Research Note

Proteinase Inhibitors from Plants As a Novel Class of Fungicides

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A purified preparation of trypsin and chymotrypsin inhibitors from cabbage foliage showed antifungal activity *in vitro*. The inhibitors suppressed spore germination and germ tube elongation of two species of phytopathogenic fungi but had no effect on a fungus that specifically attacks cabbage. Microscopic examination indicated that the inhibitors caused leakage of the intracellular contents from the susceptible species of fungi.

Serine proteinase inhibitors (PIs) are proteins or polypeptides that are ubiquitous in plants and inhibit the proteinases (e.g., trypsin and chymotrypsin) that are common in animals and microorganisms. They are thought to function as a natural phytochemical defense against predators, since they inhibit the proteinases that occur in many species of herbivorous insects and plant pathogens, and they are induced by herbivory and pathogenesis (Green and Ryan 1972; Rickauer *et al.* 1989; Geoffroy *et al.* 1990). In addition, they are able to reduce the growth and development of herbivorous insects (Richardson 1977), and they have been reported to influence the synthesis of chitin in fungi *in vitro* (Adams *et al.* 1993). However, the direct effect of plant PIs on phytopathogenic fungi has never been reported. We report that PIs from cabbage inhibited spore germination and germ tube elongation and also elicited cytoplasmic leakage in two species of phytopathogenic fungi, in an *in vitro* bioassay. However, this was not observed in a species of fungal pathogen that is specific to cabbage and contains chitin in its cell wall, suggesting circumvention of the inhibitory action. We suggest that PIs are involved in pathogenicity and should be considered a new family of antifungal compounds.

The trypsin and chymotrypsin inhibitory activity in the foliage of cabbage (*Brassica oleracea* L. cv. Superpack) originates from four proteins (12–25 kDa) that are stable over a broad range of temperature (0–100° C) and pH (4.5–7.5) (Broadway 1993) and are developmentally regulated (Broadway and Missurelli 1990). These proteins have no chitinolytic or glucanase activity, as determined by enzyme assays described elsewhere (Harman *et al.* 1993).

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A mixture of these four PIs was evaluated for its effect on the growth and differentiation of three species of phytopathogenic fungi, as determined by an *in vitro* bioassay (Lorito *et al.* 1993). We tested strains of *Botrytis cinerea,* a polyphagous fungus isolated from grapes; *Fusarium solani* f. sp. *pisi,* a pathogen of pea which does not attack cabbage; and *Alternaria brassicicola,* a pathogen specific to cabbage.

The PI preparation strongly inhibited spore germination and germ tube elongation of *B. cinerea* and *F. solani* (Fig. 1). The dose-response curves indicate that 150 μg ml^{-1} of the inhibitor produced a 50% inhibition of spore germination and germ tube elongation, whereas a much higher concentration (>600 μg ml^{-1}) was needed for complete inhibition of growth and development of *B. cinerea* and *F. solani.* Following 24-hr exposure to cabbage PIs, morphological modifications included diffuse branching and coiling of the fungal hyphae. More importantly, there was an accumulation of an amorphous exudate around the mycelia (Figs. 2 and 3). Calcofluor did not stain this exudate, which instead became fluorescent upon treatment with 4′,6-diamidino-2-phenylindole (DAPI), a specific stain for nucleic acid. This suggests that the exudate did not originate from the cell wall but was cytoplasmic in origin, and that the PIs may have modified the cell walls or membranes, thus causing leakage of the cytoplasm from the hyphae. The cabbage PIs had no effect on the cabbage pathogen *A. brassicicola.* In addition, soybean trypsin inhibitor, evaluated under the same conditions, had no effect on the growth and development of the three species of phytopathogenic fungi used in this study (data not shown). This suggests that there is specificity in the interactions of the plant PIs with the fungi.

One explanation for the observation of cytoplasmic leakage is that the trypsin and chymotrypsin inhibitors block the synthesis of chitin in cell wall, thus weakening the fungal hyphae. In support of this hypothesis, it has been reported that endogenous trypsin regulates chitin synthase by proteolytically converting the precursor zymogen into an active form (Machida and Saito 1993), and *in vitro* stimulation of microsomal chitin synthase activity of *B. cinerea* is inhibited by a range of serine PIs (Adams *et al.* 1993). Therefore, it is conceivable that selected PIs function as repressors of the activation of chitin synthesis, which would be a novel site of action for a fungicide. However, our data do not indicate the cellular

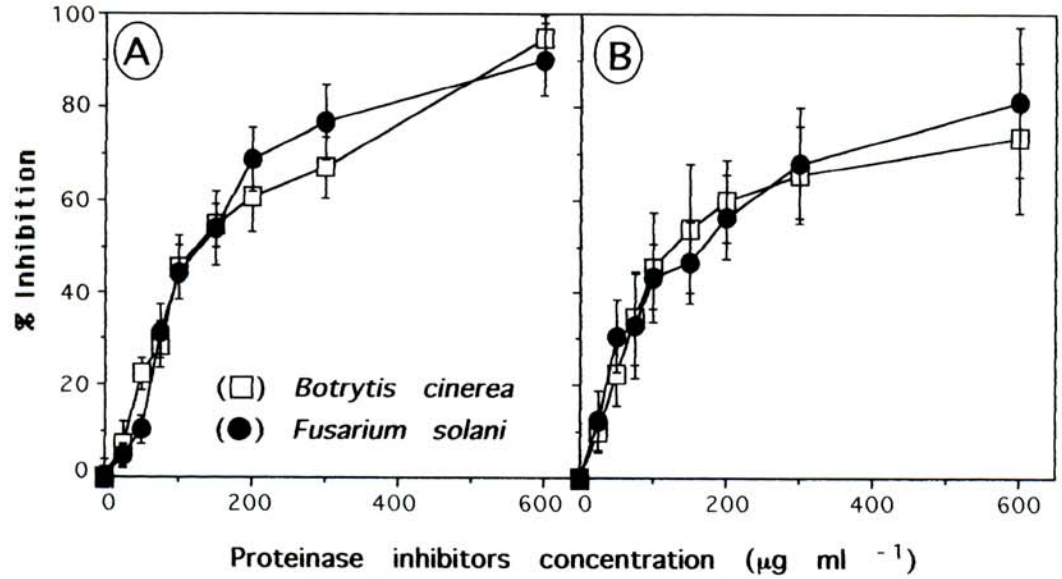

Fig. 1. Dose-response curves of inhibition of spore germination (**A**) and germ tube elongation (**B**) in the phytopathogenic fungi *Botrytis cinerea* and *Fusarium solani*. The fungi were treated with a preparation of proteinase inhibitors obtained from young cabbage leaves by affinity chromatography (Broadway 1993). There was no effect on *Alternaria brassicicola*. The bioassay consists of mixing in a sterile Eppendorf tube equal parts of the test solution with a spore suspension from the target fungus plus a liquid medium at the appropriate concentration (Lorito *et al.* 1993). After 24–30 hr of incubation at 25° C, the percentage of spore germination and germ tube elongation were determined microscopically and compared with a water control to obtain inhibition values. ED_{50} values were obtained by regression polynomial analysis of the third order with R^2 ranging between 0.95 and 0.99. Two independent experiments were conducted with at least three replicates for each treatment. Bars indicate standard errors.

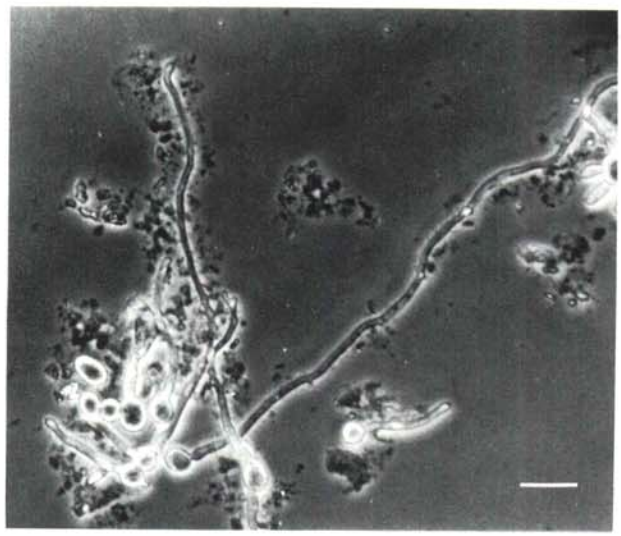

Fig. 2. Accumulation of an amorphous exudate around spores and mycelium of *Botrytis cinerea* upon treatment with a preparation of proteinase inhibitors from young cabbage leaves at a concentration of 50 µg ml^{-1}. The bioassay was performed as indicated in Figure 1. Bar = 70 µm.

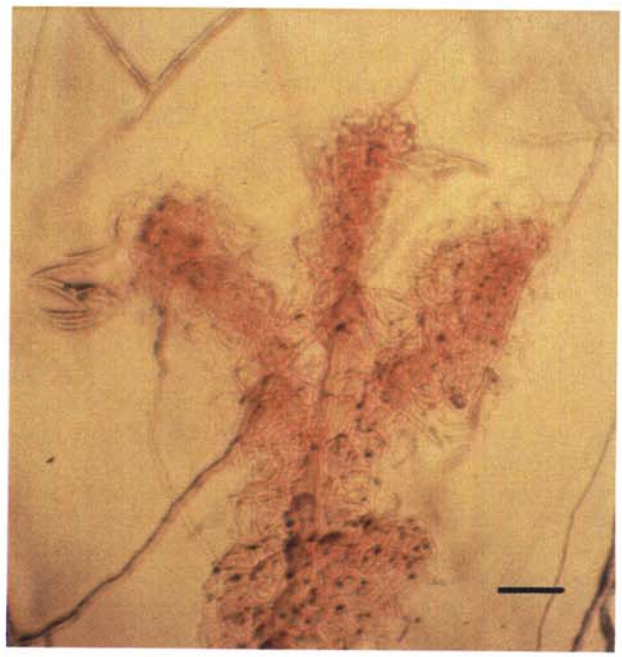

Fig. 3. Accumulation of an amorphous exudate around spores and mycelium of *Fusarium solani* upon treatment with a preparation of proteinase inhibitors from young cabbage leaves at a concentration of 50 µg ml^{-1}. The bioassay was performed as indicated in Figure 1. Bar = 70 µm.

location or the mechanism of the inhibitory action of plant PIs. Thus the reasons for the observed antifungal activity are unknown.

Another possible site of action of plant serine PIs is on the exogenous fungal proteinases involved in fungal attack of plants. A trypsin inhibitor from potato reduced the activity of exogenous proteinases found in the culture filtrates of selected fungi (Mosolov *et al.* 1976). However, there is no evidence that this inhibition influences fungal growth or development or pathogenicity.

The literature has focused on the role of PIs as a phytochemical defense against herbivorous insects. Our work is the first to indicate that PIs have the potential to function as defensive agents against fungal phytopathogens by disrupting the structural integrity of the organisms. It is possible that PIs may be involved in the inhibition of enzymes necessary for

microbial development. Interestingly, there was no apparent effect of the cabbage PIs on a host-specific pathogen (*A. brassicicola*), suggesting that there is specificity between the inhibitor and the fungus. Future work should focus on the site of action and the physiological effect of PIs on phytopathogenic fungi. The fact that soybean trypsin inhibitor was not effective against any of the three species of fungi indicates that it is important to survey a range of plant PIs for potency against selected phytopathogenic fungi and to identify the mechanism and specificity of this novel interaction.

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