PROTEASE SECRETION IN *APHANOMYCES COCHLIOIDES*.

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ABSTRACT

Protease activities have been implicated in the infection of fish and crayfish by *Aphanomyces astaci*, a pathogen of these host organisms. In an effort to characterize protease activities produced by the sugarbeet pathogen *A. cochlioides*, culture supernatants of this oomycete were tested for bulk enzyme activity and examined for protease isozyme complement. Bulk protease activity was readily detected using azocoll as a colorimetric substrate. At least 8 distinct isoforms of protease secreted by *A. cochlioides* were detected after electrophoretic fractionation in native polyacrylamide gels containing co-polymerized gelatin. A subset of the protease activities was sensitive to inhibitors of trypsin, including the proteinacious trypsin inhibitors from lima bean. Co-culture of sugarbeet seedlings in the presence of *A. cochlioides* and lima bean trypsin inhibitor resulted in increased seedling survival relative to control inoculations. The data suggest that protease activities secreted by *A. cochlioides* may be virulence determinants in the infection of sugarbeet by this pathogen.

INTRODUCTION

Root rot of sugarbeet caused by the oomycete *Aphanomyces cochlioides* is a serious disease of sugarbeet in warm wet soils (Duffus and Ruppel, 1993). Although most growing regions of the world impacted by *Aphanomyces* experience stand losses to the seedling blackroot phase of the disease, the Red River Valley of the north central U.S. has been severely affected by the chronic root rot phase of the disease in recent years. Seed treatment with hymexazol (Tachigaren™) has succeeded in retarding the seedling disease in soils with low to moderate disease pressure, but additional genetic resistance in the sugarbeet variety is required to withstand high levels of *Aphanomyces* inoculum.

The production of cell wall degrading enzymes has been implicated in the ability of rot or blight pathogens to cause disease (Walton, 1994). *A. cochlioides* is known to produce pectinase in infected sugarbeet (Herr, 1977), but definitive evidence that these are required for infection is lacking. Recently, the characterization of the pathogenesis of crayfish by *Aphanomyces astaci* revealed the potential involvement of protease activities in penetration of the crayfish cuticle (Bangyeekhun et al., 2001). In addition, protease secretion by the oomycete *Pythium insidiosum* is being investigated as a means by which this mammalian pathogen penetrates through skin (MacDonald et al., 2002). Based on these findings we investigated whether *A. cochlioides* is capable of

protease secretion and what the nature of the secreted protease(s) might be. The production of protease by *A. cochlioides* that might be involved in sugarbeet infection would provide a target for the design of pathogen control strategies based on protease inhibition.

RESULTS AND DISCUSSION

The supernatants of cultures of *A. cochlioides* comprised of pathogen, water, and autoclaved sugarbeet root slices was examined for protease activity. Digestion of azo-dye labeled collagen was used to determine the production of bulk protease activity in these supernatants. Detection of activity using the bulk assay system was sufficient to warrant further characterization of the activity using a standardized, commercial source of class-specific protease inhibitors. Using this approach, roughly one half to two thirds of the total protease activity could be assigned to trypsin-like activities. The remaining activity complement could not be inhibited by any of the other inhibitors tested.

Semi-denaturing polyacrylamide gel electrophoresis was then used to assess the number of protease isozymes comprising the activity. Samples were electrophoresed in the presence of sodium dodecylsulfate, but were not boiled prior to gel loading. On account of this, protease isozymes in the sample retained their activity. Bovine gelatin co-polymerized in the gel provided a substrate for detection of the protease isozymes. In all, at least 8 protease isozymes were detected by this method, after the gels were stained with Coomassie Brilliant Blue. When gels were co-polymerized with gelatin and the proteinacious trypsin inhibitor from lima bean, only the fastest migrating species of the isozymes disappeared from the zymogram, indicating that the trypsin-class proteases in the sample possess the lowest apparent molecular weight of the isozyme set.

Because a portion of the protease activity secreted by *A. cochlioides* was able to be inhibited by lima bean trypsin inhibitor, experiments were performed to determine if the rate of seedling damping off caused by the pathogen would be reduced in the presence of this inhibitor. Sugarbeet seed (variety ACH 9369) was sprouted in Gambourg's agar. Approximately one week following seedling emergence, 10 ml of water and one plug of mycelium of *A. cochlioides* strain 19-1z were placed into the containers. Into the test containers also was placed lima bean trypsin inhibitor at 10 ug/ml and 1 ug/ml concentrations, whereas the control containers contained no inhibitor. Containers were incubated under 18 hr daylength at 23 degrees C and, following a 5 day post-inoculation interval, were monitored daily for disease development. At least 4 independent trials of the experiment were performed where each treatment was replicated 3 times within the experiment. The results showed that presence of the inhibitor at the 10 ug/ml level could delay the onset of seedling black root at a rate of up to 50%. Upon further incubation, all seedlings eventually succumbed to the disease. Further experimentation is under way to refine the assay for the influence of protease inhibition on seedling protection from *A. cochlioides*. Future experiments will examine whether any components of genetic resistance in sugarbeet to Aphanomyces is conditioned by protease inhibition.

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