

## VESTA Suppressed Disease In Commercial Iceberg Lettuce By Establishing Favorable Soil Microbiome Communities

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### Abstract

VESTA is known to reduce disease in multiple crops. This study was conducted to determine how VESTA acts on field-grown lettuce. Soil and rhizobium samples were collected from three different lettuce fields over time from planting to harvest. V4 16S rRNA sequencing revealed differences in bacterial compositions between control and VESTA-treated plots. Members of *Sphingomonadaceae* were reduced in VESTA-treated plots. Several of the *Sphingomonadaceae* have been reported as pathogens for corky root of lettuce. Consistently, VESTA treatment reduced the incidence of disease. Previous studies have shown that corky root causing strains produce nitrite and ammonia from nitrate. The non-infectious corky root is caused by high N toxicity. The present study has revealed that VESTA acts by reducing the pathogens. Further insights into the mechanisms of VESTA activity are discussed.

Sample collection, August-October 2014

V4 16S rRNA gene sequencing, January 2015

Biological Activity Report, February 2015

### Introduction

VESTA is a fermented product formed by mixing 1:1 SOBEC and BHF10. A previous study showed that out of the total of 5,496 OTUs present in all samples tested, 525 OTUs were significantly different in their abundance between SOBEC and BHF10 products (Second Genome Report, EPAN 12-0143, 2013). VESTA has the highest disease protection activity in the field, followed by SOBEC and then BHF10 (Communicated by SOBEC, Inc.). Total of 220 OTUs showed changes in abundance when compared with the known efficacy of the products from

low to high (BHF10/SOBEC/VESTA), where 76 OTUs decreased in abundance in correlation with increased product activity and 114 OTUs increased in abundance in correlation with increased product activity in suppressing disease (Figure 4-1, Second Genome Report, EPAN 12-0143, 2013). VESTA is known to be significantly effective in disease protection for a range of crops in multiple environments. The method-of-action of VESTA on the field is not known. The purpose of this study was to discover the underlying mechanism of VESTA activity in providing disease protection to

commercial lettuce varieties grown on farmed fields in Salinas, CA.

### **Lettuce in Salinas, CA**

Salinas Valley has one of the longest growing seasons for lettuce in the country (Turini et al., 2011). Lettuce diseases cause severe losses. Downy Mildew caused by a fungus can affect up to 80% of acreage. Bottom Rot affects iceberg varieties. Bacterial diseases like corky root rot, and bacterial soft rot can affect roots, leaves and the entire plant causing severe damage and yield loss.

## **Experimental Procedures**

### **Lettuce Varieties Tested**

Several cultivars of commercial iceberg lettuce were tested, including “Oso Flaco”, “Salute”, “Rio Bravo”. See **Appendix 1** for details.

### **Sample Collection**

Samples were collected four times between planting to pre-harvest from three different fields in Salinas, CA; Merrill Spreckels 2, Merrill Norton Lot 7, and T&A Spreckels Lot 17. (1) Soil samples were collected pre-planting, (2) Soil and root samples together were collected after the first application of VESTA, (3) Soil and root samples together after the second application of VESTA, (4) Soil and root samples together were collected at pre-harvest. At each time point, five replicates of control (untreated) and VESTA-treated samples were collected for analysis. In addition, five replicates of soil and root samples were collected from dead or diseased (DORD) plants from control and VESTA-treated fields at pre-harvest. Total of **150 samples** were collected from 3 fields as follows; 5 soil samples pre-planting (3 fields x 5 samples each =**15**), 5 soil+root samples of controls and 5 soil+root of VESTA-treated at 3 time points as indicated above ((3 fields

x (5 control + 5 treated)) x3 time points =**90**), 5 soil+root of DORD control and VESTA-treated at pre-harvest (3 fields x (5 control + 5 treated) =**30**), extra 5 samples from each field were collected for pre-harvest VESTA healthy (3 fields x 5 healthy-treated =**15**). A subset (105) of the 150 samples was analyzed for this study, as described. None of the root (endosphere) samples have yet been analyzed. See **Appendix 2** for sample details.

On each field, samples were collected across different beds by gently extracting the lettuce plant from the soil, removing the loose soil around the roots by shaking and tapping to collect the rhizosphere and root (endosphere) into a 15 ml conical tube (supplied by Second Genome, Inc.). Samples were labeled with bar codes (**Appendix 1**). Samples were collected before irrigation events to facilitate soil removal by gentle shaking. After collection, all samples were kept frozen until DNA isolation.

### **VESTA Application**

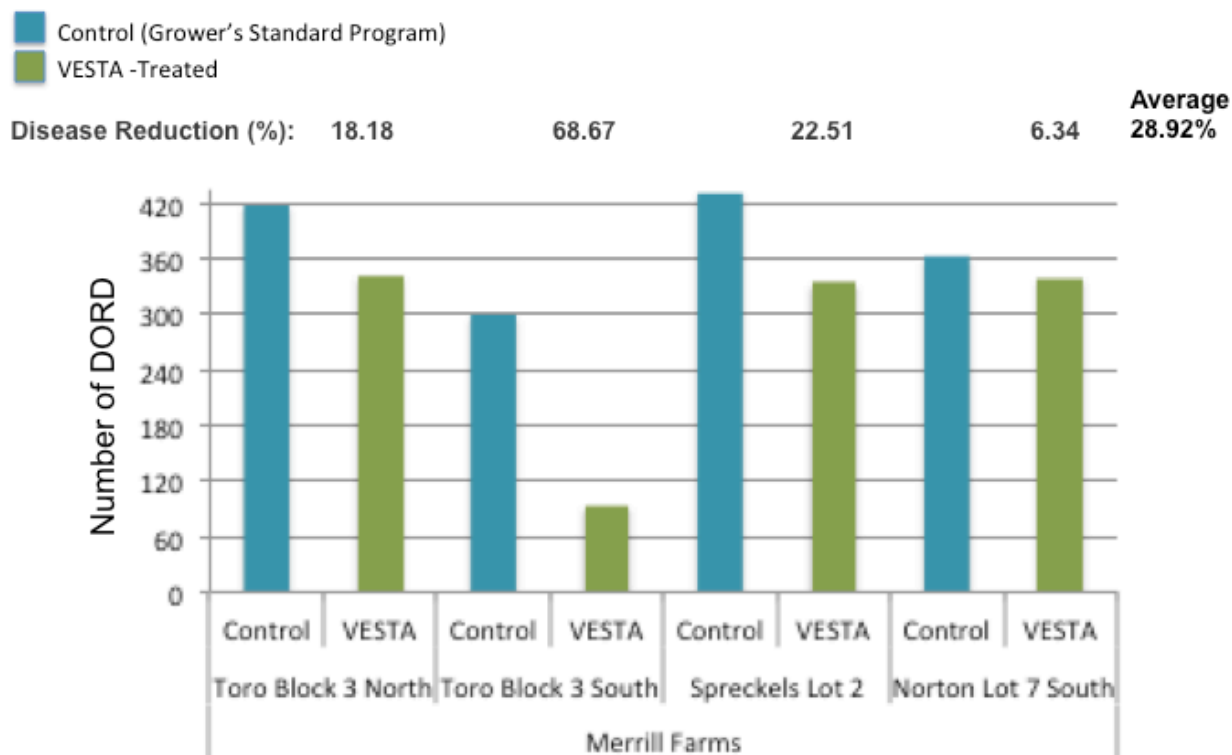
VESTA was applied through the irrigation system at the manufacturer’s recommended dosage. There were two applications of VESTA during the course of the study, and sample time points 2 and 3 were after each of VESTA applications.

### **DORD (Dead or Diseased)**

DORD plants were counted and selected based upon visual inspection. Soil and root (endosphere) samples were collected as described above.

### **Time of Study**

Sample collection spanned about three months in 2014. The first soil samples (pre-planting) were collected on Aug 1,



**Figure 1. Merrill Farms:** VESTA treatment reduced the number of DORD lettuce plants at Merrill Farms. The average affect of VESTA treatment at Merrill farms was 28.92% reduction in disease.

and the last pre-harvest samples were collected on Oct 22, 2014 (**Appendix 2**).

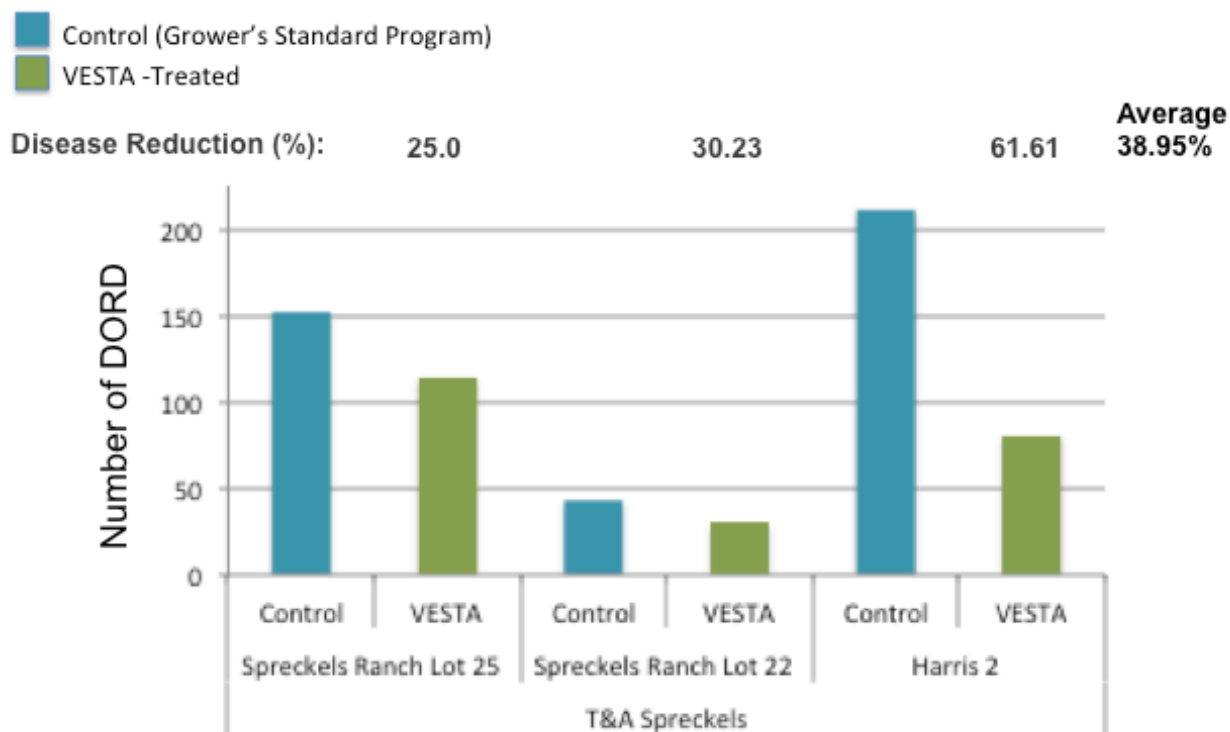
## Results and Discussion

### Disease suppression

Number of diseased or dead (DORD) plants were counted on control and VESTA treated beds. VESTA treatment reduced the incidence of disease at Merrill Farms and at T&A Spreckels. Overall, DORD plants were reduced by an average of >28% at Merrill Farms and by >38% at T&A Spreckels (Figures 1 and 2). At Toro Block 3 South and Harris 2, DORD were decreased by >60% (**Figures 1 and 2**). Rhizosphere soil samples were analyzed from untreated (control) and VESTA-treated fields.

### VESTA altered microbial composition

There were 315 significantly different taxa between soil samples collected before planting and VESTA-treated beds at TA17 field (Chapter 4, EPAN 2015-01-13). Of these 315 differences, 49 taxa were higher and 266 taxa were lower in the pre-planted soil samples (Figure 4.4, EPAN 2015-01-13). Comparison of control (untreated) and VESTA-treated soil samples from Merrill Farms showed there were 39 taxa that were higher in the control soil samples at MN7 (Figure 5.4, EPAN 2015-01-13). At MS2 farm, 4 taxa were higher, and 1 was lower in the control samples (Figure 6.4, EPAN 2015-01-13). At TA17, 8 were higher and 7 taxa were lower in control samples (Figure 7.4, EPAN 2015-01-13). These



**Figure 2. T & A Spreckels:** VESTA treatment reduced the number of DORD lettuce plants at T & A Spreckels. The average affect of VESTA treatment at T & A Spreckels was 38.95% reduction in disease

data suggested that VESTA treatment altered the presence and abundance of several different taxa.

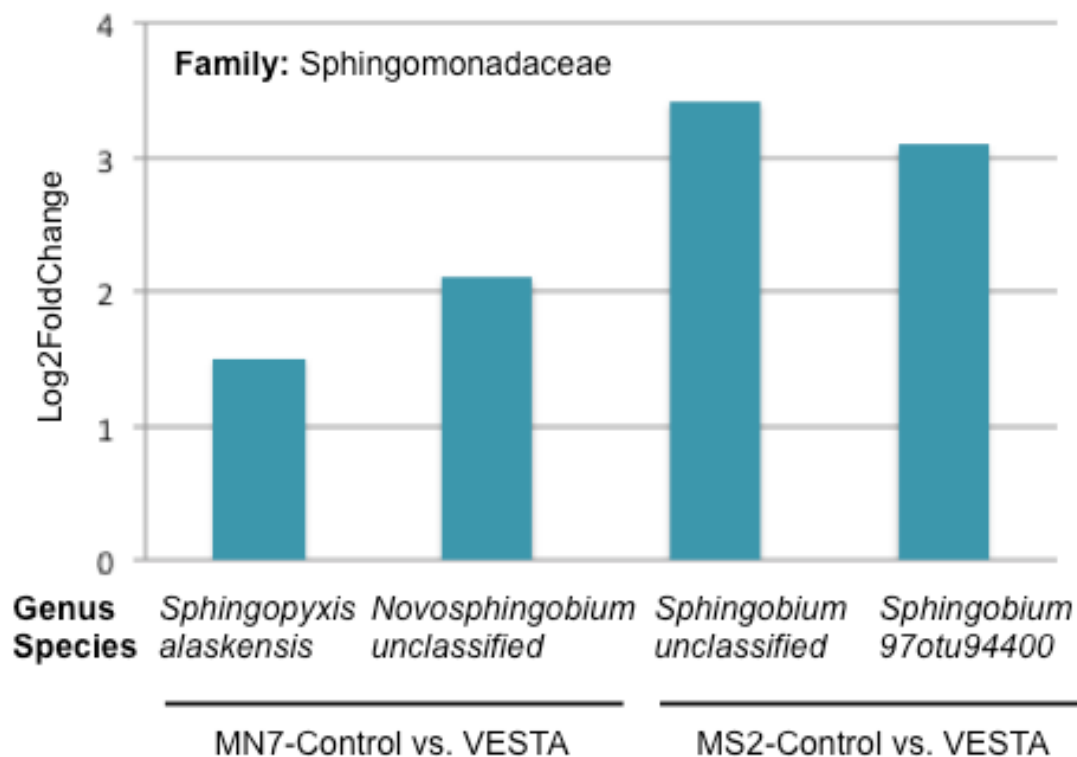
**Presence of Sphingomonadaceae family altered by VESTA treatment**

Sphingomonadaceae family was amongst those reduced by VESTA treatment. Sphingomonadaceae were higher in MN7-Control and MS2-Control (Figures 5.4 and 6.4, EPAN 2015-01-13) samples. MN7 control fields had higher levels of *Sphingopyxis alaskensis* and *Novosphingobium* (unclassified), and MS2 fields had higher levels of *Sphingobium* (unclassified) and *Sphingobium 97otu94400* (Figure 3). While there were several altered families, Sphingomonadaceae has a particular significance. Members of the

Sphingomonadaceae are known to cause corky root of lettuce.

**Corky root of lettuce**

Corky root of lettuce is an economically important disease in California. Infected lettuce roots develop yellow or brown lesions and longitudinal corky ridges. Corky root can cause 30-70% losses in yield. The most well studied pathogen for corky root is *Sphingomonadaceae suberifaciens* CA1 (formerly named *Rhizomonas suberifaciens*; van Bruggen et al., 1990; Yabuuchi et al., 1999). Several lettuce cultivars have been isolated with resistance to corky root, and the resistance is conferred by a recessive allele at a single locus (*cor*) (van Bruggen, 1997).



**Figure 3. Sphingomonadaceae species** higher (Log2FoldChange) in control (untreated) samples compared to VESTA-treated samples at MN7 and MS2 fields.

In addition to *Sphingomonadaceae* *suberifaciens* CA1, several other strains of *Sphingomonas* spp. have been shown to cause corky root of lettuce. It was reported that 16 strains of *Sphingomonas* spp., 19 isolates closest to *Sphingopyxis* spp. and 2 strains of *Sphingobium* spp. caused corky root on resistant lettuce (*cor*) cultivars (**Figure 4**) (Bull, 2009). These results have indicated that a wide range of members of *Sphingomonadaceae*, including *Sphingomonas* spp., and *Sphingobium* spp. cause corky root of lettuce.

#### VESTA – Methods of Action

VESTA treated soils at MS2 and MN7 had reduced members of *Sphingomonadaceae* (**Figure 3**). This correlates well with the significant reduction in disease observed on

those two fields. Further analysis is needed to confirm the same for T&A Spreckels. Based upon these results, there are two possible VESTA methods of action to reduce disease in lettuce. These mechanisms of action are not mutually exclusive and could function simultaneously

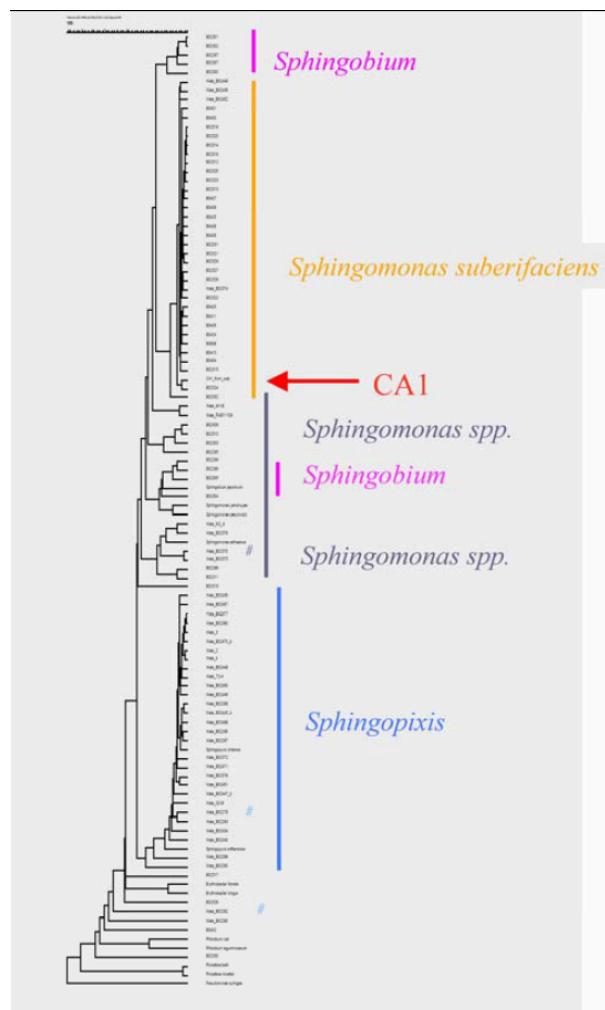
1. VESTA treatment displaces disease-causing organisms in the soil with non-disease causing organisms by:

- (a) Increasing biodiversity – the “chaos theory” (Ron Helland, SOBEC, Inc.).
- (b) Altering soil properties and thereby changing soil microbiome.

Previous report identified 525 OTUs that were more abundant in SOBEC product

than BHF10, and 114 OTUs were correlated with increase in product activity (Figure 4-1, Second Genome Report, EPAN 12-0143, 2013). A list of these 114 OTUs is attached (**Appendix 3**). It is possible that VESTA application replaces and establishes a new microbiota in the soil. In this study, the field had a strong influence on the presence and abundance of different strains; the microbiome was separated based upon where samples were collected (Figure 3.3.4, EPAN 2015-01-13). It is likely that VESTA application on different fields establishes different microbiota selected based upon the moisture and other characteristics of the particular location, thereby replacing the microbiome communities and shifting the composition to favorable consortium.

The second possibility is that communities established by VESTA actively modify soil properties and re-establish the microbiome. Corky root of lettuce has been attributed to phytotoxic components, like ammonia from decomposing lettuce or excessive fertilization (van Bruggen et al., 1989). These studies showed that lettuce corky root causing strains, all produce nitrite and ammonia from nitrate (van Bruggen et al., 1989). Autoclaved crude extract of lettuce debris from Wisconsin caused corky root symptoms (Amin and Sequeira, 1966), indicating the presence of heat stable toxic substance in lettuce debris extract. Studies performed in Salinas, CA with N (78 to 213 kg.ha<sup>-1</sup>) applied as (NH<sub>4</sub>)SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, urea, or Ca(NO<sub>3</sub>)<sub>2</sub> increased infestation of corky root pathogens, suggesting a direct relationship to NO<sub>3</sub> rather than NH<sub>4</sub> (van Bruggen and Brown, 1990). N toxicity can cause symptoms similar to corky root, as non-infectious vs. the infectious



**Figure 4.** Previously published (Bull, C., 2009) dendrogram of relationship of 16S rDNA sequences from members of the isolates linked to corky root in resistant (*cor*) cultivars, and other members of the *Sphingomonadaceae*.

symptoms.

Noninfectious corky root caused by N toxicity leads to pink or red discoloration of the root surface (van Bruggen and Brown, 1990). Similar symptoms were attributed to free ammonia, NH<sub>4</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl<sub>2</sub>, or urea (Amin and Sequeira, 1966; Grogan and Zinc, 1956). The stored DORD root samples from the current study should be compared to

controls for any discoloration, to distinguish between infectious or noninfectious symptoms.

High NO<sub>3</sub> in the soil and irrigation system could be the cause for DORD plants observed in the three fields tested in Salinas. It could be a combination of infectious and noninfectious corky root at these locations. **VESTA may work directly by reducing the causative pathogen or by reducing N toxicity.** Further studies are needed to identify the properties of the organisms present in VESTA.

One of the strains identified amongs the 114 OTUs that increase with product efficacy was *Sphingomonas wittichii* (highlighted in Appendix 3). *S. wittichii* RW1 has some rare and beneficial properties, it completely mineralizes the organic backbone of toxic dioxin pollutants, therefore cleaning the pollutants (Shabalov et al., 2014). *S. wittichii* has extensive set of catabolic enzymes for degradation of chlorinated phenolic intermediates that form during dioxin transformation. This bacterium is one of the most promising for soil bioremediation.

(2) Through molecular and biochemical interactions between other organisms and/or lettuce plants, for example by increasing plant immunity. It is possible that organisms in VESTA activate plant innate immune system. Further studies are needed to evaluate this possibility.

There was no significant difference between rhizosphere samples from DORD and healthy lettuce plants (Chapter 8, Second Genome Report, EPAN 2015-01-13). The root samples collected from DORD plants from control and VESTA-treated beds have not yet been analyzed.

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