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Ultrastructural studies of the inhibition effect against *Phytophthora capsici* of root exudates collected from two garlic cultivars along with their qualitative analysis

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ABSTRACT

The overreliance on and overuse of fungicides is not only a health hazard but also induces natural resistance in plant pathogens, resulting in an economic burden on agricultural producers and a potential threat to natural systems across the globe. It is therefore necessary to identify natural substitutes of fungicides. This study was designed to evaluate the inhibitory effect of the root exudates of two different garlic cultivars, cv. Gailiang (G064) and Cangshan (G025), against *Phytophthora capsici*, a pepper fungus. All treatments (T1 to T4, i.e., 25%, 50%, 75% and 100% root exudate concentrations) of garlic cultivar G025 showed lower inhibition effects than cultivar G064. An intervarietal inhibition effect comparison at 100% concentration (T4) exhibited a 69.24% decline in hyphal growth for G064 compared with 49.06% for G025. The mycelial growth measured in the control was found to be significantly greater compared with the garlic treatments. The results of high performance liquid chromatography (HPLC) revealed that G064 possessed a large amount of allicin compared with G025. Scanning and transmission electron microscopy showed that, compared with the controls and hyphae treated with G025, the fungal hyphae treated with G064 exudates were misshaped, fragmented and had a smaller diameter, as well as empty cytoplasmic contents in the cell wall. Thus, the root exudates of the G064 cultivar had a significant fungicidal effect on *P. capsici*.

1. Introduction

During the last few years, Phytophthora blight induced by *Phytophthora capsici* has been recorded as an increasingly severe disease in a wide range of vegetable crops, including cucurbits and peppers (Babadoost, 2004; Hausbeck and Lamour, 2004; Babadoost and Zitter, 2009). Phytophthora blight can cause up to 100% crop loss, which could bankrupt farmers (Babadoost, 2004). *P. capsici is* a soil-borne oomycete pathogen that causes disease at various growth stages of host plants, including the pepper crop. This pathogen causes root rot, stem canker, leaf blight and fruit rot in older plants. Affected plants exhibit sudden wilting and death (Sherf and Mac Nab, 1986). Over-irrigation, heavy rainfall, warm environment, a wide host range, and the ability of the pathogen to survive in soil and infested seeds are the main reasons for its spread. Previous studies have failed to identify any cultivar resistant to this pathogen; therefore, different combinations of chemical, cultural and biological control along with sanitary measures are used to manage this problem. Cultural practices such as the elimination of host weeds and rotation to a non-host crop are of limited values because of the survival of oospores of *P. Capsici* for a span of up to 10 years in soil (French-Monar et al., 2003; Hausbeck and Lamour, 2004).

The use of copper fungicides is the most effective treatment, but it is uneconomical (Li, 2002). In some crops of cucumber and pepper, resistance to a common fungicide (Mefenoxam) has been reported (Lamour and Hausbeck, 2000). Metalaxyl also has excellent fungicidal activity, but the development of induced resistance to these chemicals by some members of peronosporales has caused considerable difficulties in its continuous use. In particular, the failure of Metalaxyl to control naturally occurring Metalaxyl-resistant isolates of *P. capsici* has been reported. Therefore, mixtures of Metalaxyl with copper oxychloride are being used extensively to prevent the development of resistance to metalaxyl in pepper-growing areas (Oh and Kim, 1992). However, the continuous use of fungicides has had several long-term side negative effects on human health and the environment (Song et al., 2004; Binod and Bhupendra, 2009), including immune suppression, hormonal disruption, reproductive abnormalities, cancer and the induction of plant resistance (Ramamoorthy et al., 2001 and Gupta, 2004). Soil and ground water pollution are the main environmental effects of chemicals. Several side effects have been

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reported in many species of insects, mammals and birds by Binod and Bhupendra (2009). The use of non-selective pesticides in soils becomes toxic to beneficial soil microorganisms (fungi, bacteria and protozoa), which dominate both the structure and function of natural ecosystems (Pimentel, 1992). Consequently, the use of copper fungicides in organic agriculture has been restricted by the European Commission (European Commission, 2002).

With the increasing awareness of the harmful effects of chemical control, scientists struggle to explore alternative methods for the management of crop damage with minimum ecological hazards. Among these, plant products that are safe, easily biodegradable and eco-friendly reflect great potential compared with others. The efficacy of different plant extracts in terms of their antimicrobial properties has been identified (Afolayan, 2003; Bowers and Locke, 2000; Eksteen et al., 2001; Gulluce et al., 2003; Hol and Van-veen, 2002; Magama et al., 2003; Neuhoff et al., 2002; Rohner et al., 2004). Pretorius et al. (2002) identified crude extracts from 39 plant species and their anti-fungal potential against seven economically important plant pathogenic fungi, and considerable mycelial growth inhibition was obtained with extracts from *Aristea ecklonii*. Similarly, Demirci and Dollar, 2006 identified cabbage and garlic plant materials that are effective for the reduction of mycelial growth and disease severity in *P. capsici* of pepper.

The use of biological substances extracted from garlic (*Allium sativum*) is found to be an important anti-microbial and anti-lipidemic agent. This agent exhibits anti-bacterial, anti-fungal and anti-viral properties. Thus far, researchers have largely focussed on the aerial parts of plants (though it may not be practical to add the same to the soil). In contrast, research on the inhibition effects of root exudates is still limited because of the complexity of the methods involved in the collection of root exudates.

The present study was planned to evaluate the efficacy of root exudates collected from two different garlic cultivars for the inhibition of the P. *capsici* pathogen. Electron microscopy was used to observe the cytomorphological alterations induced by the root exudates. High performance liquid chromatography (HPLC) was used to quantify allicin, an anti-fungal substance found in the garlic root exudates.

2. Materials and method

2.1. Fungal material

The *P. capsici* culture was provided by Prof. Dr. Ma Qing, College of Plant Protection, Northwest A & F University, Yangling, China. The culture was isolated from diseased pepper plants from local fields in the Shaanxi province of China. The fungus was maintained on a potato dextrose agar (PDA) medium at 25 ± 2 °C.

2.2. Extraction and preparation of garlic root exudates

Two hundred fresh and healthy bulbs of two garlic cultivars (cv. Gailiang (G064) and Cangshan (G025)) were grown in a perlite medium at 20 °C for 10 days. During culture, the garlic plants were irrigated with distilled water to keep the growing medium wet. After 10 days, all the garlic plants were dug up, washed with tap water to remove perlite and grown hydroponically for 30 days in a growth chamber. Each hydroponic system was mainly composed of a culture plastic pot and an air pump with a time switch. The air was periodically recycled by the pump (20 min per h). The exudates were collected by a continuous root exudate trapping system (Yu and Matsui, 1994; Yu et al., 2000) in which collected water was passed drop-by-drop through active carbon to trap the exudates. Carbon washed with acetone was placed for a few minutes in an ultrasonic cleaner to submerge the active ingredient in the acetone from the carbon. The solution was evaporated with a vacuum evaporator at 40 °C and then titrated three times with ethyl acetate ($CH_3COOC_2H_5$), using 50 ml each time. The upper transparent portion of the solution mixed with sodium sulphate (Na_2SO_4) to absorb the remaining water. Evaporation was again conducted until the solution was dry. The residues thus obtained were true root exudates (Tang and Young, 1982) and were stored at −20 °C until further use.

From the 200 bulbs of each cultivar, 0.128 g of garlic root exudate dry matter was collected from G064, and 0.09 g was collected from G25. We then took an equal amount (0.09 g), measured using an analytical balance, from each cultivar and dissolved them in acetone; these solutions were mixed with 15 ml of autoclaved distilled water, obtaining stock solutions (i.e., T4-100%). Three concentrations (T1-25%, T2-50% and T3-75%) were prepared from the stock solution of each cultivar by adding the calculated amount of autoclaved distilled water. However, the stock solution was used as treatment T4-100%.

2.3. Effect of root exudates on P. capsici

2.3.1. In vitro effect of root exudates on mycelial growth of P. capsici

The inhibitory effect of different concentrations of root exudates collected from the two garlic cultivars were examined by the mycelial growth rate method. One millilitre from each concentration of root exudates was added in 15 ml of PDA in a 9-cm Petri dish and allowed to solidify. A 5-mm mycelial plug of *P. capsici* from a 7-day old culture was placed in the centre of each PDA plate under aseptic conditions. All the dishes were completely closed with plastic film bands and incubated for one week in the dark at 25 ± 2 °C. The inoculated Petri dishes were inspected after 3, 5 and 7 days to measure the colony growth of the fungus along two diameter lines with a vernier calliper. Fungal sensitivity towards the concentration of garlic root exudate was expressed in terms of the inhibition percentage of mycelial growth and was calculated according to the following formula outlined by Pandey et al. (1982):

$$\text{Inhibition}\left(\%\right) = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

2.3.2. Effect of root exudates on spore germination of P. capsici

The 10-day-old cultured fungus was used for the spore germination test. The Petri dishes were kept at 10 °C for 60 min to induce zoospore release. After the cool treatment, the dishes were returned to 25 °C for 45 min. The inoculum was adjusted to 10^{-6} zoospores per millilitre with a haemocytometer. Three replicates per treatment were used. Zoospore germination was recorded after 2–4 h of culturing under a compound microscope, and a comparison was made between the control and both cultivars when the germination rate of the control's resting spores exceeded 60%.

2.4. Electron microscopy

Mycelial plugs (1 × 1 × 3 mm) of *P. capsici* of the same age were taken from the control and both garlic cultivar root exudate treatments (T4). These mycelial plugs were prepared for both scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

In the SEM preparation, the samples were fixed 5 times in phosphate-buffered 3% glutaraldehyde dehydrate with a pH of 6.8 for 5, 10, 15, 20, 25 and 30 min, consecutively. Later, the samples were placed in increasing concentrations of alcohol (30%, 50% and