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INDUCED ALTERNATIVE OXIDASE ACTIVITY AND ATTENUATION OF VIRULENCE IN RESPIRATORY MUTANTS OF *FUSARIUM OXYSPORUM* F. SP. *BASILICUM* AND *COLLETOTRICHUM COCCODES*

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ABSTRACT

INDUCED ALTERNATIVE OXIDASE ACTIVITY AND ATTENUATION OF VIRULENCE IN RESPIRATORY MUTANTS OF *FUSARIUM OXYSPORUM* F. SP. *BASILICUM* AND *COLLETOTRICHUM COCCODES*

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Mitochondrial hypovirulence associated with cyanide-resistant alternative oxidase activity in the chestnut blight fungus offers a unique opportunity to study both mitochondrial contributions to pathogenicity and the biological control of an important disease causing agent. Recently, it has been shown that mitochondrial hypovirulence can be induced in the laboratory by the treatment of conidia with mutagens that target the mitochondria. In this way, laboratory induced hypovirulent strains can be generated for use in the biological control of virulent chestnut blight strains. I report on attempts to use mitochondrial hypovirulence in other fungal pathogens including *Fusarium oxysporum* f. sp. *basilicum* and *Colletotrichum coccodes* both serious pathogens of their respective hosts, basil and tomato.

To this end, cyanide-resistant respiration was induced in both fungi. Over 15 mutants isolated from *F. oxysporum* f. sp. *basilicum* had high levels of cyanide-resistant respiration accounting for 45 to 100% of total respiration. Twelve of those mutants were significantly less virulent and grew slower than a wild type strain. Transfer of the respiratory phenotype to a benomyl-resistant virulent strain with wild type respiration did not successfully demonstrate the cytoplasmic nature of the respiratory phenotype. However, transmission tests whereby nitrate non-utilizing *(nit)* mutants were used to form heterokaryons showed that at least one strain (Pm33 *nit3*) could transfer the

abnormal respiratory phenotype and hypovirulence to a wild type strain (Fob nitM). It appeared that resulting heterokaryon single-spore cultures likely carried mixed populations of mitochondria (heteroplasmons) as alternative oxidase levels were not as high as the parental mutant phenotype. Attempts at biological control using the respiratory mutants to control virulent strains showed little evidence of control.

High levels of cyanide-resistant respiration was also induced in *Colletotrichum coccodes*. Cyanide-resistant respiration was correlated with reduced virulence, slow growth and abnormal colony morphology in at least one of the mutants isolated.

Attempts at cytoplasmic transmission of the mutant phenotype by forcing heterokaryons between respiratory mutants (Cpm5 *nit3* and Cpm29 *nit3*) and a virulent wild type strain did not demonstrate the cytoplasmic nature of the mutation as the respiratory phenotype did not transfer. This indicates that the respiratory phenotypes were caused by nuclear mutations that induced cyanide-resistant respiration or that mitochondrial defect responsible for high levels of alternative oxidase will not freely exchange cytoplasmically in this fungus.

To my beloved wife, Zehra for her moral support

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

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Chestnut blight, caused by the filamentous fungus *Cryphonectria (=Endothia) parasitica* (Murr.) has eliminated the American chestnut (*Castanea dentate* (Mars) Birch) tree as a dominant or codominant species throughout its natural range in North America. The first infected trees were reported in 1904 in the Bronx zoo (Anagnostakis, 1982). Sprouts of the American chestnut tree have continued to grow from stumps, but before reaching maturity, they are infected, girdled and killed by the pathogen. The pathogen is still present and producing its abundant spores on the sprouts.

European chestnut (*Castanea sativa* Mill.) trees are similar to the American trees and susceptible to blight (Anagnostakis, 1987 and 1988). Blight was transported to Europe and was first observed in 1938 near Genoa, Italy, where an epidemic ensued similar to the epidemic in North America (Anagnostakis, 1987). However, by the 1960's it was obvious that chestnut blight in Europe was less devastating to the European chestnut tree population than it was to the American chestnut tree population in North America.

Discovery of Hypovirulence

Hope for control of this canker disease came in 1965 with the discovery of a variant of the pathogen showing reduced aggressiveness. Braghi, in Italy, observed European chestnut trees in which disease was in remission (Van Alfen et al.1975). The variant, later

found in France and the Pyrenes, was isolated and described as hypovirulent by Grente (Heiniger and Rigling, 1994; Nuss and Koltin, 1990). Reduced sporulation, white pigmentation in culture (compared to orange pigmentation of normal pathogenic isolates) and an abnormal growth rate were other phenotypic characteristics of the hypovirulent isolates (Heiniger and Rigling, 1994; Nuss, 1992). Furthermore, these hypovirulent isolates slowed or stopped canker development induced by virulent strains when they were inoculated around the cankers. It was suggested that the inoculation of the trees by hypovirulent isolates resulted in the conversion of resident virulent strains to the hypovirulent phenotype (Nuss, 1992; Heiniger and Rigling, 1994). Once a canker had been successfully cured by treatment with a hypovirulent strain, much of the fungal mycelium in the original virulent infection appeared to be converted to the hypovirulence phenotype.

Description of Hypovirulence

Grente and Sauret (1960) described the behavior of hypovirulent strains in culture (Anagnostakis, 1987). Hypovirulent strains segregated, yielding normal looking strains; however, normal, virulent strains never segregated to hypovirulent strains. Grente and Sauret (1969) suggested that the hyphae of the virulent strain anastomosed with hyphae of the introduced hypovirulent strain and a genetic determinant in the cytoplasm of the hypovirulent strain was transferred to the virulent strain. The cytoplasmic mode of hypovirulence transfer was genetically demonstrated by pairing auxotrophic strains of *C. parasitica*. A hypovirulent lysine auxotroph and a virulent methionine auxotroph were paired by inoculating the strains side-by-side in chestnut stems. Ninety days later,

methionine auxotrophs from the canker were recovered and found to be hypovirulent, indicating the hypovirulent phenotype was transferred from the lysine auxotroph. Additional evidence for the cytoplasmic nature of hypovirulence was provided when hypovirulent methionine and virulent arginine auxotrophic strains were paired to form a heterokaryon. Single-conidial isolates from the heterokaryon required either methionine or arginine and all were hypovirulent (Grente and Sauret, 1969; Anagnostakis, 1982).

The first hypovirulent strains of *C. parasitica* in North America were isolated from abnormal cankers from a chestnut grove near Rockford, Michigan in 1976 (Elliston et al. 1977). Fulbright et al. (1983) confirmed the presence of hypovirulent strains in different locations throughout Michigan and provided evidence for ongoing biological control.

Although it was first associated with C. *parasitic* hypovirulence has been found and studied in other fungal systems such as *Ophiostoma ulmi* (Brasier, 1983), *Sclerotinia sclerotiorum* (Boland, 1992) and *Helminthosporum victoriae* (Lindberg, 1960). In these systems, hypovirulence appears similar to hypovirulence in *C. parasitic* in that it was characterized by slow growth and abnormal colony morphology. Also, a transmissible cytoplasmic element is thought to be a factor in reduced virulence.

Hypovirulence and Vegetative Incompatibility

Though it was clear that virulent strains were converted to hypovirulent, biological control by introduced transmissible hypovirulence has been less successful in North America than in Italy and France. Vegetatively incompatible strains of the pathogen might explain the failure of some cankers to be controlled if it blocks the transfer of the cytoplasmic determinants of hypovirulence. It is thought that vegetative incompatibility

results in the failure of hyphal anastomosis between incompatible strains. Anagnostakis estimated that between five and seven nuclear genes determine vegetative incompatibility. Two vegetatively compatible strains freely undergo anastomosis if they have the same alleles at each vegetative incompatible locus (Nuss, 1992 and Anagnostakis, 1987 and 1977).

It has been thought that the ability to form anastomosis increases as the number of heterogenic alleles decreases (Anagnostakis and Waggoner, 1981). However, vegetative incompatibility may be more complex than first reported. Huber and Fulbright (1992) reported that individual vegetative incompatibility genes in *C. parasitica* may have specific effects upon the transmission of cytoplasmically carried genetic elements and that a two gene difference can be more permissive to the transmission than a particular one gene difference. Since the hypovirulence phenotype is transmitted only during hyphal anastomosis with related vegetatively compatible groups, the vegetative compatibility structure within the fungal population can affect the dissemination and persistence of introduced hypovirulence. It was reported that mixtures of different hypovirulent mycelia effectively overcome vegetative incompatibility and rapidly arrest canker development (Jaynes and Elliston, 1980).

dsRNA-Associated Hypovirulence

The answer to the question as to the nature of the cytoplasmic determinant in hypovirulent strains came partly in the late 1970's. Day et al. (1977) showed that hypovirulent strains, carried double-stranded ribonucleic acid (dsRNA) in the cytoplasm and that this dsRNA could be transferred to virulent strains via anastomosis.

The role of dsRNA and its relationship to hypovirulence was based on correlative evidence until it was shown that transformation of virulent *C. parasitica* strains with a full length complementary DNA copy of a hypovirulence-associated viral dsRNA conferred the complete hypovirulence phenotype (Choi and Nuss, 1992). Cytoplasmic dsRNA was resurrected from the chromosomally integrated cDNA copy. These dsRNA molecules were capable of converting compatible virulent strains to hypovirulence, thus establishing dsRNA as the causal agent of hypovirulence in *C. parasitica*.

There is wide variation in the expression of hypovirulence-associated phenotypes in dsRNA-containing strains of *C. parasitica*. Virulence expression of dsRNA-containing strains ranged from avirulent to almost normally virulent, indicating that different levels of hypovirulence are determined by cytoplasmically transmitted dsRNA independently of the nuclear genetic background of the recipient virulent strains (Nuss and Koltin, 1990; Nuss, 1992). In addition to reduced virulence, hypovirulent strains can exhibit several different symptoms including altered colony morphology, suppressed conidiation, reduced oxalate accumulation, reduced laccase production, reduced pigment production, as well as noted changes in several other proteins and mRNA transcripts (Nuss and Koltin, 1990).

Hypovirulence in North America

Surveys in Michigan indicated that the chestnut blight pathogen isolated from abnormal cankers had abnormal colony morphology, reduced virulence and contained dsRNA (Fullbright et al. 1983). Pathogenicity tests showed that native Michigan hypovirulent isolates of *C. parasitica* may be responsible for recovering chestnut groves in Michigan and that hypovirulence is naturally spreading (Brewer, 1995) . The most obvious

morphologic difference between European and Michigan hypovirulent strains were sporulation and pigmentation differences. Michigan strains are pigmented whereas European strains generally lack pigmentation and sporulation was not as noticeably suppressed in Michigan hypovirulent strains as in European strains (Heiniger and Rigling, 1994).

Elliston (1985) compared the cultural characteristics, pathogenicity and fruiting capacities of Italian and American dsRNA-containing strains relative to dsRNA-free strains to determine if any consistent cultural indicators of dsRNA or reduced virulence could be found. In his studies, he found that the dsRNA-containing strains all differed in culture from dsRNA-free strains and from one another. All dsRNA-containing strains were deficient in pathogenicity and fruiting capacity. He suggested that overall appearence in culture may be a useful criterion to select strains to be tested for dsRNA. Dunn and Boland (1993) reported that naturally occuring isolates of *C. parasitica* collected from different native stands of American chestnut in Ontario possessed dsRNA and were hypovirulent.

Genetic Characterization of dsRNA in Hypovirulent Strains

Double-stranded RNAs associated with different strains vary considerably with respect to size, number of bands, concentration and sequence homology. Even isolates recovered from different cankers on the same tree can contain dsRNA molecules of varying size. Most hypovirulent isolates harbor one to three large dsRNA molecules and several small molecules (Fulbright, 1990). The recent analyses of dsRNAs associated with a European (EP713) hypovirulent strain and an North American hypovirulent strain have