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THE INHIBITIVE EFFECTS OF GARLIC BULB CRUDE EXTRACT ON *FULVIA FULVA* OF TOMATO

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Abstract

The inhibitive effects of garlic bulb crude extract on *Fulvia fulva* in tomato were studied both *In vitro* and pot experiment. The results indicated that, the inhibitive rate to spore germination reached 96.08% and to mycelia growth increased to 100% when the concentrations of garlic extract reached to 40mg.mL-1 and 80mg.mL^{-1}, respectively. The preventive and curative effects were 85.32% and 83.49% in young tomato leaves *In vitro*, 76.50% and 68.91% in function tomato leaves *In vitro*, 69.92% and 69.36% in bottom tomato leaves *In vitro* when the concentration of garlic was 80mg.mL^{-1}. In pot trial, the preventive and curative effects on tomato seedlings reached 82.19% and 79.37%, respectively when garlic concentration increased to 160mg.mL^{-1}, however, the higher concentrations of garlic bulb crude extract did not show any bad effects on growth of tomato in this experiment. In conclusion, garlic bulb crude extract is effective and environmental friendly for control of leaf mold in tomato caused by *F. fulva.*

Introduction

Tomato is an economically high value crop i.e., cultivated both in greenhouses and in open field. Leaf mold incited by fungi viz., *F. fulva* in tomato, spreads rapidly under high humid conditions (Crous & Braun, 2003), especially in greenhouses. This disease infects the stem, leaf, flower and fruit of tomato, but most commonly occurs in leaf. In addition to photosynthesis, it also affects the fruit quality resulting in economic losses, i.e., yield losses up to 20% ~ 80% in China (Liang *et al.,* 2004).

The protection of tomato from pathogens has been great attention and was achieved by various synthetic fungicides. However, chemical additives and antibiotics used against fungi have many side effects; a number of resistant microorganism strains have been determined at the clinical level (Elsom, 2003; Ruddock, 2005) that force the scientists to work on the alternative material to avoid the ecological hazards. One potential method of controlling plant diseases could be the use of biological substances found in plants such as neem and garlic (Browers & Locke, 2000; Lin *et al*., 2009; Rashid *et al.,* 2004). Garlic bulb crude extract is known to possess anti-bacterial, anti-fungal and anti-viral properties (Kumar & Berwal, 1998). It contains an antimicrobial, biologically active compound-allicin. When garlic is sliced or crushed it develops its characteristic odour because cellular damage leads to mixing of the vacuolar enzyme alliin lyase (E.C.4.4.1.4) and its cytosolic substrate alliin (S-allyl-L-cysteine sulphoxide). The immediate product is thiosulphenic acid which undergoes spontaneous dimerization to diallylthiosulphinate (allicin) (Slusarenko *et al*., 2008). Allicin was shown to be the major antimicrobial substance in garlic by Cavallito & Bailey (1944). Anti-fungal activity has been demonstrated against dermatophytes (Amer *et al*., 1980), *Cryptococcus* sp. (Fromtling *et al*., 1978) and *Candidu ulbicuns* (Adetumbi *et al.,* 1986).

Other plant protection methods such as use of essential oils can be expensive due to the complexity of the preparation process. The efficacy of garlic root exudates and garlic bulb extract with water against *Phytophthora capsici* Leonian has been reported by Khan & Cheng (2010) and Su & Cheng (2009). In this study anti-fungal activities of garlic bulb crude extract were investigated against *F. fulva In vitro* and in pot trial.

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Materials and Methods

Preparation of experiments

Media: Culture medium, potato dextrose agar (PDA) contained, per liter of de-ionized water: 200 g potato, 20 g dextrose and 15 g agar with a pH of 6.0 before autoclaving.

Pathogen and culture: Pure isolate of *F. fulva* was kindly provided by Prof. Dr. Ma Qing, College of Plant Protection, Northwest A & F University, Yangling, China. The culture was maintained on the potato dextrose agar (PDA). The plugs of fungi were gently dropped at centre of the PDA plates. The plates were incubated in a moist chamber at 22^{o}C for 15 days (Ru *et al.,* 2002).

Garlic bulb crude extract preparation: The garlic bulbs were purchased from the local market of Yangling. Eight gram fresh garlic bulbs were surface-sterilized with sodium hypochlorite (NaOCl, 10%) for 10 min and then rinsed in three changed of sterile distilled water. Then it was grounded by a grinder and homogenized in 100 mL sterile distilled water to give a concentration of 80 mg·mL^{-1}. This extract was poured into a sterile 50 mL Falcon tube and centrifuged at 10.000 rpm for 10min. The supernatant was filtered through membrane filters (0.24 μm). Serial dilutions were carried out with the concentrations of 20, 40 and 60 mg·mL^{-1}. These extracts were stored in a refrigerator at 4^{o}C until subsequent use (Su & Cheng, 2009).

Bioassays

Effect of garlic bulb crude extract on mycelia growth: Inhibitory effect of garlic bulb crude extract on mycelial growth of *F. fulva* was determined by examining radial growth rate supplemented with various concentrations of garlic bulb crude extract from 20 to 80 mg·mL^{-1} (Wu, 1988; Fang, 1998). The freshly prepared garlic bulb crude extract was mixed with PDA kept at 45^{o}C and carried out for all the concentrations of garlic. An inoculum disc (5 mm dia.), taken from a 14-day-old PDA cultures of *F. fulva,* was transferred to the center of each inoculated plates. The medium without the garlic bulb crude extract served as the control (only PDA). Plates were sealed with Micropore^{TM}-tape and incubated in the dark at 22^{o}C. Each treatment was repeated three times. The diameters of the fungal colonies on all dishes were measured on the 8th day.

Effect of garlic bulb crude extract on spore germination: Effect of garlic bulb crude extract on spore germination of *F. fulva* was carried out by the spore germination method (Wu, 1988; Fang, 1998). A spore suspension of *F. fulva* was prepared by adding 10 mL of sterile distilled water to a 15-day-old Petri plate culture of *F. fulva* grown at 22^{o}C on PDA. The surface of agar was washed through a double layer of gauze with sterile distilled water. The inoculum was adjusted to a concentration of 1×10^{6} mL^{-1} with a haemacytometer. One milliliter of the garlic bulb crude extract in 20 mg·mL^{-1} and 40 mg·mL^{-1} was mixed with the same amount of *F. fulva* spore suspension. The mixture was pipetted onto clean concave slips and incubated at room temperature for 24 hours. There was a control: the sterile distilled water was mixed with spore suspension. Germination rate of spores was observed with a microscope after incubation of twelve hours. Each treatment was repeated three times.

Effect of garlic bulb crude extract on *F. fulva* in different kinds of tomato leaves *In vitro*

1. Preventive effects: Different kinds of tomato leaf (young leaf, functional leaf and bottom leaf) surface-sterilized with NaOCl (10%) were prepared into leaf disc (diameter 15 mm) with hole puncher and dipped in garlic bulb crude extract at various concentrations (20 mg·mL^{-1}, 40 mg·mL^{-1}, 60 mg·mL^{-1} and 80 mg·mL^{-1}) for 2~3 minutes and then inoculated with fungi discs (5 mm dia.) being removed from the edge of fungal colony. Leaf discs were dipped in sterile distilled water used as control. After inoculation, tomato leaf discs were then incubated in a growth chamber at >90% relative humidity (RH) and 22^{o}C, and the diameters of fungal colonies were measured until mycelium of control dish grows and covers the surface of Petri dish completely.

2. Curative effects: Sterile tomato leaf discs were prepared as described above. Each of fungi discs being removed from the edge of fungal colony located on a leaf and was kept in growth chambers. When mycelium of control dish grows completely, tomato leaf discs were dipped into a series of concentrations and processed leaf discs were kept in the same condition at >90% relative humidity (RH) and 22^{o}C before the diameter was measured. Control dish containing no garlic bulb crude extract was used to make the comparison. The experiment was repeated three times.

Effect of garlic bulb crude extract on *F. fulva* in pot trials

1. Tomato cultivation: Tomato (cv. Jinpeng No.1) seeds purchased from a local seed shop were sown in seeding trays containing sterilized soil (peat and perlite, 2:1 v/v) for germination and incubated at 22^{o}C in a light/dark cycle of 16/8 hours. After germination, the seedlings having two-leaves were transplanted into a plastic pot. Seedlings were kept in a growth chamber (RXZ-300, Ningbo Jiangnan Instrument Factory, Zhejiang Province, China) at >90% RH and 22 ^{o}C.

2. Inoculation: The seedlings at three-leaf stage were inoculated with the spore suspension (1×10^{6} mL^{-1}). The second and third leaves were sprayed using a sprayer. The two different concentrations of garlic bulb crude extract (40 mg·mL^{-1}, 80 mg·mL^{-1} and 160 mg·mL^{-1}) were sprayed 24 hours before and after the inoculation of spore suspension of *F. fulva,* respectively. Control tomato leaves were sprayed with water. After spray-inoculation, pots were then incubated in a growth chamber at >90% RH and 22^{o}C. Disease intensity was recorded at 15 days after inoculation by estimating the percentage of the affected leaf area. The affectivity of each treatment was calculated (Xu *et al.*, 2009; Portz *et al.*, 2008).

The impact of garlic bulb crude extract on growth of tomato leaves: The potential phytotoxic effects of garlic bulb crude extract on tomato leaves have been reported (Portz *et al*., 2008). Spraying leaves of 3-week-old tomato by garlic bulb crude extract containing 200~800 μg.mL^{-1} allicin led to leaf damage in category 2 (<2.5% of the leaf area showing chlorosis or necrosis). To assess whether the inhibitive concentration on *F. fulva* observed *In vitro* would affect the growth of tomato leaf, an investigation on tomato leaves was carried out.

Spray tests were carried out on maturity of tomato leaves (Li, 1981), and were divided into four different treatments. Before spraying, the height, stem diameter and leaf area of each plant were measured. Spraying with various concentrations (120 mg·mL^{-1}, 240 mg·mL^{-1} and 480 mg·mL^{-1}) of garlic bulb crude extract were carried out on the 3rd, 7th,10th and 14th day, and on the 7th and 14th day, we measured the indictors mentioned before again. However, the water was used as control.

Results

Effect of garlic bulb crude extract on mycelia growth of *F. fulva*: In order to quantify the inhibitive effect of garlic bulb crude extract against *F. fulva*, different quantities of the garlic bulb crude extract were mixed in PDA medium. The data presented in Table 1 indicates that all concentrations of garlic bulb crude extract were found to be significantly superior to the control on the inhibition of *F. fulva*. At a given concentration (80 mg·mL^{-1}) of garlic bulb crude extract loaded on the disc increased inhibition, the diameter of mycelia growth also showed a downward trend. Similar antifungal studies were carried

out by Song *et al*., (2007), Yi *et al*., (2008), Bianchi *et al*., (1997) and Su & Cheng (2009), though they controlled different pathogens. Among the treatments, the garlic bulb crude extract with a concentration of 80 mg·mL^{-1} suppressed mycelia growth of *F. fulva* (100%) completely. At 60 mg·mL^{-1}, the garlic bulb crude extract also showed a high inhibitive effect (69.59%). The significant inhibitive effects can be observed in Table 1 and Fig. 1.

Table 1. Inhibitive effects of garlic bulb crude extract on mycelia growth of *F. fulva*.

Concentration of garlic bulb crude extracts (mg·mL^{-1})	Average diameter of colony[a] (mm)	Inhibitive rate (%)
0	66.71 aA	0 eE
20	44.26 bB	33.64 dD
40	34.75 cC	47.87 cC
60	20.29 dD	69.59 bB
80	0 eE	100.00 aA

Note: [a] Diameter of colony (mm) = diameter of measure (mm) - diameter of inoculum disc (5mm). [b] Different small and capital English letters in the same row separately indicate the significant difference ($p<0.05$) and very significant difference ($p<0.01$), which analyzed with the LSD's test of the DPS statistical analysis software.

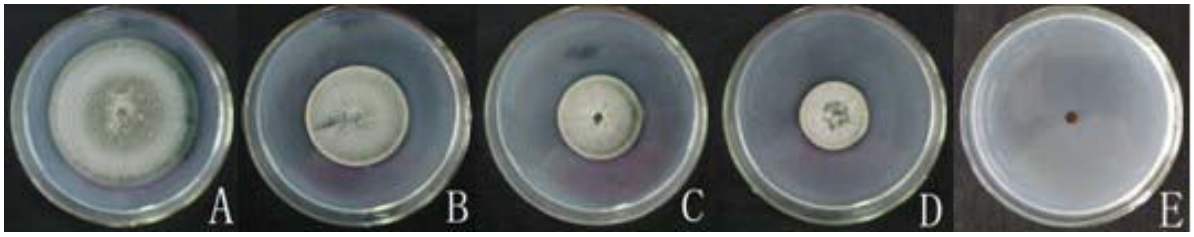

Fig. 1, Inhibitive effects of garlic bulb crude extract on mycelia growth of *F. fulva.*
A= Untreated control, B= 20mg·mL^{-1}, C= 40mg·mL^{-1}, D= 60mg·mL^{-1}, E= 80mg·mL^{-1}

Effect of garlic bulb crude extract on germination of *F. fulva* spores: The germination and inhibitive rate of *F. fulva* spores after treatment with garlic bulb crude extract is shown in Table 2. Garlic bulb crude extract caused a clear reduction in germination of *F. fulva* spores under conditions where they germinate directly with a germ tube. There was inhibition of spore germination due to exposure to garlic bulb crude extract at 20 mg·mL^{-1} and 40 mg·mL^{-1} (52.39% and 96.08%, respectively) compared with the control.

Table 2. Inhibitive effects of garlic bulb crude extract on spores germination of *F. fulva*.

Concentration of garlic bulb crude extracts (mg·mL^{-1})	Germination rate (%)	Inhibitive rate (%)
0	92.67 aA	0 cC
20	44.00 bB	52.39 bB
40	3.67 cC	96.08 aA

Effect of garlic bulb crude extract on *F. fulva* in different kinds of tomato leaves *In vitro*

1. Preventive effects: The higher the concentration of garlic bulb crude extract, the smaller diameter of mycelium in tomato leaf *In vitro*, and the largest diameter of mycelium was found in the bottom leaf. The various concentrations of garlic bulb crude extracts were used i.e., 20 mg.mL^{-1}, 40 mg.mL^{-1}, 60 mg.mL^{-1} and 80 mg.mL^{-1} (Table 3). The preventive effects on young leaf were 14.40%, 30.31%, 53.95% and 85.32%, however, on functional leaf were 14.93%, 33.14%, 57.37% and 76.50%, respectively. Effect on bottom leaves were also constantly increased (14.66%, 38.47%, 53.16% and 69.92%). Each concentration and control have shown significant differences at $p<0.05$ level. The diameter of mycelium in young leaf, functional leaf and bottom leaf were 6.82 mm, 7.64 mm and 9.15 mm, respectively, which showed the same trend with the treatment group by garlic bulb crude extract.

Table 3. Preventive effects of garlic bulb crude extract on *F. fulva* in different kinds of tomato leaf discs *In vitro*.

Concentration of garlic bulb crude extracts (mg·mL^{-1})	Young leaf		Functional leaf		Bottom leaf	
	Diameter of colony (mm)	Preventive effect (%)	Diameter of colony (mm)	Preventive effect (%)	Diameter of colony (mm)	Preventive effect (%)
Water control	6.82 aA	0.00 eE	7.64 aA	0.00 eC	9.15 aA	0.00 eC
20	5.83 bAB	14.40 dD	6.46 bAB	14.93 dBC	7.81 bA	14.66 dC
40	4.75 cB	30.31 cC	5.11 cB	33.14 cB	5.63 cB	38.47 cB
60	3.11 dC	53.95 bB	3.22 dC	57.37 bA	4.28 dBC	53.16 bB
80	1.00 eD	85.32 aA	1.77 eC	76.50 aA	2.77 eC	69.92 aA

2. Curative effects: Data shows in Table 4 that with the increase in concentration of garlic bulb crude extract the efficacy on tomato leaves *In vitro* also gone up, and the extension rate and diameter was the maximum in the bottom leaf. The concentration increased to 20 mg.mL^{-1}, 40 mg.mL^{-1}, 60 mg.mL^{-1} and 80 mg.mL^{-1} in turn, the curative effects on young leaf were 13.91%, 28.78%, 46.65% and 83.49%, respectively, on functional leaf up to 16.78%, 32.92%, 46.68% and 68.91% in turn, and on bottom leaf also increased from 10.89% to 69.36%. The group of treatment and control has shown significant differences at $p<0.05$ levels and the same trend of control effect.

Table 4. Curative effects of garlic bulb crude extracts on *F. fulva* in different kinds of tomato leaves *In vitro*.

Concentration of garlic bulb crude extracts (mg·mL^{-1})	Young leaf		Functional leaf		Bottom leaf	
	Diameter of colony (mm)	Curative effect (%)	Diameter of colony (mm)	Curative effect (%)	Diameter of colony (mm)	Curative effect (%)
0	7.33 aA	0.00 dD	7.98 aA	0.00 eD	9.49 aA	0.00 eD
20	6.28 bAB	13.91 cdCD	6.63 bB	16.78 dCD	8.45 bA	10.89 dD
40	5.19 cBC	28.78 cBC	5.34 cC	32.92 cBC	6.17 cB	34.93 cC
60	3.91 dC	46.65 bB	4.25 dC	46.68 bB	4.95 dC	47.81 bB
80	1.21 eD	83.49 aA	2.47 eD	68.91 aA	2.90 eD	69.36 aA

Effect of garlic bulb crude extract on *F. fulva* in pots: The effect on disease development by spraying tomato leaves with an application of garlic bulb crude extract containing a range of concentration 24 hours before and after inoculation with the spore suspension shown in Fig. 2 and Fig. 3, respectively. As can be seen, spraying with a high concentration of garlic bulb crude extract (160 mg·mL^{-1}) effectively reduced disease development, the affectivity of suppressing lesion development up to 82.19% and 79.37% (preventive effect in Fig. 2 and curative effect in Fig. 3), respectively, after inoculation 15 days later. Spraying with garlic bulb crude extract containing a low concentration did not completely suppress disease development. The inhibitive effects of infected leaf area were only 32.19% (prevent) and 20.69% (cure), respectively, when the concentration was 40 mg·mL^{-1}.

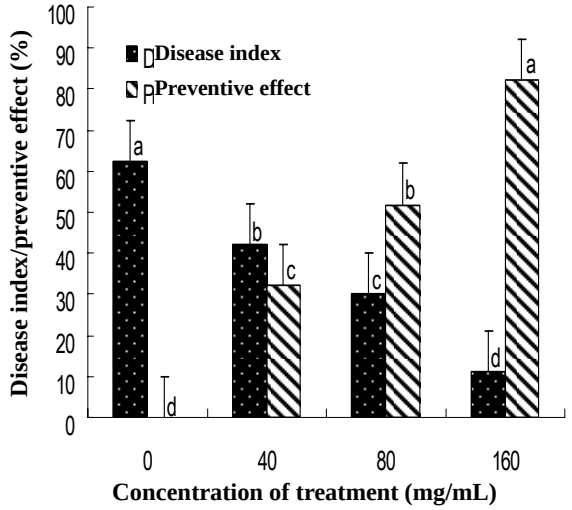

Fig. 2. The effects of spraying garlic bulb crude extract 24h before inoculation. Columns which differ significantly from one another are marked with a different letter (LSD's Test, $p<0.05$). The following figure is the same.

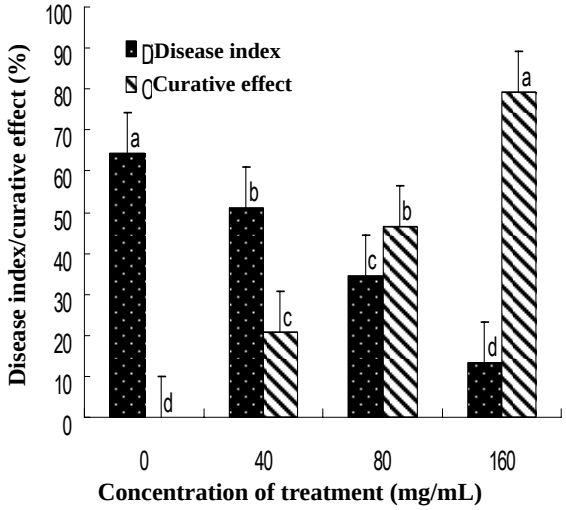

Fig. 3. The effects of spraying garlic bulb crude extract 24h after inoculation.

The impact of garlic bulb crude extract on tomato leaves: The amount of relative increase in tomato height treated by various concentrations of garlic bulb crude extract was found similar to the control (Fig. 4), and indicated that this range of concentration did not affect the growth of tomato height. The amount of relative growth of tomato stem diameter decreased at 120 mg mL^{-1}, grew slowly at 240 mg mL^{-1} and 480 mg mL^{-1}, and also decreased in the control group, so it is initially thought to be a measuring error (Figs. 5 and 6). It is observed that there is a promotion in growth of tomato stem diameter and leaf area at 240 mg mL^{-1}. This is probably because this concentration promote the activity of some enzymes on tomato, thus promote tomato growth, the growth of tomato at 120 mg mL^{-1} and 480 mg mL^{-1} were similar with that in control. It illustrates that this concentration of garlic bulb crude extract would not harm the growth of tomato, and 240 mg mL^{-1} concentration of garlic bulb crude extract even could promote the growth.

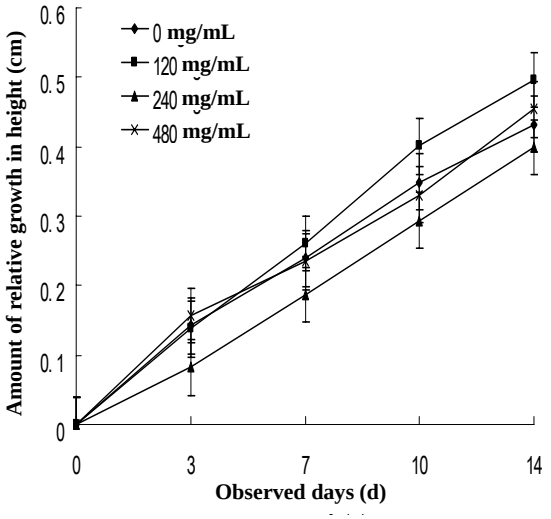

Fig. 4. Different concentrations of garlic bulb crude extracts on the amount of relative growth of tomato height.

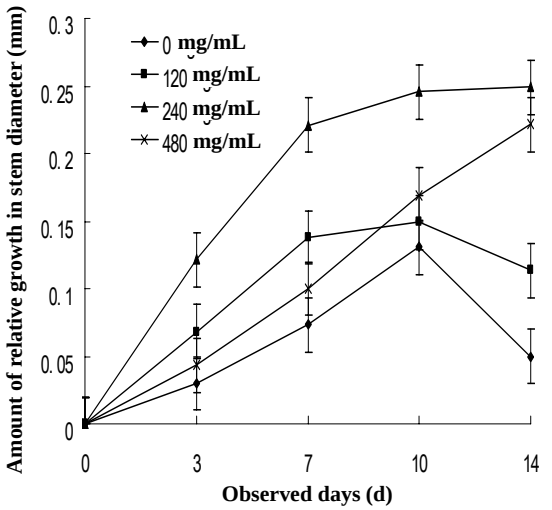

Fig. 5. Different concentrations of garlic bulb crude extracts on the amount of relative growth of tomato stem diameter.

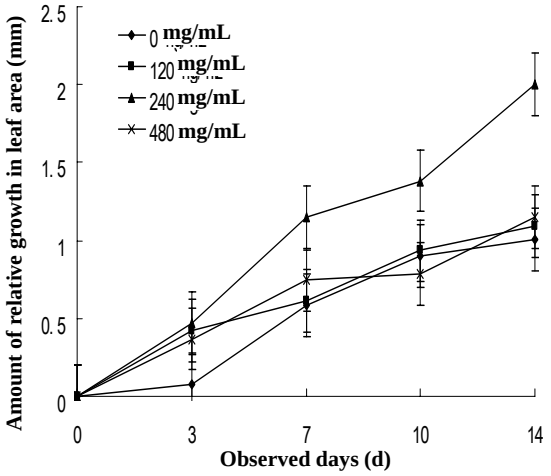

Fig. 6. Different concentrations of garlic bulb crude extracts on the amount of relative growth of tomato leaf area.

Discussion

This study demonstrated that there is an effective inhibition of garlic bulb crude extract on mycelia growth of *F. fulva*, germination of spores and on tomato leaves *In vitro* and seedlings. The inhibitive effect is proportional to the concentration of garlic bulb crude extract: the higher the concentration of garlic bulb crude extract showed the more inhibitive effects. These effects are in accordance with the results of Song (2004) and Su & Cheng (2009), who reported that garlic extract had effective inhibition on *Fusarium oxysporum* f. sp. *cucumerinum, F. oxysporum* Schl. f. sp. *nioeum* (E. F. Smith) Snyder & Hansen and *Phytophthora capsici* at 100 mg ml^{-1}, 500 mg ml^{-1} and 200 mg ml^{-1}, respectively. Daniela *et al*, (2008) also indicated that allicin in garlic juice inhibited the germination of sporangia and cysts and subsequent germ tube growth by *Phytophthora infestans* both *In vitro* and *In vivo* conditions on the leaf surface at 50 μg ml^{-1}. Similar studies were also carried out by Raouf & Khalil (2001) on the effects of aqueous extracts of 20 different plants on spore germination and vegetative growth of two pathogenic, terrestrial and zoo-sporic fungi. Disease severity in *P. infestans*. Infected tomato seedlings was also reduced by spraying leaves with garlic juice containing allicin over the range tested (55~110 μg ml^{-1}) with an effectiveness ranging from approximately 45~100%. Similarly, in growth room experiments at concentrations from 50~1000 μg ml^{-1}, allicin in garlic juice reduced the severity of cucumber downy mildew caused by *Pseudoperonospora cubensis* by approximately 50~100%.

The inhibitive action of garlic bulb crude extract on fungal growth has been attributed to the existence of allicin, as the major anti-bacterial, anti-fungal and anti-viral component (Miron *et al*., 2000). Furthermore, it has been reported that the antimicrobial substance allicin (diallylthiosulphinate) converts into oxygenated sulfur compounds, when garlic bulbs are damaged and the substrate alliin (S-allyl-L-cysteine sulphoxide) mixes with the enzyme alliin-lyase (E.C.4.4.1.4). The volatile compounds act as fungistatic or fungicidal components that disrupt fungal cell metabolism due to the oxidation of proteins (Baron & Tansey, 1977; Slusarenko *et al*., 2008). Allicin is readily membrane-permeable and undergoes thiol-disulphide exchange reactions with free thiol groups in proteins. It is thought that these properties are the basis of its antimicrobial action. Alan *et al*., (2008) reported that the reduction in disease was apparently due to a direct action against the pathogen since no accumulation of salicylic acid (a marker for systemic acquired resistance, SAR) was observed after treatment with garlic bulb crude extract in downy mildew of *Arabidopsis*. We see a potential for developing preparations from garlic for use in organic farming, e.g., for reducing the pathogen inoculum potential in planting material such as bulbs, seeds and tubers.

In addition, there is a problem on the storage environment and duration of garlic bulb crude extract. As the instability of the disulfide bonds in allicin, which is an effective antifungal ingredient in garlic, at high temperature and alkaline conditions, allicin would be hydrolyzed and loss its control effect. Therefore, garlic bulb crude extracts should be saved in acidic and low-temperature environment (pH: 5.0 and 4°C). Allinase in garlic will lose activity at 3°C for 14 days, thus affecting the synthesis of alliin and reducing inhibitive effect. So garlic bulb crude extracts should be used as soon as possible after preparation (Song, 2004).

The production process of most plant fungicides such as essential oils and allicin is complex and expensive (Wei

et al., 2009; Lin *et al*., 2008). More terrible problem is about the organic chemical residue when extraction with organic solvents.

Conclusion

This research use an easier, safer and more effective method to control fungal pathogens based on water-extracted natural inhibitor from a plant that is garlic. The use of water solution eliminates the interference of organic chemical residue, to make the antimicrobial material (allicin) directly inhibit the pathogens. Therefore, the further research will focus on the mechanism of garlic bulb crude extract against *F. fulva.*

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