VERMICOMPOST SUPPRESSION OF *PYTHIUM APHANIDERMATUM* SEEDLING DISEASE: PRACTICAL APPLICATIONS AND AN EXPLORATION OF THE MECHANISMS OF DISEASE SUPPRESSION

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ABSTRACT

VERMICOMPOST SUPPRESSION OF *PYTHIUM APHANIDERMATUM* SEEDLING DISEASE: PRACTICAL APPLICATIONS AND AN EXPLORATION OF THE MECHANISMS OF DISEASE SUPPRESSION

Allison Lara Hornor Jack, PhD

Cornell University 2012

Composts, vermicomposts and their liquid extracts can suppress plant infections caused by a variety of pathogens, however this suppression is highly variable, which limits their use in commercial crop production. In addition to the inherent variability of these materials, conflicting information abounds in the industry and extension educational materials regarding liquid compost extracts which were critically evaluated with respect to the existing scientific literature. As a case study, non-aerated liquid vermicompost extract was produced that promoted seedling growth, consistently suppressed zoospore-mediated infections of *Pythium aphanidermatum* on cucumber and maintained suppressiveness for 60 days at room temperature. As both a liquid fertilizer and a cultural practice for the suppression of seedling damping off, this material could satisfy multiple needs for organic growers.

After decades of study, we still lack critical insight into the mechanisms of action of suppressive composts. We sought to uncover potential mechanisms by which vermicomposted dairy manure suppresses *Pythium aphanidermatum* infections on cucumber by investigating the interactions between seed-associated microbial communities and *P. aphanidermatum* zoospores. We found that vermicompost-derived

seed-colonizing microbes prevented the arrival of zoospores on the seed surface and greatly reduced infection in disease suppression bioassays. When microbially modified seed exudates were collected from the bioassay apparatus and exposed to zoospores *in vitro*, fewer zoospores swam towards, encysted on and germinated in response to exudates from seeds colonized by a suppressive microbial community than to those from seeds sown in sterile sand. Combining control and modified exudates failed to restore zoospore response, indicating the presence of a toxin or repellant confirmed by the consistent lysis of zoospores in the vermicompost and combined treatment, but not the sand controls. Exposing zoospores to control and modified seed exudates that had been ethyl acetate fractionated provided evidence that the putative toxin/repellant is exclusively present in the organic fraction.

BIOGRAPHICAL SKETCH

Allison Jack (nee Hornor) was born in Mountain View, California to parents with a passion for gardening and outdoor adventures. She was given a microscope for her eighth birthday and a collection of prepared slides with the tag line “Science: first a hobby, then a career”. Much of her childhood was spent backpacking in the Sierra Nevada, rafting the canyons of the Southwest and beachcombing in the Ho Rainforest with her parents. Her participation in the Amigos de las Americas program in high school led to a summer in rural Paraguay working on a community sanitation project and a lifelong interest in organic matter management for community health. Allison later pursued a degree in biology at Reed College in Portland, Oregon where she studied forest canopy biology at the Wind River Canopy Crane Facility in Carson, Washington and tropical biology with the Organization for Tropical Studies in Costa Rica.

During two years of national service in AmeriCorps*VISTA working as a service learning coordinator at an environmental science middle school, Allison became fascinated by vermicomposting as a technology for organic waste recycling. She completed an MS in soil science at Cornell University where she researched the impact of organic transplant media amendments on rhizosphere microbial community dynamics in organic tomato production and the potential for human pathogens in liquid compost extracts. While at Cornell, she and her husband Steffen remodeled a home in the Northside neighborhood of Ithaca and became active members of the Ithaca Community Garden, where Allison volunteered as a Tompkins County Master Composter. During her doctoral studies, Allison participated in project development and

initiated an industry-university partnership with a vermicomposting company in Western NY, Worm Power, to study the disease suppressive properties of material produced at their facility. Following an interest in science communication to the public and adult and extension education, Allison participated in multiple grower outreach projects. She was a founding member of the graduate student group NWAEG at Cornell and continues to be active in the Sustainable Agriculture Education Association.

DEDICATION

To Steffen, Mom, Dad, Gram and Gramps.

Thanks for supporting my adventures in academia and always believing in me.


[1]

Pythia (Πυθία) or The Oracle of Delphi[1], after which the genus *Pythium* is named, pictured breathing what are now known to be psychoactive hydrocarbons released from a geological fault through a spring[2].

[1] Collier, J (1891) "Priestess of Delphi [Public Domain]." Retrieved May 12, 2012, from http://en.wikipedia.org/wiki/File:Collier-priestess_of_Delphi.jpg.

[2] Krajick, K. (2005) "Tracking myth to geological reality." Science **310**(5749): 762-764.

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The Department of Plant Pathology and Plant-Microbe Biology offered a challenging but supportive academic environment where someone was always ready to loan out a piece of equipment, offer research advice or engage in a deep discussion about new developments in the field. Being a part of the Nelson lab family was very special, I will always remember the day to day excitement of doing science with Mary Ann Karp, Sofia Windstam, Mei-Hsing Chen, Ellen Crocker, Rick Carr, Christina McGuire, Monica Minson and Hilary Davis. Sarah Braun, Bryan Emmett, Cyd Hamilton and others gave helpful feedback and tasty snacks during lab meetings. Julia Crane, Christine Layton, and Michael Wunsch made life on the 4th floor of the Plant Science

building more fun! Jay Worley's late night saxophone serenades and office banter kept the "I'm alone in the building" willies away.

Steffen followed me into unknown territory, designed and built the disease suppression bioassay apparatus, kept the home fires burning and washed more lab glassware than he would like to remember. He is my political advisor, my confidant, and my best friend. *Tusen takk!*

Tom Herlihy inspired me by building a vermicomposting facility that I only could have imagined in my wildest dreams. Knowing that my research was benefitting not only growers, but a new green industry fueled my drive to keep at it even when experiments failed.

NWAEG at Cornell gave me a way to meet people on campus and in the community with a shared interest in sustainable agriculture and food sovereignty. The friendship of Jennifer Gardner, Scott Perez, and Megan Schipanski made even the worst day in lab disappear into thin air.

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TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

I. The phenomenon of disease suppression

A majority of plant life relies on soil, not simply as an inert growth substrate, but as a dynamic living ecosystem in its own right, one deeply involved in global biogeochemical cycling and with more uncharacterized biodiversity that any other terrestrial ecosystem (Fitter 2005). One of the functions associated with soil microorganisms is disease suppression, or the protection of plants from disease when a pathogen is present. Disease suppressive soils play a crucial role in the creation and management of self-sustaining agroecosystems. A key goal in the practice of sustainable agriculture is to decrease the use of synthetic pesticides which creates an increased reliance on natural predators and suppressive soil microbial communities for the control of agricultural pests. Many of the soil organic matter management practices that are now known to be correlated with suppressive soils such as; cover cropping (Grunwald et al. 2000), crop rotations (Peters et al. 2003; Smith et al. 2011) and manure and composts amendments (Hoitink and Fahy 1986; Litterick et al. 2004), existed well before the advent of modern science. Amending soils with manure appears in ancient

Roman (Cato 1998) and medieval Arabic (L-Jayr 1991) texts and was a traditional practice across Asia (King 1911). The Aztec chinampas system, still in use today, has suppressive soils (Lumsden et al. 1987; Marban-Mendoza et al. 1992) potentially due to amendment with canal silt and composted aquatic plants (Thurston 1992).

During the rise of industrial agriculture with its emphasis on synthetic fertilizers, many of these soil organic matter management practices were discontinued. Some scientists warned of the unintentional consequences that could arise when organic matter was not returned to soils during agricultural production (Howard 1943), and these warnings eventually became the philosophical underpinnings of the modern organic agriculture (Heckman 2006) and soil health (Doran et al. 1996) movements. Growers today are reviving and modernizing many traditional agricultural practices in the context of sustainability. As more plant pathologists study these systems, links between soil organic matter management practices and the occurrence of suppression have been documented. The microbial ecology of suppressive soils and composts has been extensively studied (Kowalchuk et al. 2003; Benitez et al. 2007; Hjort et al. 2007; van Elsas et al. 2008; Kinkel et al. 2011). However, both a comprehensive understanding of how disease suppression occurs, and the ability to manipulate agricultural management practices to create consistently suppressive soils, remain elusive (Janvier et al. 2007). In fact, attempts to synthesize individual research findings for over 400 studies on organic soil amendments failed to find specific predictors of suppression (Bonanomi et al. 2010), so the challenge now lies in uncovering commonalities between the mechanisms at work among different cases of disease suppression in order to generate a deeper understanding of the phenomenon.

A complicating factor in translating research in disease suppression into practical tools for sustainable agriculture is that only formulations of single species of microbes can be registered as biopesticides through the US EPA and marketed legally with claims of disease control (USEPA 2005). Legal challenges to pest or pathogen control claims made by the manufacturers of unregistered materials are not uncommon (Moran 2010). This leaves little incentive for the compost industry to invest in the research and development of consistently suppressive materials since they would not be able to capitalize on that investment by marketing their material as disease suppressive. In contrast, the production of biopesticides or biocontrol agents is a major industry. However, due to the high cost of registering new biocontrol agents, relatively few fungal and bacterial species have been commercialized worldwide and almost all exhibit highly variable performance in crop production settings (Nelson 2004).

A similar regulatory hurdle exists in the medical world. Uncharacterized complex microbial communities present in the feces of healthy humans have been found to have a 90% efficacy in clinical trials for the treatment of chronic recurring *Clostridium difficile* infections (CDI) via fecal transplant (FT) procedures (Borody and Khoruts 2012). While the pharmaceutical industry has focused on individual microbial species formulated as probiotics, these introduced taxa seldom persist in the human gut after treatment has ceased. In contrast, taxa present in the donor flora can persist in the human gut for months after transplant, ostensibly due to the fact that they were introduced in the form of a stable microbial community (Borody and Khoruts 2012). Donor material for FT is widely available, but suffers the same heterogeneity in microbial community composition and lack of predictive factors as composts used for soil and plant health.

The rapidly growing field of microbial ecology will continue to contribute to our understanding of the role of complex microbial communities in the suppression of diseases in both agriculture and human medicine. However, this knowledge will need to be applied towards creating regulatory frameworks that can include complex microbial communities as amendments and therapeutic treatments in order for the promise of disease suppression to be realized.

II. Research strategies for understanding suppression

Oomycetes are fungal-like stramenopile eukaryotes that are most closely related to photosynthetic algae (Baldauf et al. 2000). Many oomycetes are pathogens of animals and plants. Plant pathogens in the genus *Pythium* are ideally suited for studies on disease suppression for several reasons. Many *Pythium* spp. are pervasive generalist pathogens that are ubiquitous in agroecosystems and constitute a major impediment to the production of a broad range of crops (Farr et al. 1989; Martin and Loper 1999). These organisms are highly susceptible to suppression by single species of bacteria (Paulitz 1991; Bowers and Parke 1993; Amer and Utkhede 2000; Bardin et al. 2003), fungi (Benhamou and Chet 1997; Georgakopoulos et al. 2002) and oomycetes (Paulitz et al. 1990; Abdelzaher et al. 1997), as well as naturally occurring assemblages of microbes present in decomposing cover crops (Grunwald et al. 2000; Conklin et al. 2002; Smith et al. 2011), soils (van Os and van Ginkel 2001; Kowalchuk et al. 2003), composts (Chen et al. 1988; Boehm et al. 1993; Ben-Yephet and Nelson 1999; Scheuerell et al. 2005) and rockwool used in hydroponic production (Postma et al. 2005). Several distinct research strategies have been employed in the investigation of *Pythium* suppressive microbial communities.

One strategy for understanding suppressive soils and compost is to search the total microbial community for individual microbial taxa that are known to be suppressive when tested alone. Certain bacterial taxa like actinomycetes, *Bacillus* spp. and fluorescent pseudomonads are often associated with *Pythium* suppression (Boehm et al. 1993; Grunwald et al. 2000; McKellar and Nelson 2003; Postma et al. 2005). Many members of these taxa are effective when used individually to challenge pathogens both *in vitro* (Carisse et al. 2003; de Souza et al. 2003) and *in vivo* (Ongena et al. 1999; Shang et al. 1999). However, we know that an individual bacterium's production of pathogen suppressing chemical compounds changes depending on the presence of different microbial competitors (Garbeva et al. 2011), a situation that is complicated to untangle (Haruta et al. 2009). The presence of a fluorescent pseudomonad in a suppressive soil may not necessarily implicate this bacterium in the observed suppression. In fact, one of the often cited examples of 'specific suppression', the role of antibiotic-producing fluorescent pseudomonads in take-all decline of wheat (*Gaeumannomyces graminis* var. *tritici*), found that *Pseudomonas fluorescens* played no role in suppression of take all in organically managed soil with high organic matter content and high microbial activity (Hiddink et al. 2005). *P. fluorescens* population density was actually higher in the non-suppressive conventionally managed soils (Hiddink et al. 2005). In addition, *Pseudomonas* spp. were thought to be responsible for the suppression of *Thielaviopsis basicola* on tobacco until a comprehensive soil microbial ecology study found multiple additional taxa with recognized biocontrol abilities at a higher prevalence in the suppressive soil (Kyselkova et al. 2009). Therefore assessing microbial communities in their entirety in the search for a core microbiome,

as daunting a task as this may be given their complexity, is essential for understanding suppression.

Another approach for identifying microbial taxa or functional genes involved in suppression is to compare the microbial communities from suppressive and conducive soils. Any taxa or functional genes exclusively present in, or present at a higher abundance in the suppressive soil have a high likelihood of participation in suppression. This approach has historically been limited by the sensitivity of the methods used for community analysis. For example, a comparison of a suppressive and conducive soil using Denaturing Gradient Gel Electrophoresis (DGGE) found no measurable differences between the microbial communities in the two substrates (Kowalchuk et al. 2003). Terminal Restriction Fragment Length Polymorphism (T-RFLP) has been used effectively to identify bacterial taxa involved in suppression (Benitez et al. 2007). However using this technique to link taxa to function, although possible, is highly complex because of the relatively short sequences that can be generated from TRFs (Benitez and McSpadden-Gardener 2009). Early attempts at using metagenomics to explore disease suppressive soils was hampered by the “needle in the haystack” approach which made it almost impossible to measure non-housekeeping genes in complex communities (van Elsas et al. 2008). Using more sensitive techniques like PhyloChip analysis can lead to higher resolution of suppressive and conducive communities and significantly narrow the taxa potentially associated with suppression (n=17 taxa) (Mendes et al. 2011). However, limitations of using this high resolution technique with rhizosphere soil include the logistical constraints of working with such a

complex community (> 33,000 OTUs). To date, this technique has only been used with one suppressive soil.

One way to limit the complexity of disease suppressive microbial communities and allow broader questions to be asked is to restrict the analysis to only those microbes that are present in the infection court at time points relevant to the inhibition of pathogenesis. Working with seeds at early time points in germination can drastically simplify the plant-associated microbial community since seeds are often the first point of contact between the host, the suppressive community and the pathogen. The area around germinating seeds, or the spermosphere, is a dynamic habitat for microbes, some of which respond rapidly to exudates and colonize the seed surface (Nelson 2004). Microbes present in the bulk soil that do not respond to seed exudates are unlikely to be involved in suppression and it is possible to remove them from the analysis via transplant. Transplant experiments where seeds are sown in a suppressive substrate for several hours, then transferred to a sterile substrate before inoculation with *Pythium* spp. have documented the important role seed colonizing microbes play in the suppression of disease (Yin et al. 2000; McKellar and Nelson 2003; Chen and Nelson 2008). Recent data show that suppressive seed-colonizing communities can be relatively simple with around 350 OTUs (Chen et al. 2012) compared to over 33,000 OTUs in rhizosphere soil (Mendes et al. 2011). The search for microbes involved in suppression can be narrowed even further through selective perturbations of the seed colonizing community with antibiotics. This approach identified 29 OTUs responsible for the observed difference in microbial communities between a suppressive seed

colonizing community and one treated with antibiotics where suppression was disrupted (Chen et al. 2012).

The system used in this study consisted of: cucumber (*Cucumis sativus* cv "Marketmore 76"), vermicomposted dairy manure (Worm Power, Avon NY) and the oomycete plant pathogen *Pythium aphanidermatum*. Transplant experiments were used to document a suppressive seed colonizing microbial community and therefore exclude the bulk compost microbial community from analysis. However, instead of investigating the seed-colonizing microbial community directly, as described above, the pathogen was used as a type of biosensor. Both *in vivo* and *in vitro* changes in the pre-infection behavior of the pathogen's motile zoospores under different experimental conditions were used to make inferences about the nature of suppression in this system. This study appears in Chapter 3.

IV. *Pythium zoospores* in the spermosphere as a model system

Pythium spp. have a complex life cycle with multiple developmental stages capable of causing disease (Figure 1). *Pythium aphanidermatum* hyphae undergo sporangiogenesis and the resulting toruloid sporangia germinate directly via the production of a germ tube or indirectly via zoosporogenesis (Matthews 1931). Recent work in the Nelson lab has called into question the ability of directly germinating sporangia to cause infection (Carr and Nelson, unpublished). Once zoospores are released they exhibit a chemotactic homing response triggered by chemical compounds present in host seed or root exudates which is an essential part of pathogenesis (Donaldson and Deacon 1993b) (Figure 2).

Figure 1. Life cycle for *Pythium aphanidermatum*, modified from (Matthews 1931)

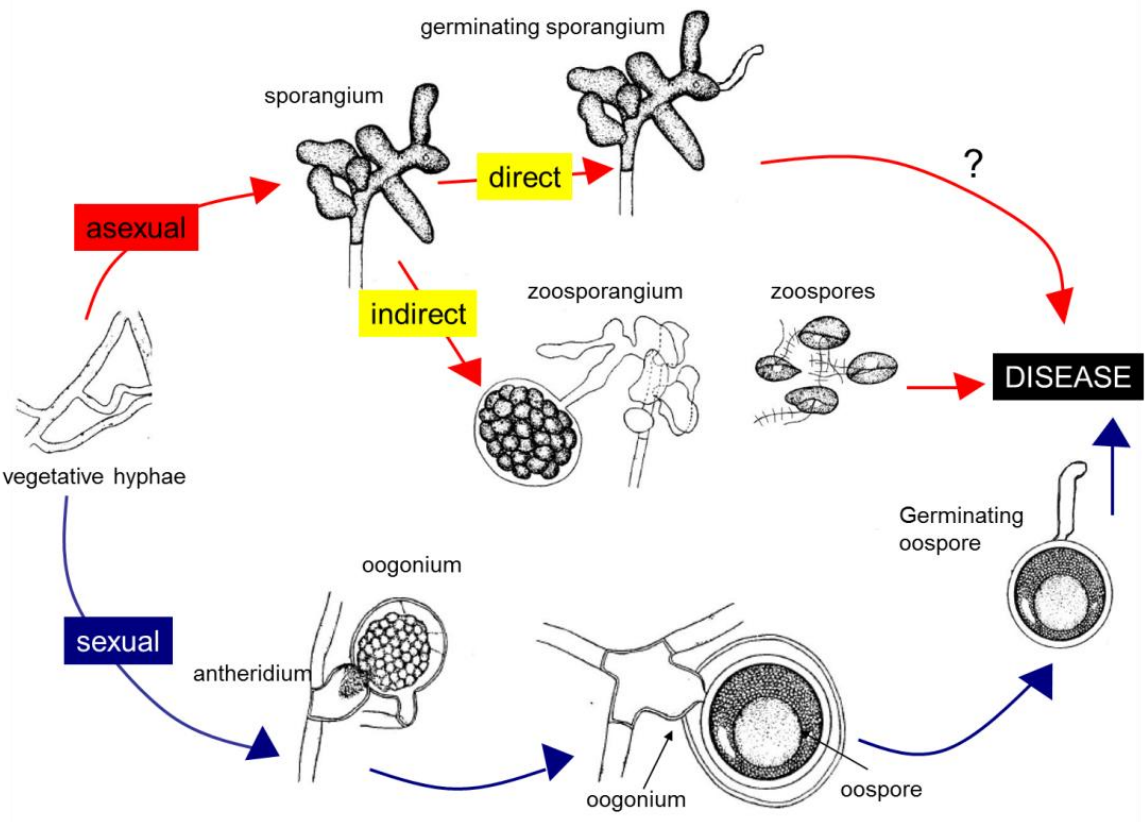
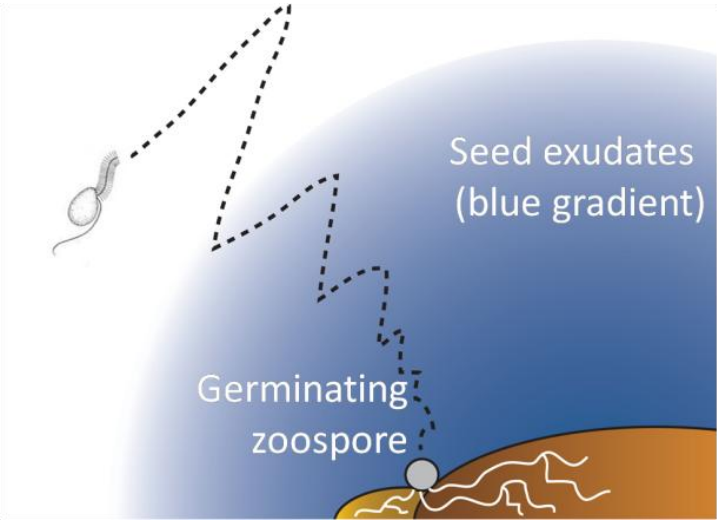


Figure 2. Schematic of zoospore chemotaxis in response to a germinating cucumber seed (swimming and encysted zoospores not to scale).

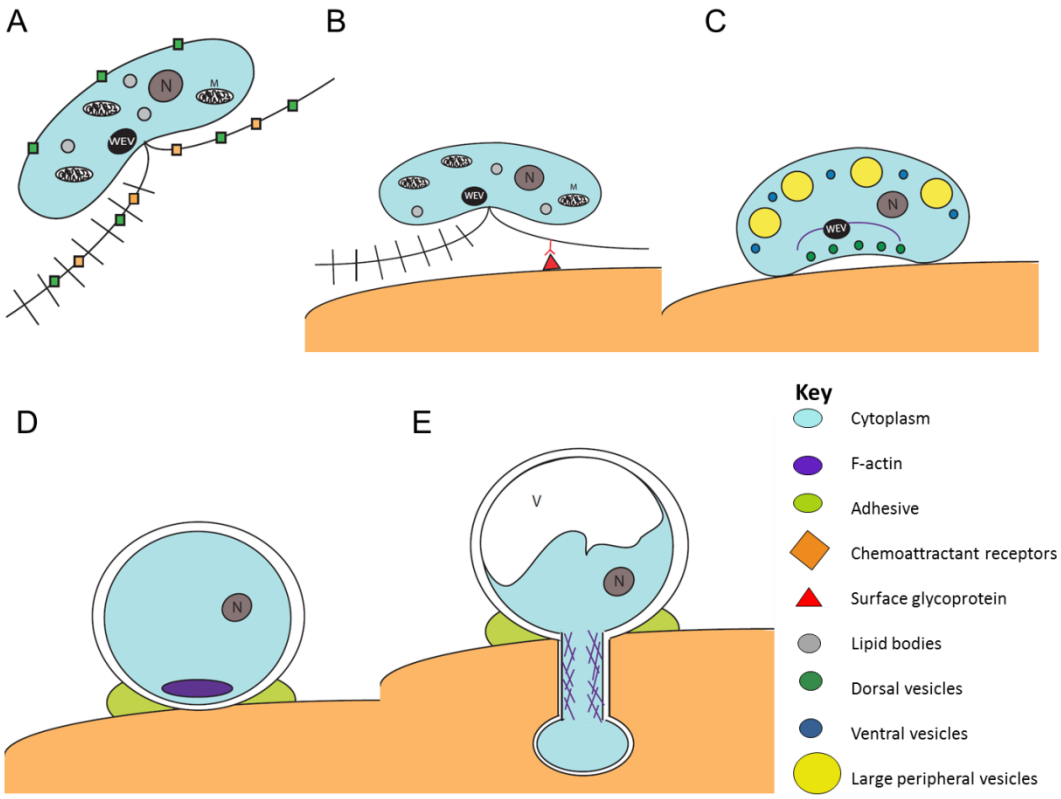


Host-derived signals are received by chemoattractant receptors present on the zoospore plasma membrane which covers both the body and flagella (Estrada-Garcia et al. 1989) (Figure 3A). Chemoattractant receptors can be species specific (Mitchell and Deacon 1986), for example plasma membrane receptors of *Aphanomyces cochlioides* bind attractant cochliophilin A (Sakihama et al. 2004) while those of *Phytophthora palmivora* bind attractant isoveraldehyde (Cameron and Carlile 1981). Signals that bind to the flagellar membrane are most likely transported to the main cell via intraflagellar transport (IFT), although this intracellular trafficking system has only been well characterized in other eukaryotes (Rosenbaum and Witman 2002). Binding of the chemoattractant to the plasma membrane receptor triggers a relatively uncharacterized signaling cascade reliant on heterotrimeric G-proteins (Islam et al. 2003; Dong et al. 2004; Hua et al. 2008). The swimming zoospore then changes direction more frequently which results in overall directional movement towards the host. Zoospores are non-assimilative and are thus limited to the energy stores present in lipid bodies (Figure 3A). They lack a cell wall and use a water expulsion vacuole to maintain osmotic regulation and lysis of the delicate plasma membrane prior to encystment (Figure 3A).

Once the swimming zoospore arrives at the host surface, it is thought that there is a physical interaction between a different set of plasma membrane receptors and glycoproteins on the host surface (Jones et al. 1991) (Figure 3B), although this interaction has not been well characterized. Saccharides on the host surface commonly act as encystment triggers (Estrada-Garcia et al. 1990); arabinoxylan for *P. aphanidermatum* (Donaldson and Deacon 1993a) and sulfated galactans for *P.*

porphyrea (Uppalapati and Fujita 2000). Once encystment begins, the zoospore docks with its ventral groove facing the host surface and sheds its flagella.

Figure 3. Schematic of zoospore pre-infection events. Key: WEV: water expulsion vacuole, N: nucleus, M: mitochondria, V: vacuole.



The usually kidney-shaped zoospore begins to become spherical and lay down a cell wall. High molecular weight glycoproteins present stored in the large peripheral vesicles are broken down as a nutrient source during encystment (Gubler and Hardham 1990) (Figure 3C). During encystment dorsal vesicles secrete a mucilaginous cyst coat (Figure 3C) and ventral vesicles secrete an adhesive which attaches the cyst to the host surface (Figure 3D) (Hardham and Gubler 1990). Once the cell wall is full formed,

cytoskeletal changes occur and F-actin concentrates at the site of germ tube emergence assisting with plant cell wall penetration (Islam 2008) (Figure 3D-E).

Because of their complex series of pre-infection events, zoospores can be used essentially as biosensors in order to dissect the biochemistry of host-pathogen interactions during both pathogenesis and suppression. To date, this strategy has been implemented exclusively in the study of plant resistance and individual biocontrol organisms. Fractioning whole plant extracts into their individual chemical components demonstrated that although a non-host species produced zoospore attractants, it also produced a "masking signal" that immobilized zoospores on contact (Islam et al. 2004). This masking signal was chemically characterized, isolated from the plant's roots and proposed as a component of this plant species' resistance to *Aphanomyces cochlioides* infection (Islam et al. 2004). *In vitro* exposure of zoospores to cell free culture supernatant can aid in determining the mechanism of suppression (Islam et al. 2005). Documenting changes in zoospore response to treated roots *in vivo* and measuring the expression of putative zoosporocidal compounds in the rhizosphere are additional important steps in determining mechanisms of biocontrol (Thrane et al. 2000). However, measuring zoospore response to exudates collected from microbially treated seeds or roots goes a step even further in linking responses from *in vitro* assays to phenomena observed *in situ* for living plant hosts (Heungens and Parke 2000; Lioussanne et al. 2008; Islam 2010). Studies of this type are not always designed in a way that can distinguish between alternate hypotheses, for example whether a biocontrol agent degrades a zoospore attractant present in plant exudates or produces a zoosporocidal toxin (Zhou and Paulitz 1993). Chapter 3 contains a combination of two approaches for

understanding suppression; 1) focusing on the seed colonizing suppressive microbial community and 2) using the zoospore as a biosensor to measure responses to microbially modified seed exudates.

IV. Disease suppressive vermicompost

Vermicomposts are distinct from traditional thermogenic composts in that their production relies on a high density of epigeic earthworms to process different types of organic wastes in a mesophilic process (Jack and Thies 2006). Thermogenic composts can take up to 9 months to fully mature and this stage usually takes place outdoors in windrows where the material is subjected to variable weather conditions. Vermicomposting can shorten curing times to 2 months or less. Due to these higher rates of material flow through facilities and the considerable higher market value for finished vermicompost, it is possible to conduct the entire process indoors which imparts a level of process control not usually associated with the composting of agricultural wastes. The integration of thermogenic and vermicomposting, through pre-composting followed by vermicomposting, can shorten stabilization time and increase overall product quality (Ndegwa and Thompson 2001). In addition, in order for vermicomposts to be used in USDA National Organic Program certified crop production without a 90 day pre-harvest interval, an initial thermogenic composting phase must be employed to assure the destruction of potential human and plant pathogens present in the initial feedstock (NOSB 2006). Vermicomposted dairy manure used in the projects described in Chapters 2 and 3 is manufactured in Avon, Livingston County NY by Worm Power, LLC one of the largest indoor vermicomposting facilities in North America (Sherman and Bogdanov 2011) which incorporates an initial thermogenic phase with a

continuous flow through vermicomposting system (Edwards 2010) and such is listed by the Organic Materials Review Institute as an allowable amendment in certified organic crop production. Demand for vermicomposts in certified organic greenhouse production is expanding as prominent growers demonstrate success in their use as tools for plant nutrient management (Yeager 2011).

Within the past two decades, interest in vermicompost amendments for plant disease suppression has grown considerably as shown by recent literature reviews (Dominguez et al. 2010; Jack 2010; Simsek-Ersahin 2011) although a majority of this interest has come from developing nations where researchers are highly invested in finding low cost effective tools for crop disease management. Efficacy has been demonstrated for a variety of plant pathogens, however variability based on feedstock, amendment rate, amended substrate and pathosystem exist (Table 1) as is the case for thermogenic composts. Very few studies have explored the mechanism of vermicompost mediated suppression or their impact on plant-associated microbial communities, with some exceptions (Robeldo et al. 2010; Jack et al. 2011). Vermicompost was chosen as a research material in the projects described here because its small particle size and profile of available plant nutrients make it highly useful for large scale use in commercial greenhouses as a transplant media amendment. In addition, the high level of process control at the facility provides a higher likelihood of the end product containing a consistent microbial community which is useful in the study of plant disease suppression.

Table 1. Disease suppressive vermicompost results in the literature, modified from Jack 2010

Reference	Feedstock	Amendment rate (%) / significant suppression + = yes, - = no					Substrate	Crop	Pathogen
(Kannangara et al. 2000) [z]	dairy manure separated solids	5 -	10 -	20 -	30 -	40 -	yellow cedar sawdust	cucumber (*Cucumis sativa* cv. 'Corona')	*Fusarium oxysporum* f. sp. *radicis cucumerinum*
(Joshi et al. 2009)	unspecified	10 t ha^{-1} [y] - (2005) 10 t ha^{-1} [y] + (2005)	- (2006) + (2006)				soil soil	French bean (*Phaseolus vulgaris* L.)	*Rhizoctonia solani* *Phaeoisariopsis griseola*
(Rivera et al. 2004)	cattle manure	25 + [x]	50 +	75 +	100 +		soil	white pumpkin (*Cucurbita maxima*)	*Rhizoctonia solani*
(Asciutto et al. 2006)	unspecified	25 - [w]	50 -	75 -	100 -		unspecified potting media	bedding ornamental (*Impatiens wallerana*)	*Rhizoctonia solani*
(Rodríguez Navarro et al. 2000)	cattle manure	10 + 10 + fert -	20 + 20 + fert +	30 + 30 + fert -	40 + 40 + fert -		black earth, chicken manure, rice husks (70:20:10)	Gerbera daisy (*Gerbera jamesonii*)	*Phytophthora dreschleri* & *Fusarium oxysporum*
(Singh et al. 2008)	vegetable waste & cattle manure	2.5 t a^{-1} +	5 t a^{-1} +	7.5 t a^{-1} +	10 t a^{-1} +		soil	strawberry (*Fragaria* x *ananassa* cv. 'Chandler')	*Botrytis cinerea*
(Rivera et al. 2001)	unspecified	25 +	50 +	75 +	100 +		soil	eggplant (*Solanum melongena* cv 'Florida Market')	*Rhizoctonia solani*
(Bhadoria et al. 2003)	unspecified	3.2 t ha^{-1} +					soil	rice (*Oryza sativa* cv. 'Pusa Basmati')	*Rhizoctonia solani*
(Wright et al. 1999)	unspecified	25 +	50 +	75 +	100 +		soil	Autumn squash (*Cucurbita maxima*)	*Rhizoctonia solani*
(Sahni et al. 2008)	vegetable waste, leaf litter	10 +	25 +	50 +			sterile field soil	chickpea (*Cicer arietinum* cv. 'Avrodhi')	*Sclerotium rolfsii*
(Villa-Briones et al. 2008)	unspecified	7.5 t ha^{-1} +	12 t ha^{-1} +				soil	tomato (*Lycopersicon esculentum* Mill.)	*Nacobbus aberrans*

Reference	Feedstock	Amendment rate / significant suppression + = yes, - = no				Substrate	Crop	Pathogen
(Szczech et al. 1993)	cattle manure	10 +	20 +	100 +		peat	tomato (*Lycopersicon esculentum*)	*Phytophthora nicotianae* var. *nicotianae*
		10 - [u]	100 + [u]			peat	tomato (*Lycopersicon esculentum*)	*Fusarium oxysporum* f. sp. *lycopersici*
		root dip [t] + [u]				peat	cabbage (*Brassica oleracea* cv. 'Ditmarska')	*Plasmodiophora brassicae*
(Szczech and Smolinska 2001)	sheep manure	50 +	100 +			peat	tomato (*Lycopersicon esculentum* cv. 'Remiz')	*Phytophthora nicotianae* var. *nicotianae*
	cattle manure	50 -	100 +					
	horse manure	50 +	100 +					
	sewage sludge	50 +	100 + [x]					
(Szczech 1999)	cattle manure	20 -	100 +			peat	tomato (*Lycopersicon esculentum* cv. 'Remiz')	*Fusarium oxysporum* f. sp. *lycopersici*
		15 +	30 +			7:2:1 pine bark compost, pine sawdust, brown coal powder		
		30 +				7:3 pine bark compost : brown coal powder		
			P. irregulare	*P. ultimum*	*R. solani*			
(Scheuerell et al. 2005)	cattle manure, food waste, paper	50	+	+	-	Sunshine mix # 1 (Sun Gro) peat perlite medium	cucumber (*Cucumis sativus* cv. "Marketmore 76')	*Pythium irregulare* *Pythium ultimum*
	dairy manure	50	-	+	-	Sphagnum peat, vermiculite	cabbage (*Brassica oleracea* cv. 'Cheers')	*Rhizoctonia solani*
	dairy manure, straw	50	+	+	-			

Reference	Feedstock	Amendment rate / significant suppression + = yes, - = no	Substrate	Crop	Pathogen
(Robeldo et al. 2010)	Dairy manure Fruit waste	50 + 50 +	rice hulls	tomato (*Lycopersicon esculentum* cv. 'Loica')	*Rhizoctonia solani*
(Singhai et al. 2011)	vegetable waste, leaf litter	20 +	sterile soil	potato (*Solanum tuberosum*)	*Streptomyces scabies*

[z] In this case a thermophilic compost made from the same feedstock was suppressive towards the pathogen
[y] Vermicompost applied in conjunction with non-aerated VC extract applied every 15 d
[x] 25 % amendment was only significantly suppressive at 15°C, not 22°C
[w] 25 % amendment had significantly higher disease than the control (non-amended)
[u] no statistics were run on these trials, however a trend of suppression was seen with vermicompost amendments
[t] Vermicompost used as a component of a root dip before transplanting, clay: vermicompost: water 3:8:5-6

Given the limited options for plant disease management in certified organic production systems, there has been a growing interest in using liquid compost extracts as a way to inoculate both below and aboveground plant surfaces with potentially beneficial microbes. This practice has not been as thoroughly scientifically investigated as have disease suppressive composts, although a significant body of literature does exist (Scheuerell and Mahaffee 2002), most of it firmly on the outer fringes of mainstream plant pathology. Non-aerated liquid compost extracts were extensively investigated in Germany in the 1980s and 90s by respected plant pathologists (Weltzien 1989), however in the US the first paper on these materials in the field of plant pathology's society journal, Phytopathology, did not appear until 1996 (Yohalem et al. 1996), with the subsequent paper in 2004 (Scheuerell and Mahaffee 2004). Scheuerell and Mahaffee's recent pioneering work has led to an increase in higher quality papers on disease suppressive liquid compost extract in recent years (Larkin 2008; Palmer et al. 2010).

Liquid vermicompost extracts are effective in certain cases in greenhouse and field studies, however most of the available studies are of the "spray and pray" variety and do not provide the kind of information needed to build a coherent body of knowledge around these materials (Table 2). In the US, an overall lack of research and extension regarding liquid compost extracts in the past has allowed for the rise of commercial entities that filled the information void with a for-profit model. Unfortunately some of these entities consistently propagate misinformation about these materials on the internet and at grower meetings (Mahaffee and Scheuerell 2006) and provide commercial testing services that purport to predict if the material will be disease

suppressive with methods that have no correlation to suppression in bioassays (Scheuerell and Mahaffee 2004). In addition, most if not all “compost tea” companies manufacture and sell expensive aeration systems even though there is no evidence that aeration is required to produce a suppressive compost extract (Cronin et al. 1996; Al-Dahmani et al. 2003; Scheuerell and Mahaffee 2006; Haggag and Saber 2007). Because of the rise of pervasive pseudoscience around these materials, some extension educators have overreacted by making snake oil comparisons and ignoring much of the scientific evidence that they can in some cases suppress plant disease (Chalker-Scott 2001; Chalker-Scott 2003; Chalker-Scott 2003b; Chalker-Scott 2007; Downer 2011). This has created a confusing situation both for people who want to make and sell liquid compost extracts and growers who want to use the material effectively in their production systems. Chapter 2 describes an applied study geared to meet compost industry and organic grower needs by revisiting the early German work on non-aerated liquid compost extracts, developing a low-cost *Pythium* suppressive non-aerated vermicompost extract and providing a rough chemical and microbiological characterization of the extraction process and the finished material.

Table 2. Suppression of plant pathogens with liquid vermicompost extracts

		Preparation of vermicompost extract:							
Ref	**Feedstock**	**Ratio VC: water**	**Aeration**	**Time**	**Additives**	**Appl. rate**	**Crop**	**Pathogen**	**S**
(Singh et al. 2003)	unspecified	1:2	shaken every 4 h	24 h	none	various[a]	*Pisum sativum*	*Erysiphe pisi*	+
							Impatiens balsamina	*Erysiphe cichoracearum*	+
(Travis and Rytter 2003)	unspecified	1:69	aerated (unspecified)	24-36 h	yes [b]	25 gal a^{-1}	*Vitis* spp.	*Erysiphe necator*	+[k]
(Scheuerell and Mahaffee 2006)	food waste, paper 1:1 v:v	1:5	none	7 d	none	unspecified	*Geranium pelargonium x hortorum* cv. 'Ringo Red 2000'	*Botrytis cinerea*	+/- [j]
		1:5	none	7 d	1.2 g powdered kelp, 2.5 ml humic acids, 3 g rock dust (in 10 L total volume)				+/-
		1:5	none	7 d	0.3 % molasses, 0.3% hydrolyzed yeast powder				-
		1:5	constant	34-36 h	No				-
		1:5	constant	34-36 h	1.2 g powdered kelp, 2.5 ml humic acids, 3 g rock dust (in 10 L total volume)				+
		1:5	constant	34-36 h	0.3 % molasses, 0.3% hydrolyzed yeast powder				-
(Joshi et al. 2009)	unspecified	1:5	initial stirring	10 d	none	1000 L ha^{-1} [c]	*Phaseolus vulgaris*	*Rhizoctonia solani*	-
								Phaeoisariopsis griseola	+
(Zaller 2006)[d]	fruit, vegetable & cotton waste	1:2	none	24 h	none	unspecified	*Lycopersicon esculentum* cv. 'Diplom F1'	*Phytophthora infestans*	+

							cv. 'Matina'		-
							cv. 'Rheinlands Ruhm'		-
(Utkhede and Koch 2004)	unspecified	~1:21	15 m h^{-1}	2 d	19.5 g soluble kelp in 16 L total volume	unspecified	*Lycopersicon esculentum*	*Clavibacter michiganensis* subsp. *michiganensis*	+
		Preparation of vermicompost extract:							
Ref	**Feedstock**	**Ratio VC: water**	**Aeration**	**Time**	**Additives**	**Appl. rate**	**Crop**	**Pathogen**	**S**
(Scheuerell and Mahaffee 2004)	vegetable waste [e]	1:30	continuous	36 h	non	soil drench	*Cucumis sativus*	*Pythium ultimum*	+/-
					5 ml bacterial nutrient solution (Soil Soup Inc. proprietary)				+/-
					1.2 g seaweed powder, 2.5 mL humic acids, 3 g rock dust (in 15 L total volume)				+
(Budde and Weltzien 1988) *	unspecified	NA	none	3 d	none	unspecified	Barley cv. 'Gerbel'	*Erisyphe graminis* f. sp. *hordei*	+
(Larkin 2008)	various [h]		continuous		Earth Tea Brewer proprietary blend [g]	1 L 216 m^{2} [f]	*Solanum tuberosum* cv. 'Shepody'	*Rhizoctonia solani*	-
								Streptomyces scabies	+ [i]
(Dagostin et al. 2011)	unspecified	NA	unspecified	NA	none	spray to run off	*Vitis* spp. cv. 'Pinot Gris' and cv. 'Chasselas'	*Plasmopara viticola*	-
					clinoptilolite[l]				-
(Orlikowski 1999) *	unspecified	1:5	NA, trade name Antifung 20 SL			soil drench	Gerbera spp.	*Phytophthora cryptogea*	+
							Hedera helix (cuttings)	*Pythium ultimum*	+
							Cyclamen	*Fusarium oxysporum* f. sp.	+

							Carnation cv. “Tanga”	*dianthi*	+
(Nakasone et al. 1999)	dairy manure, coffee chaff	1:1	None	10 d	None	*In vitro* to cultures	NA	*Botrytis cinerea*	+
								Sclerotinia scloerotiorum	+
								Sclerotium rolfsii	+
								Rhizoctonia solani	+
								Fusarium oxysporum f.sp. *solani*	+
								Hemileia vastatrix	+ [m]
								Alternaria solani	+
								Colletotrichum sp.	-

[a] Extract dried down and resuspended at 1-5 g L^{-1}
[b] Seaweed plus HydraHume-AN (118 mL), SP-85 (22 g), molasses (113 g), corn oil (1/4 t), fish hydrolysate (5 mL) to 25 gallons
[c] Applied in conjunction with soil amendment of VC 10 t ha^{-1}
[d] Small sample size, low level of naturally occurring infection during field season
[e] The vermicompost itself was not suppressive when amended at 1:3 v:v peat pearlite
[f] Rate of initial application 1 d prior to planting, additional applications in-furrow as a soil drench and sprays 4 and 8 weeks after planting
[g] Blend contains clay, blue green algae, sugar, yeast and kelp
[h] Culled produce, coffee grounds, composted horse manure, paper, straw
[i] Only in a barley – rye rotation, not in barley – clover or continuous potato
[j] At least one, but not all replicate batches/trials resulted in significant suppression of disease
[k] Only significant reduction was in severity of leaf infection, no significant reductions in incidence on leaves and clusters
[l] Ecosfera proprietary rate
[m] Spore germination, all others germ tube emergence rate
* Not a peer reviewed journal, but “Communications from the Federal Biological Institute for Agriculture and Forestry” in Germany

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CHAPTER 2. UNTANGLING THE COMPLEX WORLD OF LIQUID COMPOST EXTRACTS: A CASE STUDY ON THE DEVELOPMENT AND CHARACTERIZATION OF A NON-AERATED LIQUID VERMICOMPOST EXTRACT THAT SUPPRESSES *PYTHIUM APHANIDERMATUM* SEEDLING DISEASES OF CUCUMBER

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ABSTRACT

Liquid extracts of composts and vermicomposts can suppress plant infections caused by a variety of pathogens, however this suppression is highly variable, which limits the effective use of composts in commercial crop production. In addition to the inherent variability of these materials, conflicting information is present in the scientific research, industry information and extension educational materials regarding liquid compost extracts. These conflicts are explored and compared to the research findings reported here and in other scientific publications. Our objective for this project was to develop a consistently disease suppressive non-aerated vermicompost extract (NVE) that retains its suppressiveness over several weeks in storage. Using a simple seedling germination assay to choose the appropriate vermicompost to water ratio was crucial to avoid the phytotoxic effects of high salinity. NVE was produced at a 1:60 vermicompost to water ratio over an extraction period of 10 days with periodic minimal circulation, but not aeration, from a sump pump to re-suspend particulate matter. Dissolved oxygen levels declined from 6 to nearly 0 ppm over the extraction period, while viable bacteria showed population levels of around 10^7 cells ml^{-1}. The finished extract promoted

seedling growth and had a desirable profile of plant-available nutrients although supplementation with additional sources of nitrogen may be required for some crops. Fresh and lyophilized-reconstituted NVE consistently suppressed zoospore-mediated infections of *Pythium aphanidermatum* Edson (Fitzp.) on cucumber. Sterile-filtered NVE offered no protection compared to sand controls indicating the involvement of microbes in the observed suppression. NVE maintained suppressiveness for 60 days at room temperature after which efficacy declined significantly. As both a liquid fertilizer and a cultural practice for the suppression of seedling damping off, this material could satisfy multiple needs for organic growers. Additionally, the production of non-aerated extracts is simple, relatively low cost and this particular material has a long shelf life (60 d) compared to the 4-6 hours recommended for aerated extracts. With additional research the powdered lyophilized NVE could be developed into a seed treatment for the biologically-based management of seedling damping off.

INTRODUCTION

i. Liquid compost extracts

The use of composts for the maintenance of plant health has been central to the modern organic agriculture movement dating back to the early 1900s (Heckman 2006). Over the past century, research has shown that composts can suppress plant diseases caused by a variety of pathogens, however the high variability of suppression precludes their commercial use for biologically-based disease management (Hoitink and Fahy 1986; Litterick et al. 2004; Jack 2010; Simsek-Ersahin 2011). For management

scenarios where it is not feasible to add solid compost, i.e. applications to foliar surfaces to manage foliar diseases and as a liquid fertilizer amendment throughout the growing season, making a liquid compost extract provides an alternative means of application for plant available nutrients and potentially disease suppressive microorganisms. Like solid composts, liquid compost extracts may be disease suppressive (Weltzien 1989; Scheuerell and Mahaffee 2002), however the extreme variability along with an inability to identify predictive factors for suppression are major impediments to widespread adoption of these materials (Mahaffee and Scheuerell 2006). The uniqueness of compost feedstocks along with the multitude of methods for extract production, including complex proprietary blends of additives, make it is difficult to draw comparisons between studies in order to develop a comprehensive understanding of how the extraction process and feedstock source affect end product consistency and predictability.

Compost extracts have been prepared either through a non-aerated or aerated process (Scheuerell and Mahaffee 2002). Non-aerated compost extracts have a history in Biodynamic farming popularized by Rudolf Steiner in the early 1900s (Koepf et al. 1976) and significant plant disease suppression was documented in Germany in the 1980s and early 1990s in non-peer reviewed publications, trade journals and government research bulletins, and in peer reviewed scientific journals and dissertations as summarized in the following reviews (Weltzien 1989; Weltzien 1990; Trankner 1992). For the past several decades, non-aerated compost extracts have been shown to suppress a wide range of plant diseases (Ketterer and Schwager 1992; McQuilken et al. 1994; Zhang et al. 1998; Hibar et al. 2006; Kone et al. 2010; Dukare et al. 2011).

Approximately 10-20 years ago, actively aerated compost extracts were developed and popularized in the US by several companies; Soil Food Web, Inc., Growing Solutions Inc., EPM Inc., and Keep It Simple Inc. (Ingham 2005). The necessity of sometimes costly aeration systems and the requirements for a variety of additives and microbiological testing services was and still is heavily marketed by these companies to homeowners as well as commercial growers. However, aeration and additives were subsequently shown to be unnecessary in the production of liquid compost extracts for crop nutrient management (Pant et al. 2009; Pant et al. 2011). Although some aerated compost extracts suppressed plant diseases (Singh et al. 2003; Scheuerell and Mahaffee 2004; Scheuerell and Mahaffee 2006; Larkin 2008; Cummings et al. 2009; Segarra et al. 2009; Siddiqui et al. 2009; Palmer et al. 2010), comparisons of aerated and non-aerated extracts made from the same batch of compost show that aeration during the extraction process does not increase the finished extract's ability to suppress disease. Non-aerated extracts were either equal in suppression of disease (Al-Dahmani et al. 2003; Scheuerell and Mahaffee 2006; Xu et al. 2012), or offered significantly higher suppression of disease (Cronin et al. 1996; Haggag and Saber 2007) compared to aerated extracts made from the same batch of compost. An unfortunate situation has developed where the overall lack of research and extension education in this area has allowed much misinformation regarding aeration to be proliferated, a sentiment shared by W. Mahaffee, USDA ARS and S. Scheuerell, The Evergreen State College,

> "Due to the lack of information from the scientific community on the topic of compost tea production and utilization, [growers,

> homeowners and landscape managers] have sought and utilized information from other sources. This information void has been filled partially by a few individuals who have created and marketed simplistic solutions for plant disease control which are not supported by past and current research, but are appealing to the producer" (Mahaffee and Scheuerell 2006).

ii. Educational resources on liquid compost extracts

In the informational landscape surrounding compost extracts, much of what is produced by commercial entities (aerated compost extraction equipment producers, extract producers and extract testing services) has little to no scientific basis. However, third party educational resources on compost extracts from sustainable agriculture non-profits, federal agricultural agencies and university cooperative extension groups can contribute to the confusion over what has been scientifically validated and what hasn't. For example, resources from Appropriate Technology Transfer to Rural Areas (ATTRA, a project of the federal agency: National Center for Appropriate Technology NCAT) and the Northeast Organic Farming Association (NOFA-NY) directly recommend having extract samples tested by the commercial laboratory Soil Foodweb Inc. without any mention of third party verification of the usefulness this type of testing (Diver 2002; Gershuny 2011). The total to active bacterial and fungal ratios promoted as measurements of compost extract quality by Soil Food Web Inc. (Ingham 2005) have subsequently been shown not to correlate with the ability to suppress disease by researchers who attended a training in these techniques taught by the president of the company (Scheuerell and Mahaffee 2004). This testing method was listed in an

eOrganic resource with the description “Proposed as an index of disturbance and nutrient enrichment; inconsistency in methods has made some conclude that the method has little-to-no practical relevance” (Wander 2012). Grower resources from NOFA-NY parrot the aerated compost extract industry’s disdain for traditional non-aerated extracts without providing any rationale for the use of aeration;

> “As mentioned, an old burlap sack filled with compost and suspended in a bucket, while useful as a source of quick foliar nutrients, is a watery compost extract, not the “real deal” of fermented compost tea. To make a true fermented compost tea, you need some form of equipment and means of aeration” (Gershuny 2011).

At the time the 2011 revision of this book was published, 33 out of 35 existing peer reviewed studies on non-aerated compost extracts showed significant suppression of disease along with 7 out of 8 of the non-peer reviewed reports from the German Federal Biological Institute for Agriculture and Forestry, so the dismissal of this production method is not supported by the scientific literature.

Educational resources from the Rodale Institute have been more balanced and present information on both aerated and non-aerated extraction methods pointing out that to date more research has been done on non-aerated extracts (Ryan and Ziegler 2007). While emphasizing the need for more research on disease suppression, Ryan and Zeigler encourage growers to experiment on their own with these materials (Ryan and Ziegler 2007). On farm trials carried out by the Rodale Institute showed that while the incidence of some diseases was diminished by compost extract applications, the

incidence of other diseases was actually enhanced, clearly demonstrating the challenges of using these materials in the field (Sayre 2003). Similar mixed results for disease suppression were found in grower-initiated on-farm trials summarized by the Minnesota Department of Agriculture indicating the limitations of this practice, and the potential of these materials as valuable disease management tools for organic growers (Bailey et al. 2001; Bailey 2003).

Few university-based cooperative extension educators have tackled the complicated topic of compost extracts. Those who have rightly emphasize the importance of being skeptical of marketing information. Ventura County, CA cooperative extension plant pathologist Jim Downer has this advice,

> "Very often, snake oil products will use jargon relating to the chemistry, biology or microbiology of their products in an attempt to impress potential users with terms that sound informative but are used in a meaningless context. […] Past affiliations with universities are no guarantee that products developed after the researcher has left the institution are efficacious. Only current, published reports of efficacy in peer-reviewed journals are acceptable references" (Downer 2011).

However, after encouraging healthy skepticism, and pointing out the high variability associated with compost extracts, he then points out the existing scientific literature and encourages growers to contact their local cooperative extension offices and carry out on-farm trials (Downer 2011). Other university-based educators encourage healthy skepticism of marketing claims, but then in

what may be an overreaction to the perceived pseudoscience in this field on the internet, end up disregarding the existing scientific literature. Linda Chalker-Scott, urban horticulturalist at Washington State University Puyallup Research and Extension Center maintains a “horticultural myths” section on her website where she has written extensively on compost extracts,

> “Compost teas and extracts are traditionally used as liquid organic fertilizers, but recently have been touted as powerful antimicrobial agents capable of combating pathogens associated with foliar and fruit diseases. Anecdotal evidence abounds, but controlled, replicable experiments do not. A quick search of the Internet revealed that most of the websites containing the phrase “compost tea” are .com sites: most are selling something. The few .edu sites that do exist are cautious in regard to the miraculous properties associated with compost teas,” (Chalker-Scott 2001).

At the time in 2001, the peer reviewed literature on compost extracts was extremely limited. However, in a 2007 reassessment of the situation Chalker-Scott states that Master Gardeners cannot recommend compost tea use because “they are volunteer educators who rely on science-based information, they cannot recommend a practice or product that lacks a legitimate scientific basis,” (Chalker-Scott 2007). By that time there were 41 peer-reviewed scientific articles on aerated and non-aerated compost extracts, 33 of which showed significant suppression of disease. As it stands now, 14 out of 21 peer-reviewed scientific articles on the more recently developed aerated compost extracts show significant disease suppression in mainstream plant pathology journals

like Plant Disease, Soil Biology and Biochemistry, Phytopathology, Canadian Journal of Plant Pathology, and Journal of Plant Diseases and Protection. It is true that compost extracts have not yet been scientifically evaluated for use in landscaping, but this does not mean that no evidence exists of their effectiveness on other plant hosts.

Many certified organic growers turn to compost extracts due to their limited options for biologically based disease management, while in some cases their interest is driven by the organic ideology that biological alternatives are superior to synthetic conventional inputs (Mahaffee and Scheuerell 2006) or as part of a desire to maximize soil biological diversity. Compost extract companies can feed into this ideology by making sweeping claims that diseases present in conventionally managed fields will no longer occur if compost extracts are used (Ingham 2005). This creates a situation where the “true believers” are ripe for exploitation

> “This is the real problem I see in the world of compost tea, which is the selling of a product whose use is based on faith rather than science. As one proponent states, “There is no doubt in my mind that compost tea has already proven to be beneficial to agriculture.” Individuals with this mind-set are not open to having their beliefs challenged by scientists or anyone else. However, buying expensive “tea brewers”, purchasing ready made “tea” at several dollars a gallon, or paying a company to apply ACT in the absence of objective data sounds like snake oil rather than science,” (Chalker-Scott 2003b).

The challenge for researchers, extension educators and growers with an interest in compost extracts is to separate out the commercial hype from the scientific evidence. Commercial promotion of aerated extracts with no reference to their scientific basis seems to have drowned out the actual scientific studies that show these materials can in some cases protect plants from disease. However, evidence is growing that aeration is not required for the production of disease suppressive extracts. Since traditional non-aerated compost extracts require minimal equipment to produce on farm and can also be disease suppressive, we believe these materials are worth investigating as tools for biologically-based disease management.

iii. Case study: Development and characterization of a *Pythium* suppressive non-aerated vermicompost extract

Based on the expressed needs of the organic grower community via the Organic Farming Research Foundation the goal of this study was to develop a non-aerated vermicompost extract that is consistently disease suppressive and retains efficacy over extended storage periods based on the original protocols of Heinrich Weltzien's research group (Weltzien 1989). We chose to work with a commercially-available dairy manure vermicompost that is an allowable material in certified organic production and a seed infecting pathogen with limited control options for certified organic growers, *Pythium aphanidermatum* (Edson) Fitzp. (Martin and Loper 1999). Since the non-aerated extraction process remains for the most part uncharacterized, we aimed to document basic chemical and biological features of the process as a reference for future work in this field. Additionally, we chose to investigate the impact of freeze drying

on the suppressiveness of the extract as the first step in pursuing options for seed treatment applications.

While the production of aerated compost extracts maintains high (6-9 ppm) dissolved oxygen levels throughout the entire extraction process (Scheuerell and Mahaffee 2004; Kannangara et al. 2006), the availability of oxygen during the non-aerated extraction process has not been characterized. With this in mind, we felt dissolved oxygen along with pH and electrical conductivity or soluble salts would be relevant measurements for the characterization of the non-aerated extraction process. Under the umbrella term non-aerated compost extracts, a majority have been made with initial mixing, but then no additional agitation or stirring throughout the extraction process (Elad and Shtienberg 1994; McQuilken et al. 1994; Cronin et al. 1996; Yohalem et al. 1996; Scheuerell and Mahaffee 2004; Scheuerell and Mahaffee 2006; Ingram and Millner 2007; Joshi et al. 2009). However some methods have been reported that are more similar to the production of biodynamic preparations (Koepf et al. 1976) and include periodic mixing at different intervals throughout the extraction process (Haggag and Saber 2007; Kone et al. 2010). We chose a protocol that included periodic mixing as preliminary trials without stirring resulted in a lower extraction efficiency measured as the percent of the original dry mass of vermicompost added that remains in the finished liquid after straining (T. Herlihy, personal observation).

In their popular compost extract production manual, Soil Food Web Inc. does not recommend the use of non-aerated extracts where the dissolved oxygen levels fall below 1 ppm during production, stating that the resulting extract may be phytotoxic.

> "Anaerobic organisms are not detrimental in themselves, but their metabolic products can be extremely detrimental to plants as well as many beneficial microorganisms. Anaerobic metabolites produced are volatile organic acids […] that are very detrimental to the growth of plants and beneficial bacteria, fungi, protozoa and nematodes." (Ingham 2005).

To evaluate this claim, we chose to measure the phytotoxicity of finished extracts for a variety of vermicompost:water ratios. Additionally the idea, now pervasive in the compost extract community, that non-aerated extracts are not capable of supporting a large enough population of microorganisms to be suppressive was initiated in the same manual,

> "Not-aerated, no-nutrients added tea brews may have such low numbers of organisms in a tea that bio-films never develop and the liquid never becomes anaerobic, no matter if the liquid is never stirred or mixed or aerated. If the tea does not contain many organisms, the tea cannot have the benefits that organisms give that have been discussed previously," (Ingham 2005).

Bacterial CFU mL^{-1} for finished non-aerated compost extracts range from $1 * 10^6$ to $7 * 10^{11}$ (Haggag and Saber 2007; Kone et al. 2010) however changes in the size of the bacterial population over the course of the non-aerated extraction period are poorly characterized.

While non-aerated vermicompost extracts have in some cases been shown to inhibit phytopathogen growth (Nakasone et al. 1999) and suppress plant disease

(Weltzien et al. 1987; Budde and Weltzien 1988; Scheuerell and Mahaffee 2006; Joshi et al. 2009), the shelf life of these materials is an important factor for their effective use. Non-aerated compost extracts can be disease suppressive for up to 4 months stored at room temperature (Yohalem et al. 1994) while aerated extracts must be used immediately after extraction (Ingham 2005). The shelf life of the extract produced in this study was also evaluated as this characteristic will impact how growers are able to make and use the material.

MATERIALS AND METHODS

i. Experimental materials and non-aerated vermicompost extract (NVE) production

Cucumber seeds (*Cucumis sativus* cv "Marketmore 76", Johnny's Seeds) were sorted to remove damaged seeds, individually screened to 0.02 – 0.03 g biomass, surface disinfested with a bleach solution (0.5% sodium hypochlorite) for 5 minutes, rinsed well with sterile water and air-dried before use. Quartz sand was wet sieved to 0.5 - 1.0 mm diameter, oven dried and autoclaved 40 min for three consecutive days before use.

Pythium aphanidermatum (Edson) Fitzp. (Pa58) (Ben-Yephet and Nelson 1999) was cultured on clarified V8 plates at 27^0C. To maintain virulence and prevent bacterial contamination, cucumber seeds were inoculated with Pa58 zoospores weekly, infected seeds were overlaid with KWARP (water agar with kanamycin sulfate 0.025 mg mL^{-1}, rifampicin 0.015 µg/mL^{-1} and penicillin G 0.015 µg mL^{-1}) and hyphal tips were transferred to clarified V8. For zoospore preparation, a core borer (#15, 20 mm d) was

used to remove discs from 7 d Pa58 cultures. Each disk was placed in a 70 mm petri dish with 10 mL sterile nanopure water for 17 h at 27^0C. Liquid was then replaced with 10 mL sterile nanopure water and discs were incubated at 27^0C for an additional 7 h. Zoospores were enumerated with an Improved Neubauer Haemocytometer and diluted with sterile nanopure water if necessary. Zoospore suspensions were used immediately after preparation.

Vermicompost (Worm Power, Avon NY) was collected, stored at -20^0C and thawed at room temperature for 24 h before use in all experiments. Vermicompost was prepared from dewatered dairy manure solids which were mixed 7:1:1 with spoiled corn and hay silage and cured hot compost from previous batches. This mixture was hot composted in a forced air system for up to 2 weeks. Material was then fed to continuous flow through vermicomposting systems stocked with the earthworms *Eisenia fetida* and *Dendrobaena venata* every 3-4 days in 5 cm layers. Finished vermicompost was removed from the underside of the continuous flow through system and sieved to 10 mm 75 days after the initiation of hot composting. Preliminary data show that this vermicompost material consistently suppresses *P. aphanidermatum* on cucumber (Chapter 3).

In order to determine the optimum vermicompost to water ratio, a range of ratios were tested starting with 1:5 (m/m) or 20% by mass, which is typical for non-aerated extracts (NVE) made from thermogenic compost (Weltzien 1992), and going to 1:60 (m/m) or 1.6% by mass. Extracts were produced in open 5 gallon buckets with 60 seconds of mixing twice a day with a paint mixer over a period of 7 days at the Worm Power facility. Phytotoxicity results for these extracts (Figure 2) led us to choose the

ratio of 1:60 for further investigation and three separate batches of non-aerated vermicompost extract were prepared at Cornell by mixing 3 kg vermicompost with 180 L deionized water in a food grade plastic container for 10 days, the longer extraction period based on previous work (Trankner 1992; Cronin et al. 1996). Extracts were circulated via submersible utility pump (SHUR-DRI model SDSU6, 1/6 horsepower, Pentair Pump Group, North Aurora, IL) for 5 minutes every 12 hours with a timer. Upon completion, liquid was strained through 4 layers of cheesecloth and stored in ventilated buckets at room temperature.

ii. Chemical and biological characterization

For the characterization of extracts made with a range of initial vermicompost to water ratios, extracts produced at the Worm Power facility were transported in ventilated containers to Cornell campus within 8 h of straining. Dissolved oxygen, pH, and electrical conductivity measurements were taken. Total culturable bacteria counts (CFU mL^{-1}) were determined by plating serial dilutions of 3 replicate NVE in 0.1 M potassium phosphate buffer (pH 6.8) on 0.1 X trypticase soy agar (TSA) and 0.1 X acidified potato dextrose agar (APDA, pH 4 with lactic acid). Phytotoxicity was determined with a standard *in vitro* germination and root elongation protocol; TMECC 05.05-B (Thompson et al. 2002) with 10 cucumber seeds per treatment of water, fresh NVE and 0.2 µm sterile filtered NVE. Seeds were combined with 5 mL test liquid on a Whatman #1 filter paper in a petri dish and incubated at 27°C for 48 h. Cucumber radicle lengths were analyzed with a one-way ANOVA and Tukey's test for separation of means in Minitab 16 (Mintab Inc. State College, PA). The solubility of vermicompost in water during the extraction process was measured at the Worm Power facility by drying down solids that

were removed during straining, and weighing them. Percent solubility was defined as: [dry biomass vermicompost added to extract]/[dry biomass vermicompost removed from extract via straining through 4 layers of cheesecloth].

For the characterization of three batches of 1:60 NVE, one liter subsamples were shipped overnight to the Pennsylvania State University Agricultural Analytical Services Lab for nutrient analysis immediately after straining. Dissolved oxygen readings were taken from a 0.5 m depth every 10 minutes throughout the extraction period with a DO meter and data logging software (Traceable® Dissolved Oxygen Meter, Control Company, Friendswood, TX). Samples were removed daily from a depth of 0.5 m for pH and electrical conductivity (EC) measurements as well as enumeration of bacteria. Bacteria were enumerated and viability was assessed according to manufacturer instructions (LIVE/DEAD BacLight™ Bacterial Viability Kit, Molecular Probes Inc., Eugene, OR). Samples were syringe filtered to 0.8 µm before staining in order to remove particulate organic matter (POM) and protozoa. Significant numbers of POM-associated bacteria were lost with this filtering step, but without it the images used for counting were of poor quality. Stained samples were viewed via fluorescence microscopy with a Zeiss 450-490 nm standard fluorescein filter set and digitally imaged with 10 fields of view counted for each of 3 subsamples (Olympus DP72 digital camera, CellSens® digital imaging software, Center Valley, PA). Data were analyzed with a one way ANOVA and Tukey's means separation in Minitab 16 (Mintab Inc. State College, PA).

iii. Disease suppression

Subsamples of NVE (1200 mL) were lyophilized immediately after the 10 d extraction period and stored at -20^{0}C on argon gas (Labconco, Kansas City, MO). Lyophilized non-aerated vermicompost extract was reconstituted in sterile ultrapure water to its original concentration and incubated at room temperature for 24 h before use (designated LyNVE). Disease suppression bioassays were carried out in 12-well tissue culture plates (3 plates per treatment). Each well contained 6 mL sterile quartz sand (0.5 – 1.0 mm d), one surface disinfested cucumber seed and 1.75 mL total liquid. For NVE treated wells, 750 µL extract was added. For inoculated wells, 500 µL of a Pa58 zoospore suspension (1.2 x 10^4 zoospores mL^{-1}) was added. Non-inoculated controls were included to test seed germination. After the lids were sealed with parafilm, 12-well tissue culture plates were placed in large clear plastic containers and incubated at 27^{0}C with a 16 h photoperiod. After 3 days, lids were removed, a thin layer of water was added to the secondary container, the lid of the secondary container was replaced and sealed with parafilm to create a moist chamber. Incubation was continued for an additional 4 d at which time seedlings were removed and shoot height, health rating, seedling survival and disease incidence were recorded. Health ratings were designated as follows: 0=dead and completely rotted, 1=dead but not completely rotted, 2=cotelydon and stem lesions, 3=cotelydon lesions only, 4=stem lesions only, 5=healthy. The experiment was repeated 3 times with a total of 108 seedlings per treatment. Disease suppression bioassay data were analyzed with ANOVA (proc mixed, SAS v. 9.3, SAS Institute, Cary, NC). Binary logistic regression was used to measure the impact of NVE amendment on the likelihood of seedling survival and disease

incidence (proc genmod, SAS v. 9.3, SAS Institute, Cary NC). To determine the effect of storage time at room temperature on the disease suppressive properties of NVE, samples were tested in the disease suppression bioassay periodically up to 76 days. To track changes in suppression over time, the differential health rating was calculated as follows: ((health rating for each individual inoculated seed sown in NVE) – (average health rating for one replicate 12 well plate of inoculated seeds sown in sand). Data were analyzed using ANOVA with a Bonferroni's correction for multiple comparisons (proc glm, SAS v. 9.3, SAS Institute, Cary, NC).

RESULTS

i. Chemical and biological characterization of NVE

Components of the solid vermicompost were transferred to the liquid extract during the extraction process with an average of 30% dry biomass becoming dissolved or suspended in water (n=8 batches, mean=30% solubility, standard deviation=12.9%). A decrease in vermicompost:water ratio in the starting mixture appeared to correlate with a decrease in pH, log bacterial CFU mL^{-1} and electrical conductivity and an increase in dissolved oxygen in the 7 d non-aerated extracts (Figure 1). These trends are descriptive in nature as only one batch of each ratio was tested. Cucumber seedling radicle emergence was significantly negatively impacted only by the 1:5 vermicompost:water NVE (ANOVA, Tukey's means separation $p < 0.0001$, mean radicle length (mm) water 21.1, 1:5 NVE 9.3). However, a visual inspection of cucumber radicles revealed an absence or reduction in length of root hairs for seeds exposed to almost all batches tested except for the most dilute 1:60 ratio (Figure 2). Similar patterns of radicle emergence and root hair damage were observed when NVE samples

were filtered to 0.2 µm (data not shown). In order to avoid potential damage to root hairs and minimize soluble salts in the finished extract, the 1:60 ratio was chosen for additional experimentation. No fungal colonies were observed on APDA medium for any of the samples (data not shown).

Nutrient analysis showed that 1:60 NVE contained a range of macro- and micronutrients. Compared to a commercial synthetic 20-10-20 fertilizer, NVE had lower ammonium and nitrate levels, but higher phosphorus, potassium, and many micronutrients (Table 1). Initial dissolved oxygen was 6 ppm, which rapidly declined over the first day and stayed near 0 ppm for the remainder of the extraction period (Figure 3). Sump pump activity briefly spiked DO levels during the extraction period, however after the first day dissolved oxygen never rose above 0.2 ppm (Figure 3). Electrical conductivity rose slightly but significantly over the extraction period from 0.6 to 0.8 mmhos cm^{-1} while pH declined slightly but significantly from 8.7 to 8.3 (data not shown, regression $p < 0.0001$). Compared to the first day of the extraction period, the number of live bacterial cells were significantly highest on days 7 and 8 (Table 2, ANOVA $p = 0.01$). Overall, there was no significant change in the active bacterial population by the end of the 10 d extraction period compared to the first day. The quantity of dead bacterial cells did not change significantly over the extraction period (Table 2, ANOVA $p=0.067$).

iii. Disease suppression bioassays

All batches of both non-aerated vermicompost extract (NVE) and lyophilized – reconstituted NVE (LyNVE) significantly suppressed the symptoms of *P. aphanidermatum* seedling damping off in cucumber as measured by shoot height,

health rating, seedling survival, and seedling disease incidence (Table 3, Figure 4). Sterile NVE (StNVE), which had been filtered through a 0.2 µm filter, offered no protection from disease compared to the sand control (Table 3, Figure 4). Fresh and filter sterilized NVE treatments significantly promoted cucumber seedling growth in non-inoculated controls (Table 3). When stored at room temperature in 5 gallon ventilated buckets, extracts remained suppressive for up to 60 d after which point the level of suppression declined significantly (Table 4, ANOVA $p<0.0001$).

DISCUSSION

The non-aerated vermicompost extract developed in this study consistently suppresses *P. aphanidermatum* in cucumber in laboratory bioassays and remains suppressive for up to 60 days under storage at room temperature. The 50-gallon food grade container, PVC pipe, sump pump and timer used to construct the extraction apparatus can be purchased and assembled for under $200, this system requires only minimal electricity for periodic circulation and no nutrient additives are required. This is relatively inexpensive when compared to the cost of over $2,000 for an aerated compost extraction apparatus of a similar volume (25 gallons) plus the electricity to run it and the proprietary blend of nutrient additives recommended by the manufacturer, Growing Solutions Incorporated (Growing 2011). In addition, the NVE developed in this study could be made available to certified organic growers because liquid compost extracts made using OMRI-listed composts and without any nutrient additives may be used without restriction, whereas extracts made using nutrient additives are subject to 90 to 120 day pre-harvest intervals unless extensive human pathogen testing is carried out (NOSB 2006).

With regards to shelf life, Soil Food Web Inc. recommends using aerated compost extracts materials within 4-6 hours of production (Ingham 2002). While this particular material maintains its disease suppressive properties for up to 60 days at room temperature which could potentially ease the complications associated with using these materials in a commercial plant production setting, it is important to note that a variety of factors can impact their shelf life. Very little is reported in the scientific literature on the impacts of compost maturity, compost storage and storage of the finished extract on disease suppression. While some non-aerated extracts maintain their suppressiveness over 4 months storage at room temperature (Yohalem et al. 1994), others can lose suppressiveness after 7 days [Ketterer 1990 Dissertation] as cited in (Weltzien 1992). Compost maturity impacts the suppressiveness of extracts, with internal windrow temperatures below 50°C associated with a decline in suppressiveness towards *Botrytis cinerea* (Palmer et al. 2010) and no clear trend found for suppression of *Armillaria melea* in beech wood (Egwunatum and Lane 2009). Compost storage times from 3 months to 1 year reduced the suppressiveness of a non-aerated compost extract (Winterscheidt et al. 1990). However, this decline in extract suppressiveness can vary depending on the type of compost used with some batches making suppressive extracts for up to 18 weeks in storage and others steadily losing efficacy over time under the same storage conditions (Yohalem et al. 1996). Identifying the environmental factors responsible for declining suppressiveness during storage of both the compost and finished extracts and the pathosystem specificity of suppression will be important research objectives before clear recommendations can be made to growers.

The initial compost to water ratio of 1:5 most commonly cited for the production of non-aerated compost extracts (Weltzien 1992) was not suitable for this source of vermicompost. However a 1:60 ratio produced an extract that was not phytotoxic to cucumber. The 1:60 (1.6% vermicompost by volume) batches used in this study had an average soluble salts concentration of 1.07 mmhos cm^{-1} (Table 1), which is similar to the range of EC values (0.08 to 1.05 mmhos cm^{-1}) found for five thermogenic composts used in a 1:5 compost to water ratio (20% compost by mass) (Kone et al. 2010) which indicates that the solid vermicompost is higher in the plant available nutrients that contribute to soluble salts than traditional composts. A comparison of vermicomposted and thermogenically composted dairy manure at two facilities found that although the finished vermicompost and thermogenic compost contained roughly equivalent total nitrogen, the vermicompost had 7 – 10 times the nitrate levels (Jack et al. 2011). Highly concentrated (1:1) non-aerated vermicompost extracts (Ievinsh 2011) and 1:4 and 1:8 rinses of vermicompost can negatively impact seedling germination (Warman and AngLopez 2010). Depending on the type of feedstock used, a wide range of soil amendment rates with solid vermicompost (30%, 10%, 5%) can also inhibit or slow germination for a variety of seed types (Simsek-Ersahin et al. 2009). This further emphasizes that the high variability in composted materials has downstream impacts on liquid compost extracts and the compost to water ratio cannot be considered standard for all types of compost. We recommend that growers carry out phytotoxicity screenings in order to choose an appropriate compost to water ratio for the production of non-aerated extracts. The TMECC method 05.05-B is a straightforward phytotoxicity test with a minimal requirement for materials that could easily be carried out on-site by

growers and home gardeners when developing extract production protocols for new batches of compost (Thompson et al. 2002).

For this particular vermicompost, the nutrient profile of the 1:60 batch shows the material is suitable for use in most plant production systems, although supplementing with additional nitrogen would likely be necessary depending on the nitrogen requirements of the crop and dilution for salt intolerant crops may be necessary due to high sodium levels (56.12 ppm). The 1:60 fresh and filter sterilized NVE batches had a positive effect on plant growth with up to a 19% increase in cucumber shoot length compared to water controls. This result further contradicts the unsubstantiated claims found in the popular literature that all non-aerated extracts are inherently phytotoxic (Ingham 2005) and supports previous findings of effective nutrient management with non-aerated compost extracts (Pant et al. 2009; Pant et al. 2011). Comparisons of aerated and non-aerated extract made from the same starting batch of compost found that aeration had no impact on phytotoxicity (Xu et al. 2012). The nutrient profiles reported for 1:5 compost:water non-aerated compost extracts, not surprisingly vary widely based on the type of compost used; nitrate (below detection – 13 ppm), phosphorus (0.23 ppm - 72), potassium (195 – 421 ppm) (Kone et al. 2010; Radin and Warman 2010). Our results for a 1:60 vermicompost:water non-aerated extract fall within the previously reported ranges even with the relatively small amount of vermicompost used (1.6% by volume compared to 20% by volume for batches reported in the literature); nitrate (13 ppm), phosphorus (67 ppm), potassium (293 ppm). This high variability points again to the importance of nutrient testing during the

compost:water ratio selection process in order to produce a material suitable for specific fertility programs.

Using a sump pump and a timer allowed for defined and repeatable mixing during the extraction process although it is not known if mixing is required to achieve the solubility rates observed (30% of vermicompost remaining in solution). Available oxygen declined steeply from 6 – 0.1 ppm DO within 24 hours. Reported initial declines of dissolved oxygen in non-aerated compost extracts vary from 7.5 – 0 ppm in 30 & 60 minutes for 1:2 and 1:4 compost:water ratios respectively (Cronin et al. 1996) to 7 – 0.1 ppm in 20 hours for a 1:77 compost to water ratio (Kannangara et al. 2006). Our extract, with 10^7 cells mL^{-1}, did not meet the standard proposed by SFI for minimum active bacteria (150 µg, roughly equivalent to 10^9 cells mL^{-1} (Ingham 2005)) however it promoted cucumber seedling growth and suppressed *Pythium* seedling disease. While the industry standard involves the presence of fungi (2 µg, roughly equivalent to 10^7 cells mL^{-1} (Ingham 2005), fungi were not observed on PDA plates or during light microscopy for any of the NVE batches tested.

When used as a soil drench, the NVE developed in this study was consistently effective in suppressing *P. aphanidermatum* on cucumber and retained its efficacy through lyophilizing and reconstitution. The physical removal of microorganisms through 0.2 µm filtration eliminated suppression, indicating that suppression relies on the presence of microorganisms. This is not always the case with non-aerated compost extracts. For example, in many cases sterile filtered extracts are still fully (Yohalem et al. 1994; Cronin et al. 1996; Al-Dahmani et al. 2003) or partially (Elad and Shtienberg 1994; Haggag and Saber 2007) suppressive, while others completely lose suppression

after sterile filtration or autoclaving (McQuilken et al. 1994; Gea et al. 2009; Kone et al. 2010). These results indicate the likelihood that a variety of mechanisms are involved in the suppression of plant pathogens by non-aerated compost extracts.

Using compost extracts in organic agriculture provides biologically-based liquid fertilizer options and a potentially disease suppressive microbial community for crop production while creating economic incentives for livestock manure recycling, all of which are important components of sustainable agriculture. Linking a waste product from livestock operations with a viable market in crop production could help strengthen the regional recycling of nutrients which can cause water and soil pollution if managed improperly. However, the substantial promise of liquid compost extracts can only be realized if growers, extension educators, researchers and the compost extract industry work together to establish a reality based framework for evaluating these highly variable materials. Just as biodynamic agriculture has moved from the fringes of the agricultural scientific world to the pages of Science (Mader et al. 2002; Turinek et al. 2009), research will be an important component for the further development of liquid compost extracts.

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FIGURES AND TABLES

Figure 1. Electrical conductivity (EC) mmhos cm^{-1}, dissolved oxygen (DO) ppm, pH and average log CFU bacteria mL^{-1} of non-aerated liquid vermicompost extracts for a range of initial vermicompost to water ratios. Trends are descriptive in nature as only one batch of each ratio was tested. Extracts were strained through 4 layers of sterile cheesecloth and stored in ventilated buckets before measurements were taken.

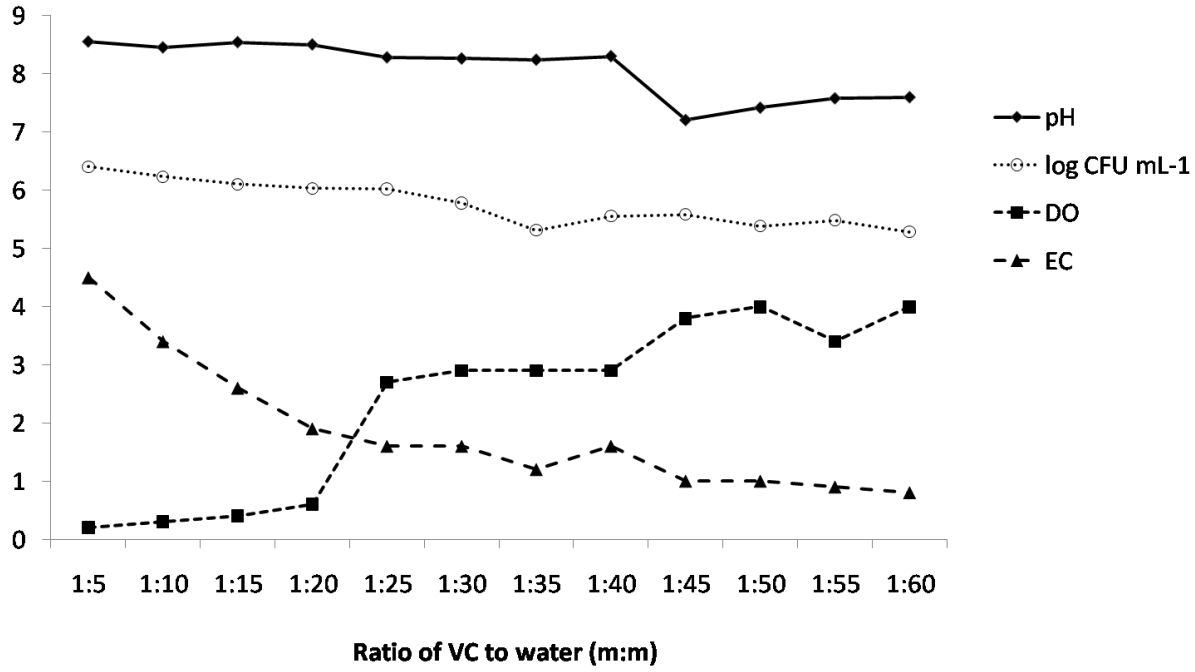

Figure 2. Representative 2 d old cucumber seedlings germinated at 27°C in A. water, B. 1:5 NVE, C. 1:30 NVE, D. 1:60 NVE. Scale bar = 1 cm

Table 1. General chemical characteristics and plant available nutrients in a 1:60 non-aerated vermicompost extract (NVE) compared to leading commercial synthetic fertilizer applied at two commonly used rates. Values are averages and ranges of 3 NVE batches.

		Commercial 20-10-20		
Measurement	**NVE**	**100 ppm N**	**200 ppm N**	**Units**
pH	7.9 ± 0.1			
soluble salts	1.07 ± 0.04			mmhos cm^{-1}
organic matter	0.09 ± 0.01			%
ammonium N	2.60 ± 1.60	40	80	ppm
nitrate N	13.31 ± 3.17	60	120	ppm
P	66.67 ± 24.33	22	44	ppm
K	293.33 ± 13.33	83	166	ppm
Ca	46.67 ± 24.33	0	0	ppm
Mg	10.00 ± 0.0	0.75	1.50	ppm
S	20.00 ± 0.0	0	0	ppm
Na	56.12 ± 3.95	0	0	ppm
Al	2.66 ± 2.44	0	0	ppm
Fe	7.61 ± 9.37	0.25	0.50	ppm
Mn	0.27 ± 0.18	0.13	0.25	ppm
Cu	0.70 ± 0.10	0.06	0.13	ppm
Zn	1.15 ± 0.35	0.13	0.25	ppm

Figure 3. Average dissolved oxygen levels (ppm) at 0.5 m depth over the 10 day extraction period for 3 180 L batches of 1:60 non-aerated vermicompost extract (NVE).

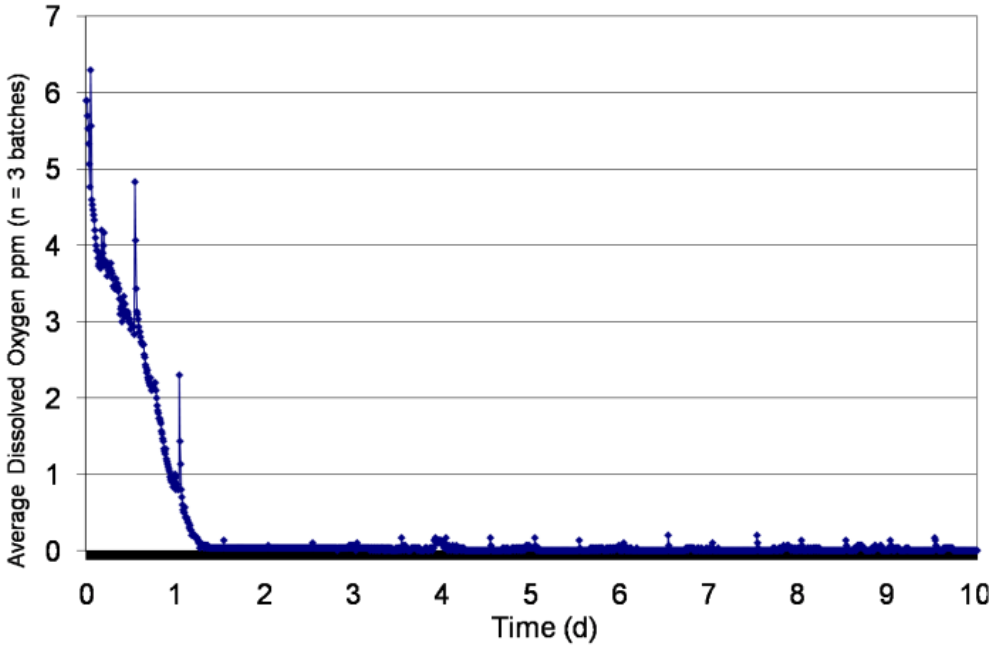

Table 2. Average log counts of live/dead bacterial cells for non-aerated vermicompost extract (NVE) over the 10 d extraction period. Each value represents 10 replicate counts from 3 individual sub-samples taken from 3 separate batches of NVE (n=27). Live cells: Tukey's means separation, ANOVA p = 0.01, dead cells ANOVA p = 0.06.

Day	**log average live cells mL^{-1}**		**log average dead cells mL^{-1}**
1	6.91	bc	3.48
2	7.25	abc	3.23
3	7.25	abc	3.17
4	7.35	abc	3.08
5	6.87	c	3.39
6	7.08	abc	3.10
7	7.43	a	2.94
8	7.45	a	3.04
9	7.30	abc	3.08
10	7.37	ab	2.99

Table 3. Health ratings for 7 day old cucumber seedlings 0 = dead and rotted, 5 = healthy. Seedlings were sown in sand and treated with lyophilized and reconstituted non-aerated vermicompost extract (LyNVE), non-aerated vermicompost extract (NVE) or 0.2 µm filter sterilized non-aerated vermicompost extract (StNVE). Inoculated seedlings received 0.5 mL of a 1.2 x 10^4 mL^{-1} zoospore solution. Bioassays were carried out in 12 well tissue culture plates with 3 plates per treatment and the entire experiment was repeated for each of 3 separate batches of NVE. Values followed by the same letter in each row are not significantly different at α=0.05 (ANOVA: shoot length, health rating; binary logistic regression, survival, incidence: ns = no significant difference detected between treatments).

Treatment	**Inoculation**	**Shoot length (mm)**	**Health rating (0-5)**	**% survival**	**% incidence**
Sand	+	26.13 B	1.62 b	60 B	98 a
NVE	+	40.00 A	3.93 a	88 A	42 b
LyNVE	+	37.66 A	4.11 a	86 A	28 b
StNVE	+	25.40 B	1.43 b	54 B	98 a

Treatment	**Inoculation**	**Shoot length (mm)**	**Health rating (0-5) ns**	**% survival ns**	**% incidence ns**
Sand	-	34.44 z	4.95	99	1
NVE	-	41.71 y	4.95	99	1
LyNVE	-	39.41 z	5.00	100	0
StNVE	-	41.99 y	5.00	100	0

Figure 4. Representative 7 day old seedlings from disease suppression bioassays in 12 well plates. Each inoculated seedling received 0.5 mL of a 1.2 x 10^4 zoospores mL^{-1} suspension. Treated seeds received 750 µL 0.2 µm sterile filtered non-aerated vermicompost extract (StNVE), fresh NVE (NVE), or lyophilized-reconstituted NVE (LyNVE).

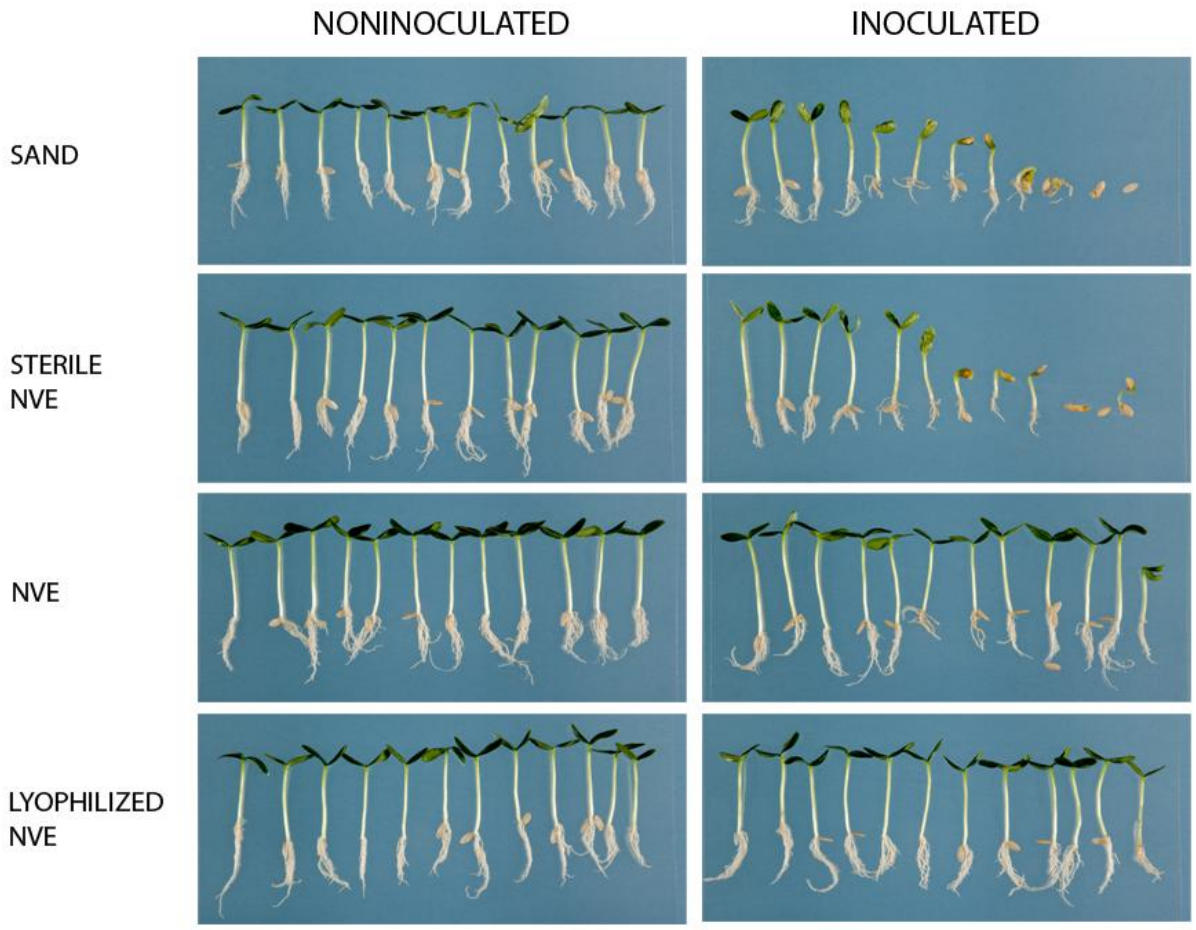

Table 4. Change in suppression of *P. aphanidermatum* over extended storage of 1:60 non-aerated vermicompost extracts. Health rating differential was calculated as follows: ((health rating for each individual inoculated seed sown in NVE) – (average health rating for one replicate 12 well plate of inoculated seeds sown in sand). Data were analyzed using ANOVA with a Bonferroni's correction for multiple comparisons (SAS v. 9.2, proc glm, $p<0.0001$). Values followed by the same letter are not significantly different at $\alpha = 0.05$.

Age of extract (d)	**Least squared mean differential health rating**
3	2.91 a
4	2.94 a
9	2.79 a
29	2.58 a
36	2.69 a
60	2.99 a
76	1.22 b

CHAPTER 3. SEED COLONIZING MICROBES FROM DISEASE SUPPRESSIVE VERMICOMPOST ALTER ZOOSPORE CHEMOTAXIS, ENCYSTMENT AND GERMINATION OF *PYTHIUM APHANIDERMATUM*

Allison L. H. Jack and Eric B. Nelson

ABSTRACT

Disease suppressive composts have been studied for decades, however we still lack critical insight into their mechanisms of action, which limits their efficacy as tools for biologically based plant disease management. We sought to uncover potential mechanisms by which vermicomposted dairy manure suppresses *Pythium aphanidermatum*-incited seedling disease on cucumber by investigating the interactions between seed-associated microbial communities and *P. aphanidermatum* zoospores. *In situ* disease suppression bioassays demonstrated that, when zoospores were forced to swim 2 mm to reach the host, vermicompost microbes that colonized seeds within 8 h effectively prevented zoospore arrival at the seed surface. This was confirmed by the low levels of zoospore DNA detected on the surfaces of seeds pre-germinated in vermicompost and transplanted to sand prior to inoculation. Exudates from seeds sown in vermicompost for 8 hours then transplanted to sand for 24 hours (microbially modified seed exudates: MMSE) were collected, sterile filtered and used for *in vitro* zoospore assays. Modification of seed exudates by vermicompost microbes that colonized seeds within 8 h inhibited the chemotaxis and encystment of zoospores relative to these responses to exudates from seeds sown in sand. In addition, the germination of mechanically encysted zoospores was reduced when exposed to MMSE. Combining

non-microbially modified seed exudate with MMSE failed to restore suppression and extensive zoospore lysis was observed. Lytic activity and inhibition of cyst germination were more prevalent in the ethyl acetate fraction of vermicompost MMSE, providing evidence for the presence of a zoosporolytic compound. We hypothesize that the observed zoospore lysis and suppression of cyst germination is likely due to a specific compound or set of compounds produced in the spermosphere by one or more members of the vermicompost-derived microbial community that colonizes seeds within the first 8 h of germination, thus protecting the seed from *P. aphanidermatum* zoospore infection.

INTRODUCTION

There has been much interest over the years in understanding the microbial mechanisms of pathogen suppression in soils naturally suppressive to disease (Mazzola 2002; Weller et al. 2002; Mendes et al. 2011) and also in soils in which suppressiveness is induced by compost amendments (van Os and van Ginkel 2001; Kowalchuk et al. 2003; Benitez et al. 2007). Although the fact that microbial communities are involved in both naturally suppressive and induced suppressive soils is clear, an understanding of the mechanisms by which these microbes prevent pathogens from infecting their hosts has been elusive (Janvier et al. 2007). One of the more common approaches for understanding the role of specific microbes and specific microbial activities in disease suppression is the comparative analysis of the bulk microbial community from disease-suppressive and non-suppressive soils (Borneman and Becker 2007; Kyselkova et al. 2009; Postma et al. 2010). The basic hypothesis driving this approach is that microbial taxa that are either present or differentially more

abundant in the suppressive soil than in the conducive soil are likely to be involved in disease suppression. This approach may provide potential candidate microbes that can be studied further for their mechanisms of disease suppression (Borneman and Becker 2007). However, the problem with this approach is that the presence, absence, or differential abundance of one or a few microbes is not likely to explain suppressiveness, since the bulk microbial community is often too complex and dynamic to detect clear differences between suppressive and conducive communities (Kowalchuk et al. 2003). Furthermore, because the infection of plants by pathogens is a spatially and temporally-dynamic process, focusing solely on the soil or compost itself would fail to detect any microbial interactions that are relevant to direct host-pathogen interactions during pathogenesis.

An alternative approach for understanding which microbes may be involved in disease suppression and also for understanding how these microbes prevent infection, is to focus on a subset of the soil microbial community most likely to interact with the pathogen during the time frame in which infection occurs. Focusing on direct microbial interactions in the infection court has been valuable in understanding mechanisms by which spermosphere bacteria suppress seed infections (Heungens and Parke 2000; Windstam and Nelson 2008a; Windstam and Nelson 2008b) as well as in providing insight into the mechanisms of compost-induced disease suppression (McKellar and Nelson 2003; Chen and Nelson 2008; Chen et al. 2012). For example, the use of this approach revealed that the compost microbes most important to the suppression of seed infection by *Pythium ultimum* are those that colonize seeds within the first 8 h after sowing (McKellar and Nelson 2003; Chen and Nelson 2008). By focusing on the

behavior of the pathogen that may be altered by specific microbial interactions in the infection court, we are able to disregard microbes present in the bulk substrate that have nothing to do with disease suppressive properties while being led more directly to the microbes and their activities that can explain disease suppression.

In our current work, we have adopted this approach to better understand the mechanisms by which *Pythium aphanidermatum* incited diseases are suppressed when seeds are sown in a vermicomposted dairy manure substrate. A number of important aspects of this system facilitate the use of this approach. First, vermicomposts, like thermogenic composts, are known to be suppressive to diseases caused by a number of different major soil-borne pathogens (Szczech 1999; Scheuerell et al. 2005; Simsek-Ersahin et al. 2009; Jack 2010). Thermogenic composts can be chemically and physically quite variable due to the use of mixed feedstocks and the variable conditions during the 6 to 9 month long mesophilic maturation period, which usually takes place outdoors under ambient weather conditions. For single feedstock composts, post-thermogenic vermicomposting in a highly engineered flow-through system can reduce the maturation period to 60 days and result in a chemically and physically uniform disease suppressive substrate. Second, *P. aphanidermatum* is an especially relevant target pathogen for our studies. Aside from being one of the most important seed- and root-infecting plant pathogens with a host range of over 650 species (Farr et al. 1989), *P. aphanidermatum*, like other *Pythium* species, is inherently sensitive to microbial competition and interference (Martin and Loper 1999), making it an ideal target for microbial-based suppression. Third, *P. aphanidermatum* is believed to infect seeds and roots largely via the formation of zoospores (Deacon and Donaldson 1993; Nelson

2006; Walker and van West 2007), which display a complex, but well-characterized homing response from the time of zoospore release to plant infection. During pathogenesis, zoospores respond to chemical cues from the host (in the form of seed or root exudates) to detect and swim to the infection court (Deacon 1996). This chemotactic response is rapidly followed by the attachment and encystment of the zoospore on the seed, radical, or root surface, followed rapidly by germination of the zoospore cyst and host penetration.

Although the exact chemical cues for the homing response are unknown, they appear to be species specific (Donaldson and Deacon 1993a; Donaldson and Deacon 1993b; Reid et al. 1995) and developmentally-specific (i.e., swimming zoospore, encystment, cyst germination) (Donaldson and Deacon 1993a; Donaldson and Deacon 1993b), and interference with these cues has been shown to be an effective means of suppressing infection by zoosporic pathogens. For example, zoospores exhibit a weaker chemotactic response to exudates from roots or seeds directly treated with microbes than they do towards exudates from untreated roots or seeds (Zhou and Paulitz 1993; Shang et al. 1999; Heungens and Parke 2000; Lioussanne et al. 2008; Islam 2010). In some cases, altered zoospore behavior appears to be due to modification of exudates by host-associated microbes (Lioussanne et al. 2008). However, it is not always clear if this chemical modification results from the degradation of a zoospore attractant, or the production of a zoospore repellant/toxin (Zhou and Paulitz 1993), or a combination of both (Heungens and Parke 2000). Therefore in order to fully understand the mechanisms by which compost microbes might interfere with

plant infection, it is necessary to examine interactions at each of these stages of pathogenic development along with the chemical cues that elicit these responses.

Building off of our previous work (McKellar and Nelson 2003; Chen and Nelson 2008; Chen et al. 2012), the goal of our current work is to understand how compost-derived microbes protect plants from infections by *Pythium aphanidermatum* as a means of elucidating mechanisms by which composts suppress plant disease. Our study attempts to draw a connection between a reduction in the colonization of cucumber seeds by *P. aphanidermatum* after zoospore inoculation with changes in zoospore behavior in response to direct microbial alterations of seed exudates that may explain the observed disease suppression. Examining interactions between the host, pathogen and microbial community in the infection court, we attempt to answer the following questions; 1) can the 8-hour seed-colonizing community explain the observed suppression?, 2) which stages of the zoospore homing response does the suppressive seed-colonizing community alter?, 3) is the altered zoospore response due to the modification of seed exudates by the seed-colonizing microbial community?, and if so, 4) does this modification of seed exudates involve (a) the degradation of a chemotactic cue, (b) the production of a zoospore repellant/lytic agent or both? It is our hope that an enhanced understanding of the mechanism of suppression will lead to a greater predictive capacity and eventually greater efficacy in the use of composts and vermicomposts for the biological management of *Pythium* species.

MATERIALS AND METHODS:

A. Experimental materials:

Cucumber seeds (*Cucumis sativus* cv “Marketmore 76”, Johnny’s Seeds) were sorted to remove damaged seeds, individually screened to 0.02 – 0.03 g biomass and surface disinfested with a mild bleach solution (0.5% sodium hypochlorite) for 5 minutes. Quartz sand was wet sieved to 0.5 - 1.0 mm diameter, oven dried and autoclaved 40 min for three consecutive days before use. Vermicompost (Worm Power, Avon NY) was collected, stored at -20^{0}C and thawed at room temperature for 24 h before use in all experiments. Vermicompost was prepared from dewatered dairy manure solids which were mixed 7:1:1 with spoiled corn and hay silage and cured hot compost. This mixture was hot composted in a forced air system for up to 2 weeks. Material was then fed to continuous flow through vermicomposting systems stocked with *Eisenia fetida* and *Dendrobaena venata* every 3-4 days in 5 cm layers. Finished vermicompost was removed from the underside of the continuous flow through system and sieved to 10 mm 75 days after the initiation of hot composting. Sterile vermicompost was prepared by autoclaving for 40 min on three consecutive days. Before use in bioassays, 500 g of vermicompost was placed in a 0.25 mm sieve and soaked in 4 L Nanopure® water for 5 minutes before being allowed to drain. This additional step was performed in order to prevent excessive bacterial growth in tubing used in the bioassay apparatus.

Pythium aphanidermatum (Edson) Fitzp (Pa58) (Ben-Yephet and Nelson 1999) was cultured on clarified V8 plates at 27^{0}C. To maintain virulence and prevent bacterial contamination, cucumber seeds were inoculated with Pa58 zoospores weekly, infected

seeds were overlaid with KWARP (water agar with kanamycin sulfate 0.025 mg mL^{-1}, rifampicin 0.015 µg mL^{-1} and penicillin G 0.015 µg mL^{-1}) and hyphal tips were transferred to clarified V8. For zoospore preparation, a core borer (#15, 20 mm diam) was used to remove discs from 7 d Pa58 cultures. Each disk was placed in a 70 mm petri dish with 10 mL sterile Nanopure® water for 17 h at 27^0C. Liquid was then replaced with 10 mL sterile nanopure water and discs were incubated at 27^0C for an additional 7 h. Zoospores were enumerated with an Improved Neubauer Haemocytometer and diluted with sterile Nanopure® water if necessary. Zoospore suspensions were used immediately after preparation.

B. Disease suppression bioassay

Bioassays were conducted in an apparatus that held matric potential (Ψ_m) at a constant -3.5 kPa in a growth chamber at 27^0C and 18 h photoperiod (Dimock growth chamber facility, Cornell University). In the apparatus, fritted glass Büchner funnels were attached to a water column held under vacuum with one end placed in an open reservoir, based on the design of Chen and Nelson (Chen and Nelson 2008). Cucumber seeds (10 per funnel) were sown in 150 cm^3 of one of three substrates in the funnels; sterile sand (0.5 – 1.0 mm d), sterile sand amended with 40% (v:v) vermicompost, and sterile sand amended with 40% (v:v) sterile vermicompost. Substrates were flooded for 30 min and 50 mL zoospore suspension (1.2×10^4 zoospores mL^{-1}) was added to inoculated funnels. Substrates were then drained and covered with ventilated Parafilm M to create a moist chamber. At 7 days seedlings were harvested and assessed for disease symptoms; shoot height, health rating, seedling survival and disease incidence were recorded. Health ratings were designated as follows: 0=dead and completely

rotted, 1=damped off but not completely rotted, 2=cotelydon and stem lesions, 3=cotelydon lesions only, 4=stem lesions only, 5=healthy. Disease incidence data (presence or absence of symptoms) were analyzed in SAS v9.3 using binary logistic regression with Bonferroni's correction for multiple comparisons. Health ratings were analyzed in SAS v9.3 using ANOVA in the general linear model with Tukey's correction for multiple comparisons.

C. *In situ* zoospore swimming bioassay

Point source bioassays based on the design of Heungens and Parke were conducted in the Büchner funnel apparatus described for disease suppression bioassays (Heungens and Parke 2000). Seeds were embedded into nylon mesh in a 4 cm diam circle before sowing to ensure their position would not be disturbed during flooding. After sowing, Erlenmeyer flasks holding the water column were raised to passively flood the substrates through the fritted glass in the Büchner funnels. After substrates were saturated (~5 min), flasks were then lowered to allow matric potentials (Ψ_m) to equilibrate at -3.5 kPa. Zoospore suspensions (5 mL, 8 x 10^4 zoospores mL^{-1}) were added to the center of the substrate and the funnel was covered with ventilated Parafilm to create a moist chamber. A portion of the seeds were destructively harvested at various hours post inoculation (hpi) to test for the presence of Pa58, and the remaining seeds were used to assess disease symptoms and survival at 9 days. For 9 d old seedlings, disease incidence (presence or absence of symptoms) was analyzed in SAS using binary logistic regression with Bonferroni's correction for multiple comparisons. Differences in Pa58 DNA on seed surfaces were analyzed using an

ANOVA in the general linear model of SAS with sliced interactions for treatment*hpi to generate a means separation.

i. Mass flow test

To ensure that zoospores added to the bioassay apparatus must actively swim to reach seeds, a 5 mL suspension of either actively swimming or mechanically encysted non-motile Pa58 zoospores (8 x 10^4 zoospores mL^{-1}) were added at a point source in the center of the bioassay apparatus based on the design of Kliejunas and Ko (Kliejunas and Ko 1974). Cucumber seeds were sown in sand with 4 cm spacing based on the design of Duniway (Duniway 1976). The viability of mechanically encysted non-motile zoospores was tested by adding 5 mL encysted Pa58 zoospores (8 x 10^4 zoospores mL^{-1}) to the center of the bioassay apparatus. For this test, cucumber seeds were sown at 1 cm spacing. After 24 hours, seeds were transplanted to funnels containing sterile sand to prevent potential infections from secondary zoospores. At 48 hpi half the seeds were removed and plated on KWARP to score for the presence or absence of Pa58. Remaining seedlings were harvested 8 d after sowing to assess disease symptoms and seedling stand. Disease incidence (presence or absence of symptoms) was analyzed in SAS v9.3 using binary logistic regression with Bonferroni's correction for multiple comparisons.

ii. Transplant bioassays

To determine whether vermicompost microbes that colonize seeds can protect seeds from zoospore infection, a transplant bioassay was carried out similar to that described by Chen and Nelson (Chen and Nelson 2008). Seeds were sown in sand and in vermicompost-amended sand in Büchner funnels as described above and allowed to

germinate 8 h before transplanting to sterile sand and point source inoculated with a zoospore suspension. One third of the seeds were assessed for seedling survival and disease symptoms at 9 d to assure the viability of zoospore inoculum and two thirds of the seeds were destructively harvested 24 h post inoculation for assessments of Pa58 biomass via quantitative PCR.

iii. Quantitative assessment of Pa58 on seeds

Cucumber seeds were removed from their respective substrates at 12, 18 and 24 hpi and gently tapped to remove adhering sand and vermicompost particles. Ten seeds were placed in the initial DNA extraction buffers (UltraClean® Soil DNA Isolation Kit, MoBio, USA) and frozen overnight at -20^{0}C before sample processing. Manufacturer's protocol for samples with high humic acids was used for DNA extraction. *P. aphanidermatum*-specific primer sets were designed using a consensus sequence generated from an alignment of 42 ITS sequences from the NCBI database and our laboratory reference strain, Pa58 (Lasergene® Megalign, DNASTAR, USA). Five potential primer sets were identified using PrimerSelect (DNASTAR, USA and subjected to a melting curve analysis. One primer pair was selected for use in quantitative PCR analysis (PaITS-F 5' AATGTACGTTCGCTCTTTCTTG 3', PaITS-R 5' GGTTGCTTCCTTTAATGTCCTA 3'). Quantitative PCR (qPCR) was carried out using an iQ^{TM}5 thermocycler (Bio-Rad, USA). Each 25 µL reaction contained 12.5 µL iQ^{TM} SYBR® Green Supermix (Bio-Rad, USA), 1.25 µL PaITS4-F and PaITS4-R (500 mM), 1 µL template and 9 µL DNase, RNase free water. *P. ultimum* mycelial DNA was used as a negative control and water was used as a no template control. Reaction conditions were 40 cycles of 95^{0}C for 15 s and 50^{0}C for 30 s. To generate a standard curve Pa58

was cultured on V8 overlaid with sterile cellophane. Mycelia were harvested after 7 d, lyophilized and weighed. DNA was extracted (see above) and quantified using a Quant-iT™ PicoGreen® dsDNA quantification kit (Invitrogen, USA) and a VersaFluor™ fluorometer (Bio-Rad, USA). DNA harvested from lyophilized mycelia was used in each qPCR plate with a range of 1 fg to 10 ng. To ensure that the presence of the cucumber seed and/or the vermicompost substrate did not interfere with DNA extraction or PCR amplification, additional treatments were used. Cucumber seeds were sown in sand or sand amended with 40% v:v vermicompost for 24 h. Seeds sown in vermicompost for 8 hours were combined with a known about of lyophilized Pa58 biomass and DNA was extracted and used to generate additional standard curves in order to rule out potential deleterious effects of residual vermicompost on DNA extraction or PCR efficiency.

D. Zoospore responses to microbially modified seed exudate (MMSE)

i. Preparation of Microbially Modified Seed Exudate (MMSE)

Seeds were sown in fritted glass Büchner funnels as described above for disease suppression bioassay and allowed to germinate for 8 h in either sand or vermicompost amended sand. Seeds were then transplanted to sterile sand for an additional 12, 18 or 24 h before being removed. The entire sand matrix of three replicate funnels was then harvested, rinsed with 1 L sterile Nanopure® water, strained through 4 layers of sterile cheesecloth, lyophilized, reconstituted in 15 mL Nanopure® water, sterile filtered to 2 µm with cellulose acetate syringe filters, lyophilized a second time and weighed. The resulting powder was stored at -80°C and reconstituted to 35 X the initial concentration in the full 150 cm^3 sand matrix present in the bioassay apparatus. This reconstitution rate was determined empirically as one that would result in high numbers of zoospores

responding to sand MMSE. Three separate batches of extracts were prepared and used in zoospore assays immediately following reconstitution.

An ethyl acetate fractionation was carried out on 0.2 µL cellulose acetate filtered sand and vermicompost MMSE. MMSE was extracted with two 500 mL portions of ethyl acetate per liter of MMSE sample. The organic layers were combined and dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo*. Residue was transferred to a tared vial with ethyl acetate, dried under a N_2 stream, and vacuum dried to constant weight. The water soluble layer was lyophilized. All samples were stored in -80^{o}C prior to use in zoospore assays.

iii. Zoospore encystment assay

A zoospore suspension was prepared as described above and 100 mL (1.2×10^4 zoospores mL^{-1}) was added to a 15 cm diam glass petri dish. Rubber gaskets (Grace BioLabs, Bend OR) were adhered to microscope slides, filled with 305 µL 0.01% agarose which was allowed to set for 25 minutes. Ten µL of 35x MMSE from each treatment was added to the agarose discs and allowed to dry for 3 minutes. Slides were then immersed in the zoospore suspension and incubated in the dark at room temperature for 30 min. Slides were removed and 4 images were acquired at 10X magnification for each treatment and used for zoospore enumeration (DP25 digital camera with DP2-BSW software, Olympus, USA). A mixture of exudate samples was prepared for an additional assay to determine whether observed differences in zoospore encystment were due to the absence of an attractant or the addition of a repellant/lytic agent in the vermicompost MMSE samples. Samples were reconstituted to create a mixture that consisted of 35 x exudates from seeds germinated in sand combined with

35 x vermicompost MMSE. The fate of zoospore cysts was monitored one hour after initial imaging at higher magnification (304X) to track the occurrence of lysis. Data were analyzed using an ANOVA with a Tukey's test for means separation (Minitab 16, USA).

iv. Zoospore germination assay

Germination rates were calculated for a) pre-encystment and b) post-encystment MMSE exposure. For pre-encystment exposure, 10 µL of the test substance was mixed with 6 mL swimming zoospore suspension for 15 minutes after which suspensions were mechanically encysted via vigorous agitation, and poured into a tissue culture well (Nunc 8 well square tissue culture plates, Thermo Scientific, USA) containing a thin layer of molecular grade low melt agarose and incubated for 1 hour prior to imaging. For post-encystment exposure, 10 µL of the test substance was mixed with 6 mL mechanically encysted zoospore suspension, immediately added to the tissue culture well and incubated for 1 hour prior to imaging. The proportion of germinated cysts (either via germ tubes or secondary zoospores) and germ tube lengths were calculated through image analysis (Olympus DP2-BSW software) for a total of 4 fields of view (~4 mm^2) with a water immersion objective (20X 0.5W Ph2, Zeiss). Germination rates were analyzed using binary logistic regression and Bonferonni's adjustment for multiple comparisons (SAS v.9.3). Germ tube lengths were analyzed using an ANOVA with Tukey's test for multiple comparisons (Minitab 16).

RESULTS:

A. Disease suppression bioassay

Inoculated seedlings in sand had 97% mortality after 7 days (Figure 1, Table 2). Seedlings sown in sand amended with 40% v:v vermicomposted dairy manure had significantly lower seedling mortality at 7 d than seedlings sown in sand with a range of 11 – 20% mortality for three different batches (Figure 1, Table 2). Seedlings sown in sterile vermicompost had a seedling mortality rate of 54% at 7 d which was significantly lower than for seeds sown in sand, but significantly higher than for seeds sown in 2 out of the 3 batches of vermicompost (Figure 1, Table 2). Mortality for non-inoculated seedlings could not be included in the statistical analysis as this measure too completely approached 0% which confounds the logistic regression procedure. The only non-inoculated treatment with any mortality was sterile vermicompost with a mortality of 3%. Seedling health rating was highest for all non-inoculated seedlings in every treatment followed by the vermicompost batches in descending order (1, 3, 2), sterile vermicompost and finally sand with the lowest health rating (Table 1).

B. *In situ* zoospore swimming bioassay

i. Mass flow test

Mechanically encysting *P. aphanidermatum* zoospores limited their ability to cause infection when added to funnels 2 cm away from germinating seeds. A greater number of seedlings survived when seeds were sown 2 cm from the encysted zoospore inoculation point than those sown 2 cm from the swimming zoospore inoculation point (Table 2) and this pattern was reflected in the proportion of seeds colonized with *P.*

aphanidermatum 48 hpi (data not shown). Mechanically encysted zoospores caused 86% seedling mortality when added directly to germinating seeds (Table 2).

ii. Transplant bioassay

To determine when a suppressive microbial community develops on seed surfaces, seeds were pre-germinated in vermicompost for 8, 12 or 24 h. Seeds exposed to vermicompost for 8 h had significantly less Pa58 DNA present at 18 and 24 hpi than seeds sown in sand for 8 hours prior to transplant and inoculation (Table 3). The vermicompost derived seed-colonizing microbial community was associated with significantly lower seedling disease incidence at 9 d (Table 3). The highest level of Pa58 colonization in seeds with the suppressive microbial community is equivalent to 95.3 ug dry mycelial biomass and 70 zoospores per seed. The presence of vermicompost on the seed surface did not appreciably affect the extraction or amplification of DNA and thus did not interfere with our ability to detect Pa58 zoospores on seeds that had been pre-germinated in vermicompost. The standard curve equation for DNA extracted from Pa58 mycelia (Ct = 28.9 + 3.15 log ng DNA, R^2 = 99.7%) and for DNA extracted from seeds sown in vermicompost combined with Pa58 mycelia (Ct = 28.2 + 3.13 log ng DNA, R^2 = 98.9%).

C. Zoospore responses to microbially modified seed exudate (MMSE)

i. Zoospore chemotaxis & encystment assay

Exudates from seeds sown in sand induced chemotaxis and encystment of zoospores, with significantly higher numbers of encysted zoospores present in response to exudates from later time points compared to those from earlier time points (24 h post

transplant > 12 and 18 h post transplant, Table 3). The number of encysted zoospores exposed to microbially modified exudates (MMSE) from seeds sown in vermicompost did not differ from the number of encysted zoospores in water for any of the time points tested (Table 3). Combining exudate from seeds sown in sand with MMSE failed to restore zoospore response (Table 4). Zoospores appeared to lyse when exposed either to seed exudates modified by the 8 h suppressive seed-colonizing microbial community or to a mixture of MMSE and non-modified exudate from sand (Figure 2). A significantly greater number of zoospore cysts lysed when cysts were exposed to vermicompost MMSE than when exposed to water or non-modified exudates from seeds sown in sand (Table 4).

A simple ethyl acetate fractionation of both the sand and vermicompost MMSE significantly impacted zoospore responses. Higher numbers of zoospores swam to and encysted on the organic fraction of sand MMSE compared to the aqueous fraction (Table 5). No differences were observed in zoospore numbers for both fractions of vermicompost MMSE and the water and ethyl acetate controls. The highest percentage of zoospore germination was observed in response to the aqueous fraction of sand MMSE followed by the aqueous fraction of vermicompost MMSE. The germination rate in response to the organic fraction of vermicompost MMSE was significantly lower than the ethyl acetate and water controls. A significantly higher proportion of zoospores lysed in response to the organic fraction compared to the aqueous fraction of vermicompost MMSE. Zoospores exposed to the ethyl acetate fraction of vermicompost MMSE lacked germ tubes and showed signs of membrane disruption (Figure 3). This lysis and consistent lack of germ tube emergence was not observed in the aqueous fraction of

the vermicompost MMSE, either fraction of sand MMSE or in the water or ethyl acetate controls (Figure 3).

iii. Zoospore germination assay

Pre-encystment incubation of zoospores with exudates from seeds sown in sand resulted in a significantly higher germination rate than that observed for zoospores exposed to water or MMSE from seeds sown in vermicompost (Table 3). No difference in germination percentages was observed between cysts exposed to water or to MMSE from seeds sown in vermicompost and no differences were observed among the 12, 18 and 24 h time points. For post-germination exposure, cyst germination rates declined over time for both sand and vermicompost MMSE, however 24 h vermicompost MMSE germination rates were significantly lower than those observed in response to 24 h sand MMSE indicating a treatment effect at this time point (Table 3). No significant differences in germ tube lengths between treatments were observed (data not shown, $p=0.299$).

DISCUSSION

Examining the tripartite interactions among germinating seeds, the seed-colonizing microbial community recruited from a disease-suppressive substrate, and *P. aphanidermatum* zoospores has offered several insights into the nature of vermicompost-mediated disease suppression. First, our results confirm the important role the spermosphere microbial community plays in disease suppression and form part of the growing body of evidence that microbes with the ability to rapidly colonize host surfaces directly modulate the activities of soil pathogens (McKellar and Nelson 2003; Chen and Nelson 2008; Chen et al. 2012). Detection of Pa58 DNA on seed surfaces

using qPCR indicates that zoospores were able to swim approximately 2 cm in sterile sand at -3.5 kPa matric potential and begin to colonize their hosts within 12 hours after inoculation. In contrast, the presence of the 8 hour vermicompost spermosphere microbial community greatly reduced colonization of germinating seeds within a 24 h time period and provided almost complete protection from disease. It is important to note here that the qPCR assay cannot distinguish between viable and non-viable zoospores. It is possible that the low levels of Pa58 DNA detected in the spermosphere of seeds colonized by vermicompost microbes was not an indication of low levels of infection but in fact a reflection of DNA from non-viable or lysed zoospores as those frequently observed in the zoospore attraction and encystment assays. In addition, it is not clear if the documented increase in Pa58 biomass over time on seeds sown in sand is due to the arrival of additional zoospores or to the rapid growth of Pa58 mycelia as it colonized the host, or a combination of the two. However, taken as a whole, the qPCR and transplant bioassay data show that the observed disease suppression in bulk vermicompost (61% reduction in disease incidence compared to sand) appears to be almost entirely due to the 8 hr seed-colonizing microbial community (67% reduction in disease incidence compared to sand). This does not, however, exclude the possibility that microbes in the bulk vermicompost also play a role in the suppression of disease. In fact, microbial community analyses have shown that many of the same bacterial taxa correlated with disease suppression are present in both rhizosphere and bulk soil (Benitez et al. 2007) Instead our results validate focusing on plant-associated microbes as a way to simplify the search for mechanisms of disease suppression.

A second insight from these results is that assessing suppressive microbial communities indirectly via monitoring pathogen response is a viable approach to understanding disease suppressive microbial communities. Since chemotaxis and encystment of *P. aphanidermatum* zoospores occur in direct response to chemical compounds present in the host seed's exudates (Donaldson and Deacon 1993a; Donaldson and Deacon 1993b), we hypothesized that any reduction in chemotaxis and encystment observed with the presence of a suppressive seed colonizing microbial community would be due to these microbes somehow modifying the seed exudates. We found that zoospores did in fact respond differently to exudates collected from seeds colonized by a suppressive microbial community, with fewer encysted zoospores observed in the *in vitro* assays. It's tempting to conclude that the presence of a lower number of encysted zoospores indicates that both chemotaxis and encystment were inhibited by vermicompost MMSE. However, it is possible that zoospores actively swarmed around the vermicompost MMSE test compound, but did not encyst or attach to the agarose and therefore were not present in the final counts for the assay. In addition, zoospores may have actively swum towards and clustered around germinating seeds in the *in situ* assays, but then lysed before encysting leaving their DNA to degrade before it could be detected via qPCR. So we cannot exclude the possibility that the zoospore chemotactic response was not inhibited, however encystment and germination were significantly impacted.

Previously this "pathogen as biosensor" approach has been applied successfully to interactions between zoospores, plant hosts and individual biocontrol agents. For example, exudates collected from cucumber roots colonized with *Pseudomonas* spp.

attracted fewer *P. aphanidermatum* zoospores than exudates from untreated roots in capillary assays (Zhou and Paulitz 1993). In addition, exudates from roots colonized with the arbuscular mycorrhizal fungus *Glomus intraradices* attracted fewer *P. nicotianae* zoospores than water, indicating the presence of a repellant (Lioussanne et al. 2008). HPLC characterization of the control and microbially modified exudates identified isocitric acid and proline as potential zoospore repellants in this system (Lioussanne et al. 2008). We found evidence of a repellant by exposing *P. aphanidermatum* zoospores to combinations of control and microbially modified cucumber seed exudates. Had mixing exudates from both treatments restored zoospore attraction and encystment, this would have provided evidence that an important chemotaxis and/or encystment cue was missing from the MMSE. Instead, zoospore chemotactic and encystment response to the mixture of exudates was no different than that to vermicompost MMSE, indicating the presence of a zoosporocidal toxin or repellant.

Microbially modified root exudates can differentially impact the stages of zoospore pre-infection events. For example, exudates from *Bacillus cereus*-treated tobacco roots, including both antibiotic-producing (zwittermicin A and kanosamine) and antibiotic mutant strains, reduced the number of *Pythium torulosum* zoospores actively swimming towards roots and successfully encysting on roots (Shang et al. 1999). However, only the antibiotic producing strain reduced zoospore cyst germination indicating that multiple routes of interference with zoospore pathogenesis may be present (Shang et al. 1999). Additional evidence for the existence of a dual mechanism is the fact that both antibiotic-producing and antibiotic mutant strains of *Burkholderia*

cepacia incubated *in vitro* with pea seed exudates eliminated attraction for *P. aphanidermatum* zoospores (Heungens and Parke 2000). However only the antibiotic-producing strain caused zoospore lysis, prevented cyst germination and reduced germ tube growth (Heungens and Parke 2000) indicating that *B. cereus* both reduced important attractants and produced a zoosporocidal toxin. We found evidence of this type of dual mechanism as zoospores exposed to the aqueous fraction of vermicompost MMSE after ethyl acetate fractionation responded with a low level of attraction and encystment indicating that attractants may have been degraded during microbial modification of cucumber seed exudates. Germlings exposed to the organic fraction of vermicompost MMSE had significantly lower rates of germination and lysis than those exposed to the aqueous fraction of the same exudate, indicating that the toxin/s or repellant/s may be concentrated in the organic fraction. This provides evidence that the low levels of chemotaxis and encystment in response to the aqueous fraction of vermicompost MMSE are thus due to the absence of an attractant in microbially modified exudates which might be an additional mechanism of pathogen suppression.

Additional evidence for the presence of a zoosporocidal toxin was generated through microscopic observation of germinating zoospores in the zoospore encystment assay. Zoospores exposed to exudates from seeds sown in sand formed germ tubes while a high proportion of those exposed to vermicompost MMSE appeared to lyse during the process of encystment, or if encysted did not germinate. Without an in-depth chemical analysis of vermicompost MMSE it is impossible to know which lytic compound is being produced in the spermosphere of seeds colonized with this suppressive community. The observed lysis is morphologically similar to that of

Phytophthora cactorum zoospores exposed to the zwittermycin- producing biocontrol bacterium *Bacillus cereus* UW85 (Gilbert et al. 1990), *Aphanomyces cochlioides* zoospores exposed to cell free *Lysobacter* sp. SB-K88 culture filtrate or its metabolite xanthobaccin A (Islam et al. 2005), and *Phytophthora capsici* zoospores exposed to the *P. fluorescens* metabolite rhamnolipid B (Kim et al. 2000), indicating that a wide range of compounds could be responsible for lysis in our pathosystem. In all of these cases, lysis is characterized by highly granulated cysts with no visible cell wall whose contents appear to spill into the surrounding medium.

Although a wide range of bacteria could be responsible for the changes in zoospore pre-infection events observed in our study, previous work has documented the presence of compost-derived bacteria (gammaproteobacteria, *Pseudomonas* spp.) known to produce zoospore lytic compounds in the cucumber spermosphere and identified as crucial for the suppression of *Pythium ultimum* (Chen et al. 2012). In addition to lytic activity, vermicompost MMSE reduced the incidence of germination but not germ tube length in mechanically encysted zoospores. Not all compounds known to reduce zoospore encystment are necessarily also lytic (Folman et al. 2004) so it is possible that more than one active compound is present in the spermosphere of seeds colonized with the vermicompost-derived suppressive microbial community. While pre-encystment exposure to vermicompost MMSE did not significantly reduce rates of cyst germination compared to a water control as we initially predicted, this assay was not sensitive enough to document lysed zoospores, which were only observable under 304X magnification with the non-immersion objective. Therefore calculating the rate of germination as: (the number of germinated cysts) / (the total number of cysts), may

have been inaccurate if many more zoospores had initially been present and those that had lysed were not included in the total count. Since the only structures of *Pythium aphanidermatum* susceptible to lysis are those lacking a cell wall, i.e. zoospores, vesicles formed during zoosporogenesis, and zoospore cysts in early stages of development it would be interesting to explore the impact of vermicompost MMSE on additional developmental stages of the pathogen. Based on the observed zoospore lysis we could hypothesize that the plasma membranes of vesicles formed during zoosporogenesis may also be susceptible as has been observed previously for *Phytophthora* spp. (Meyer and Linderman 1986; Norman and Hooker 2000). And based on the observation of non-lytic effects, i.e. reduction of germination after the formation of a cyst cell wall, sporangiogenesis may also be negatively impacted.

While the zoosporocidal compounds produced by the vermicompost derived spermosphere microbial community may be unique to this particular substrate and pathosystem, this study demonstrates the usefulness of our experimental approach in uncovering mechanisms of suppression. The strategy of focusing exclusively on the spermosphere microbial community while using the pathogen's zoospores as biosensors to make inferences about changes in that community could be applied to the investigation of suppression for other soil-borne oomycete pathogens. This approach documented the general mechanism involved in vermicompost-mediated suppression of *Pythium aphanidermatum* on cucumber and provided clear directions for additional investigation, including follow-up chemical analyses of vermicompost MMSE. Given the demonstrated relative simplicity of early seed-colonizing microbial communities; 350 bacterial OTUs (Chen et al. 2012) compared to over 33,000 bacterial OTUs in

rhizosphere soil (Mendes et al. 2011), this microbial habitat is a good candidate for metagenomic analysis in the continued study of disease suppression.

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FIGURES AND TABLES

Figure 1. Representative 7 day old cucumber (*Cucumis sativus* cv. “Marketmore 76”) seedlings from disease suppression bioassays. Surface disinfested cucumber seeds were sown in A) sand amended with vermicomposted dairy manure (40% v:v), B) sterile quartz sand, and C) sand amended with sterile vermicompost (40% v:v). Each group of 10 inoculated seedlings received 6 x 10^5 *Pythium aphanidermatum* zoospores. Matric potential (Ψ_m) was held constant at -3.5 kPa. Scale bar = 5 cm.

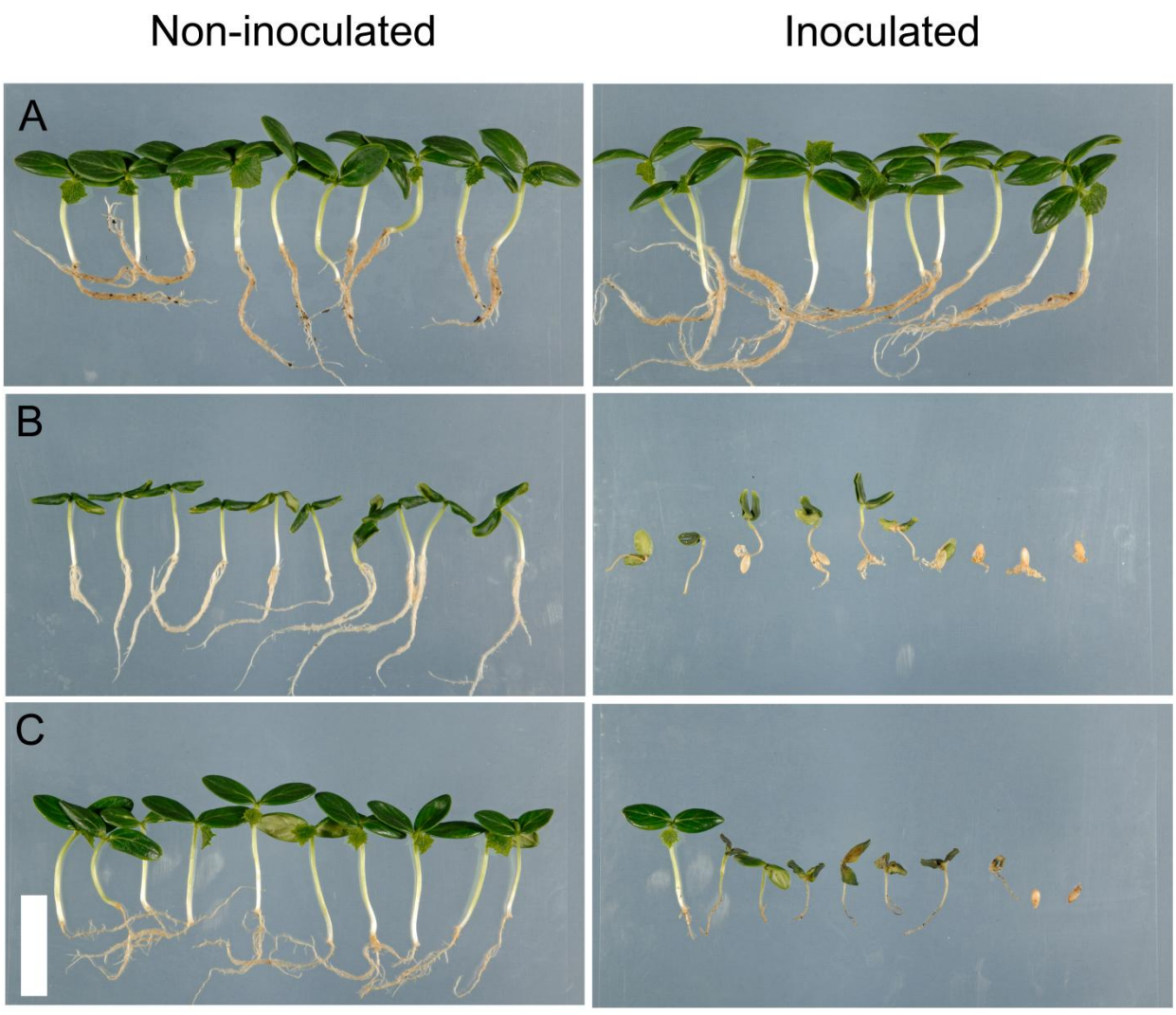

Table 1. Mortality (%) of cucumber seedlings from disease suppression bioassays at 7 d (n=90 seeds for all treatments except for sterile vermicompost n=60 seeds). Seedling mortality means followed by the same letter are not significantly different (binomial logistic regression with Bonferonni's correction for multiple comparisons $p<0.0001$). Health ratings least squared means followed by the same letter are not significantly different (ANOVA with Tukey's correction for multiple comparisons, $p<0.0001$). Health ratings for individual seedlings were designated as follows: 0=dead and completely rotted, 1=damped off but not completely rotted, 2=cotelydon and stem lesions, 3=cotelydon lesions only, 4=stem lesions only, 5=healthy.

Treatment	**Inoculation**	**Seedling mortality (%) at 7 d**	**lsmeans health rating at 7 d**
Vermicompost Batch 1	-	0	5.00 A
	+	11 C	4.38 B
Vermicompost Batch 2	-	0	5.00 A
	+	20 B	4.05 C
Vermicompost Batch 3	-	0	5.00 A
	+	11 C	4.25 BC
Sand	-	0	5.00 A
	+	97 A	0.78 E
Sterile vermicompost	-	3	4.83 AB
	+	28 B	3.35 D

Table 2. Seedling mortality (%) at 8 days. Surface sterilized cucumber seeds were point source inoculated with $6x10^4$ zoospores that were either actively swimming, or had been mechanically encysted. At 48 hpi seedlings were transplanted to sterile sand to avoid contact with secondary inoculum produced from encysted zoospores. Means followed by the same letter are not significantly different (binomial logistic regression with Bonnferoni's correction for multiple comparisons, $p<0.0001$).

Type of inoculum	**Inoculum to seed distance (cm)**	**Mortality (%)**
Swimming	2	96 A
Encysted	2	27 C
Encysted	0	93 B

Table 3. *In vitro* and *in vivo* responses of *Pythium aphanidermatum* zoospores to cucumber seed exudates that have been modified by the seed-colonizing microbial community developed after 8 h of germination in vermicompost. Values followed by the same letter in each column are not significantly different (α=0.05).

Treatment		*in vitro*			*in situ*	
		Chemotaxis/ encystment[a]	Germination [b] (exposure pre-encystment)	Germination[b] (exposure post-encystment)	Arrival/ colonization[c]	Infection[d]
		Average # encysted zoospores per 1 mm^2	Germination rate (%)	Germination rate (%)	Pa DNA pg 10 $seeds^{-1}$	Disease incidence at 9 d (%)
S	12 h	16.58 BC	55 A	63 A	8.49 ABC	98.8 A
	18 h	20.78 B		59 AB	14.43 AB	
	24 h	39.77 A		52 B	16.37 A	
V	12 h	9.74 CD	46 B	55 AB	1.07 C	31.1 B
	18 h	7.07 D		51 B	0.36 C	
	24 h	5.08 D		35 C	0.63 C	
Water		2.26 D	43 B	27 D	na	na

[a] Zoospore chemotaxis and encystment response to seed exudates modified by the 8 hour seed colonizing microbial community derived from vermicompost and harvested 12, 18 or 24 h after colonized seeds were transplanted to sterile sand. Each value is an average of 4 fields of view from 1, 2 and 3 replications respectively for 3 different MMSE batches (n=6, p<0.0001).

[b] Binary logistic regression for: pre-encystment exposure (n=3, p < 0.0001, post-encystment exposure (n=3, p<0.0001). An average of 400 total zoospores from 4 fields of view and 3 replications were used to calculate germination percentages for each treatment – time point combination with a total of over 9,000 individual zoospores scored.

[c] Least squared means of Pa DNA for seeds removed from point source bioassay experiment at 12, 18 and 24 h. Each point is an average of 2 funnels within 3 full repetitions of the qPCR assay (n=6). (treatment p< 0.0001, total treatment*hpi p=0.101, significant individual treatment*hpi interactions all p<0.001).

[d] Seedling disease incidence for seeds sown in vermicompost or sand, transplanted to sand at 8 h, point-source inoculated with $6x10^4$ zoospores, and incubated at a matric potential (Ψ_m) of -3.5 kPa for 9 d with 16 h photoperiod at 27^0C (n=30). (p<0.0001)

Table 4. Zoospore encystment assay results of 24 h MMSE from seeds originally sown in sand and vermicompost and a mixture of the two types of exudates. Means were calculated by counting 4 fields of view for 2, 3 and 3 replicates respectively of 3 different batches of MMSE. Means followed by the same letter in each column are not significantly different (n=8, ANOVA $p<0.0001$). Over 1,000 total zoospores were scored for lysis.

Seed exudate treatments	**Mean encysted zoospores per 1 mm^2**	**Proportion of lysed zoospores (%)**
Sand	31.1 A	15 B
Vermicompost	13.1 B	34 A
Mixture of sand and vermicompost	13.1 B	44 A
Water (no seed)	2.7 C	2 B

Figure 2. Representative *P. aphanidermatum* zoospore germlings exposed to microbially modified seed exudate (MMSE) in the zoospore encystment assay. A) 24 h MMSE from seeds sown in sand, B) 24 h MMSE from seeds sown in vermicompost amended sand, C) a 1:1 mixture of A & B (304X magnification).

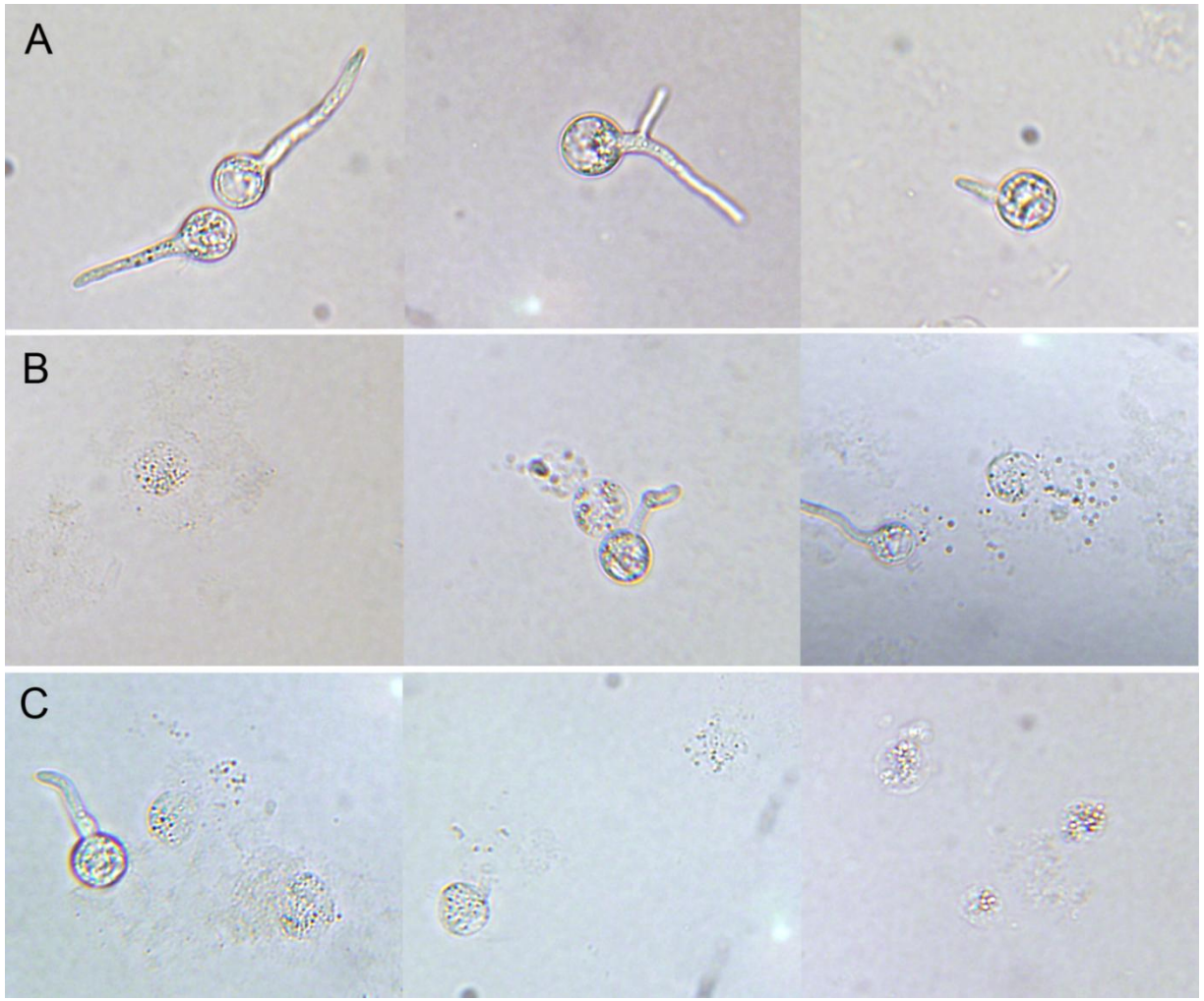

Table 5. Zoospore encystment assay results for different chemical fractions (extracted with ethyl acetate and water) of 24 h MMSE from seeds originally sown in vermicompost or sand for 8 h. Means followed by the same letter in a single column are not significantly different (n=5, ANOVA $p < 0.0001$). Over 1,200 zoospores were scored for germination and lysis (n=5, binary logistic regression $p < 0.0001$).

Seed exudate treatments	lsmeans encysted zoospores per 1 mm^2	Proportion of germinated zoospores (%)	Proportion of lysed zoospores (%)
Sand - organic fraction	30.2 A	66 C	6 B
Sand - aqueous fraction	10.9 B	94 A	5 B
Vermicompost - organic fraction	3.0 C	35 D	16 A
Vermicompost - aqueous fraction	4.6 C	81 B	2 B
EtOAc (no seed)	7.5 C	58 C	6 AB
Water (no seed)	1.6 C	86 AB	6 AB

Figure 3. Representative *Pythium aphanidermatum* zoospore germlings exposed to fractionated microbially modified seed exudate (MMSE) in the zoospore encystment assay. A) EtOAc fraction of sand MMSE, B) aqueous fraction of sand MMSE, C) EtOAc fraction of vermicompost MMSE, D) aqueous fraction of vermicompost (304X magnification).