48 h in brown paper bag. D- Induced samples at 0 h. E- Induced samples at 24 h transportation containers. F- Induced samples at 48 h transportation containers.
Figure 7

A- Control samples peeled at 48 h from brown paper bag. B- Induced sample peeled at 48 h from transportation container.
Figure 8

A

B
Figure 9

A

B
Control samples did not contain nitrile compounds.
Induced soil samples contained acetonitrile in all six samples.
Conversion of TRP → IAN, the nitrile is released and binds to plants tissue to potentially delay fruit ripening.
Figure 13

Table 1

An integrated ion count showed ethylene concentrations differed in control and ethylene induced soil samples. Soil exposed to ripening fruit ethylene concentration was six time higher than control samples.

<table>
<thead>
<tr>
<th>Samples:</th>
<th>Ethylene (IIC)*</th>
<th>Cyanide (IIC)*</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene Induced Soil</td>
<td>58912 ± 1832</td>
<td>91442 ± 2347</td>
<td>≤0.01%</td>
</tr>
<tr>
<td>Control Non-Induced Soil</td>
<td>9658 ± 416</td>
<td>80004 ± 1239</td>
<td>≤0.01%</td>
</tr>
</tbody>
</table>