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CHARACTERIZATION OF THE *STENOTROPHOMONAS MALTOPHILIA* C3 ANTAGONISM SYSTEM AGAINST *BIPOLARIS SOROKINIANA* ON TALL FESCUE

by

Zhongge Zhang

A DISSERTATION

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Under the Supervision of

Professor Gary Y. Yuen

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DISSERTATION TITLE

Characterization of the Stenotrophomonas maltophilia C3 Antagonism System against Bipolaris sorokiniana on Tall Fescue

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Zhongge Zhang, Ph. D.

University of Nebraska, 1999

Advisor: Gary Y. Yuen

Stenotrophomonas maltophilia C3 inhibited the germination of conidia and the growth of germtubes of *Bipolaris sorokiniana* (Sacc.) Shoemaker on tall fescue (*Festuca arundinacea* Schreb.) leaves. It reduced the incidence and severity of leaf spot disease caused by *B. sorokiniana* under growth chamber and field conditions. Chitinolysis was found to be one mechanism of action. Transposon mutants of C3 that are chitinase deficient or have reduced chitinase production colonized grass to lower levels and provided reduced disease protection in comparison to the wild type. Chitinase activity on grass leaves and bean blossoms colonized by C3 was higher than on the non-colonized plant parts; the addition of chitin with C3 further increased chitinase activity. When a chitinase-minus mutant was applied to plant parts with or without chitin, no additional chitinase activity was detected over the controls. Extracts from C3-colonized plant parts had antifungal activity, and this property was related to

chitinase activity. Chitinase production by C3 was induced *in vitro* by chitin or chitin-containing fungal cell walls. Chitinolytic fractions of C3 in broth cultures, that were partially purified by chitin affinity chromatography, were more antifungal than non-chitinolytic fractions. They also exhibited strong exochitinase and slight endochitinase activities. When the major chitinolytic fraction was subjected to SDS-PAGE, five proteins were revealed (25, 32, 48, 65 and 75 KDa). Only the N-terminal amino acid sequence of the 32-Kda protein showed homology to known bacterial chitinases. Five chitinase-active bands were detected when the gels were probed with a fluorescent chitin substrate. These bands appeared only as 32-Kda and 48-Kda proteins when re-electrophoresed in denaturing gels, suggesting that the smaller proteins were cleavage products of the larger proteins. Several methods were identified that increased disease control efficacy: application of C3 cells in combination with chitin, application of cells in combination with fluid from a chitin-containing broth culture, and use of C3 cells induced to produced chitinase. Thus, improvements in biocontrol can be achieved with knowledge of the modes of action.

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Chapter I

Literature Review

BIOCONTROL OF NECROTROPHIC FUNGAL FOLIAR PATHOGENS

Necrotrophic plant pathogens can only utilize nutrients released from dead tissues and, therefore, have to kill the host tissue before they can live inside the tissue and use nutrients. The life cycle of necrotrophic pathogens is generally composed of three stages: the infection stage during which healthy host tissues are invaded and killed; the saprophytic and dissemination stage during which hyphal growth in host tissues and sporulation occur; and the survival stage during which mycelia, sclerotia or spores persist in a dormant state in plant debris or soil. Generally, infection by spores of necrotrophic pathogens is stimulated by exogenous nutrients such as leachates, pollen grains, insect honeydew, and organic and inorganic dust (2), and penetration depends on production of cutinolytic and cell wall-degrading enzymes. After infection, tissue surrounding the infection site is killed by the toxins or enzymes produced by the pathogen. The pathogen subsequently invades the freshly killed tissues and utilizes the nutrients.

There are different strategies of biocontrol that can be applied to address each of the three life cycle stages. A pathogen may be more susceptible to biocontrol during a particular stage. The first strategy is to degrade or inactivate pathogen survival structures by the antagonist. Some mycoparasites such as *Trichoderma spp* and *Sporidesmiun sclerotvorium* are capable of parasitizing, macerating, or rotting sclerotia (14, 29). Reduction of viability and infectivity of the sclerotia may effectively hinder the epidemics of single cycle diseases, in which sclerotia are the primary inoculum and there is no secondary infection

phase. Reduction in numbers of survival structures, however, provides litter level of benefit in suppressing foliar diseases in which the primary inoculum is produced from sclerotia or mycelia in debris.

The second strategy for biocontrol of necrotrophic diseases is to suppress pathogen infection during the initial pathogenic stage by inhibiting spore germination and germ-tube growth. This strategy has been shown to be effective in preventing infection through wounds of cut flowers and on postharvest produce. On cut rose, Redmond et al (70) found *Exophiala jeanselmei* was capable of preventing infections by *Botrytis cinerea* as effectively as the fungicide ipodione. Some microbial antagonists also have strong potential for biological control of postharvest diseases of citrus, stone fruit and apples, where wounds made during harvest and processing are the main infection sites (22, 91). Several biocontrol products for postharvest disease biocontrol have been commercialized, or are close to commercialization.

The third strategy is to suppress saprophytic growth and sporulation of the pathogen on the surfaces of necrotic tissues, which therefore prevent initiation of secondary infection. The exploitation of antagonistic interactions during the saprophytic stage has the advantage of a significantly longer antagonist-pathogen interaction as compared to interactions during the infection process of fungal spores (26). Conceivably, a longer interaction would potentially increase the effectiveness and reliability of biocontrol by the antagonist. However, the environmental conditions in the phyllosphere are

complex and characterized by low nutrient availability, rapidly fluctuating temperature and moisture, uv radiation and the deposition of agrochemicals (2). These harsh conditions require the antagonist to be highly ecologically competent in order to establish on the leaf surfaces. Therefore, a powerful antagonist should be tolerant to or be adapted to the rapidly changing nutritional and microclimatic conditions of the phyllosphere and have a short lag phase for regrowth. Thus far, bacteria and yeasts have not been found to be effective in inhibiting necrotrophic foliar pathogens during the saprophytic stage of the pathogens owing to their sensitivity to low humidity. A few fungal antagonists have been reported to suppress pathogen colonization of necrotic tissues. Kohl et al (48) compared antagonists to suppress sporulation of *B. allii* on dead onion leaves and only one antagonist, *Ulocladium atrum*, consistently germinated and colonized the dead leaves and significantly suppressed *B. cinerea* leaf colonization and sporulation by 90%. Sutton and Peng (84) showed that *Gliocladium roseum* reduced sporulation incidence and fruit infection of strawberry in the field. The antagonist is able to penetrate the green leaf and to colonize epidermal cells without detrimental effects on the host plant (85). Once the leaf is drying, the antagonist progressively colonizes the dead leaf and excludes *B. cinerea* (84).

MECHANISMS FOR BIOLOGICAL CONTROL OF FOLIAR PATHOGENS

Thorough understanding of the mechanisms by which antagonists suppress disease is critical for the development of reliable and effective biocontrol agents and the improvement of biocontrol methods. In the past two

decades, the mechanisms involved in antagonism against soilborne pathogens were extensively studied (86, 88). The majority of reports on foliar pathogen biocontrol, however, have been limited to descriptions of the phenomenon of disease suppression, and less attention has been paid to the mechanisms responsible for disease suppression. One reason for this is that the study of foliar disease biocontrol overall lags behind that of soilborne disease biocontrol. Another reason may be due to the difficulties in studying the mechanisms behind many foliar disease biocontrol systems. Most biocontrol agents of above-ground diseases are fungi, which are more difficult to investigate by genetic analysis than bacteria. A third reason is that competition, a complicated process involving multiple gene products, is thought to be one of the major modes of action in foliar disease suppression. Nutrient competition is less amenable to investigation by molecular techniques than other mechanisms in which a single factor is usually involved.

For a given antagonist-pathogen-host system, multiple mechanisms may be involved in disease suppression. Although a number of mechanisms for foliar disease biocontrol have been proposed, most have not been tested under *in vivo* conditions. Continued study is needed to elucidate the importance of these mechanisms in disease suppression by the antagonists in the field and to determine their effects on growth and development of the host plants.

Competition. In competition, two species are negatively affected by each other due to the common need for some limiting source. Competition can be determined by measuring both antagonist and pathogen populations after

application. Marois and Broome (59) found that when the yeast *Exophiala jeanselmei* was applied to newly-opened rose flowers in the absence of *B. cinerea*, its population growth rate was higher than when applied with the pathogen. The pathogen growth rate, however, was also reduced when applied with the yeast, and thus biocontrol was achieved. Using ^{14}C-labelled glucose and amino acids, Edwards and Blakeman (24) showed that, in the presence of *B. cinerea* and the antagonist *Sporobolomyces sp*, most nutrients were utilized by the antagonist. Competition is mostly studied with respect to carbon and nitrogen as nutrients. It is also possible for microelements such as Fe^{3+} to be involved in competition.

Competition is likely to be an effective mechanism for disease suppression when the antagonist is added to environments where few other organisms are present or where a large supply of nutrients is available. Such situations include petals and stigmas of newly- opened blossoms, pruning wounds, freshly-cut tree stumps, and young leaves.

Antibiosis. Antibiosis, the production of organic compounds of low molecular weight by one microorganism that inhibit other microorganisms, is one of the most common interactions among microorganisms in nature (27). There are numerous studies dealing with the importance of antibiosis in soilborne pathogen biocontrol (Fravel, 1988) and some are well characterized (86), but only a few examples have been identified in connection with the biocontrol of fungal foliar pathogens. *B. subtilis* CL27 produced two antibiotics of high activity against *B. cinerea* (51). *Epicoccum nigrum* produced antibiotic compounds

effective against *S. sclerotiorum* (34) and *Monilinia laxa* (58). Isolates of *Chaetomium globosum* that produced higher levels of antibiotic in culture were more antagonistic than those producing lower levels of antibiotic to the apple scab pathogen *Venturia inaequalis* on apple seedlings (16). These investigations, while implicating the involvement of antibiotics, are lacking as to biochemical and genetic analysis of the antibiotics.

To be effective, antibiotics must be produced in sufficient quantities and must persist at the site of interaction. A high level of antibiotic production would require multiplication to high population density, favorable environmental conditions, and nutrient availability. Thus, the likely site for antibiosis to occur would be where succession was still in the early stages of development (60). However, antibiotics are usually not stable on leaf surfaces for long periods due to microbial breakdown or adsorption by leaf surface components (44). Furthermore, resistance to the antibiotics in the pathogen population can develop in response to repeated application of the antibiotic-producing antagonist (52).

Parasitism. Mycoparasitism, the parasitism of one fungus by another, depends on contact between antagonist and host, the secretion of enzymes, and the active growth of the mycoparasite into the host (1). Mycoparasitism has been widely reported against necrotrophic soilborne pathogens. *Sporidesmium sclerotivorum* is capable of parasitizing sclerotia of *Sclerotinia minor*, the causal agent of leaf drop of lettuce (1). This parasite produces a huge number of conidia, which germinate through the soil. The germ-tubes can penetrate into

sclerotia of the pathogen and produce haustoria inside the sclerotia (1). *Coniohyrium minitans* parasitized sclerotia of *S. sclerotiorum*, reducing sclerotia by 90% in the field (28, 90). Mycoparasitism for foliar pathogen control is mostly exploited for biocontrol of biotrophic pathogens. Examples include most powdery mildews, which are parasitized by *Ampelomyces quisqualis* (5), and rusts, which are parasitized by *Verticillium lecanii* (81). High levels of parasitism, achieved by frequent application of the mycoparasite, lead to inactivation of rust and powdery mildew mycelia due to enzymatic degradation and reduction of pathogen sporulation.

Mycoparasitism may be limited for suppression of foliar pathogens in that survival of the parasite requires high humidity, the interaction relies on direct parasite-host contact, and parasitism is a slow process.

Destructive lysis is proposed to be an important mechanism for fungal pathogen biocontrol by bacterial antagonists. The interaction between antagonist and pathogen does not require direct contact. Alternatively, the antagonist secrets lytic enzymes that can degrade the cell walls of the fungal pathogen at a distance. These enzymes include chitinase, glucanase, and protease. Because chitinases have been implicated in many biocontrol systems, the importance of chitin and the role of chitinases will be elaborate as below.

Induced resistance. Some nonpathogenic microorganisms, such as certain plant growth-promoting rhizobacteria, can activate physiological responses through the entire plants, making them more resistant to subsequent pathogen

infection. This phenomenon is termed induced systemic resistance (45). Biological control through induced resistance has been reviewed by van Loon et al (87). Induced resistance has been perceived as a potential mechanism of antagonism and, therefore, extensively studied in connection with soilborne disease biocontrol. However, less information is known about its role in biocontrol of fungal foliar pathogens by epiphytic microbes. A bacterial culture containing its metabolites induced resistance in wheat against powdery mildew (19). Wilson et al (92) showed that the yeast *Candida saitoana* induced production of chitinase, known to play a role in the defense of plants against pathogens.

Induced resistance as a mechanism of biocontrol may have the advantage that, once host resistance has been induced, high population densities of the antagonists may no longer be required. It represents a new direction for research on biocontrol of foliar necrotrophic pathogens.

Alteration of leaf surface properties. Some epiphytic microorganisms, e.g. *Pseudomonas spp,* can change wettability of the leaf surface (11). Such changes can affect leaf wetness duration, nutrient distribution, and the attachment of the pathogens to the leaf surfaces (11). *Bacillus brevis*, which was effective against grey mold of Chinese cabbage, decreased periods of leaf wetness by causing water drops to spread and dry. Leaf wetness after overhead irrigation was four times shorter for leaves treated with *B. brevis* (75, 76). It is assumed that reducing water availability by changing surface activity may suppress a wide range of fungi because spore germination and infection

by all foliar pathogens depends on a minimum duration of free moisture on the surface.

Inhibition of pathogen enzyme production. Zimand et al (94) reported that in the presence of the antagonist *T. harzianum*, *B. cinerea* produced reduced levels of various hydrolytic enzymes (e.g. polygalacturonase, pectin lysase, and pectinolytic enzymes) that are important for pathogen infection. This finding suggests a new mechanism by which antagonists can interfere with the pathogenesis by inhibiting the production of pathogenicity factors. More studies are needed to evaluate the role of this mechanism in biocontrol of foliar pathogens.

OCCURRENCE, PRODUCTION AND DEGRADATION OF CHITIN

Chitin is an insoluble, linear β-1,4-linked homopolymer of N-acetylglucosamine (GlcNAc) of varying chain lengths. The chains are stabilized by hydrogen bonds as a highly ordered crystalline structure. Chitin is frequently associated with proteins and may be stabilized further by additional inorganic compounds (65).

Chitin is a major structural component in the cell walls of the true fungi (hyphochytridiomycetes, chytridiomycetes, zygomycetes, ascomycetes, brasidiomycetes, and deuteromycetes), but is absent in the oomycetes (4). Chitin molecules are exposed at the hyphal tips, but are cross-linked and probably overlaid by other polysaccharides and protein layers in mature cell walls (89). Chitin is also present in the exoskeletons of insects, crustaceans,

nematodes, and protozoa (31). Plants, vertebrates and prokaryotes, however, have not been found to contain chitin.

Chitin is the second most abundant biodegradable polymer in nature after cellulose. The annual production of chitin is estimated to be 10^8 tons (65), and the total amount of steady-state chitin in nature is thought to be 10^{10}-10^{11} tons (31). With such enormous annual production of chitin, recycling is needed to prevent a sink in the global carbon and nitrogen. Mineralization of chitin relies heavily on microbial degradation. Breakdown of chitin releases carbon and nitrogen for microbial growth (17). Microorganisms involved in chitin mineralization include prokaryotic organisms (such as gliding bacteria, pseudomonads, vibrios, photobacterium, enteric bacteria, actinomycetes, bacilli and clostridia) and eukaryotic organisms such as fungi (31). Degradation of chitin by microorganisms occurs in diverse locations: oceans, estuaries, freshwater, soils, and the gut of herbivorous and carnivorous animals (31). The rate of degradation depends on population size and activity of chitinolytic microbes, environmental conditions such as temperature, the size of the chitin particles, and purity of chitin (31).

PRODUCTION AND CLASSIFICATION OF CHITINASES

Chitinases catalyze the hydrolysis of chitin. All organisms containing chitin produce chitinases; a major role of chitinases in these organisms is modification of their structural chitin components, as in the cell walls of fungi or in the exoskeletons of insects and invertebrates (32). Some chitin-lacking organisms, such as bacteria, vertebrates (e. g. marine fish) and plants, also

synthesize chitinases. In plants, the primary role of chitinases is thought to provide resistance (15). In other organisms, chitinases serve to digest chitin for nutrition (31).

According to its catalytic properties, a chitinase is categorized into one of three principal types: endochitinase, exochitinase and β-1,4-N-acetylglucosaminidase (36, 72). Endochitinase (EC 3.2.1.14) cleaves chitin randomly at internal sites over the entire length of the chitin microfibril. The products are soluble, low-molecular weight GlcNAc oligomers such as chitobiose, chitotriose and chitotetraose. Exochitinase (EC 3.2.1.14) cleaves chitin in a stepwise fashion resulting in the release of chitobiose as the sole product. β-1,4-N-acetylglucosaminidase (EC 3.2.1.30) cleaves chitin or its oligomers in an exotype fashion, with GlcNAc monomers being the end products.

By 1993, 301 glycosyl hydrolases were identified. Based on amino acid sequence similarities, these hydrolases were classified into 35 families (37). Chitinases can be placed into two families, 18 and 19. Based on comparisons of amino acid sequence features, chitinases have been separated into five classes, I to V. Classes I, II and IV are of plant origin (33). These chitinases share a homologous catalytic domain, in addition to the signal peptide found in all chitinases. Class I chitinases found in both monocots and dicots, consist of a cysteine-rich region (signal peptide) next to the catalytic domain (39). The cysteine-rich domain is important for binding to chitin but not for catalytic activity (67). Most of the class I chitinases contain a carboxyl-terminal signal peptide