CHAPTER ONE

INTRODUCTION

INTRODUCTION TO THE SEED-*PYTHIUM ULTIMUM* -*ENTEROBACTER CLOACAE* MODEL SYSTEM

The *Pythiaceae* is a family within the Oomycetes that belong to the Stramenopiles, a very diverse group of eukaryotic microorganisms (2, 115). Much of the notoriety of *Pythiaceae* stems from their aggressiveness as plant pathogens and one such infamous plant pathogen is *Pythium ultimum* Trow that exhibits a world wide distribution and a broad host range. *P. ultimum* causes large losses to a wide variety of crop plants, especially targeting seeds and seedlings (51, 88, 154).

Pythium infections can be effectively controlled by applying the Gram negative bacterium *Enterobacter cloacae* (Jordan) Hormachae & Edwards to below ground plant parts (63, 88, 97, 101, 117, 119, 162, 165, 166). *E. cloacae* belongs to the *Enterobacteriaceae* family which are facultatively anaerobic rods with a world wide distribution. The bacterium is a common component of seed and root associated microbiota and *E. cloacae* is in some cases as efficacious in controlling *Pythium* as commercial pesticides commonly applied to seeds (31, 34, 43, 57, 58, 84, 97, 101, 128, 162, 165). The tri partite interaction between seeds, *P. ultimum* and *E. cloacae* has served as a model biocontrol system and has contributed greatly to our understanding on the ecology of soilborne plant pathogens, microbial inoculants in the spermosphere, and the intricate role that seeds play in driving biocontrol interactions.

Despite the understanding gained from years of research on this biocontrol system, there are still unresolved questions. For instance, some plant species such as cucumber and cotton are particularly supportive of *Enterobacter*-induced *Pythium*

suppression whereas little or no suppression occurs in the spermospheres of other plant species such as corn and pea (63, 101). Seeds play a central part in determining the outcome of the interaction between *Pythium* and *E. cloacae* as they release powerful regulators during germination which modulate *Pythium* pathogenesis and *E. cloacae* expression of biocontrol. In order to understand how seeds influence the biocontrol outcome of *Pythium* and *E. cloacae* interactions in the spermosphere, it is paramount that we understand when and how seed exudates drive *Pythium* disease development before we can answer when and how *E. cloacae* must express its biocontrol activities for *Pythium* suppression to be successful. We will start by examining the general nature and characteristics of seed factors involved in supporting pathogenesis before focusing on the specifics of *Pythium* disease development and control of this pathogen by *E. cloacae*, both of which are regulated in part by seeds.

PYTHIUM BIOLOGY AND PATHOGENESIS IN THE SPERMOSPHERE

Pythium ultimum biology in the spermosphere

Planting seeds exposes the embryonic plant to a dynamic set of soil microbes for the first time and the effects of this initial and continuous interaction can prove to be very damaging for plant health if *Pythium* spp. or other soilborne plant pathogens are present (99). Seeds and seedlings are very susceptible to infections caused by *Pythium* species which are especially adept at responding to these juvenile stages of plant growth. Typical symptoms of *Pythium* infection include seed decay, pre-, and post-emergence damping-off (51, 150, 154).

As seeds are sown they start to rapidly take up water from the surrounding medium (11, 30, 145). Coupled to this water uptake is leakage of seed solutes referred to as exudates, which give rise to the spermosphere (99). Major classes of compounds that have been detected in seed and root exudate includes sugars and their alcohols (5,

6, 18, 50, 64, 65, 82, 95, 109, 129, 132, 133, 138, 141, 142, 147, 153, 161, 163, 167), amino acids (5, 6, 50, 64, 95, 120, 132, 138, 141, 147, 153, 161, 167), aliphatic and aromatic organic acids (6, 18, 64, 65, 71, 75, 161, 167), fatty acids and lipids (81, 92, 136), flavonoids and phenolic compounds (9, 19, 27, 70, 121, 149, 160), volatiles (39, 47, 100, 107, 113, 118, 137), and a number of inorganic nutrients (18, 80)

By releasing primarily carbon and nitrogen rich compounds, the seed greatly influences the volume of substrate surrounding it and the spermosphere has been regarded as analogous to the rhizosphere (129, 142-144). A key difference is that the spermosphere is rapidly established after sowing, but it is dynamic and short lived; for individual seeds it is defined in a strict temporal manner that lasts until the seed has germinated, at which point it is considered a seedling. Furthermore, the nature and composition of root exudates is based on the photosynthetic assimilation of carbon whereas the source of seed exudate components are primarily preformed compounds found in seeds (15, 33, 60, 69, 99). There are several excellent reviews on seed biology and the physiological characteristics that affect water uptake dynamics and exudate release during seed germination which is why these aspects will not be covered in great detail (10, 11, 99).

The spermosphere represents the infection court for *Pythium* as the exudate released by germinating seeds have profound impacts on the ecology of this pathogen (140, 142, 144, 156). *Pythium* has evolved the ability to survive in soil long term in a state of dormancy during which metabolic activity is greatly reduced (78, 154, 158, 169). Survival of *P. ultimum* is primarily realized by resistant survival structures such as oospores and sporangia (51, 154, 157). Saprophytic growth is not considered a survival strategy for *Pythium* spp. since mycelia tend to be short lived and *Pythium* spp. are poor competitors in soil. Only under certain circumstances when the activity of other microbial competitors is greatly reduced due to environmental conditions such

as low oxygen tension due to soil saturation is *Pythium* able to sustain saprophytic growth (51, 88). Because of their role as survival structures, oospores and sporangia of *P. ultimum* are considered to be the propagules responsible for initiating primary infection and both have been found to be viable for up to 11-12 months in soil (83, 158).

Oospores are considered constitutively dormant structures meaning that even if the environment is conducive for germination, only approximately 10% or less will germinate (154). The nature of this dormancy is not well understood. Sporangia on the other hand are exogenously dormant in field soil because of unfavorable environmental conditions, also called fungistasis (78, 140). This dormancy is broken by seed exudates released from appropriate hosts upon sowing. *P. ultimum* sporangia are able to germinate directly to resume active growth and tend to be attracted to sites of increased exudation, and there exists a correlation between increased exudation and greater disease (49, 50, 90, 138, 144, 147, 148). Once sporangia are committed to germinating they depend on exogenous nutrients being available. If the source of substrate is insufficient to support further growth the germlings are subjected to rapid microbial lysis (154). However, it has been reported that *P. ultimum* has survival mechanisms which allow survival of germinated sporangia (157). When nutrient levels are low germling growth ceases, and the protoplasm can retract from the tip of the germtube, laying down septa as it moves into the parent sporangium which is then capable of re germinating.

Although not considered a primary initiator of disease, the importance of mycelial growth lies in the ability of *P. ultimum* to rapidly grow towards the host once sporangia and oospores have germinated, and prolific mycelial growth is beneficial for host colonization and secondary spread (41, 42). In fact, *P. ultimum* hyphae can grow up to 1 mm/hr on suitable nutrients (42, 156). Simple as well as complex sources of

carbon commonly found in seed exudates are capable of supporting ample mycelial growth of *P. ultimum in vitro* (42, 53). When applied as a sole carbon source, glucose, fructose, sucrose, maltose, mannose, dextrin, cellobiose, and starch greatly increase *P. ultimum* biomass (5, 36, 53, 94). Vegetable oils as well as sterols stimulate prolific mycelial growth of *Pythium*, even more so than simple sugars (52, 54, 55).

Apparently, *P. ultimum* does not have any vitamin requirements, and although most phytopathogenic *Pythium* spp. prefer complex nitrogenous sources such as casein hydrolysates, and peptone, they are also able to grow using inorganic nitrogen as a nitrogen source (4, 5, 72, 126). The issue with interpreting the value of various substrates for supporting *P. ultimum* mycelia growth is that most are tested as sole carbon sources. Therefore, when presented with a complex nutrition source such as seeds and their exudates, it is difficult to predict which substrates will be important in supporting seed colonization by *Pythium*.

Seed exudates are essential both for initiating *Pythium* pathogenesis as well as sustaining further disease development, since both sporangial germination and host colonization have an absolute requirement for exudates. If exudates are removed by pre-germinating seeds or through microbial consumption then *Pythium* sporangial germination and host colonization becomes greatly reduced and disease fails to develop, demonstrating that seed exudates are critical for pathogenic development (43, 50, 110, 111, 144, 162). Furthermore, it establishes that sporangial germination and seed colonization are essential stages in pathogenesis and if they are interrupted the pathogen will be unable to cause disease.

Any parameter that affects seed exudation will also influence *Pythium* disease development, as changes in exudation dynamics impact the pathogen's physiology and development. Additionally, fluctuations in seed exudation will also impact the spermosphere size. An important spatial aspect of the spermosphere is heterogeneity.

Seeds do not exudate in a uniform manner across the seed surface because seed water uptake is not even through the seed coat (120, 138, 144). For instance, broad beans (*Vicia faba*) release more exudate through the micropylar region whereas peas (*Pisum sativum*) have increased exudate release through the site of radicle emergence and pathogen activity is concomitantly increased at these sites (120, 144). The spermosphere size is critical to many soilborne pathogens, not just *Pythium*, with larger spermospheres giving rise to greater levels of infection (140).

It is difficult to quantify the size of the spermosphere, especially considering the significant heterogeneity over the surface of a single seed as well as the differences between seeds. Nevertheless, most of the attempts at measuring the spermosphere size have utilized pathogenic behavior as a marker for delineating the borders of this microbiologically active site. These are best exemplified with *Fusarium solani* chlamydospores and *P. ultimum* sporangia. Although the latter were shown to germinate more when closer to bean seeds, the spermosphere size was estimated to be extending as far as 8-12 mm from the surface of the seed, depending on soil moisture (156). Using *F. solani* chlamydospore germination as an indicator, Short and Lacy (1974) showed that the pea spermosphere extended 3-7 mm away from the seed and that size depended on cultivar used, moisture level and temperature (144).

There are a number of both intrinsic and extrinsic factors that affect the quality and quantity of exudates released by seeds. The most obvious intrinsic factor that affects exudation is plant species and cultivar. The amount of exudate that is released by different plant species is related to the total seed weight, where heavier (larger) seeds with more storage material release more exudate than lighter (smaller) ones (63, 137, 167). Cultivar differences that may contribute to differential exudation and thus disease susceptibility are seed coat color, structural features and integrity (138, 151, 152). Extrinsic factors that greatly influence seed exudation and accordingly impacts

Pythium disease development are primarily soil moisture and temperature (39, 47, 50, 100, 107, 138, 141, 147, 156, 161). Differences in exudation due to factors discussed above have been detected specifically for amino acids (50, 85, 124, 138, 141, 147, 161, 167), sugars (50, 85, 124, 138, 141, 142, 147, 161, 167), organic acids (71, 81, 161, 167), flavonoids and phenolics (9, 121), and volatiles (39, 47, 100, 137). At this point we can only speculate about the importance of differential exudation of specific components to *Pythium* pathogenesis and disease development, as the only concrete association between changes in exudation and *Pythium* disease is that there is a relationship between increased exudation and disease susceptibility.

Temporal dynamics of Pythium ultimum pathogenesis in the spermosphere

Given the inherent characteristics of the spermosphere, especially the speed by which its formation is initiated, *Pythium* has to be extremely adept at responding quickly to environmental stimuli that signal conditions permissive for infection (3, 47, 98, 139, 140, 144, 155). Perhaps the speed of the response is also an evolved trait that compensates for *Pythium*'s lack of direct competitiveness with other soil organisms under non-pathogenic conditions. Characteristic of *P. ultimum* is its rapid response of pre-infection stages such as sporangia activation and germination to seeds and roots followed by equally fast tropic responses, colonization, and ultimately plant infection and death. The extremely short response time of various stages in *Pythium* pathogenesis indicates that there is an important temporal dynamic to seed infection.

It is widely recognized that seeds are at risk from *P. ultimum* infection very soon after planting, usually within the first 12-24 hr (99). Our current understanding is that sporangia of *P. ultimum* germinate directly in the spermosphere within 1-1.5 hr with maximum germination occurring 3-4 hr after exposure to seeds (48, 57, 63, 77, 91, 104, 156). Studies on the exudate release dynamics of seeds demonstrate that the major peak in exudation occurs within the first hr after sowing with a secondary,

smaller peak around 6 - 8 hrs, with the bulk of exudate release being completed within 12 hrs (142, 145). The exudate release detected within the primary peak is principally due to exudate leakage occurring within the first 5-10 min and this initial peak is clearly critical for stimulating sporangial germination (122, 145). Because there is an unknown lag time between sporangia host perception and the appearance of a sporangial germ tube, it is not currently known which temporal fractions of seed exudates signal sporangia to germinate or if this temporal dynamic differs between plant species.

Once sporangia have germinated, an equally rapid germ tube growth at a rate of up to 300 μm/hr takes place (42, 156). Following this aggressive trophic growth towards its host, *P. ultimum* may colonize seeds as early as 2 hr after planting, with nearly 100% seed colonization occurring within 12-24 hr of planting (91). While the timing of embryo infection by *P. ultimum* is unknown for many plant species, complete infection of some species can take place within 48 hr (35, 59, 159). This swift response to and infection of seeds is one of the more conspicuous hallmarks of *Pythium* damping-off diseases. This results from the quick release of seed exudates containing biochemical cues that trigger a developmental change in *Pythium* species that allow them to resume active growth and initiate plant infection, as they are usually present in soil as dormant propagules.

A critical aspect of this temporal development is that it provides an important reporting system on the presence of seed exudate molecules that elicit rapid developmental responses common to *Pythium* diseases. The manifestation of *Pythium* developmental responses allows us to identify when seeds release these molecules. Such pre-infection events represent critical stages in pathogenesis and, if altered, greatly affect subsequent disease development (43, 110, 111, 142). Despite their importance, however, temporal responses of pathogens to seeds or roots are rarely

studied within an ecologically meaningful time-frame and there are few examples of temporal responses reported in the literature. Moreover, many studies on seed exudation and its effect on disease development seldom take the temporal dynamic of *Pythium* behavior into account when sampling exudate. Consequently, data on nature of seed exudates and their temporal release dynamics during pathogenic development of *Pythium* in the spermosphere is more or less non existent.

Plant derived fatty acids elicitors of Pythium sporangial germination

Because of the rapid germination response of *P. ultimum* sporangia to plants, there has been much interest in determining the factors that trigger their germination. Although much of the early literature pointed to sugars, amino acids, and volatiles as the primary exudate components responsible for stimulating sporangium germination and initiating *Pythium*-seed interactions in soil (20, 38, 72, 73, 98, 103, 118, 119), it is now clear that these observations were based on artificial *Pythium* responses, as it has since been demonstrated that media on which sporangia are reared determines subsequent germination response (104, 105). Sporangia produced on living plant tissue fail to germinate in response to sugars, amino acids, and other organic acids, but remain responsive to unfractionated seed exudates.

When fractionating cotton seed exudates it was found that unsaturated long-chain fatty acids (ULCFA) present in seed exudates serve as the primary elicitors of *P. ultimum* sporangium germination (136). Linoleic (C18:2) and oleic acid (C18:1) are the most common fatty acids in seeds and constitute more than 50% of the fatty acid pool in seed lipids from many plant species (29, 62, 89). Both linoleic and oleic acid are among the most abundant seed and root exudate fatty acids, which are also the two most important sporangium germination elicitors (81, 114, 136). Direct relationships exist between ULCFA concentrations and *P. ultimum* sporangium germination where increasing the dose of ULCFA results in increase germination level (136, 165). Any

reduction or removal of these elicitors results not only in reduced germination of *P. ultimum* sporangia but subsequently, reduced infection of seeds (57, 63, 91, 165). Correspondingly, removal of these elicitors by pre-germinating seeds, dramatically reduces sporangium germination and seed infection (99). Although ULCFA have proved to be very active germination stimulants in cotton seed exudates it is not yet known if and at what concentrations they are released by other seeds during times that correspond with sporangial germination of *P. ultimum*.

While the molecular nature of fatty acid induced germination has eluded discovery there are some interesting observations regarding lipid acquisition in *Pythium* and *Phytophthora* and the central role of plant derived fatty acids and lipids in spore germination and development of eukaryotic microorganisms (1, 46, 52-55, 114). For instance, *Thielaviopsis basicola* endoconidia and chlamydospores only germinate in the rhizosphere or in soil amended with fatty acid and lipid extracts from bean rhizospheres (114). In contrast to true fungi that are capable of producing their own sterols, *Pythium* and *Phytophthora* must rely on exogenous sterols for their development (52, 54, 55). Production of oospores and zoospores requires a supply of sterols and oomycetes have developed elicitins such as cryptogein, which are sterol binding and carrying proteins, for the specific purpose of securing sterols (76, 93). Therefore, an important goal for *Pythium* must almost certainly be to secure access to sterols during the infection process in order to assure proper development of survival propagules that serve as primary inoculum for initiating a new disease cycle. These elicitins are so critical to *Pythium* and *Phytophthora* spp. that plants have learned to recognize these molecules as pathogen specific proteins that induce defense responses (40, 59, 66, 67, 125). Furthermore, these elicitins are also capable of binding free fatty acids such as linoleic acid (112).

ENTEROBACTER CLOACAE CONTROL OF *PYTHIUM* INFECTION

E. cloacae distribution and proliferation in the spermosphere

E. cloacae has proved to be very effective in protecting seeds against *Pythium* infection on a number of different plant species when the bacterium is applied to seeds and roots (43, 63, 97, 101, 165, 166). The ability of *E. cloacae* to exploit exudate resources for supporting its growth and activity in the spermosphere have been considered factors contributing to the success of the bacterium in controlling *Pythium* diseases. Judging from the rapid disease development of *Pythium* in the spermosphere, *E. cloacae* must be active very soon after sowing in order to be the successful biocontrol agent that it is.

E. cloacae attaches to seed coats quickly after planting seeds with maximum attachment occurring within 3-5 hr (57). Even though *E. cloacae* attaches over the entire surface of the seed coat, cells exhibit a preference for groves and cracks on the coat as these are sites of increased exudation on the seed coat (138). Attachment of *E. cloacae* is facilitated by exudate, explaining the spatial preference as well as the increased number of cells found on whole seeds compared to excised seed coats (57). The effective colonization ability of *E. cloacae* is confined to seeds as the bacterium does not colonize the developing roots to a large extent. When seeds treated with *E. cloacae* were planted in non sterile soil, only the upper 25% of the root was colonized with 90% of the total surface area colonized by the bacterium was within the first 2 cm from the seed (101, 130).

Carbohydrates and amino acids released from germinating seeds are important substrates that encourage spermosphere colonization by *E. cloacae* (132, 135). Exudate substrates and their role in sustaining *E. cloacae* growth and proliferation in the spermosphere are best understood for carbohydrates. *In vitro* growth of *E. cloacae* is supported by a variety of mono- and oligo-saccharides that are constituents of seed

storage carbohydrates and commonly released by germinating seeds (129, 134, 135). The significant role of exudate carbohydrates in supporting colonization by *E. cloacae* is best exemplified by bacterial strains with defective carbohydrate metabolism. The mutant strain A-11 with an impaired phosphofructokinase gene (*pfkA*) was unable to grow on a number of different seed exudate sugars (arabinose, galactose, glucose, maltose, ribose, and sucrose), but could grow on fructose, glycerol, amino acids, and organic acids (16, 133). A-11 proliferation was greatly reduced in the cucumber and radish spermospheres, compared to the wildtype strain. However, proliferation in pea, soybean, sunflower, and corn spermospheres was not impacted within 24-45 hr of sowing (16, 133, 135). The *pfkA* mutation has the greatest impact on *E. cloacae* growth rate in the spermospheres of seeds such as cucumber and radish because they released low quantities of carbon sources that supported growth of A-11 (eg. fructose and amino acids) (16). Mutants are less affected in the spermospheres of pea, soybean, sunflower, and corn whose seeds release relatively high levels of carbohydrates, particularly fructose (up to 4000-fold) and amino acids, during the first 24-96 hr of seed germination. Such high levels of fructose support the growth of *pfkA* mutants at wild-type levels. For example, adding fructose to cucumber and radish seeds at quantities similar to those released from pea seeds over a 96 hr period, resulted in spermosphere populations of the *pfkA* mutant equivalent to wild-type levels. Furthermore, complementation of the *pfkA* mutation with a homolog cloned from *E. cloacae* 501R3 restored the nutritional phenotype as well as spermosphere colonization to near wild-type levels (133). Other catabolic mutants have been described that show similar reductions in spermosphere colonization (131). Most likely these catabolic genes and pathways play key roles in the competitiveness of *E. cloacae* in the spermosphere.

While the role of carbohydrate metabolism in spermosphere colonization and biological control is readily apparent, the role of exudate amino acids in affecting these activities in *E. cloacae* has not been elucidated. *E. cloacae* is able to grow *in vitro* and in soil on several amino acids commonly found in seed exudates (127, 132). Mutants auxotrophic for seven different seed exudate amino acids were reduced in their ability to proliferate in the spermosphere of corn, cucumber, and pea. This reduced colonization could be rescued in some mutants by applying casamino acids along with the bacteria to the spermosphere (127).

Fatty acid degradation as a control mechanism of E. cloacae

E. cloacae significantly reduces *Pythium* pre-emergence damping-off of carrot, cotton, cucumber, lettuce, radish, sunflower, tomato, and wheat (63, 97, 101, 166) despite the fact that the bacterium does not produce antibiotics, a common mechanism of control for *Pseudomonas*, *Bacillus*, and *Gliocladium* spp (44, 88). Also, *E. cloacae* does not parasitize *Pythium* hyphae, induce systemic resistance, nor does it produce siderophores, toxins, cell-wall degrading enzymes, or other *Pythium* inhibitory substances in the spermosphere that would explain its seed protective abilities (58, 79, 102, 106, 166). Yet, *E. cloacae* can rapidly reduce the colonization of seeds and subsequent seed infection by *P. ultimum* (57, 97, 98, 101).

The only biocontrol mechanism of *E. cloacae* that has not been excluded and which has also been supported by experimental evidence is competition. Although competition for nutrients has been suggested as mode of action for other biocontrol agents, it is best understood under iron limiting conditions where siderophore producing *Pseudomonas* spp are able to outcompete pathogens such as *Pythium* and *Fusarium* for iron (17, 28, 31, 32, 61, 165). *E. cloacae* serve as the only example where competition for carbon substrates in the spermosphere has been demonstrated.

UCLFA's would be logical targets for substrate competition, knowing their role as inducers of *P. ultimum* sporangial germination (136). It has been noted that *E. cloacae* is particularly effective in taking up and metabolizing plant derived oleic acid, a common ULCFA found in seed exudates that stimulates sporangium germination (68, 136). More direct evidence for fatty acid competition as a control mechanism of *E. cloacae* comes from studies with cotton in which mutants of *E. cloacae* that were unable to transport or degrade fatty acids (*fadL*Δ, *fadAB*Δ) were generated (165). Both mutants were incapable of metabolizing fatty acids as well as degrading germination stimulants in cotton exudate collected for 4 hr. The most crucial phenotype of the mutants was that they were unable to protect plants from *Pythium* incited damping-off (165). Bacterial fatty acid degradation, degradation of germination stimulants and control of *Pythium* infection were all restored when complementing the mutants with functional copies of the *fad* genes. Whether or not *E. cloacae* operates by the same biocontrol mechanism in the spermosphere of other plant species remains to be established. However, it is clear that *E. cloacae* fatty acid degradation is a critical trait for suppressing *Pythium* infection in the cotton spermosphere.

At this juncture we lack insight into when during *Pythium* pathogenesis in the spermosphere *E. cloacae* expresses activities that are related to controlling *Pythium* and if these activities are required during specific disease developmental stages in order for plant protection to be successful. The fact that *E. cloacae* fatty acid uptake and degradation are critical biocontrol traits in the cotton spermosphere in conjunction with the role of ULCFA as sporangial germination elicitors suggests that *E. cloacae* primarily targets the sporangial germination stage in *Pythium* pathogenesis, even though we can not exclude *E. cloacae* affecting subsequent disease stages (136, 165). Furthermore, several studies focusing on exudate collected *in vitro* point out that one key to the success of *E. cloacae* lies in its ability to rapidly degrade germination

stimulants in the seed exudates of a number of different plant species, thereby rendering the exudate unable to induce sporangia germination (63, 96, 166).

Seed exudate components with potential to regulate plant species specificity of E. cloacae protection

The seed itself holds a central role in regulating the disease outcome in the interaction between *P. ultimum* and *E. cloacae* (63, 101). Although *E. cloacae* is effective in suppressing *P. ultimum* development and infection in the spermosphere of many plant species there are others such as corn and pea seeds that are not protected (45, 63, 102). The difference between seeds that are protected by *E. cloacae* versus the ones that are not protected, lie in the exudates they release and whether not the bacterium quickly metabolizes the germination stimulatory activity of it. For example, *E. cloacae* is able to degrade sporangia germination stimulants in 2 hr exudate from carrot, cotton, cucumber, lettuce, radish, sunflower, and wheat but not from corn or pea exudate (63). *E. cloacae* is also unable to suppress *Pythium* damping-off of corn and pea, whereas the other seeds are well protected. Yet again, due to the experimental approach it is impossible to determine whether this association between degrading germination stimulants in seed exudate and the ability to protect seeds is due to a true cause and effect relationship or results from indirect effects. In other words, the data suggests that the bacterium interferes with sporangial germination in the spermosphere of seeds that are protected by *E. cloacae* and that this is what is causing disease reductions. However, there is no conclusive link since it can not be excluded that *E. cloacae* is also able to interfere with subsequent disease stages such as seed colonization.

An interesting relationship that has been observed for seeds that are differentially protected by *E. cloacae* is that they have extremely different rates and levels of sugar release during germination. The seeds that release low levels of

carbohydrates during the first 24 hrs of germination, such as cucumber, radish and sunflower are protected by *E. cloacae* (129, 133). In terms of total quantity, corn and pea seeds release 1700-3000 times more sugar than cucumber and 300-500 times more than radish seeds during the first 24 hrs of seed germination. Corn and pea release up to 4000 times more fructose during the first 96 hrs after sowing than cucumber and radish seeds. In general, the former seeds released several orders of magnitude more of most sugars than cucumber and radish. Fructose, glucose and sucrose were the predominant sugars found in exudate from corn and pea and these sugars are also able to completely disrupt *E. cloacae* suppression of *Pythium* damping-off when coated onto cucumber seeds (101).

Assuming that *E. cloacae* fatty acid degradation is the general biocontrol mechanism operating in the spermosphere, then the association between increased sugar levels in exudate and diminishing suppressive capacity by the bacterium makes sense. Fatty acid uptake and catabolism in *Escherichia coli*, a close relative to *E. cloacae*, is under strict transcriptional control and both exogenous long-chain fatty acids (LCFA) and simple sugars like glucose are involved in this regulation.

Degradation of LCFA is accomplished through the action of a number of different proteins encoded for by the *fad* regulon (22, 24, 74). Uptake of LCFA is facilitated through the action of two proteins, FadL a transporter, and FadD, an acyl-CoA synthetase, which has been extensively reviewed (8, 13, 23). FadL is a outer membrane protein with a high affinity for LCFA and it has been proposed that it binds LCFA which then diffuses into a fatty acid binding pocket that is extracellularly located and this induces a conformational change of the protein that facilitates transport across the outer membrane (12, 87, 164). To date there has been no satisfactory explanation for how fatty acids get transported across the periplasmic space, although it has been suggested that there may be a protein that acts as a H^+ and

fatty acid co-transporter (7, 8). Uptake of fatty acids is greatly increased when cells as well as membranes are energized (8, 86). This is partly due to the process of vectorial acylation, where acyl CoA synthetase that is associated with the inner membrane binds and activates fatty acids to their fatty acyl-CoA form, a process that requires ATP (13, 14, 23, 168). The activation of LCFA's contributes to their unidirectional transport into the cell. Even though all fatty acid substrates must be activated by acyl CoA synthetase before subsequent catabolism through the β-oxidation, only LCFA uptake is mediated through an active transport system by FadL (23, 24, 86). β-oxidation is realized by the activity of 6 enzymes of which 5 are located in a multienzyme complex organized in a $\alpha_2\beta_2$ heterotetrameric structure and these enzymes are encoded for by the *fadAB* operon. The gene *fadE* which is also part of the *fad* regulon, encodes the 6^{th} protein required for fatty acid degradation.

LCFA transport, activation and degradation are under strict transcriptional regulation because LCFA are viewed as less attractive carbon sources for sustaining bacterial growth and metabolism. The repressor protein FadR has a central role in regulating both fatty acid degradation and biosynthesis (25, 108, 146). If there is exogenous LCFA present then the activated form (fatty acyl-CoA) binds to FadR to relieve repression of *fadD*, *fadL*, *fadAB*, and *fadE* (26, 123). The *fad* regulon is thus induced by LCFA, whereas *fabA* which is involved in synthesizing fatty acids is activated by FadR in the absence of LCFA. FadR therefore has a unique role as a repressor and activator in the global control of fatty acid metabolism in cells. Part of the *fad* regulon is also governed by catabolite repression and is under additional transcriptional control through the cAMP receptor protein (CRP) in a fashion analogous to the glucose-lactose diauxie (37). *fadAB*, *fadD*, and *fadE* expression is greatly activated by CRP which only occurs when glucose or other simple sugars are not available (21, 116). Even under conditions where LCFA is present in growth

medium, the presence of glucose will take precedent in controlling *fadAB* and *fadD* transcription, thereby keeping fatty acid degradation and vectorial acylation shut down. Additionally, under conditions of high osmolarity expression of *fadL* is repressed by the protein OmpR (56). Because of the inducible nature of the genes contained in the *fad* regulon there is a lagtime before expression is fully upregulated in response to exogenous LCFA.

Fructose, glucose, and sucrose are the predominant sugars found in corn and pea seed exudate in amounts that correspond up to 200 mM during the first 96 hrs of seed germination if assuming that the spermosphere extends 10 mm from the seed surface (129). These three sugars directly impact *E. cloacae* fatty acid degradation and either sugar in increasing concentrations between 1-4 mM leads to a concomitant decrease in linoleic acid degradation and as a result increasing levels of *Pythium* sporangial germination (Windstam and Nelson, *unpublished*). Concentrations above 5 mM were enough to shut down linoleic acid degradation all together. Spiking 4 hr cotton seed exudate with increasing amounts of glucose produced a similar result, where the bacterium was unable to degrade germination stimulants in the presence of increasing glucose levels, resulting in increased sporangia germination.

Clearly sugar repression could be responsible for the differential control of *Pythium* infection observed by *E.cloacae* but there are still many unresolved questions before this conclusion may be stated with a degree of confidence. For example, data is lacking on the amounts of sugars that are released by different seeds in a temporal fashion that corresponds to *E. cloacae* control of *Pythium*. Considering that the bulk of exudate release occurs during the hours immediately following sowing, it stands to reason that the majority of the sugars detected by Roberts et al (1999, 2000) within 24 hrs of planting seeds should be from seed exudate within the first few hours (129, 133). Furthermore, ULCFA availability during the first hours of sowing is completely

unknown and it is plausible that seeds that do not support *E. cloacae* suppression of *Pythium* release an exorbitant amount of ULCFA that exceeds the metabolic capacity of *E. cloacae*, thus allowing sporangia germination elicitors to escape degradation.

The current model explaining the differential disease outcome of the tri partite interaction between seeds, *Pythium* and *Enterobacter* for corn and cucumber can be seen in figure 1. It is assumed that both corn and cucumber seeds release ULCFA since *Pythium* sporangia are stimulated to germinate in response to exudate from both seeds (63). As less corn seed exudate is required for eliciting the same germination rate as cucumber it is believed that corn seeds release more ULCFA germination stimulants in their exudate. If sporangia are present in the corn and cucumber spermosphere, germination will take place, followed by seed colonization and plant disease. Although there are differences in the exudates released with respect to sugars and amino acids, it is unknown what effect these compounds have on sporangial germination, colonization and infection. If *E. cloacae* is also present in the spermosphere, disease will not develop on cucumber seeds, but corn will succumb to *Pythium* infection. In the cucumber spermosphere *E. cloacae* is believed to degrade ULCFA germination elictors and thus interrupt sporangial germination and subsequent disease. In the corn spermosphere the bacterium is unable to prevent sporangial germination which could be due either to sugars repressing fatty acid degradation or fatty acids being released at such quantities that *E. cloacae* simply can not successfully degrade enough to negate sporangial germination, or a combination of these two.

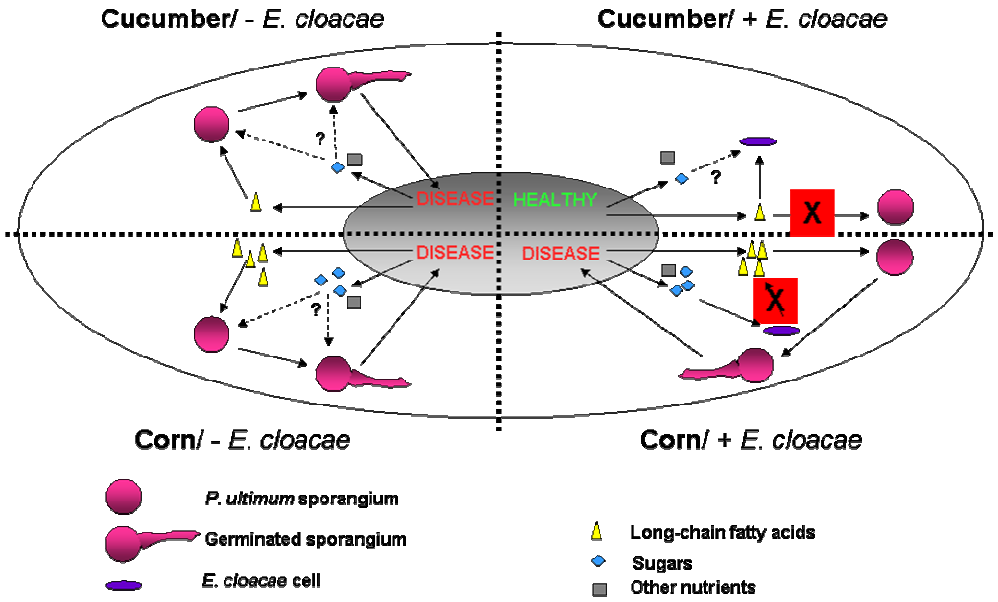

Figure 1. Current model of the tri-partite interactions in the spermosphere between seeds, *P. ultimum*, and *E. cloacae*. The solid line denotes the spermosphere and interactions to the left is between the seed and *P. ultimum* in the corn and cucumber spermosphere. Both seeds are able to induce sporangia germination and are consequently susceptible to *Pythium* infection. Because corn seed exudate is more stimulatory to sporangia germination than cucumber seed exudate it is believed that corn seeds release more germination stimulants in the form of ULCFA (63). To the right is depicted the interaction between seeds and *P. ultimum* when *E. cloacae* is also present. The differential disease control observed on corn vs cucumber is believed to be due to corn seeds either releasing quantities of ULCFA that exceeds *E. cloacae* degradative capacity or release of sugars that are known to repress LCFA degradation.

STATEMENT OF PROBLEM AND HYPOTHESES

There is much circumstantial evidence suggesting a role for seed exudate UCLFA and sugars as important regulators of the interaction between *P. ultimum* and *E. cloacae* in the spermosphere. Unresolved questions in this important model system for biological control relate to the chemistry of seed exudate and the temporal development of *Pythium* and biocontrol expression of *E. cloacae*. The main aim of this dissertation is to resolve some of these parameters and how they explain the differential biocontrol of *Pythium* infection. Corn and cucumber seeds were chosen as the seeds that experiments were based on, as they are both susceptible to *Pythium* infection but *E. cloacae* displays differential control of seed infection on these seeds. Based on this problem and current background information that is available, the following hypotheses have been formulated:

1. Corn and cucumber seeds release UCLFA at times that correspond to temporal sporangia germination
2. *E. cloacae* is able to reduce sporangial germination in the cucumber but not the corn spermosphere and this explains the differential suppression of *Pythium* infection
3. Corn seeds release sugars at much higher quantities than cucumber, and these repress transport and degradation of UCLFA, alternatively
4. Corn seeds release UCLFA at much higher quantities than cucumber and it exceeds the metabolic capacity of *E. cloacae* or is released at times when *E. cloacae* is not active

RESEARCH OBJECTIVES

(i) Describe the temporal disease development of *P. ultimum* in the corn and cucumber spermosphere

(ii) Evaluate the ability of *E. cloacae* to express its biocontrol activity during disease development of *Pythium*, and assess the contribution of bacterial fatty acid metabolism to *E. cloacae* activities

(iii) Determine the temporal release of UCLFA and sugars from corn and cucumber seeds during *P. ultimum* disease development and *E. cloacae* biocontrol

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CHAPTER 2

DIFFERENTIAL INTERFERENCE WITH *PYTHIUM ULTIMUM* SPORANGIUM ACTIVATION AND GERMINATION BY *ENTEROBACTER CLOACAE* IN THE CORN AND CUCUMBER SPERMOSPHERE

ABSTRACT

This study was conducted to better understand how *Enterobacter cloacae* prevents seed infections by *Pythium ultimum* on some plant species but not others. We examined how the activity of *E. cloacae* on two differentially protected plant species, corn and cucumber, affected very early interactions between *P. ultimum* and the seed. Exudates released by cucumber and corn seeds within 30 and 15 min after sowing were sufficient to induce significant *P. ultimum* sporangial activation. Maximum levels of activation occurred 1.5 to 3 hrs and 30 min, respectively, after exposure to the seed. Subsequent germ tube emergence occurred between 2 to 2.5 and 1 to 1.5 hrs with maximum frequency reached 5 to 6 hrs and 4 hrs after exposure to cucumber and corn seeds, respectively. In the presence of a wild type strain of *E. cloacae*, sporangial activation in the cucumber spermosphere was reduced from ~60% to ~15%. No such reduction was observed in the corn spermosphere. In the presence of either a fatty acid transport (*fadL*) or fatty acid degradation (*fadAB*) mutant of *E. cloacae*, no reductions in sporangia activation in the cucumber spermosphere were observed. Decreased sporangial activation in the cucumber spermosphere correlated with only small reductions in seed colonization incidence by *Pythium*, but consistently suppressed further *Pythium* biomass development. *E. cloacae* did not affect *Pythium* seed colonization incidence nor biomass developing on corn. The reductions in *P. ultimum* biomass on cucumber seeds treated with *E. cloacae* was found to be directly due to the

ability of the bacterium to interrupt *Pythium* sporangial activation. These observations reveal that the components of seed exudate stimulating activation of *Pythium* sporangia are released very early during seed germination, and these exudate fractions are powerful regulators of early interactions between *E. cloacae* and *P. ultimum.* Additionally, interactions between *E. cloacae* and the seed directly after sowing that result in disrupting early *P. ultimum* pathogenic developmental stages directly relate to reduced pathogen biomass.

INTRODUCTION

The Oomycete pathogen, *Pythium ultimum*, is notorious for rapid pathogenic development in response to germinating seeds (11). Sporangia, together with oospores, serve as dormant survival stages, each requiring seed exudates to break dormancy (10). Sporangia can transition from quiescence to active growth in as little as 1.5 to 2 hrs after exposure to a seed (8, 18); maximum germination occurs as early as 3 to 4 hrs after exposure to seeds. Exudates from many plant species can induce these rapid responses (6, 20). Although the specific exudate elicitors from most plants are unknown, the elicitors from cotton seed exudates are known to be unsaturated long chain fatty acids (ULCFA) (10, 11, 17). Other exudate molecules, such as saturated fatty acids (SFA), sugars, amino acids, and organic acids do not elicit sporangium germination (13, 14, 17).

Once sporangia have germinated, high levels of seed colonization and subsequent infection is observed within 24 to 48 hrs, resulting in seed and seedling death (3, 4, 9, 10, 22). However, if exudate stimulants that are released within 2 to 4 hrs of sowing are removed by physical or biological means to prevent sporangia from germinating, little or no seed colonization and disease development occurs (5, 8, 12, 15).

Enterobacter cloacae is a seed associated bacterium that protects a number of plant species from *P. ultimum*-induced pre-emergence damping-off (5, 6, 9, 12) by eliminating the sporangium stimulatory activity of seed exudates (6, 20). This is known to occur when *E. cloacae* is coated onto seeds of carrot, cotton, cucumber, lettuce, radish, sunflower, tomato, and wheat but not on seeds of corn or pea (6, 12). On cotton *E. cloacae* accomplishes this inactivation through the degradation of ULCFA (19). One hypothesis explaining the reduced ability of *E. cloacae* to abolish sporangium germination stimulatory activity of exudates in the spermosphere of corn or pea is that *E. cloacae* fails to degrade ULCFA released into the spermosphere. Alternatively, *E. cloacae* may retain its ability to degrade ULCFA but the timing and level of ULCFA exudation from corn and pea may exceed the capacity of *E. cloacae* to degrade released ULCFA, thus providing adequate levels for sporangial stimulation.

Currently, we do not know how quickly sporangium germination and subsequent disease development is initiated on a number of different hosts, and whether reduced disease development accompanying the removal of exudate stimulants is due solely to reductions in sporangium germination, seed colonization, or both. Since most of our current knowledge of how seeds stimulate sporangium germination comes from work with *in vitro* collected seed exudates (6, 17, 19, 20), we have not been able to fully appreciate the temporal dynamics of sporangial responses and how these responses relate to the temporal exudation of stimulatory molecules.

To better understand the mechanisms underlying the differential protection of plants by *E. cloacae*, we have chosen to focus our studies on the dynamics of *P. ultimum* during early stages of pathogenesis in the presence and absence of *E. cloacae* on two differentially-protected hosts. Our reasoning is that by defining the behavior of *P. ultimum* in response to seeds of two differentially-protected plants, we will be in a better position to test our above hypotheses. Our goals are to identify how early *E.*

cloacae interacts with *P. ultimum* and at what stage of pathogenesis the interactions are most significant. A further goal is to better understand the potential role of fatty acid metabolism in these early stages of disease development. The specific objectives of our study were to: (*i*) establish the baseline temporal sporangial activation and germination patterns of *P. ultimum* in response to seeds, and (*ii*) determine if *E. cloacae* is able to reduce sporangium activation and affect subsequent pathogenesis events in the spermosphere, and whether or not fatty acid transport and degradation are important biocontrol traits expressed during these early interactions.

MATERIALS AND METHODS

Plant Material

Corn (*Zea mays* cv. Northern X-tra Sweet) and cucumber (*Cucumis sativus*, cv. Marketmore) seeds were sorted by discarding cracked, deformed, or discolored seeds. Seeds were then surface sterilized for 3 minutes in 0.05% sodium hypochlorite with 1-2 drops of Tween® 20, rinsed with sterile deionized water (sdw), and blotted dry. Seeds weighing between 160 to 200 and 24 to 30 mg were used for all corn and cucumber assays, respectively. Germination rates of each species were estimated based on three replicates of 100 seeds each. For imbibition assays, each well of Corning® 12-well tissue culture plates received 2 ml of washed sterile sand (0.5-1 mm particle size), a seed, followed by another 2 ml of sand to cover the seed. Sdw was added to each well to bring the final water content (WC) to 18% (wt/wt). This water content corresponded to a matric potential of -2 kPa (Chen, *unpublished*). At 0, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 hr after sowing, 10 seeds were removed from the sand, rinsed and blotted dry, and individually weighed. Three replicate assays were performed for each plant species.

Production and germination of Pythium ultimum sporangia

Pythium ultimum P4 was routinely grown on a mineral salts medium (SM+L) containing 0.1% α-phosphatidylcholine. Sporangia produced on this media mimic the behavior of sporangia produced on live plant tissue (13). Agar discs (4 mm diam.) were cut from 5 day old cultures kept at 27°C. Discs were leached twice in darkness for ten minutes each followed by a 3 hr leaching by adding leaching buffer (0.01 M $Ca(NO_3)_2 \times 4\ H_2O$, 0.04 M $MgSO_4 \times 7\ H_2O$, 0.05 M KNO_3, pH 5.8) and replacing with fresh buffer at the end of each leaching period. Discs were rinsed twice with sdw and kept at 24°C for 2 days in darkness.

When preparing sporangia for biomass experiments with defined levels of inoculum, entire (9 cm diam.) SM+L plates were leached as described above. These sporangia were harvested by adding 500 µl sdw per plate, scraping sporangia from the surface of the culture, and filtering through sterile cheesecloth. Sporangia were collected by centrifuging the suspension and washing twice with sdw. Concentrations of sporangia were adjusted after determining the number of sporangia using a heamocytometer. Five µl of sporangial suspension was then added to the surface of water agar discs (4 mm diam.) and allowed to absorb. Sporangial germination rates were determined by staining with 0.03% acid fuchsin in 85% lactic acid and viewing microscopically at 100-250X.

Production of Enterobacter cloacae cells

E. cloacae strains used in this study were 501 (wt), L1 (*fadL*Δ), and 31 (*fadAB*Δ). *E. cloacae* L1 is unable to transport long-chain fatty acids and strain 31 is unable to degrade fatty acids (19). Tryptic soy broth (TSB, BD Diagnostics, NJ, USA) amended with rifampicin (100 µg/ml) and kanamycin (50 µg/ml) when appropriate, were inoculated with *E. cloacae* cells and grown for 18 hr at 30ºC. Cells were harvested by spinning, washed twice in Tris buffered saline (TBS, pH 7.2), and

resuspended in TBS. Cell density was adjusted to 10^9 cfu/ml at OD_{600} to make a stock cell suspension. The stock was diluted to make suspensions that would give final concentrations of cells at 10^4, 10^6, or 10^8 cfu/cm^3 substrate at 18% WC.

***P. ultimum* sporangial activation and germination assays**

Activation experiments were designed to establish when sporangia first perceive exudate stimulants whereas the germination experiments were designed to assess sporangial germ tube emergence in response to seeds. Glass beads (volume 1 cm^3, 0.1 mm diam.) were added to wells of Corning® 12-well tissue culture plates and sdw was added, adjusting the WC to 18%. A sporangial disc was added to each well, and a surface sterilized seed was placed on the surface of the disc. Seeds were covered with an additional 3 cm^3 of glass beads and watered to make the final WC 18%.

For activation assays, sporangial discs were harvested at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hours after exposure to cucumber seeds or at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, and 4 hours after exposure to corn seeds. After retrieval from the glass bead matrix, sporangial discs were incubated for an additional time so that the spermosphere activation time and subsequent germination (i.e. germ tube penetration and extension) time totaled 6 and 4 hrs for cucumber and corn, respectively.

For the sporangial germination assays, sporangial discs were retrieved, and rinsed, and immediately assessed for germination. Sporangial discs in wells containing substrate and water, but no seeds served as negative controls. Five replicates were prepared for each treatment, and each experiment was repeated 3 times. At each sampling time, seeds were removed, blotted dry and the seed weight was recorded.

Activation assays were also performed with *E. cloacae* cells added at 10^4, 10^6, or 10^8 cfu/cm^3 substrate. A second set of experiments tested *E. cloacae* strains 501, L1, or 31 at 10^8 cfu/cm^3 substrate. Sporangia discs in wells containing no seeds but with *E. cloacae* 501 added at a rate of 10^8 cfu/cm^3 substrate served as negative

controls. Positive controls consisted of sporangia discs exposed to seeds, with *E. cloacae* 501 cells added after disc harvest to match the approximate amount of cells from the maximum concentration used in the study. The design of the controls allowed us to check the level of sporangial activation but also to demonstrate that *E. cloacae* does not have a negative effect on sporangia germination resulting from activation during the incubation period. Three replicates were prepared for each treatment, and each experiment was repeated 3 times.

Seed colonization incidence

Seeds were prepared in Corning® 12-well tissue culture plates as described above. *E. cloacae* strains 501, L1, or 31 were added at 10^8 cfu/cm^3 substrate. Seeds in wells containing no sporangia but with *E. cloacae* 501 added at a rate of 10^8 cfu/cm^3 substrate served as negative controls. Seeds inoculated with *P. ultimum* sporangia served as positive controls. Seeds were removed at 0, 1, 2, 4, 6, and 8 hours after sowing, rinsed and placed on 1% water agar plates amended with 15 µg/ml rifampicin and 15 µg/ml penicillin G (WARP). Plates were incubated at 27°C and the percentage of colonized seeds was assessed after 48 hr by observing characteristic colony growth emerging from seeds. Six replicates were prepared for each treatment and the experiment was repeated three times.

Quantification of P. ultimum biomass in the spermosphere by qPCR

To equate DNA levels with mycelial biomass equivalents, *P. ultimum* was grown for 6 days at 27°C on potato dextrose agar plates overlaid with cellophane (PDA, Difco, USA). Mycelium was harvested, weighed, frozen, and lyophilized. After determining dry weights, lyophilized mycelia were stored at -20°C. DNA was extracted using the Qiagen DNeasy plant mini kit (Qiagen sciences, MD, USA) according to manufacturer's instructions for optimized yield. The DNA was then

quantified using the Quant-iT™ PicoGreen® dsDNA assay kit (Molecular Probes, OR, USA).

To prepare standards for qPCR analysis, *P. ultimum* was grown as described above. Mycelial mass was harvested, ground in liquid nitrogen, and transferred to 4 ml of extraction buffer (0.05 M ethylene diamine tetracetic acid [EDTA], 0.1 M Tris, 0.5 M NaCl, 0.7% β-mercaptoethanol, 0.25% sodium dodecyl sulfate [SDS]). Mycelia were lysed at 65ºC for 1 hr after which 1.3 ml 5 M potassium acetate was added, and samples were incubated on ice for 20 min. Samples were centrifuged (3000 rpm, 10 min) and 3.2 ml isopropanol was added to supernatants and kept on ice for 30 min. Samples were centrifuged and pellets containing DNA were air dried and re-suspended in TE buffer (0.01 M Tris, 1 mM EDTA). The suspension was treated with 10 µg RNAse A at 65ºC for 10 min. The DNA was then extracted using phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) followed by chloroform:isoamyl alcohol (24:1, v/v) and ethanol precipitation of the DNA. DNA was redissolved in 10 mM Tris (pH 8.0) and the quality and integrity of the DNA was evaluated by gel electrophoresis. DNA was quantified using the Quant-iT™ PicoGreen® dsDNA assay kit and split into aliquots stored at -80°C.

For biomass determinations directly in the spermosphere, seeds were prepared in glass beads and Corning® 12-well tissue culture plates as described above. Non inoculated seeds were used as negative controls, and inoculated seeds served as positive controls for all experiments. Treatments consisted of inoculated seeds with either *E. cloacae* strain 501 (wt) or 31 (*fadAB*Δ) added at 10^8 cfu/cm^3 at 0 hr for the first and at 2 hrs for the second experiment. Total *P. ultimum* biomass was measured on both corn and cucumber in the first experiment, but only on cucumber for the second. In a third series of biomass experiments, cucumber seeds were inoculated with 0, 100, 300, or 600 sporangia per seed (sporangia obtained as described above). At 4,

8, and 12 hours after sowing, seeds were removed, rinsed, cut into 6-8 pieces, transferred to cryo-vials and frozen in liquid nitrogen. Seeds were kept at -80°C until extracted. Seed pieces were ground with a mortar and pestle and glass beads in liquid nitrogen. DNA was extracted using the Qiagen DNeasy plant mini kit according to manufacturer's instructions for optimized yield. DNA samples were kept at -20°C until analyzed. Three replicates were prepared for each treatment and each experiment was repeated twice.

The amount of *P. ultimum* DNA on and in seeds was measured in triplicate in 96-well plates (Phenix, NC, USA) using quantitative PCR (qPCR). qPCR reactions were prepared by mixing 7 µl water, 0.5 µl 10 µM primer AFP276 (5'-TGTATGGAGACGCTGCATT-3') (7), 0.5 µl 10 µM primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (21), 10 µl Platinum® SYBR®Green qPCR SuperMix-UDG with ROX (Invitrogen, CA, USA), and 2 µl template. Samples were amplified using the ABI Prism® 7000 sequence detection system (Applied BioSystems, CA, USA) with the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. A dissociation protocol starting at 60°C was used to assess the presence of primer-dimer formation and other artifacts. Quantification was achieved by comparing cycle threshold (C_T) values in samples to C_T values of exogenous controls of *P. ultimum* standard DNA ranging from 1.5 fg to 75 ng. A curve of *Pythium* standard DNA amounts vs C_T values was established for every 96-well plate analyzed and the curve formula was used to calculate the amount of *Pythium* DNA in seed samples. All regression coefficients were ≥0.98, where the majority were above 0.99 [data not

shown]. Slopes from the standard curves were used to calculate the efficiency (E) of the PCR reaction using the formula $E = 10^{[-1/slope]} - 1$. The average efficiency was 106% (± 3).

Statistical analysis

The PROC GLM procedure in SAS v9.1 (SAS Institute, Cary, NC) was used for statistical analyses. All assays were analyzed individually and thereafter pooled and re-analyzed. Datasets for inoculated corn and cucumber seeds were also pooled and analyzed for comparison. Seed imbibition, sporangium activation, sporangium germination, *Pythium* seed colonization incidence, and *Pythium* seed colonization biomass data were analyzed using two-way ANOVA with 'water uptake', 'activation percent', 'germination percent', 'colonization percent' and 'amount DNA' as response variables, respectively. 'Time' and 'treatment' were used as continuous and categorical predictors, respectively. Additionally, simple linear regression (SLR) was performed on all response variables but 'water uptake' (pooled datasets) and treatments individually using 'time' as a continuous variable. This was followed by multiple regressions with 'treatment' as additional predictor variable and ANCOVA analysis for comparison of treatment slopes. DNA amount in *Pythium* mycelia was analyzed using SLR with 'amount DNA' as the response variable and 'mycelia weight' as the predictor. qPCR standard calibrations of *Pythium* DNA vs CT values were also analyzed using SLR for estimation of the curve formula. Percentage data were transformed (arcsine ($\sqrt{(p)}$) where p is the proportion) to stabilize the normality and variance when necessary. The response variable 'amount DNA' in the *Pythium* biomass data sets was transformed (-log (pg DNA +1)) to achieve normality. Appropriate diagnostic plots were established to ensure that all assumptions of the tests were fulfilled, and to check for influential points. Insignificant terms ($\alpha = 0.05$)

were dropped in all tested models. Results referred to as significant are comparisons with $p < 0.05$ unless otherwise mentioned.

RESULTS

Seed characterizations

Since exudation dynamics are directly related to imbibition behavior (11), we used seed imbibition and subsequent seed germination rates to gauge exudation rates and assure viability and seedling quality. The germination rates of both cucumber and corn seeds were >99%. Both cucumber and corn seeds rapidly imbibed a significant amount of water within the first 0.5 hr and continued to take up water over the 12 hr sampling period (data not shown). When imbibition rates were expressed as mg H_2O/mg seed weight, no differences were observed between corn and cucumber seeds during the first four hours of imbibition. Beyond four hours, significantly greater amounts of water were taken up by corn seeds than by cucumber seeds. Corn seeds weighed an average of 163 (± 2.5) mg, which is about 7 times greater than the weight of cucumber seeds (25 (± 0.6) mg). Therefore, expressing imbibition rates as mg H_2O/seed revealed that the overall amount of water imbibed by corn seeds greatly exceeded that of cucumber as early as 0.5 hr after sowing **(Figure 2.1)**.

Activation and germ tube emergence of P. ultimum sporangia in the spermosphere

Sporangial activation experiments were designed to assess when sporangia perceive an exudate stimulus that ultimately translates into a germinated sporangium. Exudate released by cucumber during the first 30 min of seed germination was sufficient to induce significant sporangial activation, as compared to the negative (no seed) control **(Figure 2.2)**. Maximum sporangial activation in the cucumber spermosphere reached levels of 60-70% within 1.5 to 3 hrs of exposure to the seed. In contrast, sporangial activation in the corn spermosphere was observed as early as 15

min after exposure to the seed, with maximum levels of activation (80-100%) observed as early as 30 min after exposure to the seed **(Figure 2.2)**.

A significant level of germ tube emergence (direct evidence for sporangial germination) did not occur in the cucumber spermosphere until 2 to 2.5 hrs after sowing **(Figure 2.3)**, reaching 60-70% by 5 to 6 hr after exposure to the seed. In contrast, significant germ tube emergence was observed from sporangia in the corn spermosphere as early as 1 to 1.5 hrs after exposure to corn seeds, with maximum germination incidence (>90% germination) by 4 hours after exposure to cucumber seed **(Figure 2.3)**.

Sporangial activation and germ tube emergence in response to corn and cucumber seeds increased linearly over time until the response was maximized **(Table 2.1)**. This linear relationship was highly significant ($p < 0.0001$) in both spermospheres but the rate of increase was significantly lower in the cucumber than in the corn spermosphere **(Table 2.1)**.

Effects of cell density and fad mutants of E. cloacae on P. ultimum sporangial activation in the spermosphere

These experiments focused on sporangial activation and not sporangial germination. We reasoned that the exudates that are responsible for inducing initial sporangia activation (which sets the germination process into motion) are the exudate fractions that *E. cloacae* must inactivate to efficiently reduce sporangial germination. Adding *E. cloacae* cells to cucumber seeds resulted in significant reductions in sporangial activation, but only when *E. cloacae* was applied at the highest rate of 10^8 cells/cm^3 substrate **(Figure 2.4A)**. Significant reductions were also observed at all sampling points up to 6 hrs. *E. cloacae* reduced sporangial activation by approximately 45% in the cucumber spermosphere. No reductions of sporangial

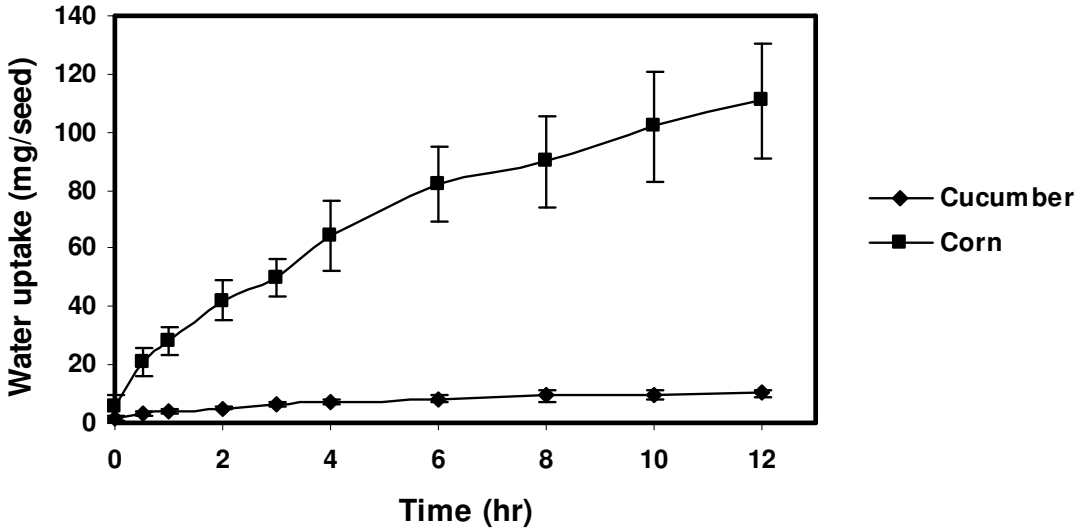

Figure 2.1. Cucumber and corn water uptake dynamics during the first 12 hours of seed germination. Seeds were germinated in sand at 18% water content. Each point with the error bars represents the mean ± S.D. of 30 observations from 3 repeated experiments.

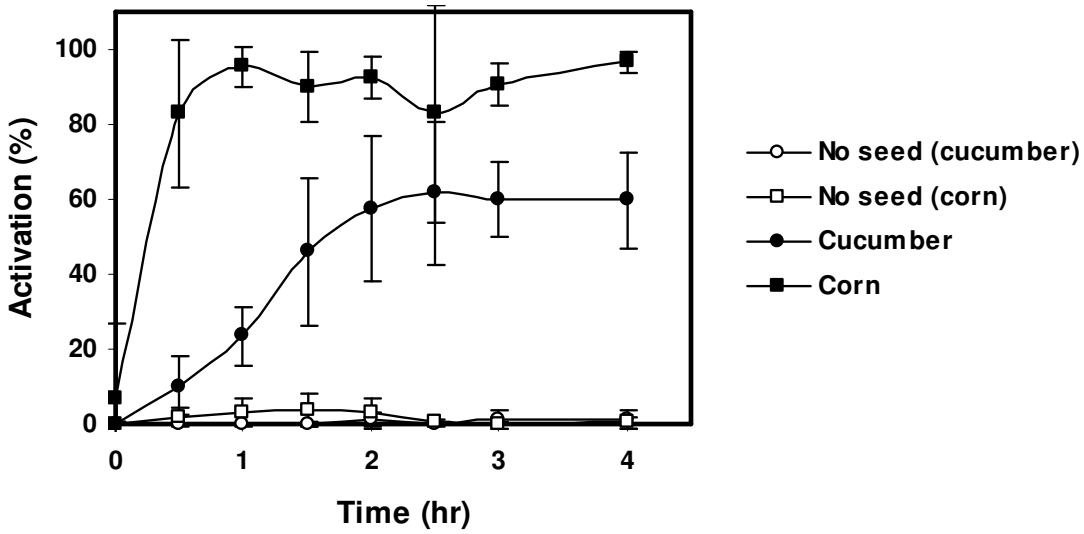

Figure 2.2. *Pythium* sporangia activation in the corn and cucumber spermosphere at 18% water content during the first 4 and 6 hours of seed germination, respectively. Activation was assessed as sporangia germination where the sporangia were exposed to seeds for various periods of time and then allowed to incubate in the absence of seeds. Each point with the error bars represents the mean ± S.D. of 15 observations from 3 repeated experiments.

activation by *E. cloacae* were observed in the corn spermosphere at any sampling time **(Figure 2.4B)**.

Mutants of *E. cloacae* defective in fatty acid transport (strain L1, *fadL*Δ) or β-oxidation (strain 31, *fadAB*Δ) did not significantly reduce sporangial activation in the cucumber spermosphere at any timepoint tested **(Figure 2.4C)**. Furthermore, the rate of increase over time of activated sporangia in the presence of *fad* mutants did not differ from the positive control **(Table 2.2)**. However, the wildtype strain 501 significantly reduced overall sporangial activation as well as the rate of activation increase over time in the cucumber spermosphere **(Table 2.2)**. No significant changes in sporangial activation by any of the *E. cloacae* strains were observed at any time in the corn spermosphere **(Figure 2.4D; Table 2.2)**.

P. ultimum colonization of seeds treated with E. cloacae

The incidence of corn and cucumber seeds colonized by *P. ultimum* increased at a constant rate, reaching a maximum colonization incidence (100%) by 6 hr after sowing **(Figure 2.5A and B)**. No significant differences in colonization incidence or rate of increase were observed between corn and cucumber seeds. Neither of the *E. cloacae fad* mutants reduced seed colonization incidence by *P. ultimum* in the cucumber spermosphere. *E. cloacae* wildtype strain 501 only reduced *Pythium* colonization incidence in the cucumber spermosphere at the 8 hr sampling point. Despite this, the rate of increase in cucumber seed colonization incidence was significantly lower for seeds treated with *E. cloacae* strain 501 compared to the inoculated control seeds **(Table 2.2)**. No reductions in seed colonization incidence were observed by any of the *E. cloacae* strains in the corn spermosphere **(Figure 2.5B and Table 2.2)**.

Table 2.1. Regression statistics of *P. ultimum* sporangial activation and germination response to cucumber and corn seeds over time

Assay	r^{2a}	LS Means[c]
Activation		
Cucumber	0.822***[b]	23.65 b
Corn	0.803***	97.84 a
Germination		
Cucumber	0.922***	15.59 b
Corn	0.910***	72.16 a

[a] Regression coefficient of the regression line for response (activation, biomass) vs time

[b] Significance level of the F-test testing the association between the response variable and time; ** = $p<0.01$, *** = $p<0.001$

[c] LS means estimates from ANCOVA analysis comparing the slopes of corn and cucumber seeds for the activation and germination assays

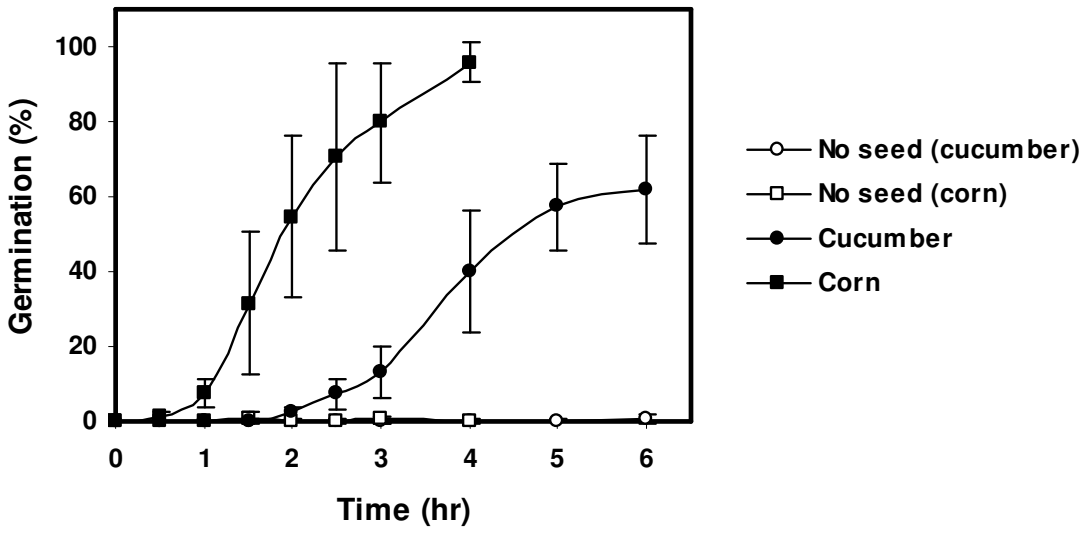

Figure 2.3. *Pythium* sporangia germination in the corn and cucumber spermosphere at 18% water content during the first 4 and 6 hours of seed germination, respectively. Each point with the error bars represents the mean ± S.D. of 15 observations from 3 repeated experiments.

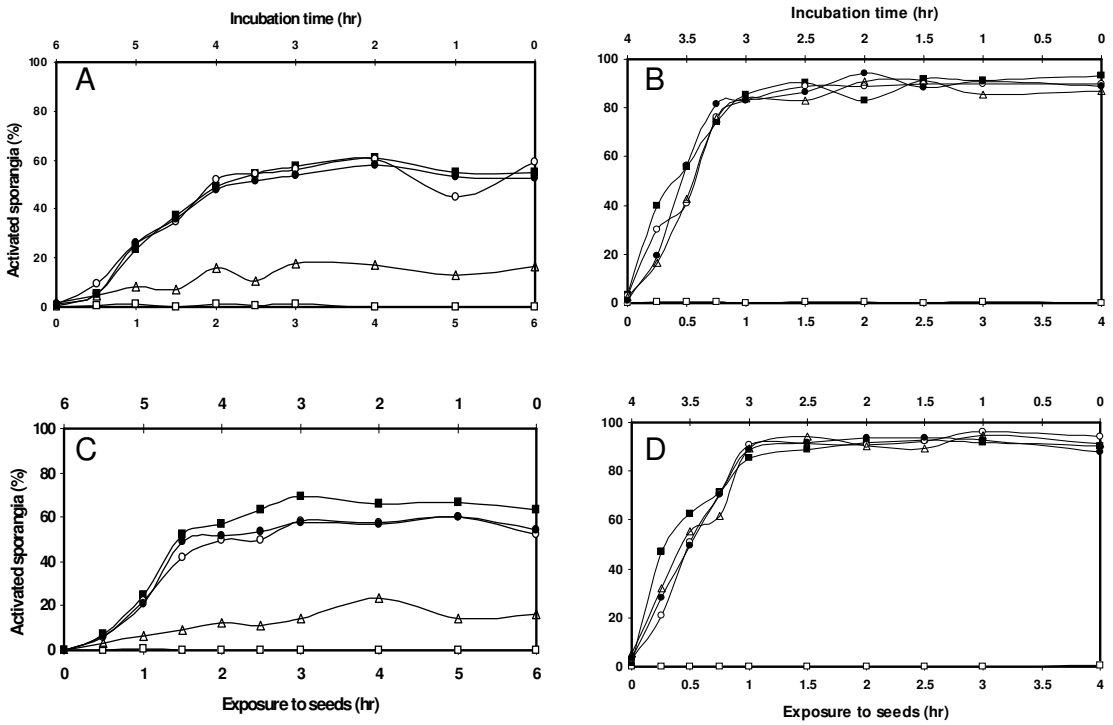

Figure 2.4. *Pythium* sporangia activation in response to cucumber (A, C) and corn seeds (B, D) in the presence of *E. cloacae* L1 (*fadL*Δ), 31 (*fadAB*Δ) or 501 (wt) at 18% water content during the first 4 to 6 hrs of seed germination. Activation assays in the absence (□) and presence (■) of a seed. Sporangia activation in the presence of *E. cloacae* 501 at 10^4 (○), 10^6 (●), and 10^8 (Δ) cfu/cm^3 substrate in the cucumber (A) and corn (B) spermosphere. Sporangia activation in response to cucumber (C) and corn (D) seeds treated with *E. cloacae* L1 (○), 31 (●) or 501 (Δ) applied at 10^8 cfu/cm^3 substrate. Each point represents the mean of 9 observations from 3 repeated experiments. Note the different x-axis.

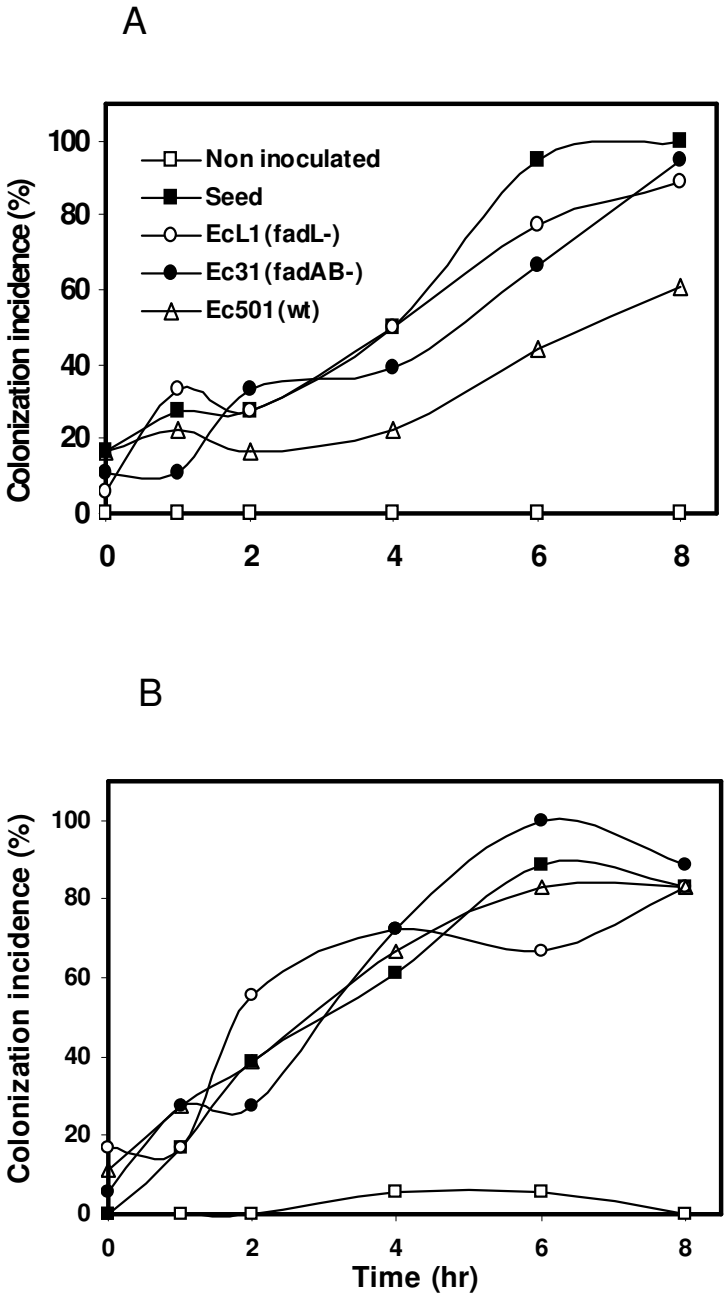

Figure 2.5. Frequency of cucumber (A) and corn (B) seeds colonized by *Pythium ultimum*. Treatments consisted of seeds that were inoculated and not inoculated with *Pythium*. Additionally, inoculated seeds were treated with *E. cloacae* strain L1 (*fadL*Δ), 31 (*fadAB*Δ), or 501R3 (wt). Each point represents the mean of 3 observations from 3 repeated experiments, where each observation was based on 6 replicates.

P. ultimum biomass development of corn and cucumber seeds treated with E. cloacae

Whereas colonization incidence provides little information on the absolute amount of *P ultimum* colonization of seeds, quantitative estimates of *P. ultimum* biomass provide a better indication of the overall level of seed colonization. Increasing amounts of *P.ultimum* mycelia was linearly correlated with increasing amounts of DNA ($r^2 = 0.920$, $p < 0.0001$) which is why DNA was used as a biomass estimator. *P. ultimum* biomass was detected on both cucumber and corn seeds as early as 4 hr after sowing **(Figure 2.6A and B)**. At this point 43 and 75 pg *P. ultimum* DNA could be detected on cucumber and corn seeds, respectively. By 8 hrs, approximately a 10-fold increase of *P. ultimum* biomass was detected (501 pg and 721 pg DNA on cucumber and corn seeds, respectively). However, biomass levels on corn did not differ significantly from those on cucumber. No further increase in *P. ultimum* biomass on cucumber seeds was observed between 8 and 12 hr. On the contrary, by 12 hr after sowing, levels of *P. ultimum* biomass on corn seeds were significantly greater than those observed at 8 hr after sowing and significantly greater than levels on cucumber seeds at 12 hr (3470 pg and 677 pg for corn and cucumber, respectively). No *P. ultimum* DNA was detected on non-inoculated seeds at any of the sampling times.

The effect of the *E. cloacae* strain 31 on *Pythium* biomass development on cucumber seeds was variable **(Figure 2.6A)**. Significantly lower levels of *P. ultimum* biomass developed on strain 31-treated seeds by 4 and 8 hrs after sowing. By 12 hr after sowing no significant difference in *P. ultimum* biomass between treated and non-treated seeds was observed. Furthermore, the linear rate of biomass increase was not significantly lower in the presence of strain 31 than in the positive control **(Table 2.2)**. When *E. cloacae* wildtype strain 501 was applied to cucumber seeds, *Pythium* biomass was significantly reduced over that observed on non-treated seeds at all time points tested **(Figure 2.6A)**. Additionally, the rate of biomass increase over time was

significantly lower on *E. cloacae* wt-treated seeds than on non-treated seeds and not different from the non inoculated control seeds **(Table 2.2)**. *P. ultimum* biomass on corn was not significantly reduced in the presence of any of the three *E. cloacae* strains **(Figure 2.6B and Table 2.2)**.

Relationship between sporangium activation and P. ultimum biomass on cucumber seeds

Two approaches were used to determine whether the observed reduction in *P. ultimum* biomass by *E. cloacae* on cucumber seeds was due solely to reduction of sporangial activation or whether *E. cloacae* interacts with *P. ultimum* after sporangial activation and germination to reduce seed colonization. In the first approach, *E. cloacae* cells were added to cucumber spermospheres only after sporangia were fully activated (2 hr). We reasoned that if disruption of sporangial activation is the principal cause of reduced *P. ultimum* biomass, adding *E. cloacae* cells after full activation should result in biomass levels that do not differ from those observed on non-treated seeds. Furthermore, biomass levels should be significantly greater than on seeds where *E. cloacae* was introduced at the time of sowing. Our results support this hypothesis. Adding wild type cells of *E. cloacae* 2 hrs after sowing resulted in no significant decreases in *Pythium* biomass as compared to non-treated seeds at any sampling time after inoculation **(Figure 2.7A and Table 2.3)**. The rate of the linear increase in *Pythium* biomass over time was significantly higher when *E. cloacae* cells were introduced 2 hrs after sowing than when cells were added at the time of sowing ($p < 0.0001$, data not shown). Strain 31 of *E. cloacae* behaved similarly to the wild type strain.

The second approach involved manipulating the number of activated and germinated sporangia by controlling the total number of sporangia in the inoculum.

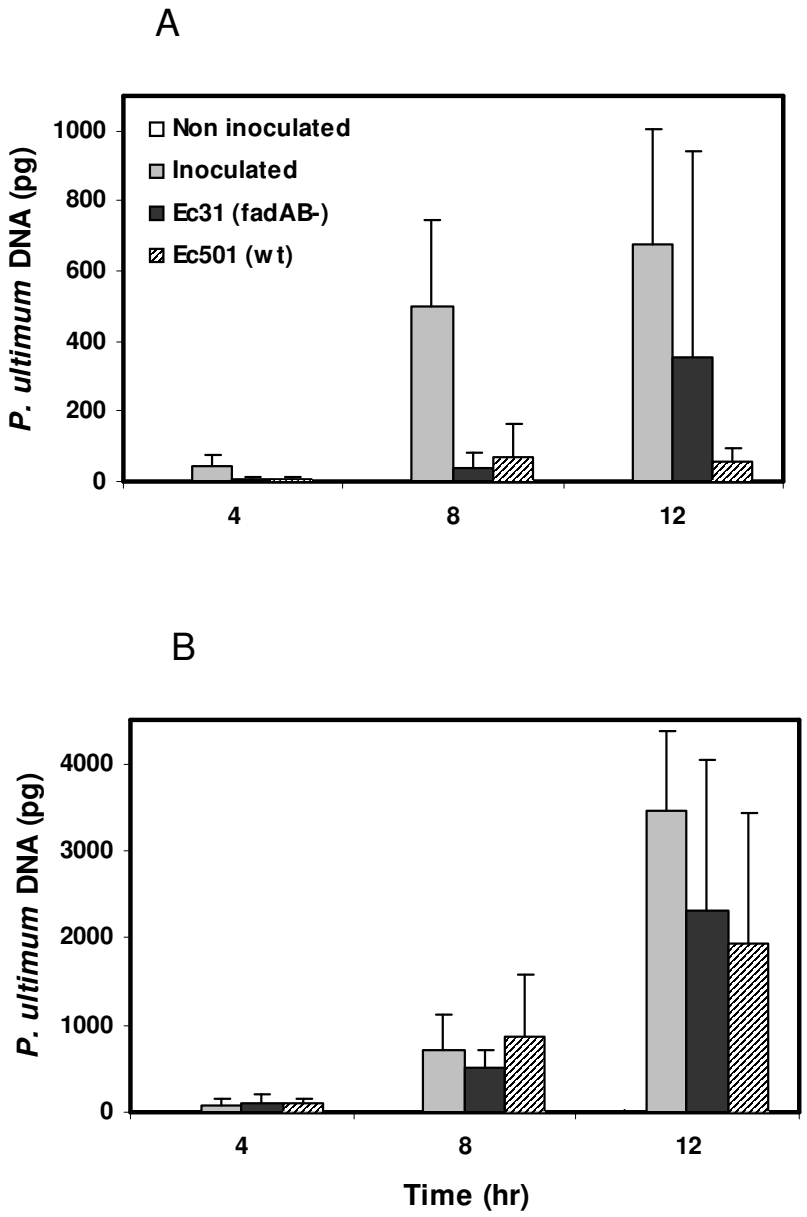

Figure 2.6. *Pythium ultimum* biomass development on cucumber (A) and corn (B) seeds in the presence of *E. cloacae* strain 31 (*fadAB*Δ) or 501 (wt). Seeds were exposed to *Pythium* for 4, 8, and 12 hours after which amounts of *Pythium* DNA was determined using qPCR. Each point with the error bars represents the mean ± S.D. of 6 seeds from 2 repeated experiments. Note the different y-axis.

Table 2.2. Regression statistics of *P. ultimum* sporangial activation, seed colonization incidence and biomass development on cucumber and corn seeds treated with *E. cloacae* strains L1 (*fadL*Δ), 31 (*fadAB*Δ) or 501 (wt) at 10^8 cfu/cm^3 substrate

	Activation		**Colonization**		**Biomass**	
Treatment	**r^{2a}**	**LS Means[c]**	**r^2**	**LS Means**	**r^2**	**LS Means**
Cucumber						
Non inoculated	0.047 (ns)[b]	0.106 c	0.000 (ns)	0.000 a	0.000 (ns)	0.000 b
Inoculated	0.920***	34.05 a	0.829***	52.78 a	0.547***	406.83 a
Inoculated/+ L1	0.853***	28.21 a	0.654***	47.22 a	Nd[d]	Nd
Inoculated/+ 31	0.873***	28.21 a	0.709***	42.59 ab	0.150 (ns)	131.22 a
Inoculated/+ 501	0.538***	7.14 b	0.532***	30.56 b	0.111 (ns)	44.02 b
Corn						
Non inoculated	0.035 (ns)	0.079 b	0.004 (ns)	1.85 b	0.000 (ns)	0.000 b
Inoculated	0.783***	53.54 a	0.814***	48.15 a	0.797***	1420.92 a
Inoculated/+ L1	0.906***	47.35 a	0.788***	53.71 a	Nd	Nd
Inoculated/+ 31	0.954***	47.96 a	0.555***	51.85 a	0.593***	972.26 a
Inoculated/+ 501	0.860***	48.66 a	0.797***	51.85 a	0.417**	971.65 a

[a] Regression coefficient of the regression line for response (activation, colonization, biomass) vs time

[b] Significance level of the F-test testing the association between the response variable and time; ns = non significant, ** = $p<0.01$, *** = $p<0.001$

[c] LS means estimates from ANCOVA analysis comparing the slopes of treatments for corn and cucumber. Estimates with different letters are significantly different

[d] Not determined

We predicted that, if biomass development was dependent on the numbers of activated/germinated sporangia in the spermosphere, there should be a direct correlation between sporangium dosage and biomass development. Exposing cucumber seeds to increasing dosages of sporangia ranging from 0 to 600 sporangia per seed resulted in increasing levels of *P. ultimum* biomass on cucumber seeds either at 4, 8, and 12 hr after sowing **(Figure 2.7B and Table 2.4)**. *P. ultimum* biomass levels did not differ for individual sporangia doses between the 4, 8 or 12 hr samplings. Furthermore, biomass levels at each of the sampling times were approximately 10% of those from experiments where sporangial inoculum was not manipulated **(Figures 2.6 and 2.7A)**.

DISCUSSION

The current study was aimed at describing the temporal dynamics of the earliest pre-infection stages of *Pythium* pathogenesis that influence disease development, with the long term goal of better understanding the mechanisms underlying differential suppression of *Pythium* damping-off by *E. cloacae* on different hosts. Previous work had identified the sensing of ULCFA by sporangia and the subsequent germination of sporangia and colonization of seeds as key pre-infection stages in pathogenesis (6, 12, 17, 20). Complementary to these findings was the recognition that degradation of seed exudate fatty acids by *E. cloacae* not only eliminated sporangium germination, but also eliminated seed colonization and disease development (19, 20).

Our approach in the current work was to better define how soon sporangium germination is set into motion in the spermosphere after exposure to a seed and to examine the impact of *E. cloacae* on this and subsequent pre-infection responses. This temporal framework can then provide the basis for exploring the seed exudate

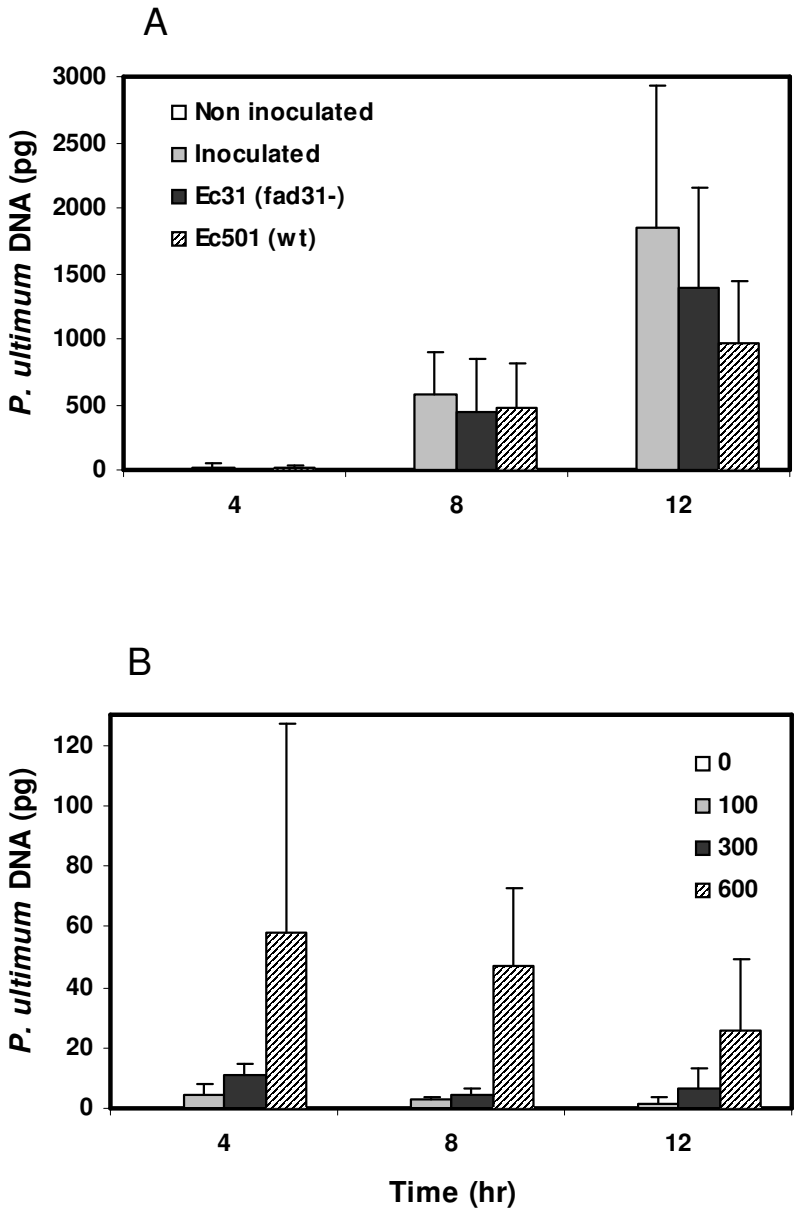

Figure 2.7. *Pythium ultimum* biomass development on cucumber seeds when *E. cloacae* strain 31 (*fadAB*$^{-}$) or 501 (wt) were added at 10^8 cfu/cm^3 substrate at 2 hrs post inoculation (A). *P. ultimum* biomass on cucumber seeds inoculated with 0, 100, 300, 600 sporangia/seed (B). Seeds were exposed to *Pythium* for 4, 8, and 12 hours after which amounts of *Pythium* DNA was determined using qPCR. Each point with the error bars represents the mean ± S.D. of 6 seeds from 2 repeated experiments.

Table 2.3. Regression statistics of *P. ultimum* biomass in and on cucumber seeds inoculated with *E. cloacae* strains 31 (*fadAB*Δ) or 501 (wt) at 2 hrs post sowing

Treatment	r^{2a}	LS Means[c]
Non inoculated	0 (ns)[b]	-0.000 b
Inoculated	0.575***	813.96 a
Inoculated/+31	0.605***	610.34 a
Inoculated/+501	0.600***	485.34 a

[a] Regression coefficient of the regression line for biomass vs time

[b] Significance level of the F-test testing the association between the response variable and time; ns = non significant, *** = $p<0.001$

[c] LS means estimates from ANCOVA analysis comparing the slopes of treatments. Estimates with different letters are significantly different

molecules that differentially regulate activities of *E. cloacae* in spermospheres of different plant species. The rapid sporangium activation dynamic observed in the corn and cucumber spermosphere is completely unrivaled among soilborne pathogen propagules that are considered dormant according to current knowledge. Together with the temporal sporangial germination and seed colonization this generates predictions about the tri partite interaction between seed, *Pythium* and *Enterobacter*.

E. cloacae must express its biocontrol activity within 30 min and 2 to 2.5 hrs of corn and cucumber seed germination, respectively, if interfering with sporangial activation and germination is critical for reducing *Pythium* infection. Once sporangia are activated and thus slated for germ tube emergence, then *E. cloacae* is unable to interfere with the sporangial germination response. This is why the temporal exudate fractions that elicit activation are the most critical ones for the bacterium to inactivate in order to affect subsequent germination. If the ability of *E. cloacae* to reduce seed infection is expressed during *Pythium* seed colonization, then this activity must be taking place within 4-6 hrs after sowing corn and cucumber seeds. Furthermore, the ability to metabolize fatty acids should be critical during the same windows of time if it is a key trait for reducing *Pythium* infection.

The bacterium is clearly active in interfering with *Pythium* host perception in the spermosphere within the above predicted time frame since adding *E. cloacae* to cucumber seeds reduces *P. ultimum* sporangial activation, and this is significant already within an hour of sowing seeds **(Figure 2.4A)**. Furthermore, fatty transport and degradation were critical traits for *E. cloacae* in reducing *Pythium* sporangia activation in the cucumber spermosphere since both *fadL* and *fadAB* mutants were unable to interrupt the activation response **(Figure 2.4C)**. None of the *E. cloacae* strains were able to interfere with *Pythium* sporangia activation in the corn spermosphere **(Figure 2.4B and D)**. In order to determine whether or not this

differential interference with sporangium activation in the cucumber and corn spermosphere explains the differential biocontrol we followed the seed colonization behavior of *Pythium* on *E. cloacae* treated seeds.

E. cloacae reduced neither *Pythium* colonization nor biomass development on corn seeds where the bacterium is also unable to reduce sporangia activation **(Figure 2.6 and Table 2.2)**. Even though the bacterium is able to decrease *Pythium* sporangial activation rates in response to cucumber and also is able to consistently suppress pathogen biomass it could not be concluded that the reduced sporangial activation directly resulted in suppressed *Pythium* colonization levels. The possibility of *E. cloacae* expressing activities relevant to biocontrol during *Pythium* colonization and biomass development could not be excluded by the intial experimental approach. Furthermore, *E. cloacae fadAB* mutants also suppressed initial *Pythium* biomass development on cucumber indicating that *E. cloacae* may have the ability to retard *Pythium* colonization that is independent of interfering with sporangial activation and does not depend on fatty acid degradation.

The bacterium did not significantly suppress *Pythium* biomass when adding *E. cloacae* cells to the cucumber spermosphere after full sporangial activation had taken place (2 hr post sowing) **(Figure 2.7A and Table 2.3)**. This demonstrates that *E. cloacae* interference with sporangial activation in the cucumber spermosphere directly impacts *Pythium* pathogenesis by reducing the pathogen seed colonization level. Since we did not quantify the number of *E. cloacae* attaching to seeds, it could be argued that cells added at 2 hrs post sowing simply failed to colonize seeds which would then result in normal Pythium biomass levels. Since sporangial germ tube emergence does not take place at a significant rate by 2 hrs, *E. cloacae* should not be competitively excluded by the pathogen. Furthermore, the system is devoid of any other

Table 2.4. Regression statistics of *P. ultimum* biomass in and on cucumber seeds inoculated with 0 – 600 sporangia per seed

Time (hr)	r^{2a}	LS Means[c]
4	0.297**[b]	18.23 a
8	0.637***	13.55 a
12	0.438***	8.46 a

[a] Regression coefficient of the regression line for biomass vs number of sporangia
[b] Significance level of the F-test testing the association between the response variable and time; ** = $p<0.01$, *** = $p<0.001$
[c] LS means estimates from ANCOVA analysis comparing the slopes of treatments. Estimates with different letters are significantly different

microorganisms that could prevent bacterial colonization so there is no apparent reason for why colonization should fail. Additional support for the association between reduced sporangial activation and decreased Pythium biomass was the finding that increasing sporangia levels in the *Pythium* inoculum resulted in increasing biomass levels on cucumber seeds (**Figure 2.7B and Table 2.4**). Demonstrating that suppressed *Pythium* colonization levels directly result from *E. cloacae* interruption of sporangial activation offers a possible explanation for why *E. cloacae* is unsuccessful in protecting corn. The bacterium is simply unable to reduce sporangial activation in the corn spermosphere which leads to sporangial germination and subsequent seed colonization. From the current data it seems like *E. cloacae* is not able to significantly interfere with *Pythium* disease development once sporangial germination has been initiated which is why this is the key stage in pathogenesis where *E. cloacae* biocontrol must be expressed for successful disease reduction.

Comparison of reports on cucumber and corn exudate fatty acids suggests why *E. cloacae* is unable to interrupt sporangial activation in the corn spermosphere. Corn seeds induce a higher rate of sporangium activation and germination than cucumber which suggests that corn may release higher quantities of the ULCFA's that have been identified as the primary germination stimulants in seed exudate (17, 19). This was confirmed in a recent study where corn seeds were found to release a total of 0.2 µg of oleic and linoleic acid per seed by 15 min of sowing which was about 4 times more than cucumber (Windstam and Nelson, *unpublished*). Considering that fatty acid degradation is critical for reducing sporangial activation and that *E. cloacae* is unable to perform this task in the corn spermosphere, one explanation may be that the bacterium does degrade ULCFA but the timing and level of ULCFA exudation may exceed the capacity of *E. cloacae* to degrade released ULCFA, thus leaving residual levels that are adequate for sporangial stimulation.

Bacterial fatty acid uptake and degradation is accomplished by the genes contained in the *fad* regulon (1). All genes within the regulon are under strict transcriptional control that is alleviated when exogenous long chain fatty acids (LCFA) are present. Being inducible genes, there is a certain lag time before actual expression of *fad* genes takes place and given the extreme rapidity with which *Pythium* sporangia become activated in the corn spermosphere it does make sense that *E. cloacae* cells may not be able to degrade fatty acids released by corn fast enough to abolish sporangial activation. However, some of the genes in the *fad* regulon are transcriptionally repressed in the presence of glucose and other simple sugars despite the presence of inducing LCFA in a fashion analogous to the lactose-glucose diauxie in *Escherichia coli* (2, 16).

An alternative explanation for the differential ability of *E. cloacae* to eliminate sporangium germination stimulatory activity of exudates is that the bacterium fails to degrade ULCFA released into the corn spermosphere due to catabolite repression. *E. cloacae* degradation of linoleic acid or germination stimulants in seed exudate is increasingly reduced in the presence of increasing levels of glucose, fructose, and sucrose (Windstam and Nelson, *unpublished*). Additionally, it was found that those three sugars are the pre dominantly released sugars by corn seeds with glucose representing 10% of the total exudate release 15 min after sowing. Just glucose alone was detected at 31 µg per corn seed at 15 min and this amount had doubled by 30 min. In contrast, cucumber released 0.7 µg and 2 µg of total sugars by the same timepoints,. Fructose was the predominant sugar released by cucumber, but the amount never exceeded 0.8 µg. With such large differences just on the basis of glucose and fructose exudation, sugar repression emerges as the most likely candidate as the explanation of *E. cloacae* plant species specific biocontrol.

In summary, activation assays have provided us with a timeline of sporangia host perception and have identified when essential exudate components that regulate *Pythium* pathogenesis and *E. cloacae* control activities are being released by germinating seeds. *E. cloacae* interrupts sporangial activation in the cucumber but not corn spermosphere and this explains the differential control of these plant species as it subsequently leads to decreased pathogen biomass. Bacterial degradation of ULCFA germination elicitors during the sporangial activation is key for successfully interfering with this stage in *Pythium* disease development. Seed exudate fatty acids and sugars are potent modulators of *Pythium* and *E. cloacae* activities in the spermosphere and the excessive release of sugars by corn suggests that catabolite repression of *E. cloacae* fatty acid degradation takes place in the corn spermosphere, thus explaining why corn is not protected by the bacterium. Sporangial germination following the activation event is critical for *Pythium* resuming growth and being able to colonize the seeds. This aspect is important not only for the seed infection progress but also for secondary spread. It is poorly understood which host factors drive the colonization and infection process and this is yet another area where shedding some light on these developmental stages would allow us to better understand pathogenesis of *Pythium* spp.

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CHAPTER 3

TEMPORAL EXUDATION OF FATTY ACIDS AND SUGARS FROM IMBIBING CORN AND CUCUMBER SEEDS: IMPLICATIONS FOR SUGAR REGULATION OF FATTY ACID DEGRADATION BY *ENTEROBACTER CLOACE*

ABSTRACT

The bacterium *Enterobacter cloacae* is able to protect cucumber but not corn seeds from *Pythium ultimum* incited infections. The mechanism underlying this protection is believed to involve fatty acid competition between *P. ultimum* and *E. cloacae* that may be regulated by sugars found in seed exudates. This study focused on determining the fatty acid and sugar content of corn and cucumber seed exudate as previous studies have shown that certain exudate sugars obstruct the ability of *E. cloacae* to degrade fatty acids and thus prevents the bacterium from inhibiting *Pythium* sporangia germination (Windstam and Nelson, *unpublished*). Fatty acids were readily detected in corn and cucumber seed exudate at 1.22 and 0.28 µg/seed, respectively, as early as 15 min of initiating imbibition. The most common unsaturated fatty acids were oleic and linoleic acid detected at a maximum of 0.87 and 0.26 µg/seed in corn, respectively. Oleic and linoleic acid content of corn exudate was approximately four times greater than for cucumber. The predominant saturated fatty acids in exudates from both seeds were palmitic and stearic acids. Sugars constituted a significant portion of corn seed exudate, accounting for 41% of the total dry weight, whereas only 5% of the cucumber seed exudate was comprised of sugars. Glucose, fructose, and sucrose accounted for 71-83% and 37-60% of all exudate sugars detected in corn and cucumber; where corn released up to 1140 times more of these three sugars than cucumber. When assuming that the spermosphere extends 10 mm away

from the seed surface, corn releases a total amount of 1.6 M of glucose, fructose, and sucrose within 15 min of initiating imbibition which exceeds the amount needed in order to shut down *E. cloacae* fatty acid degradation by almost three orders of magnitude. Our results demonstrate that elevated levels of sugars found in the spermosphere of some plant species such as corn can explain the lack of degradation of unsaturated fatty acid germination stimulants by *E. cloacae* and lead to a higher degree of *P. ultimum* sporangium germination, which leads to greater disease development.

INTRODUCTION

Germinating seeds are powerful stimulators of microbial activities in soil by virtue of the exudate molecules that are released during germination. Seed exudates contain a variety of compounds such as sugars, amino acids, organic acids, flavonoids, fatty acids, proteins, and inorganic nutrients (3, 20). These exudates give rise to the spermosphere, an important infection court for soil borne plant pathogens (19, 20), many of which exist in soil in a dormant state and require stimuli provided by germinating seeds to resume active growth (19).

One such pathogen is the Oomycete *Pythium ultimum*, which causes seed and seedling damping-off on a number of different plant species. *P. ultimum* has the capacity to switch from a quiescent to an active state of growth with extreme rapidity in the presence of a proper host (20). Seed exudates are critical in facilitating that switch as dormant survival propagules such as oospores and sporangia are dependent on exudates to become activated (19, 34, 35). *P. ultimum* sporangia are activated to germinate by the exudation of molecules released from corn seeds within the first 15 min after sowing (Windstam and Nelson, *unpublished*). Germ tubes emerge 45 to 75 min later, reaching a maximum level within 3-4 hours of sowing (16, 36) (Windstam

& Nelson, *unpublished*). Unsaturated long chain fatty acids (ULCFA) present in cotton seed exudates have been shown to be the most stimulatory elicitors of sporangium germination (31). Once sporangia have germinated, rapid mycelial growth ensues resulting in host colonization and infection within 24 to 48 hrs (8, 18, 39). Sporangial activation and germination and seed colonization are critical stages in *Pythium* pathogenesis, and if any of these is eliminated then disease will not develop (10, 16).

Pythium seed infections are reduced when seeds are coated with the bacterium, *Enterobacter cloacae* (10, 14, 18, 37, 38). *E. cloacae* achieves such control by effectively interrupting the sporangium activation in the cucumber spermosphere (Windstam and Nelson, *unpublished*) by the degradation of ULCFA (37). This effectively suppresses *Pythium* seed colonization (Windstam and Nelson, *unpublished*) and subsequent disease development.

Recent studies have demonstrated the central role of the plant in regulating the activities of seed-associated bacteria, including *E. cloacae* (6, 9, 14, 15, 27, 33). For example, seeds such as cotton and cucumber are effectively protected by *E. cloacae* whereas seeds such as pea and corn are not. In the corn spermosphere, *E. cloacae* is not able to interrupt *Pythium* sporangial activation as it does in the cucumber spermosphere (Windstam and Nelson, *unpublished*). The differential ability to interrupt *Pythium* sporangial activation is believed to be due to differences in corn and cucumber seed exudates with respect to exudate molecules that regulate the bacterial degradation of ULCFA (Windstam and Nelson, *unpublished*).

Genes within the *fad* regulon of *Enterobacteriaceae* are responsible for uptake and degradation of long chain fatty acids (LCFA) from the environment (4). Many of these genes are transcriptionally induced in the presence of exogenous LCFA, and thus require a lag time before expression can be detected (4, 5). Because LCFA are

considered less attractive sources of carbon for sustaining bacterial growth and metabolism, these genes are under additional transcriptional regulation by the cAMP receptor protein. Therefore, *fad* genes are not transcribed in the presence of LCFA if glucose is also present in the environment or if the extracellular osmolarity is high (11, 25). *In vitro* studies with *E. cloacae* have demonstrated that fructose, glucose or sucrose in excess of 5 mM shuts down degradation of fatty acids and germination stimulants in seed exudate which results in increased sporangial germination of *Pythium* (Windstam and Nelson, *unpublished*).

Since seed exudates contain both fatty acids and sugars it is likely that sugars could serve as regulators of fatty acid degradation in the spermosphere (27, 28, 31) and thus explain the differential activity of *E. cloacae* in corn and cucumber spermospheres. Based on previous studies (27, 28), we expect greater levels of sugars released from corn than from cucumber seeds. Therefore, we hypothesize that corn seeds release much greater quantities of sugars that are repressive to *E. cloacae* fatty acid degradation than cucumber seeds during the first few hours of seed germination. Alternatively, corn releases such large quantities of ULCFA compared to cucumber, that bacteria may not be able to degrade enough fatty acids to reduce sporangial germination.

It needs to be mentioned that the qualitative and quantitative changes in fatty acid and sugar exudation during the first few hours of seed germination are virtually unknown. Typical exudate analyses are conducted many hours beyond this critical time frame (1, 19, 27). The objective of our study were to analyze the fatty acids and sugars released during the first few hours of corn and cucumber seed exudation in order to relate the amount of specific compounds within either class of compounds to their direct effect on UCLFA degradation by *E. cloacae*.

MATERIALS AND METHODS

Production and germination evaluation of Pythium ultimum sporangia

Pythium ultimum P4 was routinely grown on a salt mineral medium (SM+L) amended with 0.1% soy lecitin (α-phosphatidylcholine, Sigma, USA) since previous work has demonstrated that sporangia produced on this media mimic sporangia produced on plant tissue (22). Six mm (in diam.) agar discs were cut from 5 day old cultures kept at 27°C. These discs were leached twice in darkness for ten minutes each followed by a 3 hr leaching by adding leaching buffer (0.01 M $Ca(NO_3)_2 \times 4\ H_2O$, 0.04 M $MgSO_4 \times 7\ H_2O$, 0.05 M KNO_3, pH 5.8) and replacing with fresh buffer at the end of each leaching period. Discs were rinsed twice with sdw and kept at 24°C for 2 days in darkness. Ten µl of seed exudates dissolved in 10 mM potassium phosphate buffer (PPB, pH 6.0) at 0, 0.5, 1, 2, 5, 10, and 20 µg/µl were added to leached discs that were allowed to incubate for 3 hours at 24°C. Discs were stained with 0.03% acid fuchsin in 85% lactic acid and the numbers of germinated and non germinated sporangia were enumerated at 250X. Sporangia counts were made across an entire diameter length of each *P. ultimum* disk.

Collection of seed exudate

Corn (*Zea mays* cv. Northern X-tra Sweet) and cucumber (*Cucumis sativus*, cv. Marketmore) seeds were sorted by discarding cracked, deformed, or discolored seeds. Seeds were surface sterilized for 3 minutes in 0.05% sodium hypochlorite, rinsed with sterile deionized water (sdw), blotted and dried. Three g of seeds were added to 30 ml of sdw in 125 ml flasks, and seed exudate was collected by shaking the flasks at 150 rpm at 24°C for 0.25, 0.5, 1, 2, 4, and 6 hrs. Exudate was harvested by decanting the solution through sterile cheesecloth into 50 ml tubes. Five µl of 25 mM butylated hydroxytoluene (BHT, Sigma, USA), an antioxidant, was added to each tube. Ten 10 µl aliquots of the exudate solution was spotted onto plates of potato dextrose agar

(PDA, Difco, USA) and TSA and assessed for contamination after 48 hours. Non contaminated solutions were pooled, lyophilized and stored frozen under an atmosphere of argon. Four replicate flasks were prepared for each treatment. The exudate collection was repeated three times.

Seed exudate fatty acid extraction, derivatization and analysis

Aqueous seed exudate (30 ml) was spiked with 30 µg of tridecanoic acid and acidified with H_2SO_4 to make the pH ~3.5 before extraction using 500 mg Oasis® HLB solid phase extraction columns (Waters Corp., MA, USA). Columns were conditioned and samples extracted using a 2-D elution protocol with $CHCl_3$:MeOH (2:1, v/v) and MeOH according to manufacturers instructions. All solvents used were of HPLC grade. Samples were evaporated and redissolved in 2 ml MeOH with 1% H_2SO_4, and spiked with 30 µg of nonadecanoic acid. Samples were vortexed and incubated at 85°C for 50 min. After incubation, 1.2 ml water was added and methyl esters were extracted twice with 2 ml hexane. The organic fraction was dried over anhydrous Na_2SO_4, evaporated and re dissolved in methylene chloride, and 1-2 µl injections were made into a HP5890 (Hewlett-Packard) gas chromatograph (GC), equipped with a DB-225 column (20-m length; 0.18-mm inner diameter; 0.2-µm film thickness) and a flame ionization detector (FID). The injector and detector temperatures were 230ºC. The oven starting temperature was set at 150ºC and held for 1 min, followed by increasing the temperature to 240ºC at 4ºC/min, that was held for 10 min. Peak areas and retention time were integrated with a HP3393A integrator. Three replicates were analyzed for each treatment and the experiment was repeated twice. Subsequently, samples from the same seed and time were pooled and 2 µl aliquots were injected into a HP 6890 GC equipped with a Supelco Equity 5 column (30-m length; 0.25-mm inner diameter; 0.25-µm film thickness) and an Agilent HP 5973 mass spectrometer detector (MS). The same parameters used above were

employed for the GC-MS analysis. Peak areas and retention time were integrated using Agilent ChemStation software. Fatty acids were identified on the basis of retention times and mass spectrograms compared to a standard mix and a library of mass spectrograms. Individual peaks were quantified by comparing the peak area to the peak areas of the internal standards.

Seed exudate sugar derivatization and analysis

Seed exudate sugars were derivatized and analyzed as described by Roberts et al. (1997, 1999) (26, 27). In short, 10-100 µl aliquots of 40 mg/ml exudate were lyophilized in 250 µl glass inserts in 2 ml autosampler vials. 25-50 µl N-methyl bis [Trifluoroacetamide] and 25-50 µl pyridine was added and vials capped with Teflon septa. Vials were heated at 65°C for 2 hrs with occasional vortexing. 2 µl aliquots were injected into a HP5890 GC-FID equipped with an Ultrawax 2 column (25-m length, 0.2-mm inner diameter, 0.33-µm film thickness). The injector and detector temperatures were set at 250 and 300°C, respectively. The initial oven temperature of 75°C was held for 1 min, and the oven ramped at 10°C/min to the final temperature of 225°C that was held for 2 min. Peak areas and retention times were integrated with a HP3393A integrator. Individual sugars were identified and quantified by comparing the retention time and peak areas to external standards. Three replicates were analyzed for each treatment and the experiment was repeated twice.

Statistical analysis

Exudate yields, sporangia germination rates, individual fatty acid and sugar components were analyzed with ANOVA using the PROC GLM procedure in SAS v9.1 (SAS Institute, Cary, NC). Probit analysis using Minitab v14.2 (Minitab Inc., PA) was used for estimating the EC_{50} values (exudate concentration that stimulates 50% sporangia germination). Means were separated using students t-test and pairwise comparisons were done by LSD (least significant difference) tests. Germination data

were transformed (arcsine (squareroot(p)) where p is the proportion of germinated sporangia) to stabilize the normality and variance when necessary. Diagnostic plots were performed for all the data to ensure that assumptions of the tests were fulfilled. Insignificant terms ($\alpha = 0.05$) were dropped in all tested models.

RESULTS

Corn and cucumber seed exudate yields

Corn and cucumber seeds differed dramatically in their exudation characteristics. Individual corn seeds released 384 µg of exudate within the first 15 min of imbibition compared to the 42 µg from cucumber during that same period (**Table 3.1**). By 6 hrs, over 1800 µg/seed of exudate was released from corn. This represents approximately 1% of the total starting corn seed weight. In contrast, exudates released within 15 min from cucumber seeds represents the bulk of the exudate liberated over entire 6-hr sampling period. By 6 hrs, cucumber seeds released only 68 µg/seed of exudate which did not differ significantly from the amount of exudate released within 15 min.

P. ultimum sporangial germination in response to corn and cucumber seed exudates

Corn seed exudate was significantly more stimulatory to sporangial germination than cucumber seed exudate. The EC_{50} (exudate dose required to stimulate 50% sporangial germination) values for corn seed exudate are consistently lower than those for cucumber. Also, corn exudate collected from all time points (from 15 min up to 6 hr) induced between 80 and 100% germination when applied at concentrations of 2-5 µg/µl (**Figure 3.1**). Considering that 10 µl of exudate solution is added to each sporangial disc when evaluating the germination response this equates to 50 and 20 µg of total exudate, which at 15 min represents 5-13% of all exudate released by an individual seed. On the other hand, cucumber seed exudate collected

Table 3.1. Amount of bulk exudate released by corn and cucumber during the first 6 hrs of seed imbibition.

	Exudate yield			
	µg/seed		**mg/g seed**	
Collection time	**Corn**	**Cucumber**	**Corn**	**Cucumber**
15 min	384 (± 28)[a]	42 (± 20)	2.30 (± 0.17)	1.67 (± 0.79)
30 min	493 (± 57)	50 (± 6)	2.96 (± 0.34)	2.02 (± 0.23)
1 hr	739 (± 106)	49 (± 6)	4.43 (± 0.64)	1.98 (±0.26)
2 hr	1001 (± 65)	63 (± 20)	6.01 (± 0.39)	2.5 (± 0.79)
4 hr	1399 (± 271)	72 (± 28)	8.40 (± 1.62)	2.88 (± 1.11)
6 hr	1844 (± 259)	68 (± 11)	11.07 (± 1.55)	2.71 (± 0.43)

[a] Mean exudate yield (n=3) ± S.D.

for 15 min induces a maximum of 39% sporangial germination when 200 µg, representing 480% of exudate released by an individual seed, is supplied.

Both corn and cucumber seed exudate becomes more stimulatory as the collection time is increased **(Table 3.2)**. EC_{50} values for corn seed exudate were 68% lower at 6 hr than the initial value at 15 min (1.38 and 0.44 µg/µl, respectively). The 56% decrease in cucumber exudate EC_{50} values was equally drastic over this same period (25.04 and 10.95 µg/µl for the 15 min and 6 hr time points, respectively).

Fatty acids in seed exudates

Both corn and cucumber seeds released significant levels of fatty acids (FA) as early as 15 min of initiating imbibition **(Table 3.3)**. Although corn released 4-7 times more FA than cucumber over 6 hr of germination, the release of FA by cucumber represented a greater proportion of the total exudate. Total FA (w/w) was equivalent to 0.11-0.32 % and 0.4-0.66 % of the total weight of cucumber and corn seed exudate, respectively (data not shown). FA amounts detected by 15 min did not increase significantly for either plant species over the 6 hr sampling period.

The most abundant ULCFAs consistently detected in corn and cucumber seed exudate were oleic (C18:1) and linoleic (C18:2) acid. Corn seeds released 92 and 83 ng of C18:1 and C18:2 per seed, respectively, within 15 min of sowing **(Figure 3.2)**. Over the same 15 min period, cucumber exudate contained 7 ng/seed of C18:1 and 41 ng/seed of C18:2. By 6 hrs corn seeds released 870 and 255 ng/seed of C18:1 and C18:2, respectively, whereas the release of these two fatty acids from cucumber seeds fluctuated over time but did not significantly increase by 6 hrs. Inconsistent trace amounts of myristoleic (C14:1) and palmitoleic (C16:1) acid were also detected in cucumber seed exudate (data not shown). C14:1 was only detected at 4 hrs and C16:1 at 4 and 6 hrs at rates that did not exceed 3 ng/seed.

Table 3.2. Dose response relationship between corn and cucumber seed exudate and *Pythium* sporangial germination

	EC_{50}[a] (µg/µl)	
Collection time	**Corn**	**Cucumber**
15 min	1.38 (± 0.03)[b]	25.04 (± 1.06)
30 min	1.28 (± 0.03)	17.48 (± 0.66)
1 hr	1.24 (± 0.02)	13.12 (± 0.32)
2 hr	0.89 (± 0.02)	12.87 (± 0.30)
4 hr	0.73 (± 0.02)	12.87 (± 0.27)
6 hr	0.44 (± 0.02)	10.95 (± 0.20)

[a] The amount of exudate required to induce 50% sporangial germination as estimated by probit dose-response regression

[b] Mean EC_{50} value (n=9) ± S.E.

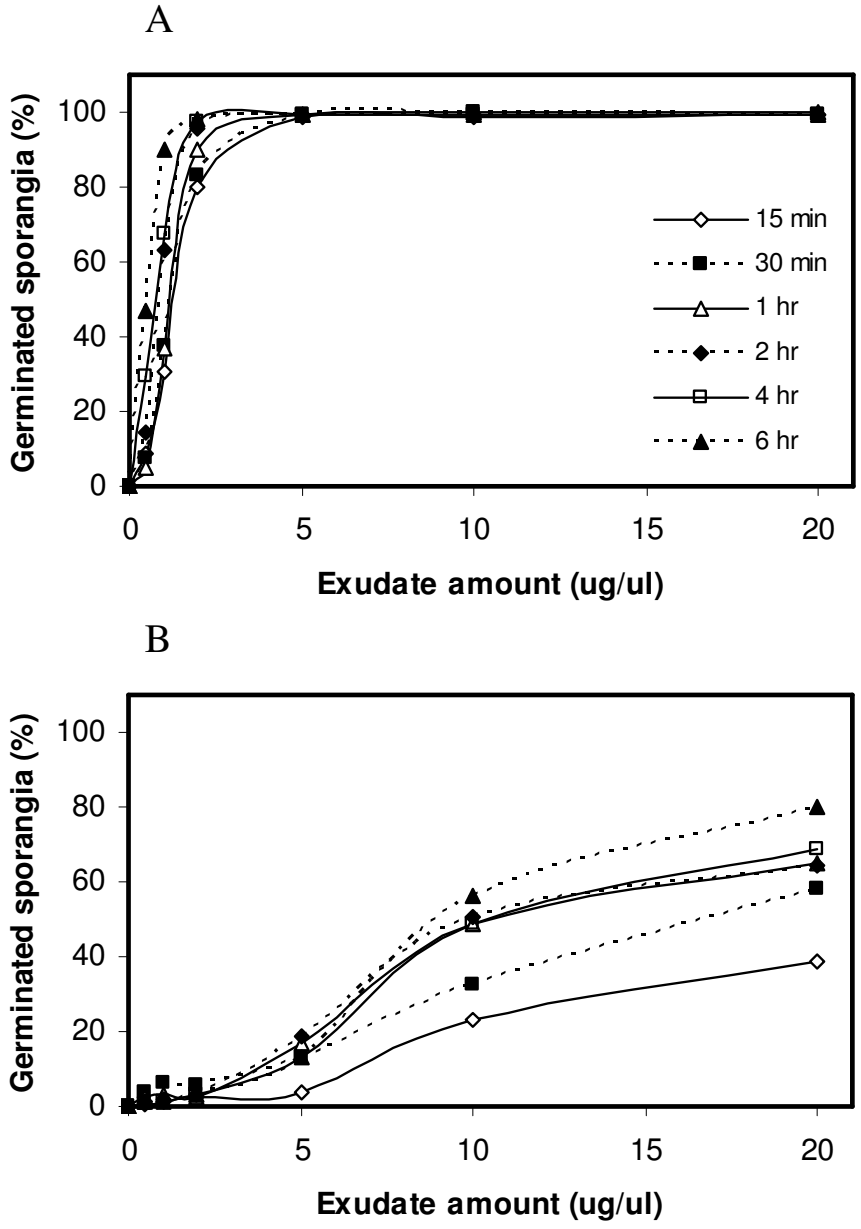

Figure 3.1. *P. ultimum* sporangial germination in response to corn (A) and cucumber (B) seed exudate. Each marker represents the mean of 9 observations.

Medium and long-chained saturated fatty acids (SFAs) predominated in both corn and cucumber exudate, and made up approximately 70-80% of all FAs detected. The most abundant SFAs found in both corn and cucumber exudate were palmitic (C16:0) stearic (C18:0), and lauric (C12:0) acids **(Figure 3.3)**. Other SFAs found in both exudates included caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), and behenic (22:0) acid, but these rarely represented more than 10% of total FAs.

Sugars in seed exudates

Corn seed exudates contained extremely high amounts of sugars compared to cucumber. Corn seeds released 94 µg of sugars per seed within 15 min compared to 0.7 µg/seed from cucumber **(Table 3.3)**. By 6 hrs corn and cucumber seeds released 729 µg/seed and 3.4 µg/seed, respectively. Corn seeds released up to 214 times more sugar than cucumber over the 6 hr sampling period. Sugars accounted for up to 41% of the total corn seed exudate (w/w) whereas it was less than 5% of the total dry weight of cucumber seed exudate.

Glucose, fructose and sucrose were the most abundant sugars detected in corn seed exudates at all collection times **(Figure 3.4A)**. These three sugars were also significant in cucumber exudate even though their total contribution to the exudate sugars was greater in corn than in cucumber seed exudates **(Figure 3.4B)**. Combined levels of glucose, fructose, and sucrose accounted for 71-83% of all corn exudate sugars whereas the same three sugars comprised only 37-60% of all cucumber sugars, depending on the time sampled.

Glucose, the most abundant sugar found in corn seed exudate, accounted for 31-46% of all exudate sugars. The glucose released within 15 min of imbibition accounted for nearly 10% of the total dry weight of exudate released by 15 min. By 6 hr, glucose accounted for 16% of the total dry weight of exudate. The greatest levels of fructose and sucrose (89 and 169 µg/seed, respectively) were detected after 6 hr.

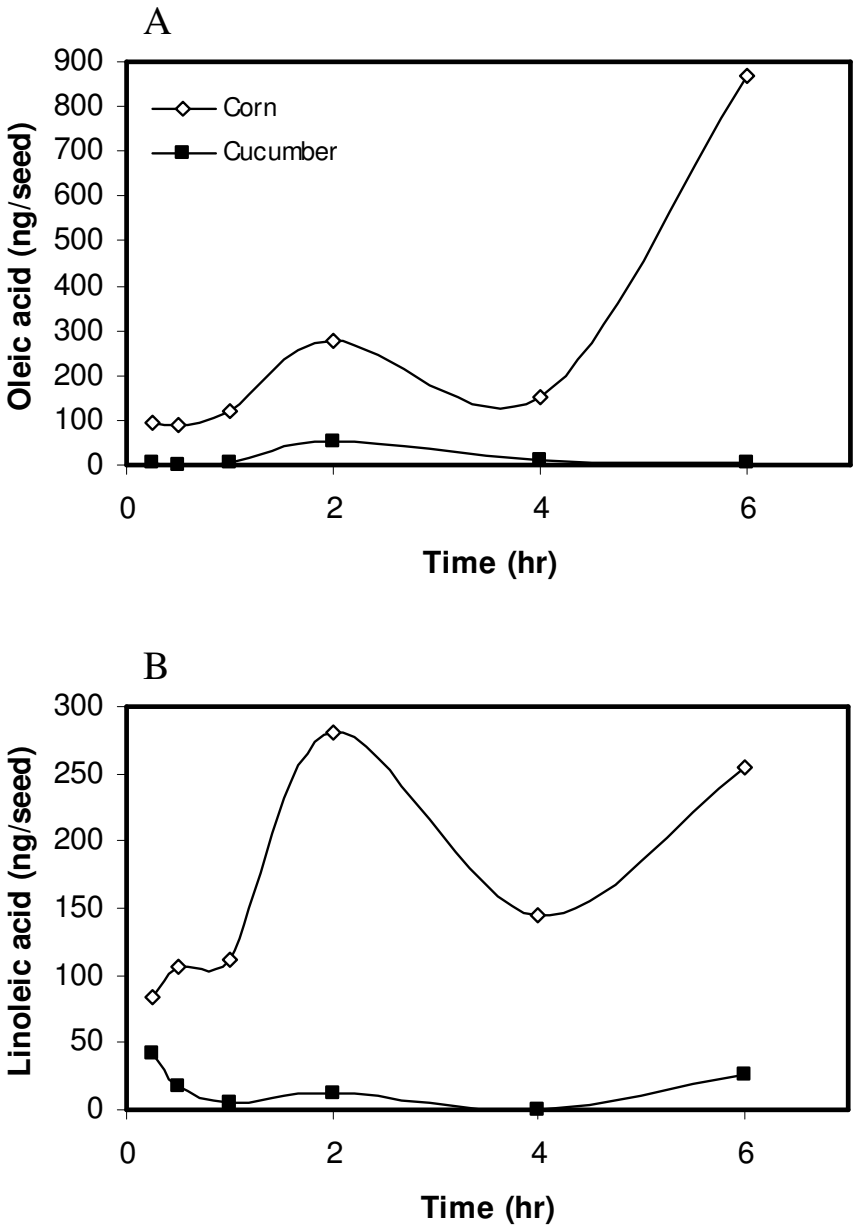

Figure 3.2. Oleic (A) and linoleic (B) acid released by corn and cucumber seeds during the first 6 hrs of germination. Each marker represents the mean of 6 observations. Note the different y-axis.

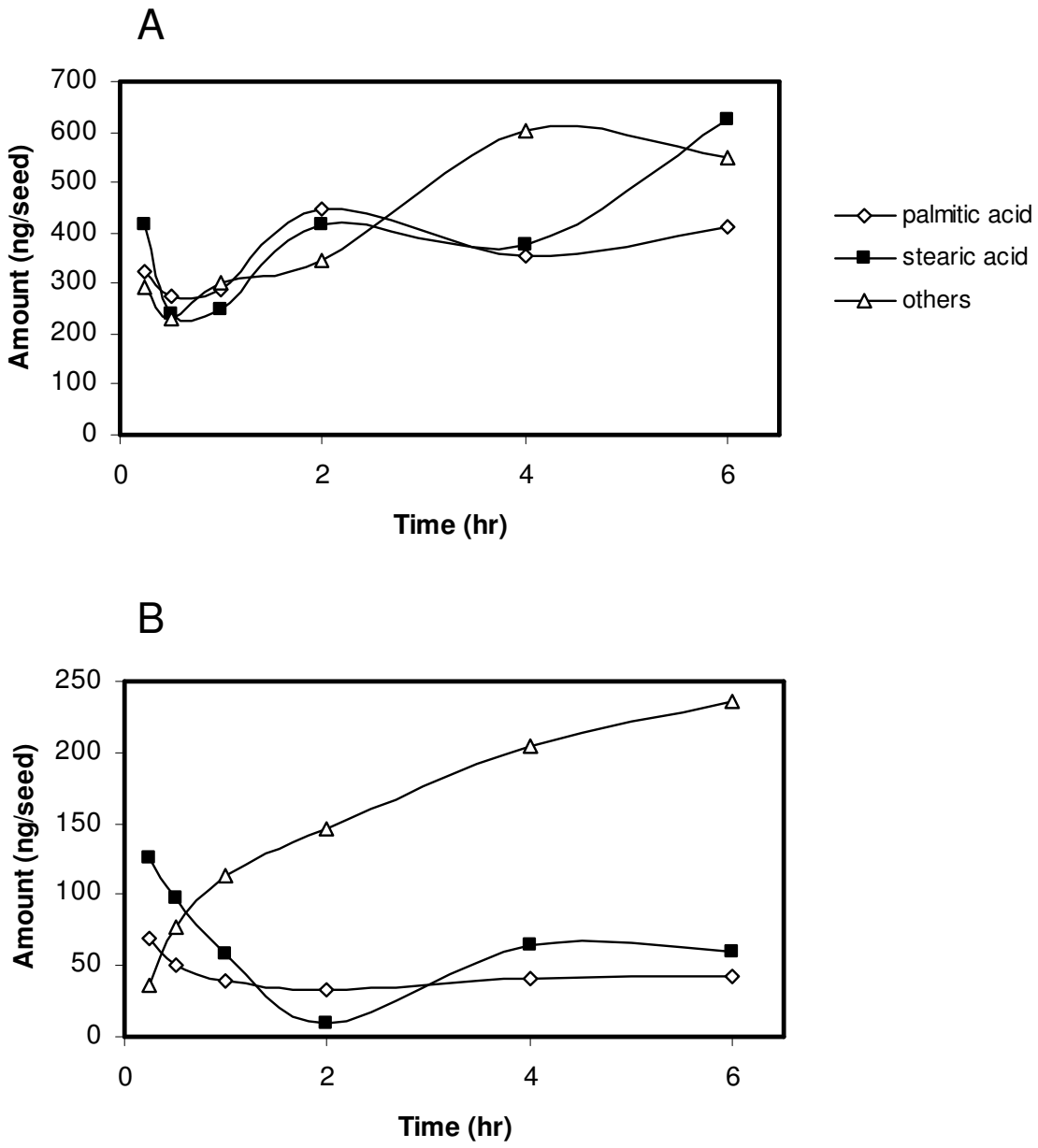

Figure 3.3. Saturated fatty acids released by germinating corn (A) and cucumber (B) seeds during the first 6 hrs after sowing. Others mean caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), and behenic (22:0) acid. Each marker represents the mean of 6 observations. Note the different y-axis.

Other sugars detected in corn seed exudate included arabinose, cellobiose, galactose, lactose, maltose, mannitol, rhamnose, ribose, trehalose, and xylose. However, the combined levels of these sugars were similar to levels of either fructose or sucrose, but significantly lower than for glucose.

Fructose was the most abundant sugar released in cucumber exudates **(Figure 3.4B)** comprising between 24-37% of all cucumber exudate sugars. The bulk of fructose was released within the first 30 min of germination (seeds released 0.27 and 0.78 µg/seed at 15 and 30 min, respectively). Although glucose and sucrose were found to be significant components of cucumber seed exudate, they were detected at lower concentrations than for fructose (0.029 and 0.018 µg/seed detected at 15 min of seed imbibition). Cucumber seed exudates contained all the sugars also found in corn seed exudate, albeit at lower levels, with the exception of mannose and lactose, which were absent in corn seed exudate.

DISCUSSION

Fatty acids and sugars are seed exudate components that have the ability to regulate *E. cloacae* control of *Pythium* sporangial germination. We analyzed the temporal fatty acid and sugar release of corn and cucumber seeds to generate data on differences in seed exudation between these two seeds in order to explain how underlying differences in corn and cucumber exudation might result in differential control by the bacterium. The success of *E. cloacae* as a biocontrol agent stems from its ability to degrade ULCFA germination stimulants released by cucumber and cotton which effectively reduces *Pythium* sporangial activation and germination which consequently suppresses subsequent disease development (37) (Windstam and Nelson, *unpublished*). Furthermore, common exudate sugars such as fructose, glucose, and sucrose in excess of 5 mM completely shuts down *E. cloacae* degradation of linoleic

acid or germination stimulants in seed exudate *in vitro* which results in high levels of *P. ultimum* sporangial germination (Windstam and Nelson, *unpublished*).

The primary hypothesis we formulated was that corn seeds release much greater quantities of sugars that are repressive to *E. cloacae* fatty acid degradation than cucumber seeds during the first few hours of seed germination, which would explain the inability of *E. cloacae* to reduce sporangial activation. Alternatively, corn releases such large quantities of ULCFA compared to cucumber, that bacteria may not be able to degrade enough fatty acids to reduce sporangial activation.

Present data on fatty acid and sugar release by seeds combined with observations on the effect sugars have on *E. clo*acae fatty acid degradation favors sugar repression of fatty acid degradation as an explanation for why *E. cloacae* is unable to interfere with *Pythium* sporangial activation in the corn spermosphere. Simple sugars such as fructose, glucose and sucrose blocks the degradation of linoleic acid when supplied in amounts exceeding 5 mM with lower concentrations reducing degradation of linoleic acid by *E. cloacae* (Windstam and Nelson, *unpublished*). As a result of the reduced fatty acid degradation by *E. cloacae*, *Pythium* sporangia are able to germinate at high levels. The combined levels of glucose, fructose, and sucrose found in corn seed exudate by 30 min correspond to 1.6 M **(Figure 3.4)** if assuming a spermosphere that is extending 10 mm from the seed surface which is almost three orders of magnitude more than is required for shutting down fatty acid degradation completely. Furthermore, even though corn releases 4 to 5 times more of the germination stimulatory ULCFA's linoleic and oleic acid than cucumber, the levels of two ULCFA are too low to result in a reduced rate of fatty acid degradation by the bacterium (Windstam and Nelson, *unpublished*).

It is also important to note that extremely high amounts of fructose, glucose, and sucrose are released in corn seed exudate by 15 min after imbibition is initiated

(Table 3.3 and Figure 3.4). Exudate released by corn seeds within this timeframe is enough to initiate significant sporangial germination which *E. cloacae* is unable to interfere with (Windstam and Nelson, *unpublished*). These sugars are therefore present at times corresponding to when the bacterium is incapable of reducing sporangial activation. The temporal release of such a great amount of sugars in the corn spermosphere allows us to conclude that sugar repression of *E. cloacae* ULCFA degradation is the most likely reason why *E. cloacae* is unable to interfere with sporangial activation and thus unable to protect corn seeds from *Pythium* infection.

The ability of *E. cloacae* to protect cucumber seeds against *Pythium* infection lies in the significantly lower amounts of repressive sugars released. Depending on the time point sampled cucumber seed exudate contains up to 1140 times less fructose, glucose, and sucrose, respectively, than corn seeds. At such minor quantities sugars are no longer repressing degradation of ULCFA germination stimulants and the bacterium is therefore able to drastically reduce exudate induction of sporangial activation in the cucumber spermosphere.

It is not unusual that sugars have such a profound impact on activities and gene expression of plant associated bacteria (2, 12, 13, 24, 27, 28, 32). Specific sugars such as glucose can either up regulate or down regulate the biosynthesis of antibiotics involved in control of Oomycete plant pathogens which are produced by primarily *Pseudomonas* spp. (12, 13, 24, 32). Sugars greatly influence *E. cloacae* biocontrol activities in the spermosphere and carbohydrate metabolism is an essential function to the bacterium for its growth and colonization of seeds (26-30). It has been shown for *E. cloacae* that knocking out key genes, such as *pfkA* encoding phosphofructokinase, in sugar catabolic pathways limit bacterial growth and colonization of certain seeds such as cucumbers (27, 28). The *pfkA* mutant was not affected in its colonization of corn, which could be explained by the fact that corn releases great quantities of

fructose which was one of the few sugars the mutant strain was still capable to grown on. Cucumber on the other hand, releases significantly less fructose than corn and the quantities were clearly limiting to *E. cloacae* colonization.

Previous studies have implicated sugars as important regulators of *E. cloac*ae biocontrol activities, primarily through association (14, 21). *E. cloacae* is unable to protect seeds such as corn and pea that tend to release great quantities of sugars during their germination (14, 27). When adding sugars such as glucose to seeds that are normally protected by *E. cloacae* it abolishes bacterial biocontrol resulting in greatly increased *Pythium* infection levels (21). Recent and present studies explain how sugars may be involved in regulating *E. cloacae* biocontrol activities and when during *Pythium* pathogenesis this regulation occurs. The main stage in *Pythium* disease development targeted by the bacterium is the induction of sporangial activation elicited by exudate, which occurs very soon after sowing but is different depending on the seed species (Windstam and Nelson, *unpublished*). Sugars within this temporal exudate fraction are critical for determining whether or not ULCFA degradation is repressed. The quantities of fructose, glucose, and sucrose released by corn seeds during sporangial activation are large enough to repress fatty acid transport and degradation in *E. cloacae* which is why bacterial biocontrol is not expressed on corn. Cucumber on the other hand releases significantly less sugar than corn and at levels that are no longer repressive. High levels of exudate sugars released by some plant species can therefore explain why some plant species are not protected by *E. cloacae*.

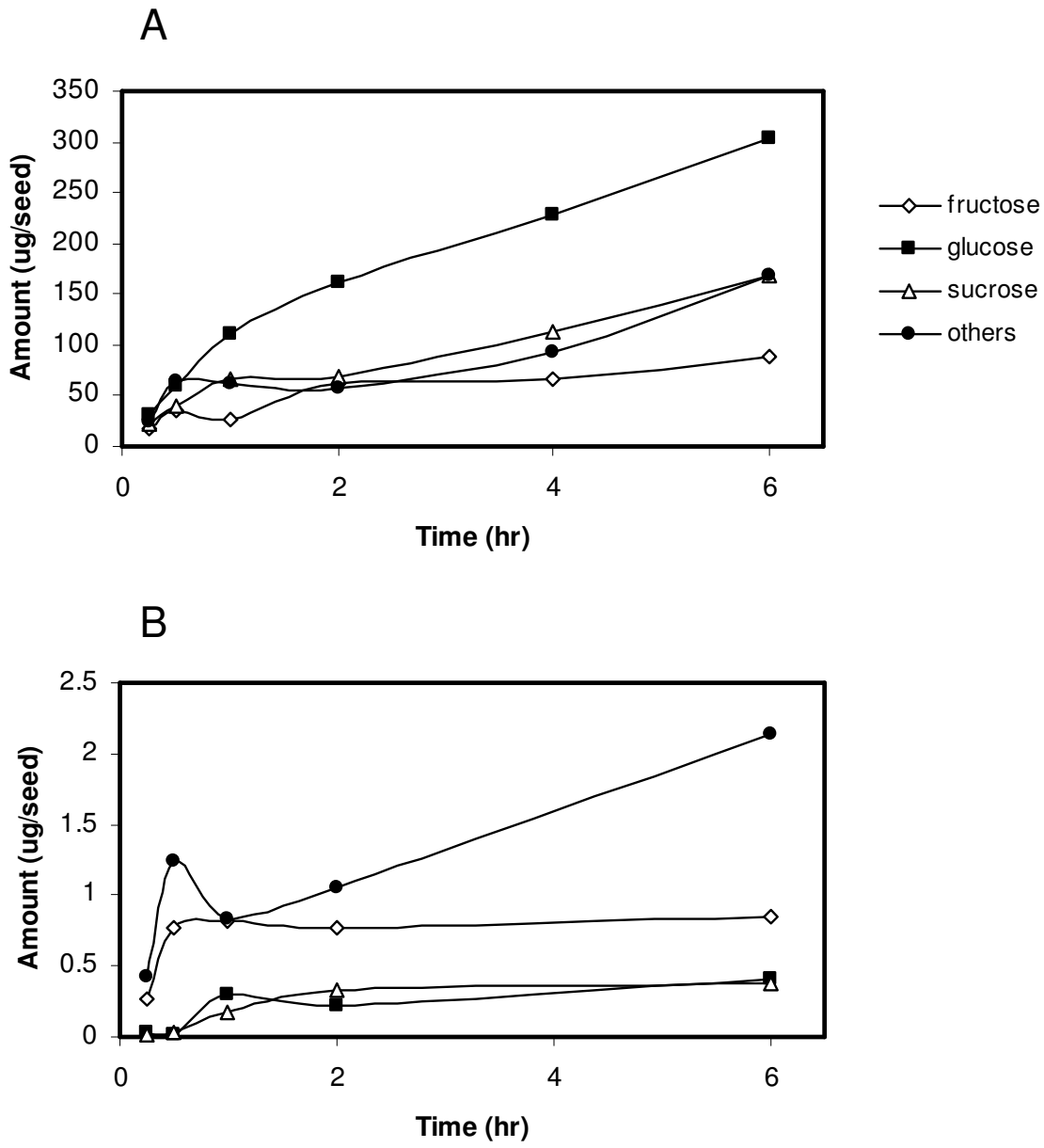

Figure 3.4. Fructose, glucose, sucrose, and other sugars released by germinating corn (A) and cucumber (B) seeds during the first 6 hrs after sowing. Other sugars mean arabinose, cellobiose, galactose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, trehalose, and xylose for corn. The same sugars except lactose and mannose are included in the same category for cucumber. Each marker represents the mean of 6 observations. Note the different y-axis.

Table 3.3. Total amount of saturated and unsaturated fatty acids (C8-C22) and sugars released by corn and cucumber seeds

	Fatty acids (ug/seed)		**Sugars (ug/seed)**	
Collection time	**Corn**	**Cucumber**	**Corn**	**Cucumber**
15 min	1.22 (± 0.588)[a]	0.279 (± 0.150)	93.7 (± 49.1)	0.739 (± 0.154)
30 min	0.943 (± 0.371)	0.245 (± 0.139)	202 (± 73.4)	2.08 (± 1.06)
1 hr	1.04 (± 0.433)	0.220 (± 0.049)	167 (± 84.0)	2.12 (± 0.357)
2 hr	1.81 (± 1.28)	0.250 (± 0.053)	350 (± 82.6)	2.50 (± 0.551)
4 hr	1.59 (± 0.422)	0.319 (± 0.088)	501 (± 143)	Nd[b]
6 hr	2.65 (± 2.48)	0.365 (± 0.184)	729 (± 224)	3.41 (± 1.07)

[a] Mean amount (n=6) ± S.D.
[b] Not determined

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GENERAL CONCLUSIONS

Not everything that I attempted during my six years as a doctoral student is covered in the chapters of this dissertation. There is plenty of material and experiments that either ended up in the appendix or in a desk drawer. My aim with the general conclusions is to cover what I have learned from the collected work that went in to writing the preceding chapters, both the presented and the unseen as the latter also helped shape my thinking about the biological system that I worked with. Hopefully it can also help any future student in the field to identify pertinent questions and experimental approaches that are feasible, instead of repeating some of my mistakes.

Starting out I was convinced that creating and utilizing biosensors for reporting expression of bacterial genes involved in sugar and fatty acid metabolism would help answer why *E. cloacae* is not able to protect some seeds like corn against *Pythium* infection. Because sugars and fatty acids are key exudate molecules that are capable of regulating biocontrol activities of *E. cloacae* that directly impacts *P. ultimum* pathogenesis I reasoned that it was important to attempt to understand what the bioavailability of these compounds were and when during seed germination they modulated expression of *E. cloacae*. Biosensors offered the opportunity to study the latter in natural environments such as the spermosphere.

Through Steven Lindow I acquired sucrose and fructose reporting *Erwinia herbicola* biosensors. I got both ice nucleation, *inaZ,* and *gfp* reporter constructs and Dr Lindow also very generously donated reporter vectors which could be used to fuse promoters of my own choice to either *gfp* or *inaZ*. I started out working with the sugar responsive *inaZ* biosensor in cultures amended with standardized concentrations of various sugars as well as following the expression of *inaZ* in the spermosphere of corn

and cotton, as I initially focused on these two hosts. Very soon I learned that due to the vastly different ranges of sucrose and fructose that the promoter responded to, it was practically impossible to achieve any specificity in the detection of each sugar as well as reliable sugar concentration estimates. There was also the issue of a certain lagtime required before significant *inaZ* reporter gene expression could be detected in response to sucrose and fructose. For instance, in sand microcosms amended with sucrose that induce full *inaZ* expression in culture, expression of the reporter gene increased until 10 hrs after inoculating the cosm with biosensor cells. Even though at this point I lacked an appreciation for how quickly *Pythium* sporangia become activated by seeds and thus how quickly *E. cloacae* cells are required to express their biocontrol activities for reduced *Pythium* infection to result, I did know that 10 hrs was way beyond the timeframe required for the pathogen sporangia to germinate and colonize seeds.

Parallel to the sugar biosensor trials I was attempting to generate *E. cloacae* biosensor strains with the *inaZ* reporter gene fused to the *fadAB* promoter. A benefit of using *inaZ* is that it is a very sensitive reporter which is very useful when examining the expression of *fad* genes as they are usually only up regulated by 8-10 fold in the presence of exogenous LCFA. Unfortunately I was never able to generate bioreporter strains and a couple of years later I learned that when working with sticky end cloning you loose the sticky ends of the PCR generated promoter fragment and the linearized vector when you gel purify. My advice to any future student interested in creating biosensor strains is to avoid gel purification altogether or to link adapters to the blunt ended fragments that result from the gel purification step.

Working with the sugar responsive *inaZ* biosensor made it painfully clear that I did not fully comprehend the temporal aspects of *Pythium* pathogenesis and in order to understand how and when *E. cloacae* controls *Pythium* infection, I needed to more directly examine the disease development of the pathogen on the differentially

protected hosts that I had chosen to study. Preliminary studies showed that the temporal sporangial development was quite different when comparing different hosts such as corn and cucumber. At this point I also decided to focus on these two hosts, primarily because they displayed less variability in their sporangial germination induction, imbibition, exudation, and germination rates than other species that I tested (cotton and pea). Unraveling the temporal sporangial development in the corn and cucumber spermosphere made me wonder which temporal exudate fractions from these two seeds actually set germination in motion. I also reasoned that if *E. cloacae* is to be able to reduce *Pythium* sporangial germination in the spermosphere it needs to degrade the germination stimulants of the temporal seed exudate fractions that activate sporangia and slate them for germination.

Assessing the hitherto unknown temporal sporangial activation in the corn and cucumber spermosphere revealed a drastically compressed timeframe for *Pythium* sporangial development. For instance, exudate released by corn seeds within 30 min of sowing was enough to fully activate sporangia whereas it takes exudate released by corn for 2 hrs to elicit full activation. Based on these observations we predicted that *E. cloacae* would have to degrade the exudate germination stimulants released within 30 min and 2 hrs of sowing corn and cucumber, respectively, if reducing sporangial germination is a prerequisite for the biocontrol of the bacterium.

It turns out that adding *E. cloacae* to the spermosphere does result in significantly reduced sporangial activation in response to cucumber, but not corn. Bacterial fatty acid transport and degradation was found to be critical traits for reducing sporangial activation in the cucumber spermosphere. In order to determine whether or not this differential in terms of *E. cloacae* ability to interfere with sporangial activation had any bearing on further disease development of *Pythium*, I

decided to study the seed colonization behavior of the pathogen in the presence of the bacterium.

As it turns out, *E. cloacae* did suppress *Pythium* colonization and biomass generation in and on cucumber seeds, but not on corn seeds. Further experiments (*E. cloacae* spike and sporangial dose experiments) showed that *E. cloacae* interference with sporangial activation was directly related to reductions in *Pythium* biomass on cucumber.

Once the timeline for *Pythium* sporangial development and seed colonization in the spermosphere of corn and cucumber was established and it was determined that *E. cloacae* biocontrol is primarily due to its ability to interfere with sporangial activation, I started examining the temporal exudate release of the two hosts. I had attempted to collect sequential corn seed exudate by placing seeds in a column filled with glass beads and then drip irrigating the column and collecting the effluent at hourly intervals, but found that it was difficult to accurately quantify the amount of exudate released by the seeds. This was mainly due to the low number of seeds utilized (only 5) so experiments were scaled up to work with 3 g aliquots of seeds and collect exudate in a cumulative fashion for up to 6 hrs. Both corn and cucumber released exudate within 15 min of starting imbibition but corn seeds release greater exudate quantities than cucumber. Cucumber seeds essentially release the bulk of their exudate within the first 30 min of sowing whereas corn seeds continue to release increasing amounts of exudate over the first 6 hrs. Furthermore, corn seed exudate is more stimulatory to *Pythium* sporangial germination than cucumber meaning that less corn seed exudate is required than cucumber exudate for eliciting a certain germination level.

Subsequent analysis of exudate fatty acids showed that corn indeed releases 4 to 5 times more ULCFA than cucumber which may explain why corn seed exudate is

more stimulatory than cucumber. The levels of ULCFA in corn though are not so high as to explain why *E. cloacae* is unable to degrade fatty acids in corn exudate. Not yet published data on the effect of fructose, glucose, and sucrose on *E. cloacae* degradation of linoleic acid had demonstrated that either sugar in concentrations of 5 mM or higher would effectively shut down bacterial fatty acid degradation and thus prevent the bacterium from reducing the resulting *Pythium* sporangial germination. At this point we knew when *E. cloacae* expresses its biocontrol activities on cucumber and when the bacterium fails to effectively interfere with *Pythium* disease initiation and development.

Utilizing above timeline we could analyze the sugars released by corn and cucumber seeds at timepoints during seed germination that are relevant for *E. cloacae* biocontrol to determine if corn released sugars at quantities that were sufficient for repressing degradation of ULCFA and thus allow sporangial germination to take place in the corn spermosphere. Within 15 min corn seeds do release large quantities of fructose, glucose, and sucrose that exceed the amount required for repressing *E. cloacae* fatty acid degradation whereas cucumber releases several orders of magnitude less of repressive sugars.

I still believe that examining the expression of *E. cloacae fad* genes in the spermosphere of differentially protected plants is the most direct way of testing if different host seeds regulate this biocontrol trait differently. With the advent of quantitative PCR there is now also an appropriate tool for measuring gene expression albeit by destructively harvesting the spermosphere. A benefit of using biosensors, especially those employing *gfp* with a short halflife, is that it allows one to examine expression of genes of interest in a non destructive manner in the same population of cells across time. It would also allow the assessment of reporter gene expression in a spatial manner. The major hurdle on such a project would be if there is enough

sensitivity in detecting the *gfp* expression in response to exogenous LCFA since the up regulation is low and there may be potentially high background fluorescence when viewing *E. cloacae* cells in and on other biological materials such as soil and seeds.

Ultimately this should be linked to chemical data analyzing the effects of *E. cloacae* on the fatty acid and sugar profile of differentially protected seeds in order to measure the direct effects of *fad* gene expression of the chemical exudate composition. Since there is considerable variation to contend with when working with seeds, a recommendation would be to scale up experiments as much as possible to minimize the amount of variability. With the current data collected it would also be very useful to spike the cucumber spermosphere with accurate amounts of sugars found in corn exudate to see if this abolishes bacterial interference with sporangial activation and pathogen biomass suppression.

APPENDIX

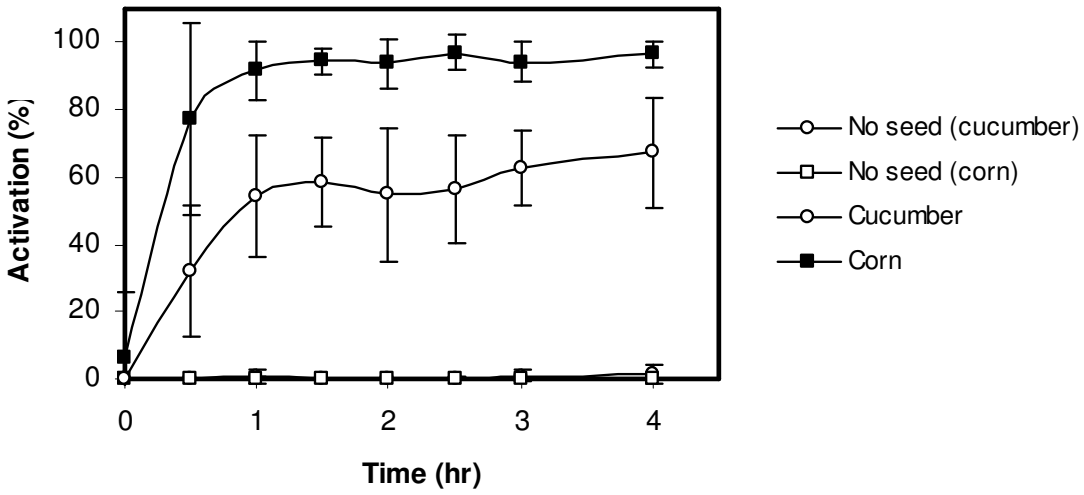

Figure A1. *Pythium* sporangia activation in the corn and cucumber spermosphere at 10% water content during the first 4 and 6 hours of seed germination, respectively. Each point with the error bars represents the mean ± S.D. of 15 observations from 3 repeated experiments. See details on materials and methods on page 45-46.

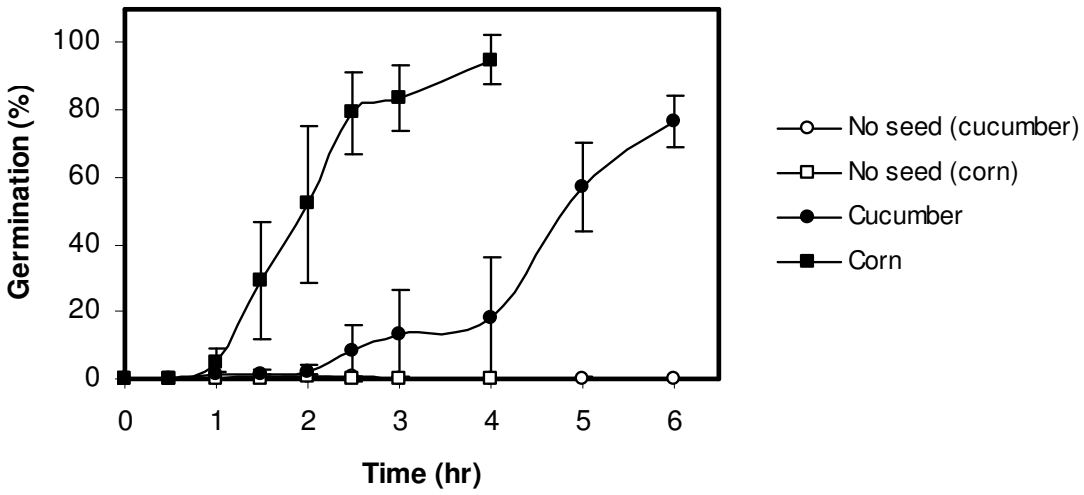

Figure A2. *Pythium* sporangia germination in the corn and cucumber spermosphere at 10% water content during the first 4 and 6 hours of seed germination, respectively. Each point with the error bars represents the mean ± S.D. of 15 observations from 3 repeated experiments. See details on materials and methods on page 45-46.

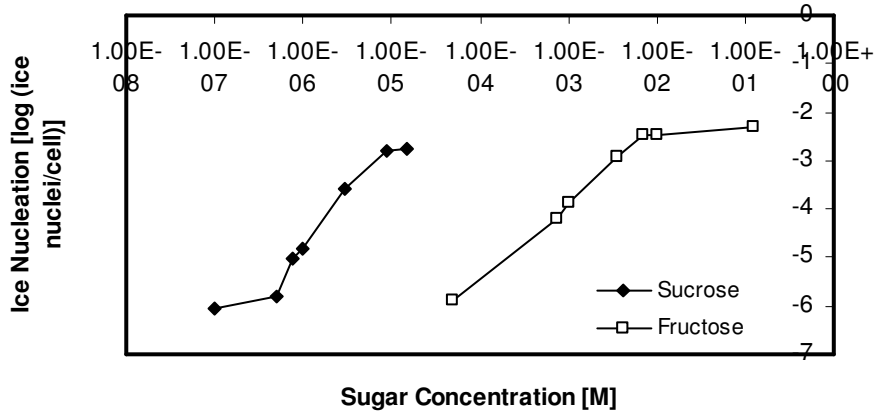

Figure A3. The ice nucleation activity of a fructose and sucrose responsive promoter *srcY* of *Erwinia herbicola* fused to the reporter gene *inaZ*, in response to standardized concentrations of either sugar (3). Bioreporter cells were grown in Minimal A medium amended with 0.2% casamino acids for eighteen hours (2). Cells were harvested by spinning and rinsed twice with Tris buffered saline (TBS, pH 7.2). Cells were then incubated with inducing sugars in TBS for 3 hours and thereafter harvested. Harvested cells were diluted, plated onto LB, and evaluated by the droplet freezing assay for assaying the ice nucleating activity. InaZ expression was assessed by the droplet freezing assay (1). Forty 10µL droplets were placed on a foil sheet, overlaid with paraffin, floating on a coolant bath at -7°C. After incubation for 5 minutes the number of frozen droplets was enumerated. Fractions of frozen droplets were used to calculate the ice nucleation activity which was normalized to a nuclei/cell basis and log transformed. Each point is the mean of 3 replicates.

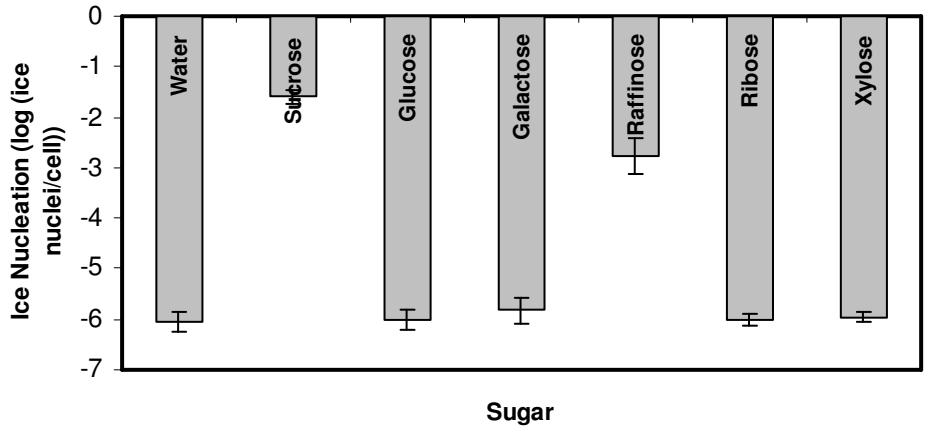

Figure A4. InaZ expression in response to glucose, galactose, raffinose, ribose, and xylose. Water served as the negative control and sucrose as the positive control. Each bars with error bars represents the mean ± S.D. of 3 observations. See legend of figure A3 for methodology.

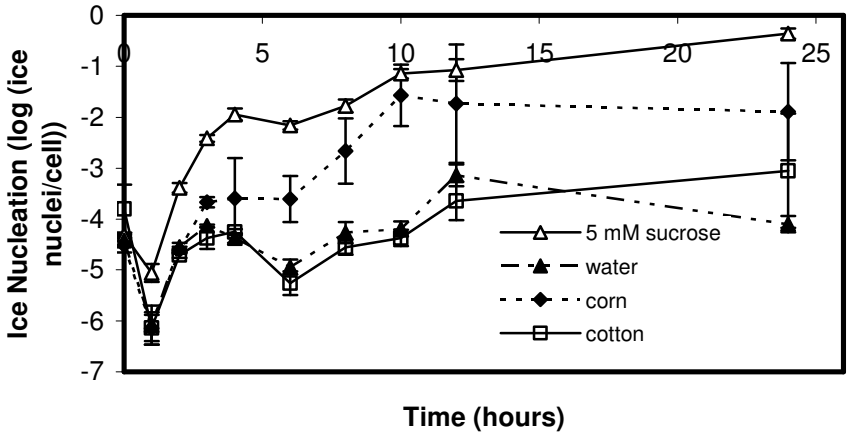

Figure A5. *InaZ* expression of a fructose and sucrose responsive promoter of *E. herbicola* in the corn and cotton spermosphere. Seeds were sown in 4 ml sterile sand (particle size 0.5-2 mm) and inoculated with bioreporter cells at a final water concentration of 18% (v/v).Water served as the negative control and sucrose as the positive control. Each marker with error bars represents the mean ± S.D. of 5 observations. See legend of figure A3 for methodology.

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1. **Loper, J. E., and S. E. Lindow.** 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. Applied And Environmental Microbiology **60:**1934-1941.

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3. **Miller, W. G., M. T. Brandl, B. Quinones, and S. E. Lindow.** 2001. Biological sensor for sucrose availability: Relative sensitivities of various reporter genes. Applied and Environmental Microbiology **67:**1308-1317.