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Proteomic analysis on zoxamide-induced sensitivity changes in *Phytophthora cactorum*

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ABSTRACT

Zoxamide is an important fungicide for oomycete disease management. In this study, we established the baseline sensitivity of *Phytophthora cactorum* to zoxamide and assessed the risk of developing resistance to zoxamide using ultraviolet irradiation and fungicide taming methods. All 73 studied isolates were sensitive to zoxamide, with effective concentrations for 50% inhibition of mycelial growth ranging from 0.04 to 0.29 mg/L and mean of 0.15 mg/L. Stable zoxamide-resistant mutants of *P. cactorum* were not obtained from four arbitrarily selected isolates by either treating mycelial cultures with ultraviolet irradiation or adapting mycelial cultures to the addition of increasing zoxamide concentrations. However, the sensitivity of the isolates to zoxamide could be easily reduced by successive zoxamide treatments. In addition to displaying decreased sensitivity to zoxamide, these isolates also showed decreased sensitivity to the fungicides flumorph and cymoxanil. Proteomic analysis indicated that some proteins involved in antioxidant detoxification, ATP-dependent multidrug resistance, and anti-apoptosis activity, are likely responsible for the induced decrease in the sensitivity of *P. cactorum* to zoxamide compared to controls. Further semi-quantitative PCR analysis demonstrated that the gene expression profiles of most of above proteins were consistent with the proteomic analysis. Based on the above results, *P. cactorum* shows low resistance risk to zoxamide; however, the fungicidal effect of zoxamide might be decreased due to induced resistance when this fungicide is continuously applied.

1. Introduction

Zoxamide is the only benzamide fungicide that is highly effective against oomycete pathogens [1]. This fungicide disrupts the microtubule cytoskeleton by binding to β-tubulin [1,2]. Currently, this fungicide is widely used on potatoes, vine crops, and other vegetables [3]. Many benzimidazole and N-phenylcarbamate fungicides, which share a similar mode of action with zoxamide, have faced serious resistance problems with many fungal pathogens [1,4,5]. The risk of *Phytophthora* spp. resistance to zoxamide was previously considered low because zoxamide resistance in *Phytophthora* spp. has not been reported anywhere in the field until recently [1,3,6] and because attempts to obtain highly resistant isolates to zoxamide in *Phytophthora infestans* and *Phytophthora capsici* have been unsuccessful [6,7]. Recent studies have reported that stable zoxamide-resistant isolates of *P. capsici* were obtained by treating either mycelial cultures or zoospores with UV irradiation and subsequently selecting on zoxamide amended agar media [3]. A further study suggested that zoxamide resistance is controlled by two recessive genes and that zoxamide resistance occurred when at least one pair of these alleles was homozygous. This result would imply that if sexual reproduction occurred in the same field, then the risk of pathogen resistance to zoxamide might be high [8].

Phytophthora cactorum is a widespread and highly damaging oomycete pathogen. This pathogen is capable of infecting many hosts, such as apples, strawberries, rhododendron, and silver birch, and of causing significant economic losses [9]. Methods such as crop rotation, cultural practices and genetic resistance have been used to manage *P. cactorum*; however, chemical control has been shown to be more effective [10,11]. Nevertheless, a limited number of fungicides are available, and fungicide resistance is a major challenge. For example, phenylamide fungicides have provided excellent disease control in the past; however, the efficacy of these fungicides has

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decreased because of the broad emergence of resistant subpopulations of *P. cactorum* [12,13]. The resistance risk of *P. cactorum* to zoxamide is unknown. *P. cactorum* can easily produce zoospores through sexual reproduction in the field due to its homothallic character. If the resistance of *P. cactorum* to zoxamide is controlled by a recessive gene, then the resistance risk of *P. cactorum* to zoxamide might be high. Moreover, the biochemical and molecular mechanisms of zoxamide-resistance in oomycete pathogens remain poorly understood.

The mechanisms that may confer fungicide resistance have been studied using a variety of methods [14]. Global approaches, including cDNA micro-arrays and proteomics, may complete classical analyses by characterizing the different pathways involved in chemoresistance acquisition. Compared to transcriptome profiling analysis using a Deep SAGE sequencing approach and microarray analysis, proteomic analysis can directly reveal the proteins with significantly different expression levels [15,16]. Proteomic analyses have been used to study the modes of action for fungicides [17,18]. A systematic approach to studying chemoresistance using proteomics has also been used in medicine [19–21]. However, no study regarding the overall protein expression in zoxamide-resistant isolates has yet been reported.

In this study, we were interested in assessing the resistance risk of *P. cactorum* to zoxamide and in exploring the mechanism of developing resistance by proteomic analysis. The specific objectives were to (i) determine the baseline sensitivity of natural populations of *P. cactorum* to zoxamide, (ii) assess the potential for this sensitivity to change in *P. cactorum* using ultraviolet irradiation and fungicide taming methods, and (iii) explore the mechanism of zoxamide sensitivity changes in *P. cactorum* using proteomic analysis.

2. Materials and methods

2.1. P. cactorum isolates

Panax notoginseng plants with typical symptoms of *P. cactorum* infection were collected from fields with no history of zoxamide application in 2009, 2010, and 2011 in Wenshan, Yunnan Province, China. The leaves or roots of *P. notoginseng* with lesions were cut into 0.5-cm diameter pieces, disinfected in 0.5% NaClO for 3 min, rinsed three times by shaking in sterile water, and plated on carrot agar medium (CA) (200 g of boiled carrot and 15 g of agar in a total volume of 1 L of distilled water) [17]. After 3 days of incubation at 25 °C in the dark, the mycelium was isolated from the edge of the culture and transferred to a new CA plate. In total, 73 isolates were obtained, and all isolates were transferred as single zoospores to obtain pure cultures.

2.2. Fungicides

Technical-grade zoxamide (97.5% a.i.; Gowan Company, LLC, Yuma, AZ, USA) and flumorph (kindly provided by the Shenyang Research Institute of Chemical Industry, Shenyang, Liaoning, China) were dissolved in methanol (Bodi Chemical Company, LLC, Tianjin, China) to prepare the stock solutions. The stocks were stored at 4 °C in the dark. The final concentration of methanol in any tested agar media was limited to 0.1% (vol/vol), which was the level at which no inhibition of mycelial growth of *P. cactorum* was observed in a preliminary test. This methanol concentration was used throughout the study when the agar media were amended with the fungicides. The same volume of methanol used to dissolve the fungicides was used as a control. Fungicide stock solutions were serially diluted and added to the CA medium to prepare the agar plates amended with varying levels of zoxamide.

2.3. Baseline sensitivity of P. cactorum to zoxamide

Mycelial growth inhibition was used to determine the sensitivity of *P. cactorum* to zoxamide according to Lu et al.'s method [22]. For each of the 73 isolates of *P. cactorum*, a fresh plug (5 mm in diameter) was taken from the growing edge of a CA culture and transferred onto CA amended with zoxamide at 0, 0.02, 0.05, 0.1, 0.15, 0.2, 0.5 and 1.0 mg/L. Each treatment was replicated three times. After incubation at 27 °C in the dark for six days, two measurements were taken for each colony at perpendicular angles, and the average of the two measurements was used for analysis. The diameter of the mycelial plug (5 mm) was subtracted from the colony diameter. A regression equation was derived by correlating the log of the zoxamide concentration and the probit of the percent inhibition of the average radial mycelial growth of *P. cactorum*. The effective concentration for 50% inhibition (EC_{50}) of radial mycelia growth of *P. cactorum* was calculated from this regression equation [22]. A histogram of the EC_{50} for all isolates was plotted, and the shape of the frequency distribution was analyzed by examining the curve shape, range and mean values of the EC_{50}, as well as the ratio of the highest and lowest EC_{50} values.

2.4. Generation of zoxamide-resistant mutants of P. cactorum

Ultraviolet irradiation and fungicide taming methods were used to generate isolates that were less sensitive to zoxamide according to Bi et al. and Young et al.'s methods [3,7]. Four isolates of *P. cactorum* were randomly selected from natural populations to generate the less sensitive isolates. Mycelial plugs were treated with ultraviolet (UV) irradiation (method I) or subjected to zoxamide taming (method II) as described below. In method I, mycelia of 5-day-old wild-type isolates were treated with UV light (20 W, 254 nm) at 20 cm in the vertical direction for 30 min at 25 °C, followed by 30 min of incubation in the dark to avoid light-repairing of DNA damage. A 5-day-old culture of the same isolate without UV treatment was used as a control. To select the isolates with less sensitivity to zoxamide, mycelial plugs (5 mm in diameter) were cut randomly from the UV-treated cultures using a cork borer and were placed on CA plates containing zoxamide at 4 mg/L with the mycelial side in direct contact with the medium. The cultures growing from the plug were subsequently transferred to zoxamide-free CA plates and then tested for fungicide sensitivity. In method II, mycelia of *P. cactorum* isolates were first cultured on the CA plates containing zoxamide at 1.0 mg/L and then transferred to a series of CA plates with increasing zoxamide concentrations in the media, i.e., 2, 3, and 4 mg/L, at each transfer. A small piece of agar was transferred from the fastest growing area of the colony to the freshly prepared zoxamide-amended medium. Colonies that survived at the final concentration of zoxamide were transferred to zoxamide-free plates for one generation, and then their sensitivity to zoxamide was tested. This experiment was performed three times with three replicates for each isolate. Each replicate contained 20 plates, and each plate was inoculated with 30 pieces of mycelia plugs.

2.5. Characterization of isolates with less sensitivity to zoxamide

2.5.1. Sensitivity determination

The cultures growing on zoxamide-free CA plates were transferred onto new CA plates amended with zoxamide at concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/L and incubated for 4 days in the dark at 25 °C. The diameters of the colonies were measured perpendicularly, and the two measurements on each plate were averaged. The effective concentration of zoxamide for 50% mycelial growth inhibition (EC_{50}) was determined from a dose–response (zoxamide concentration and mycelial growth) regression equation. The ratio of the EC_{50} of a less sensitive isolate to the EC_{50}