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INFLUENCE OF GARLIC ROOT EXUDATES ON CYTO-MORPHOLOGICAL ALTERATION OF THE HYPHAE OF *PHYTOPHTHORA CAPSICI*, THE CAUSE OF *PHYTOPHTHORA* BLIGHT IN PEPPER

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Abstract

Studies were carried out under *In vitro* conditions to investigate the inhibition effect of garlic root exudates on the hyphae of *Phytophthora capsici* and some other specific observations were made using scanning and transmission electron microscope (SEM & TEM) to detect the possible alteration in growth, shape and cell structure of the pathogen affected by the use of garlic root exudates. Four concentrations of garlic root exudates i.e., 0.02% w/v, 0.04% w/v, 0.06% w/v and 0.08% w/v were used. It was noticed that mycelial growth of *P. capsici* was significantly effected by garlic root exudates and reduction in mycelial growth was found directly proportional to the increase in exudates concentration. The maximum inhibition (%) in growth of *P. capsici* was observed at 0.08% w/v garlic root exudates when compared with the control. Scanning electron microscopy (SEM) revealed cyto-morphological alteration of hyphae treated with garlic root exudates. Exudates-treated hyphae reflected alteration in their growth pattern and were found either collapsed, damaged or thinner when compared with control. During Transmission electron microscopy (TEM), a general increase in vacuolization was observed with consequent reduction of cytoplasm of the treated fungal cells. These results suggest that use of garlic root exudates is a promising, effective and environment-friendly management measure against *Phytophthora* blight of pepper and thus, may be used in the production of organically grown vegetables.

Introduction

Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops based on consumption, nutrition and cash value for farmers. In general, soil-borne diseases are considered more difficult to manage due to complex physical, chemical and biological factors of the soil. *Phytophthora* blight induced by *P. capsici* Leonian is one of the most widely spread and devastating soil-borne diseases. The pathogen attacks roots, stems, leaves and fruit of the pepper and causes 70% to 100% yield loss (Liu & Lu, 2003). At present various chemical, biological and cultural methods are being used to manage this disease. The most effective control has been achieved with the use of synthetic fungicide such as Metalaxyl, a cheap and widely applied formulation (Li, 2002). However, these chemicals are reported to persist in the soil, not easily degrade and can cause toxicity to humans and domestic animals (Lingk, 1991). This pesticidal toxicity gradually cause resistance in the pathogens. In China, the same has been observed in some pathogens associated with pepper and cucumber after 2-3 years of metalaxyl applications (Wang *et al*., 2002). Due to the persistent effects and hazards posed to health and environment by the use of these chemicals, researchers are now looking for some safe and environment friendly methods to manage *Phytophthora* blight. The long term aim of our research is to develop and evaluate new alternative methods for managing soil-borne fungal pathogens to replace chemical fungicides.

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The use of natural products for the control of fungal diseases in plants is considered an interesting alternative to synthetic fungicide due to their lesser negative impact on the environment (Brunelli, 1995). Some plant products have been found non-phytotoxic, more systemic, and biodegradable than synthetic pesticides (Madanagu & Ebena, 1994). Extracts of peanut, onion (*Allium cepa*), welsh onion (*Allium fistulosum* L.), ginger (*Zingiber officinale* Roscoe) and green pea (*Pisum sativum* L.) have inhibitory effects on *Phytophthora* blight of pepper (Lee *et al*., 1990). However, among the natural fungicide substances, garlic extract has been found most active in various trials to manage various phytopathogenic fungi (Tariq & Magee, 1990).

Garlic (*Allium sativum*), being a common plant, is used extensively in medicines, aquaculture, stock raising as human food because of its antibacterial, antiviral and nutritional functions (Zhou *et al*., 2008). China is the worlds largest producer and exporter of garlic (Meng *et al.,* 2006). Root exudates are the largest source of allelochemical input into the soil environment. Although, studies have been carried out on the use of garlic bulb juice to assess its efficacy against *P. capsici* (Su & Cheng, 2009) but still very little work has been done on the inhibitory effects of garlic root exudates on *P. capsici*. Therefore, the objective of present study was aimed to evaluate the toxic effects of root exudates of garlic on *P. capsici* under *In vitro* conditions. The purpose was to investigate some environmentally safe and effective management measures for *Phytophthora* blight of pepper.

Materials and Methods

Pure isolate of *P. capsici* were obtained from Prof. Dr Gong Zhen Hui, Vegetable Research Institute Laboratory, College of Horticulture, Northwest A& F University, Yangling, China. The culture was maintained on potato dextrose agar (PDA) medium at 20ºC.

Collection of garlic root exudates: One hundred bulbs of garlic were grown in the perlite at 20ºC for 10 days. During this, garlic plants were irrigated with distilled water to keep the perlite wet. After 10 days, all the garlic plants were dug up, washed with tap water to remove perlite and grown by means of hydroponic system for 30 days in growth chamber. Each system was mainly composed of a culture plastic pot and a pump with a time switch. The air was periodically recycled by the pump (two hours every day). Water was changed at 15 days interval. Root exudates were collected by a continuous root exudates trapping system (Yu & Matsui, 1994; Yu *et al*., 2000). Collected water was passed by active carbon to trap the exudates, drop by drop. Carbon was washed with acetone and put for few minutes in ultrasonic cleaner to submerge the active ingredient in the acetone from carbon. The solution was evaporated with vacuum evaporator at 40ºC and then titrated with ethyl acetate (CH3COOC2H5) three times by using 50 ml each time. Upper transparent portion of solution was used to mix it with Sodium sulphate (Na_2SO_4) to absorb the remaining water. Evaporation was again carried out till dryness. The residue thus obtained is widely believed to be true root exudates (Tang & Young, 1982) and was stored at -20ºC till further use.

Preparation of treatments: In total, 0.285 gm root exudates (dry matter) was collected. The concentrations were prepared with autoclaved water by 0.02% w/v (T_1), 0.04% w/v (T_2), 0.06% w/v (T_3) and 0 .08 w/v (T_4). Autoclaved water served as control (T_0). Each treatment was repeated three times.

Culture experiment: The effect of various concentrations of garlic root exudates was examined by the mycelial growth rate method. One ml of each of the above mentioned four concentration of root exudate was added in 15 ml of PDA in a 9-cm diameter Perti dish and was allowed to solidify. A five mm mycelial plug of *P. capsici* from 7-day old culture plate was placed in the center of each PDA plate under aseptic conditions. All the dishes were completely closed with plastic film bands and incubated in the dark place at 25ºC for one week. Inoculated Petri dishes were inspected after 3, 5 and 7 days for measuring colony growth of the fungus along two present diam., lines with vernier caliper. Obtained growth values were converted into inhibition percentage of mycelial growth in relation to control. Fungal sensitivity towards exudate concentration was expressed in terms of percentage of mycelial growth inhibition and was calculated according to the following formula given by Pandey *et al*., (1982):

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

Electron microscopy: Mycelial plugs (1 x 1 x 3 mm) of *P. capsici* culture of same age from control and garlic root exudate treated treatment (0.8% w/v) were prepared for SEM and TEM.

In SEM preparation, the samples were fixed in phosphate-buffered 3% glutaraldehyde dehydrated having pH 6.8 for five times starting from 5, 10, 15, 20, 25 and 30 minutes, respectively. Later on, samples were put in alcohol having concentration of 30, 50 and 70% for 15 minutes and similarly, for the concentration of 80, 90, 95 and 100% for 20 minutes. The graded aqueous series of acetone (25, 50, 75, and 100%) and critical point dried with CO_2 using acetone as an intermediate fluid. The pieces of agar were sputter-coated with plutonium by JFC-1600 auto fine coater. Fungal hyphae of both, the control and treated treatments, were examined under JSM-6360LV SEM (JEOL Ltd. Made in Japan) and placed in Main Hi-Tech Research Laboratory Building of Northwest A&F University, China.

For TEM preparation, samples were fixed in phosphate-buffered 3% glutaraldehyde, post fixed in phosphate- buffered 1% Osmium tetroxide, dehydrated in graded series of ethanol following the procedure described by Alberto Bianchi *et al*., (1997). Plugs were embedded in a plastic fix coating and dried with Hitachi Critical Point Dryer. Ultra-thin sections were cut with LKB ultra tome, stained with uranyl acetate and lead citrate and examined with JEOL model JEM-1230 electron microscope.

Statistical analysis: The experiment was designed in complete randomized design and replicated thrice for each treatment. Statistical analysis was performed with the data processing system 2.0 statistical software and results were subjected to analysis of variance and mean separated by Duncan's multiple 2 range test (p=0.05).

Results

Effect of garlic root exudates on mycelial growth of *P. capsici*: The inhibitory effects of garlic root exudates on mycelial growth of *P. capcisi* are given in Table 1. All the concentration of garlic root exudates showed inhibitory effect on mycelial growth of *P. capsici* when compared with only PDA-grown *P. capsici* (control) (Fig. 1). However, the treatment of garlic root exudates with 0.08 % w/v conc. showed highly significant effects (53.65% inhibition) followed by 0.06% w/v conc. that also had good inhibitory effects (38.96 %) on the pathogen growth.

Table 1. Inhibition effect of garlic root exudates on the growth of *P. capsici* after 7 days.

Treatments	Concentration	Mean colony diameter (cm)	Inhibition (%)
T0 (control)	(0.00) 0%	67.67	0.00 e
T1	(0.02% w/v) 25%	55.05	18.64 d
T2	(0.04% w/v) 50%	51.09	24.50 c
T3	(0.06% w/v) 75%	41.30	38.96 b
T4	(0.08% w/v) 100%	31.42	53.65 a

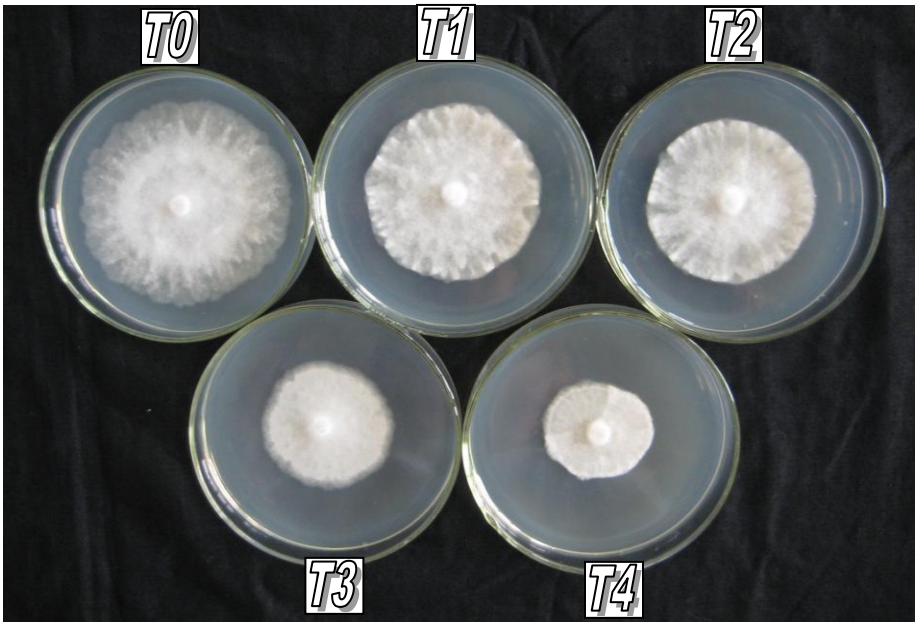

Fig. 1. Colony diameter of *P. capsici* showing differences obtained using garlic root exudates. The biggest diameter is control whereas the smallest is garlic root exudates treatment with 100% (0.08% w/v) root exudates.

Scanning and transmission electron microscope observations: Mycelial growth of *P. capsici* was observed under electron microscope and with treatment of 0.08% w/v garlic root exudates with exhibited morphological alterations in the hyphae of *P. capsici*, which were found collapsed. Abnormal hyphal swelling, curling, short branching and accumulation of protoplasm in mycelia was observed, whereas, no such changes were noticed in the mycelia developed in control treatment. Hyphae of garlic root exudates treated treatments were fragmented and had smaller diam., as compared to control (Fig. 2). The number of hyphae grown on garlic root exudates were also lesser than hyphae developed in control treatment.

Alterations of the cell structure of *P. capsici* were clearly observed in treatment with garlic root exudates (0.08% w/v) when compared with control (Fig. 3A & B). The main difference was increase in size and number of vacuoles in the hyphal cells. Also, great

variation was found in the cell wall structure (Fig. 3C & D). The hyphae treated with garlic root exudates showing strongly thicken cell wall whereas the control revealed thin. The tips of the hyphae were different from control treatment showing vacuolization (Fig. 3E & F) and nucleus was very clear in control treatment, however, in garlic root exudates treated tip the nucleus seemed disappeared.

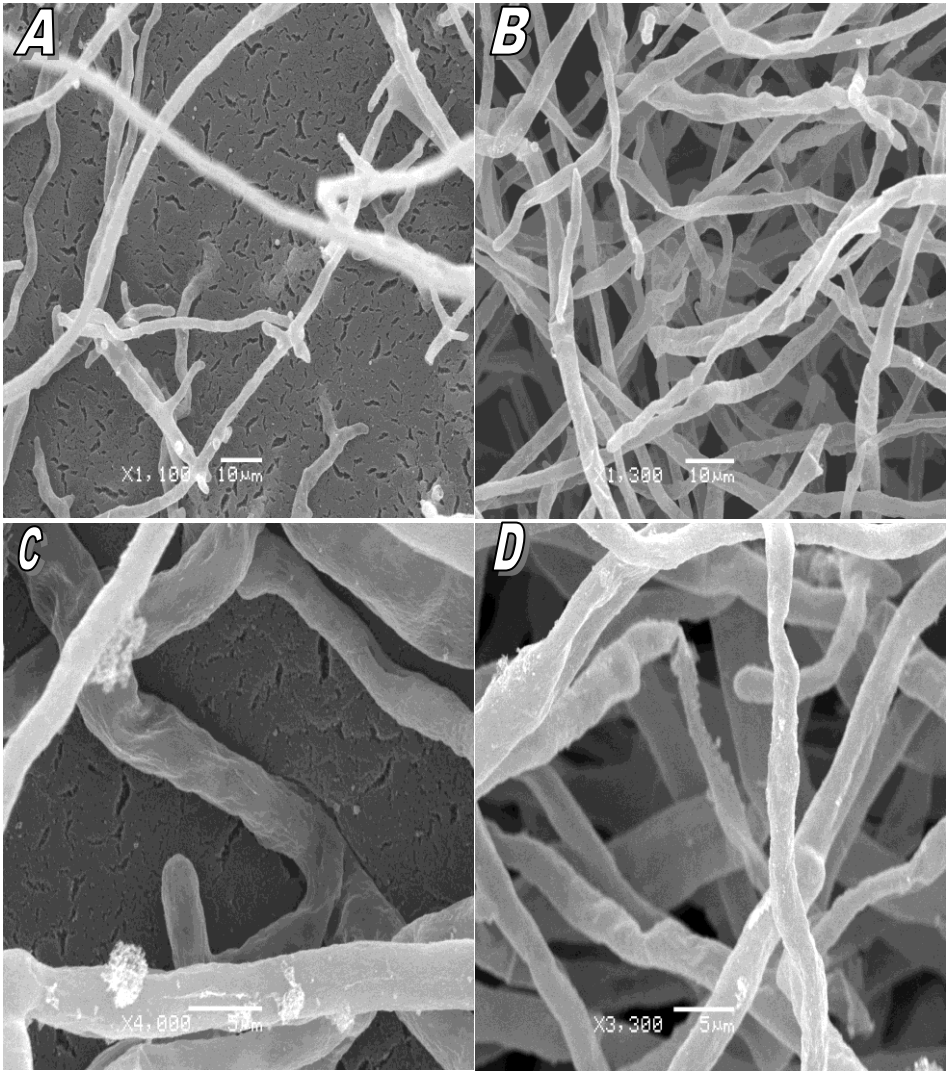

Fig. 2. Scanning Electron Microscopy. (A) Collapsed and few in number hyphae treated with garlic root exudates (0.08% w/v); (B) hyphae of control treatment (both seen on same frequency i.e., 10 µm): (C) Hyphae changing their shape and showing collapsing leading to death after treatment with garlic root exudates (0.08% w/v); and (D) Hyphae of control. Scale bars = 5µm

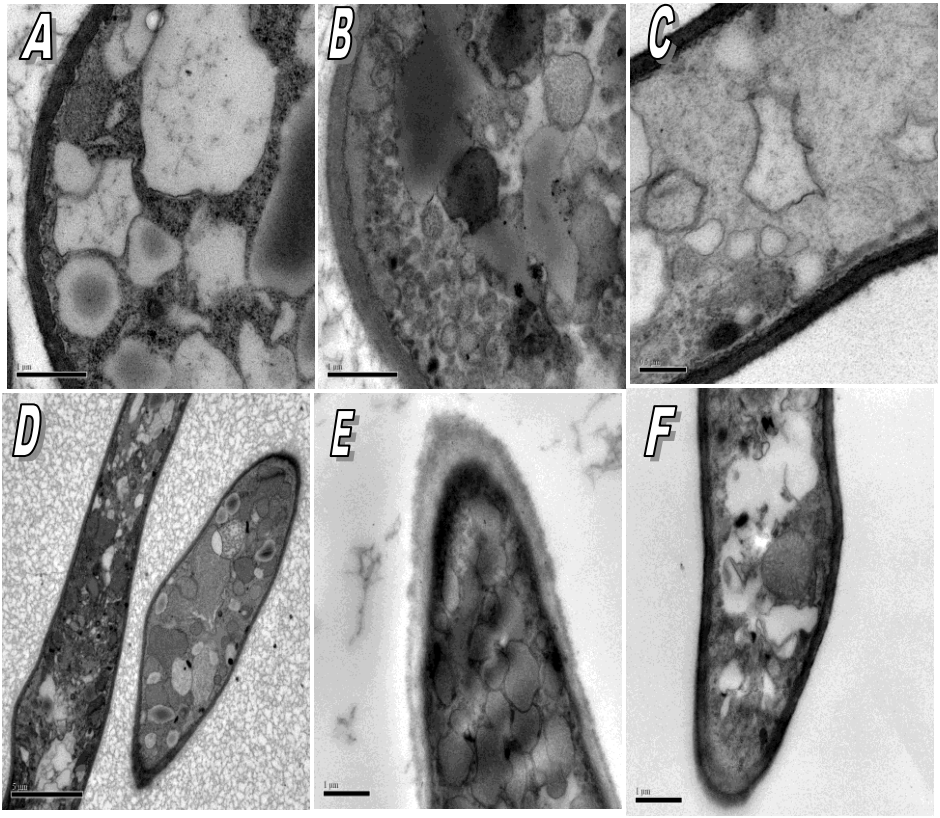

Fig. 3. Transmission Electron Microscopy. (A) Section of hyphae of similar age of control treated with garlic root exudates (0.08% w/v) showing anomalous distribution of electron dense granules; (B) hyphae of control: (C) Hyphae treated with garlic root exudates (0.08 % w/v) showing strongly thicked wall with the same magnification and age as that of control; (D) Cell wall of the control: (E) Tips of the hyphae treated with garlic root exudates @ 0.08% w/v, the increase in the size of vacuole can be seen; (F) tips of the hyphae of control treatment. Scale bars =1µm.

Discussions

Many years of increasing use of chemicals have created a situation leading to an ecological imbalance and the increase of multiple multi-resistant pathogenic micro-organisms (Levy, 1997). On the other hand, large reservoir of natural fungicides exists in plants, which serve as safe and effective alternative to synthetic fungicides (El-Mougy *et al*., 2007). Some plants have allelopathic potential by releasing allelochemicals to their surroundings that have either deleterious or beneficial effects on other plants growing in the vicinity. Auto toxicity and deleterious allelopathic effects among the same plant species have been documented in a number of plant species and is believed to be involved in natural- and agro-ecosystems (Singh *et al*., 1999 & Yu, 1999). Roots release an array of chemicals that are exuded into rhizosphere and bring about significant ecological effects (Bertin *et al*., 2003; Bais *et al*., 2004). Root mediated interactions, especially the allelopathic ones, are very common in the rhizosphere soil. Furthermore, allelochemicals in the soil are known to inhibit biological nitrogen fixation process

thereby reducing N availability. The root exudates contain low molecular weight compounds, which include amino acids, organic acids, sugars, an array of secondary metabolites and high molecular weight compounds like mucilage and proteins (Badri & Vivanco, 2009).

For successful development of new, more effective and environment friendly strategies to control *Phytophthora* blight disease of pepper by using garlic through intercropping with pepper crop, a more thorough understanding of the host pathogen interaction is necessary. During these investigations, we found that root exudates of garlic showed 53.65% inhibition in mycelial growth of *P. capsici*. Similar results have already been found in previous studies about various fungi by using leaf extracts of some medicinal plants (Cruz *et al*., 1998; Schwan *et al*., 1998). Fiori *et al*., (2000) observed that essential oil of *C. citratus*, *A. conyzoides* and *E. citriodora* provide 100% inhibition in the mycelial growth of *D. bryoniae*. Dried plant material of garlic, alfalfa and cabbage inhibit mycelial growth and disease severtity caused by *P. capcisi* (Fikret & Dolor, 2006; Su & Cheng, 2009).

The cyto-morphological modifications, particularly, the accumulation of lipid bodies and thickening of cell wall induced by garlic root exudates, are similar to those produced by some synthetic fungicides and garlic extracts (Alberto *et al*., 1997; Hippe, 1991). Increase in size and number of vacuole formation along with other alternations might also, in turn, modify the activity of membrane enzymes involved in the formation of cell wall, causing anomalous development. Garlic root exudates used in this experiment demonstrated a good antifungal activity against *P. capsici*. In the present study, garlic root exudates have been found effective in reducing mycelial growth and modification in cell structure of hyphae of the pathogen. These advantageous garlic root exudates disintegrate in nature and do not leave any toxic residues in the environment, thus, have great potential to incorporate in the package of organic pepper cultivation.

Conclusion

This study proved that garlic root exudates can be used as a useful, cost-effective and environment friendly management strategy for *Phytophthora* blight of pepper but it may not be practical to add garlic root exudates into the soil. In the further study, we should concentrate on garlic and pepper intercropping to observe the inhibitory effect of garlic root exudates on this fungus. Moreover, studies for screening some promising garlic cultivars producing higher concentration of inhibitory root exudates against *P. capsici* seems imperative.

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