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INHIBITORY EFFECT OF CRUDE EXTRACTS DERIVED FROM AROMATIC PLANTS AGAINST WHITE MOLD OF *BRASSICA JUNCEA* VAR. *TUMIDA*

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A B S T R A C T

The objective of this study was to evaluate antifungal activity and resistance inducing potential of crude extracts derived from neem (*Azadirachta indica*) and ginger (*Zingiber officinale*) against three isolates of *Sclerotinia sclerotiorum*, the causal agent of mustard white mold under *in vitro* and *in vivo* conditions. In addition, enzymatic tests were carried out to assess the effect of crude extracts on activities of resistance-inducing enzymes in mustard leaves. The results showed that ethanol extracts of neem and ginger at concentration 2 g/l were able to reduce mycelial growth of the pathogen (isolate 3) by 61.5 and 44.3%, respectively. The ethanol extracts of neem and ginger at concentration 2 g/l reduced infection radius on plant leaves from 9.7 in control to 3.1 and 3.4, respectively, due to antifungal efficacy. In addition, ethanol extracts of neem and ginger at concentration 2 g/l decreased infection radius (isolate 1) on plant leaves from 9.5 in control to 2.1 and 2.3, respectively, seven days after application. Enzymatic analyses showed significant increase in level of chitinases, β-1,3-glucanase, Phenylalanine ammonia lyase and Peroxidase due to application of ethanol extracts of neem and ginger.

Keywords: Ethyl acetate, Ethanol, Chitinases, PPO, PAL.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a soil borne ubiquitous fungus which can infect over 400 dicotyledonous plant species (Boland and Hall, 1994). This pathogen has recently become a serious threat to tuber mustard cultivation in Hangzhou and other mustard growing parts of Zhejiang province. Tuber mustard, *Brassica juncea* var. *tumida*, is an economically and nutritionally important *Brassica* plant which is grown in many fields of Hangzhou, center of Zhejiang province, in south east China. Initial symptoms of white mold appear as water soaked spots on leaves or stems. Later the lesions on the leaf extend to petiole and infect the stem. Lesions on the stem appear as a pale grey to white lesions on the stem at or above the soil surface. As disease advances it spreads to upper branches. Finally stem girdles at the point of infection, leading to wilting and death of the plant. Black sclerotial bodies are produced on or inside the hollow stem (Ojaghian, 2009). Although no study has been done to evaluate yield loss caused by *S. sclerotiorum* in Hangzhou, white mold of mustard is reported to cause yield losses up to 40 percent in India (Chattopadhyay *et al.,* 2003). Some management methods such as biological control (Chandra et al., 2007) and bio-fumigation (Ojaghian et al., 2012a) have been effective against Sclerotinia diseases. However, the majority of local mustard growers prefer application of fungicides mostly iprodione and dichloran (Ojaghian *et al.,* 2015). Due to increasing concerns about the environment, human health and development of fungicide resistance several researches have been conducted in Zhejiang province to

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introduce sustainable control methods in order to reduce fungicides such as iprodione and dichloran applied in mustard fields.

Many reports are available showing that aromatic plants synthesize secondary metabolites such as terpenes, tannins, flavonoids, alkaloids, and polypeptides (Castillo *et al.,* 2010). These bioactive compounds may play an important role in protecting plants from pests or preserving the integrity of plants during environmental stresses such as high temperatures and dehydration (Lira *et al.,* 2007). Many studies report that medicinal plants have potential to be considered as novel sources of antifungal agents against different plant diseases (Abou-Jawdah *et al.,* 2002; Hernández et al., 2007). The crude plant extracts derived from medicinal plants such as *Robinia pseudoacacia* (Zhang et al., 2008), *Agapanthus africanus* (Tegegne *et al.,* 2008), *Aloe vera* (Jasso de Rodríguez *et al.*, 2005), *Bucida buceras* and *Olinia ventosa* (Mahlo *et al.,* 2010) were found to be effective against a variety of fungal plant pathogens. According to previous researches, derivatives of neem (*Azadirachta indica*) have potential to significantly inhibit a number of plant pathogenic fungi *in vitro* such as *Drechslera oryzae*, *Fusarium oxysporum*, *Alternaria tenuis* (Govindachari et al. 1998), *Fusarium moniliforme*, *Fusarium nivale*, *Fusarium semitectum* and *Drechslera hawiinesis* (Sitara et al., 2008). Ojaghian et al. (2014) showed that crude extracts derived from neem, ginger, cinnamon and rosemary have antifungal and resistance inducing potential against carrot rot caused by *S. sclerotiorum.* Many studies report that ginger has antifungal effect against plant pathogens (Islam et al., 2006; Suleiman and Emua, 2009; Islam and Faruq, 2012).

Therefore, the objective of this study was to evaluate antifungal activity and resistance inducing potential of crude extracts derived from neem and ginger against three isolates of *S. sclerotiorum*, the causal agent of mustard white mold under *in vitro* and *in vivo* conditions. In addition, enzymatic tests were carried out to assess the effect of crude extracts on activities of resistance-inducing enzymes in mustard leaves.

MATERIALS AND METHODS

***S. sclerotiorum* isolates and Plant materials:** Three isolates of *S. sclerotiorum* were chosen from the isolates collected from infected tuber mustard fields in Hangzhou. These isolates had already shown high aggressiveness against mustard plants under greenhouse conditions (data not published). The isolates were designated as 1, 2 and 3, respectively. They were routinely cultured on Carrot dextrose agar (CDA, infusion of 200 g of carrot, 20 g of dextrose, and 15 g of agar for 1 L of medium).

Dried ginger rhizomes were purchased from Huisong Pharmaceuticals (Hangzhou, China) and neem leaves were kindly provided by Dr. Auwal Hassan (Maiduguri University, Nigeria). The plant materials were ground into a fine powder and passed through a 24-mesh sieve.

Extraction of plant materials: In order to achieve extraction, milled plant materials (1000 g) were added to 5 L ethyl acetate (EA) in five Erlenmeyer flasks. The flasks were placed in an ultrasonic cleaner (Kunshan-500B) for 1 min to increase solubility efficacy and remained on the laboratory desk for 24 h. After getting filtered through Whatman filter paper No 1, the solvent was evaporated using a rotary evaporator. In addition, the ethanolic extraction was performed as explained above. The obtained extracts were remained in amber glass bottles at 4(±1) °C until used. The average yields of ginger and neem extracts obtained from EA and ethanol were about 5.1-6.3 and 5.5-6.8%, respectively (Ojaghian et al., 2014).

Efficacy of crude plant extracts against mycelial growth: The inhibitory effect of plant crude extracts was determined in volatile and contact phases against mycelial growth of the pathogen isolates. In volatile effect test, 100, 200 and 500 µl of each crude extract were loaded to sterile filter papers (10 mm diameter, Whatman filter paper No. 1) at concentration of 200 µg/ml and placed in Petri plates (90 mm). The lid of each plate was replaced with bottom of another CDA plate inoculated centrally with 5mm mycelial discs taken from the edge of 4 days old culture of three isolates of *S. sclerotiorum*. In all treatments, the pairs of each Petri dish were sealed together in sterile conditions with paraffin tape to prevent them from any infection and escape of volatile materials. Because volatile substances always move upwards, the plant extract sections were always in the lower sections and the CDA containing section with the organism was inverted over the lower section (Ojaghian *et al*, 2015). There were three sets of controls in this test including equal amount of EA, ethanol and sterile distilled water (SDW). This experiment was repeated three times with five repetitions for each treatment.

In order to assess contact phase efficacy of plant extracts against the pathogen, plant crude extracts were added to

molten CDA medium to obtain a final concentration of 0.5, 1 and 2 g/l. Because EA is not water soluble, EA extractions were firstly dissolved in 5 ml Tween 20 and then added to the culture medium. The medium was poured into the Petri dishes. These plates were centrally inoculated with 5mm mycelial plug taken from the edge of 4-day culture of three isolates of *S. sclerotiorum*. There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20. This test was conducted three times with three repetitions for each treatment.

The plates were incubated at 22°C in both volatile and contact phase trials. The radial growth of *S. sclerotiorum* in all plates was calculated six days after inoculation and percent inhibition of radial growth in each treatment was by the formula $I = (C-T)/C \times 100$, where I= percent growth inhibition, C = colony diameter of pathogen in control, and T = colony diameter of pathogen in treatment (Ojaghian *et al.,* 2012).

Antifungal effect of plant crude extracts: Tuber mustard (*B. juncea* var. *tumida*) seeds were bought from Feng Seeds company (Ningbo, Zhejiang province, China). After disinfection in 2% hypochlorite sodium for 2 min, the seeds were washed with autoclaved tap water and dried on sterile filter papers. The seeds were sown in each plastic pot filled with field soil pasteurized at 75 ± 5 C for 1 h, topped with 1 cm of vermiculite and were watered as needed for 70 days (Ojaghian *et al.,* 2018).

The mustard plants were sprayed with the extracts dissolved in SDW at concentrations 0.5, 1 and 2 g/l using sterile medical syringes, so that the leaves were coated all over. The EA extractions were firstly dissolved in 5 ml Tween 20 and then added to SDW. There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20. Four hours after spraying, leaves of plant were cut off the stems for evaluation of disease severity. A 5mm mycelial plug was placed at the center of each leaf with the mycelium towards leaf surface. The leaves were placed in 200 mm Petri plates on two filter papers soaked with sterile water. These filter papers were used to provide necessary humidity in the plates. The plates were remained at 20-22 °C. The infection (water soaked) radius on leaves was determined seven days after inoculation. This experiment was repeated four times within four consecutive weeks for three isolates in a completely randomized block design.

Evaluation of crude extracts against mustard white mold over time: This experiment was conducted to assess the development of acquired resistance caused by plant extracts against mustard white mold over time. In this test, 70-day mustard plants were sprayed with the extracts dissolved in SDW at concentrations 0.5, 1 and 2 g/l as explained above. The EA extractions were firstly dissolved in 5 ml Tween 20 and then added to SDW. There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20. After leaving plants in laboratory conditions (24-26 °C) for seven days, the leaves were cut off the plants and were inoculated as explained above and remained at 20-21 °C. The infection radius on leaves was determined seven days after inoculation with pathogen isolates. This experiment was repeated three times within three weeks for three isolates in a completely randomized block design.

Protein extraction: One, three and six days after sprayed with crude extracts at the concentration of 2 g/l, 70-day mustard leaves were cut off the plants. This concentration had already shown the best inhibitory effect against the disease at previous tests. According to Roberti *et al.* (2008), the leaves were weighed and ground in liquid nitrogen to fine powder in a pre-chilled mortar and pestle. Using 20 mM sodium acetate buffer (pH=5.2, 1 mL for 1 g fresh weight) containing polyvinylpolypyrrolidone (1 %, Sangon Chemical Company, Shanghai, China), total proteins were extracted and incubated at 4 °C for 1.5 min under continuous gentle stirring. Afterwards, the extracts were centrifuged twice at 4 °C at 8000 g for 25 min. In next step, using a Syringe Filter Unit (GV Millex®, Millipore, USA), the supernatant was filtered. The filtered material was then concentrated and desalted using a Centrifugal Filter Unit (Ultrafree®, Millipore, USA). According to Bradford (1976), protein concentrations were evaluated by the protein–dye binding technique. There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20.

Activity assessment of chitinases, β-1,3-glucanase and peroxidase: The 70-day plants were sprayed with crude extracts at the concentration 2 g/l because this concentration showed the best efficacy against the pathogen when disease severity was assessed over time. As previously explained (Ojaghian *et al.,*

2017), activities of β-N-acetyl hexosaminidase, endochitinase and chitin 1,4-β-chitobiosidase as well as β-1,3-glucanase and peroxidase were evaluated after one, three and six days of inoculation with *S. sclerotiorum* (isolate 1) with three replications. There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20.

Spectrophotometry analysis of Phenylalanine ammonia lyase (PAL), Polyphenoloxidase (PPO) and Peroxidase (POD): The 70-day plants were sprayed with crude extratcts at the concentration of 2 g/l. Activity of PAL, PPO and POD was measured after three and six days of inoculation with *S. sclerotiorum* (isolate 1) as explained by Ojaghian *et al.* (2013). There were two sets of controls in this test including SDW and SDW including equal amount of Tween 20. These experiments were repeated two times and there were three replicates in each treatment.

All enzyme extraction procedures were conducted at 4° C. To assess the activity of PAL, 1 g of leave tissue obtained from three plants was macerated and mixed with 2 ml extracting buffer [0.2 M boric acid buffer containing 10% (w/v), Polyvinylpolypyrolidone (PVPP), 1 mM EDTA and 50 mM β mercaptoethanol, pH 8.8]. For PPO and POD, 2 g of leave tissue obtained from three plants for each replication was macerated with 10 ml of 100 mM sodium phosphate buffer (pH 6.4) containing PVPP (0.2 g), homogenized and centrifuged at 8000 g at 4° C for 30 min, and the supernatant was collected and used for enzyme assay.

In order to assay PAL, the enzyme extract (300 µl) was incubated with 1 ml 0.02 M L-phenylalanine and 2 ml of the PAL extracting buffer at 24 °C for 2 min, and absorbance at 290 nm was measured in an ultraviolet spectrophotometer. The PAL activity was expressed as U290, where U290 = 0.01ΔOD290/mg protein/min.

To determine PPO activity, 100 µl of enzyme extract was incubated with 2 ml of 0.05 M phosphate buffer (pH 7.0) and 0.5 ml of 0.5 M catechol at 24 °C for 2 min, and the absorbance at 398 nm was measured with an ultraviolet spectrophotometer. The PPO activity was expressed as U398, where U398 = 0.01ΔOD398/mg protein/min.

The POD activity was determined using guaiacol as substrate. The reaction mixture consisted of 0.1 ml of crude extract and 2 ml of guaiacol (8 mM in 100 mM sodium phosphate buffer, pH 6.4) and was incubated for 30 min at 30 °C. The increase in absorbance at 460 nm was measured after 1 ml H_2O_2 (24 mM) was added. The activity of POD was expressed as U460, where U460 = 0.01ΔOD460/mg protein/min.

DATA ANALYSIS

The effects of different treatments in each experiment were determined by the analysis of variance (ANOVA) using SAS software (SAS 8.2, 1999-2001; SAS Institute Inc., Cary, NC) in completely randomized design tests. Before running the statistics, the homogeneity of variance was proved using Hartley's F_{max} test. Means of treatments were separated using Fishers' LSD test.

RESULTS

Effect of crude plant extracts against radial growth of the pathogen; Volatile phase: After six days of inoculation, the results showed no inhibitory effect ($P < 0.05$) at 100 µl for EA and ethanol extracts of ginger and neem (Table 1, Figure 1). The results of all experiments on both controls were significantly similar, therefore; only data related with SDW have been presented in this manuscript.

Table 1. Percentage of radial growth inhibition of *S. sclerotiorum* (three isolates) on carrot dextrose agar by volatile compounds resulting from 100, 200 and 500 µl of crude plant extractions at concentration 200 µg/ml after 6 days.

	Radial growth inhibition (%)*											
	Neem						Ginger					
	Ethyl acetate			Ethanol			Ethyl acetate			Ethanol		
	100	200	500	100	200	500	100	200	500	100	200	500
Controls	0	0	0	0	0	0	0	0	0	0	0	0
Isolate 1	NI *	NI	22.7±3.9^a	NI	NI	29.6±6.2^a	NI	8.4±1.4^a	25.8±5.2^a	NI	20.2±5.3^a	39.7±12.3^a
Isolate 2	NI	15.2±3.6	35.1±6.4^b	NI	12.3±4.7	36.7±5.4^b	NI	21.3±0.8^b	47.6±15.7^b	NI	31.5±11.2^b	52.4±25.4^b
Isolate 3	NI	NI	25.5±4.1^a	NI	NI	27.7±4.3^a	NI	9.1±1.5^a	22.7±7.9^a	NI	23.5±4.6^a	40.9±23.2^a

*NI: Not inhibited. Within columns, means followed by a common letter do not differ significantly at the $P < 0.05$ level of confidence according to Fisher's test. Values in the table indicate means ± standard error. This experiment was repeated three times and each treatment was replicated five times.

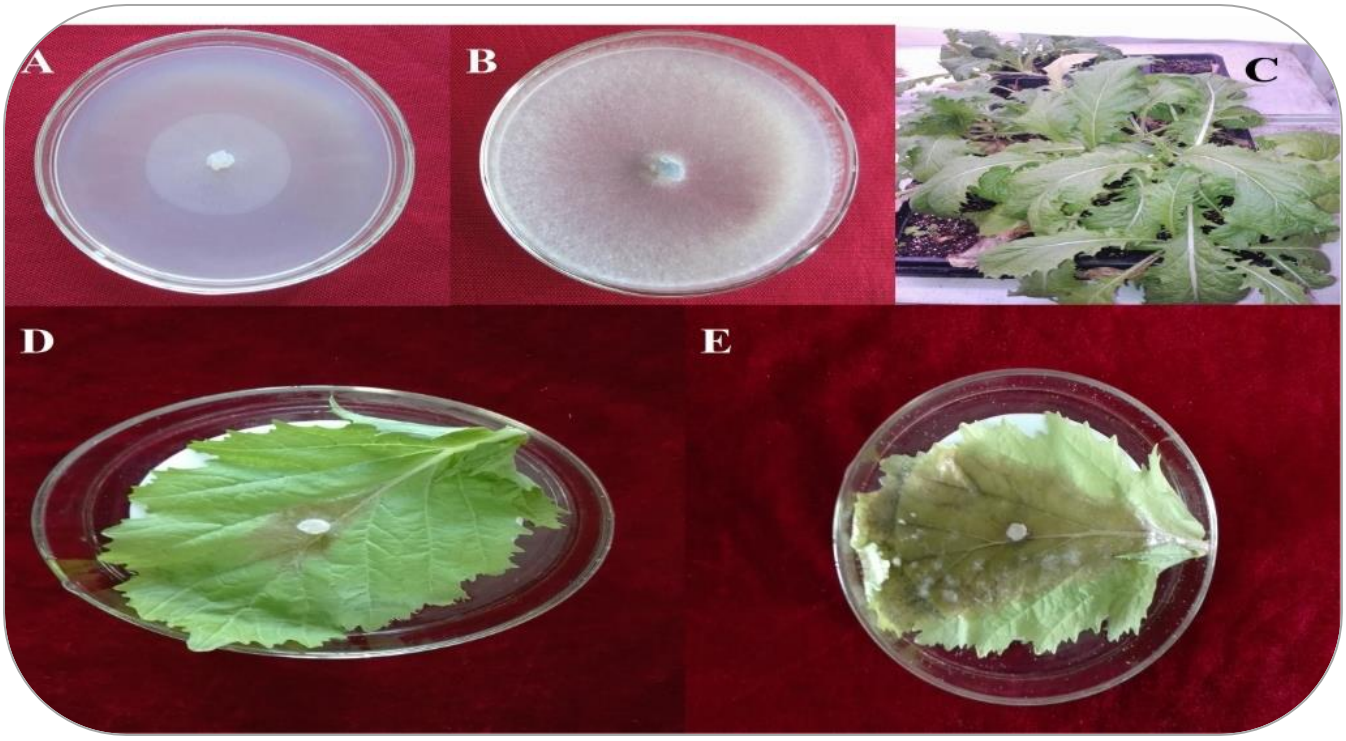

Figure 1. Inhibitory effect of ginger ethanol extract against white mold of tuber mustard (*B. juncea* var. *tumida*) caused by *Sclerotinia sclerotiorum* (isolate 2). Compared with control (B), the radial growth of the pathogen was significantly reduced (A) by volatile materials after five days of inoculation. Due to antifungal efficacy, ethanol extract of ginger (2 g/l) reduced infection radius caused by the pathogen (D) on plant leaves cut off from 70-day plant (C) after seven days compared with the control (E).

At volume of 500 µl, all treatments significantly reduced radial growth of the pathogen compared with control. A positive correlation was observed between volume of the crude extracts and inhibitory efficacy against mycelial growth of the pathogen. Radial growth of the isolate 2 was reduced more than other two isolates (Table 1). At volume of 200 µl, EA and acetate extracts of neem markedly decreased mycelial growth of isolate 2 but no inhibitory effect was observed in isolates 1 and 3.

Contact phase: The EA and ethanol extracts of neem and ginger were able to reduce mycelial growth of all tested isolates (Table 2) in all concentration ($P > 0.05$). A positive relation was observed between concentration of the crude extracts and inhibitory efficacy against mycelial growth of the pathogen isolates. EA extract of neem, as well as ethanol extract of ginger, at concentration 0.5 g/l reduced mycelial growth of three isolates in statistically similar level but isolate 2 was inhibited more than other isolates at concentration 1 and 2 g/l. In addition, isolate 2 was inhibited more than isolates 1 and 3 as treated by ethanol extract of neem at concentration 0.5 g/l and 1 (Table 2).

Antifungal effect of plant crude extracts: This experiment was conducted to assess antifungal efficacy of crude extracts against the pathogen isolates four hours after spraying.

For isolate 1, the highest efficacy was found to be in ethanol extract of neem (2 g/l) and ginger at 1 and 2 g /l with statistically at par results (Table 3, Figure 1). EA extracts of neem and ginger (1 and 2 g /l) and ethanol extract of neem were the next treatments in order of efficacy with statistically similar performance (Table 3).

For isolate 2, ethanol extract of ginger (1 and 2 g/l) and neem (2 g/l) showed the highest antifungal efficacy against mustard white mold with statistically similar performance followed by EA extract of ginger (2 g/l) (Table 3).

For isolate 3, the highest inhibitory effect was observed due to spraying ethanol extract of ginger (1 and 2 g/l) and neem (2 g/l) as well as EA extract of ginger (2 g/l) in a statistically similar level. EA extract of ginger (1 g/l) and ethanol extract of neem (1 g/l) were the next treatments in order of superiority (Table 3).

Table 2. Inhibitory effect of crude plant extractions (contact phase) against radial growth of *S. sclerotiorum* (three isolates) at different concentrations (g/l) after 6 days.

	Percent inhibition of radial growth*											
	Neem						Ginger					
	Ethyl acetate			Ethanol			Ethyl acetate			Ethanol		
	0.5	1	2	0.5	1	2	0.5	1	2	0.5	1	2
Controls	0	0	0	0	0	0	0	0	0	0	0	0
Isolate 1	25.4±5.3[a]	27.5±4.3[a]	47.8±4.2[a]	17.2±5.2[a]	38.5±10.1[a]	62.5±15.1[a]	19.7±1.5[a]	25.2±3.8[a]	55.4±19.7[a]	22.7±6.2[a]	34.7±4.9[a]	42.4±10.8[a]
Isolate 2	27.1±2.8[a]	43.5±7.1[b]	64.2±5.8[b]	24.1±6.8[b]	55.4±13.9[b]	63.1±11.6[a]	18.2±0.7[a]	14.8±3.7[b]	38.9±10.2[b]	20.8±7.2[a]	46.2±11.3[b]	55.7±6.1[b]
Isolate 3	23.9±6.4[a]	30.8±5.5[a]	44.9±7.2[a]	18.8±8.5[a]	34.9±5.2[a]	61.5±17.3[a]	17.9±1.6[a]	16.2±4.9[b]	34.7±12.4[b]	19.8±4.3[a]	31.7±5.8[a]	44.3±12.8[a]

* Within columns, means followed by a common letter do not differ significantly at the $P > 0.05$ level of confidence according to Fisher's test. Values in the table indicate means ± standard error. This experiment was repeated three times and each treatment was replicated five times.

Table 3. Antifungal efficacy of crude plant extracts at 0.5, 1 and 2 g /l against mustard white mold four hours after spraying

			Infection radius (cm) [a]		
			Isolate 1	Isolate 2	Isolate 3
controls			9.7±0.3 [a]	8.5±1.4 [a]	9.5±0.2 [a]
Neem	Ethyl acetate	0.5	9.4±0.5 [a]	8.1±1.6 [a]	9.1±0.5 [a]
		1	5.1±1.4 [b]	8.2±1.3 [a]	8.1±2.3 [ab]
		2	4.9±2.5 [b]	6.1±2.4 [b]	6.2±1.8 [b]
	Ethanol	0.5	9.2±2.4 [a]	8.2±0.9 [a]	9.3±1.6 [a]
		1	5.4±1.6 [b]	7.3±1.5 [ab]	6.1±1.2 [b]
		2	3.1±0.5 [c]	2.4±0.8 [c]	3.5±1.1 [c]
Ginger	Ethyl acetate	0.5	9.3±3.1 [a]	8.2±3.1 [a]	9.4±2.8 [a]
		1	5.1±2.8 [b]	6.3±2.4 [b]	4.7±0.9 [bc]
		2	4.8±1.6 [b]	4.5±0.8 [bc]	3.4±1.6 [c]
	Ethanol	0.5	9.2±3.4 [a]	8.2±1.9 [a]	9.2±1.7[a]
		1	3.2±0.7 [c]	2.5±0.4 [c]	3.8±1.4 [c]
		2	3.4±0.6 [c]	2.3±0.7 [c]	3.5±0.6 [c]

*All the means within a column followed by the same letter are not significantly different at the $P<0.05$ level of confidence according to Fisher's test. This experiment was replicated four times.

Inhibitory effect of crude extracts against the disease seven days after spraying: The objective of this test was to determine whether the crude extracts can induce resistance against mustard white mold after seven days of spraying. EA extracts of neem and ginger were not able to reduce diseased incidence in all tested isolates (Table 4).

For isolate 1, ethanol extract of ginger and neem (2 g/l) were the most effective treatments with statistically similar performance followed by ethanol extract of ginger (0.5 g/l) (Table 4).

For isolate 2, the least disease severity was observed due to application of ginger extract (1 and 2 g/l) and ethanol extract of neem (2 g/l) with statistically similar results. Ethanol extract ginger (1 g/l) reduced infection radius to 4.3 cm (Table 4).

For isolate 3, ethanol extract of ginger (1 and 2 g/l) showed the highest antifungal effect with statistically similar results, and ethanol extract of neem (2 g/l) was the next treatment in order of efficacy (Table 4).

Table 4. Resistance induction against mustard white mold due to application of crude plant extracts at 0.5, 1 and 2 g /l seven days after spraying

			Infection radius (cm) [a]		
			Isolate 1	Isolate 2	Isolate 3
controls			9.5±0.4 [a]	8.9±2.3 [a]	10.2±2.5 [a]
Neem	Ethyl acetate	0.5	9.2±2.1[a]	8.5±1.4[a]	9.9±3.4[a]
		1	9.4±1.6[a]	8.6±3.2[a]	10.3±3.8[a]
		2	9.3±0.9[a]	8.4±2.7[a]	9.8±1.7[a]
	Ethanol	0.5	4.1±1.7[b]	7.2±1.3[ab]	5.5±2.1[b]
		1	3.3±0.3[bc]	5.3±1.8[b]	5.9±1.5[b]
		2	2.1±0.2[c]	3.8±0.9[c]	3.7±0.6[c]
Ginger	Ethyl acetate	0.5	9.4±2.7[a]	8.6±2.4[a]	10.1±3.2[a]
		1	9.3±2.1[a]	8.2±3.1[a]	9.7±0.4[a]
		2	9.4±3.6[a]	8.8±2.5[a]	9.8±3.1[a]
	Ethanol	0.5	3.2±0.5[bc]	4.3±1.6[bc]	4.8±1.2[bc]
		1	4.5±1.2[b]	3.7±0.2[c]	2.3±0.4[d]
		2	2.3±0.4[c]	3.3±0.4[c]	2.5±0.6[d]

*All the means within a column followed by the same letter are not significantly different at the $P<0.05$ level of confidence according to Fisher's test. This experiment was replicated four times.

Activity assessment of chitinases, β-1,3-glucanase and peroxidase: The results showed that ethanol extracts of neem and ginger were able to increase ($P>0.05$) level of resistance related enzymes, except for peroxidase, after three and six days (Table 5).

The highest level of β-N-acetyl hexosaminidase was found in ethanol extract of neem and ginger after six days. Ethanol extract of ginger was the only treatment which increased endochitinase activity after three and six days. Activity of chitin 1,4-β-chitobiosidase markedly increased due to application of ethanol extracts of ginger and neem after one, three and six days with statistically at par level. Ethanol extract of neem was the most effective treatment in increasing β-1,3-glucanase after six days, and no change was observed in peroxidase level after one, three and six days of crude extracts application compared with controls (Table 5).

Evaluation of PAL, PPO and POD: After three days of inoculation, significant increase was observed in PPO level due to application of ethanol extracts of neem and ginger with statistically similar results. The treatments did not change level of POD compared with controls. However PAL level markedly increased due to application of ethanol extract of ginger.

After six days of inoculation, the highest level of PPO was found to be due to application of ethanol extract of neem followed by ethanol extract of ginger, and EA extracts of neem and ginger were the next treatments in order of efficacy with statistically similar performance. The crude extracts did not change POD level after six days of inoculation. The highest level of PAL activity was observed due to application of ethanol extract of ginger followed by ethanol extract of neem, and EA extracts of neem and ginger were the next treatments in order of superiority with statistically similar results (Table 6).

Table 5. Effect of crude extracts of neem and ginger (2 g/l) on resistance related enzymes in tuber mustard leaves after one, three and six days of spraying on 70-day plants.

Enzyme activity after one day (U/mg protein) [a]					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-β-chitobiosidase	β -1,3-glucanase	Peroxidase
Control 1	0.00635 [a]	0.00432 [a]	0.00343 [a]	2.81 [a]	2.34 [a]
Control 2	0.00638 [a]	0.00437 [a]	0.00348 [a]	2.77 [a]	2.47 [a]
Ginger (ethanol)	0.00637 [a]	0.00443 [a]	0.00541 [b]	2.86 [a]	2.55 [a]
Ginger (EA)	0.00716 [ab]	0.00440 [a]	0.00352 [a]	2.79 [a]	2.58 [a]
Neem (ethanol)	0.00642 [a]	0.00437 [a]	0.00549 [b]	2.65 [a]	2.63 [a]
Neem (EA)	0.00644 [a]	0.00445 [a]	0.00341 [a]	2.85 [a]	2.34 [a]

Enzyme activity after three days (U/mg protein) [a]					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-β-chitobiosidase	β -1,3-glucanase	Peroxidase
Control 1	0.00637 [a]	0.00436 [a]	0.00346 [a]	2.84 [a]	2.37 [a]
Control 2	0.00649 [a]	0.00438 [a]	0.00342 [a]	2.73 [a]	2.48 [a]
Ginger (ethanol)	0.00811 [b]	0.00584 [b]	0.00551 [b]	4.64 [ab]	2.36 [a]
Ginger (EA)	0.00632 [a]	0.00431 [a]	0.00443 [ab]	2.76 [a]	2.58 [a]
Neem (ethanol)	0.00803 [b]	0.00422 [a]	0.00547 [b]	5.12 [b]	2.61 [a]
Neem (EA)	0.00686 [a]	0.00441 [a]	0.00349 [a]	2.67 [a]	2.63 [a]

Enzyme activity after six days (U/mg protein) [a]					
	β-N-acetyl hexosaminidase	Endochitinase	Chitin 1,4-β-chitobiosidase	β -1,3-glucanase	Peroxidase
Control 1	0.00643 [a]	0.00438 [a]	0.00352 [a]	2.79 [a]	2.45 [a]
Control 2	0.00638 [a]	0.00442 [a]	0.00347 [a]	2.85 [a]	2.40 [a]
Ginger (ethanol)	0.00923 [c]	0.00592 [b]	0.00545 [b]	5.23 [b]	2.62 [a]
Ginger (EA)	0.00627 [a]	0.00424 [a]	0.00457 [ab]	2.68 [a]	2.78 [a]
Neem (ethanol)	0.00935 [c]	0.00436 [a]	0.00557 [b]	7.58 [c]	2.54 [a]
Neem (EA)	0.00639 [a]	0.00429 [a]	0.00335 [a]	2.77 [a]	2.43 [a]

[a] All the means within a column followed by the same letter are not significantly different at the $P>0.05$ level of confidence according to Fisher's test. U: one unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of substrate per min. There were two sets of controls in this test including SDW (C1) and SDW including equal amount of Tween 20 (C2).

Table 6. Effect of ethanol and ethyl acetate (EA) crude extracts of neem and ginger on Phenylalanine ammonia lyase (PAL), Polyphenoloxidase (PPO) and Peroxidase in tuber mustard leaves after three and six days of inoculation with *Sclerotinia sclerotiorum* (isolate 1).

	Enzyme activity					
	PPO		POD		PAL	
	After 3 days	After 6 days	After 3 days	After 6 days	After 3 days	After 6 days
Control 1	67.1±8.2 [a]	66.4±11.8 [a]	5.3±1.4 [a]	5.2±2.1 [a]	9.7±2.7 [a]	9.3±1.5 [a]
Control 2	68.5±12.1 [a]	70.8±17.3 [a]	5.2±1.5 [a]	5.4±1.5 [a]	10.3±2.2 [a]	9.5±1.8 [a]
Ginger (ethanol)	156.5±27.4 [b]	165.2±18.7 [b]	5.4±0.7 [a]	5.5±0.5 [a]	21.5±9.6 [b]	52.9±12.8 [c]
Ginger (EA)	109.9±26.2 [ab]	105.4±21.5 [ab]	5.2±1.5 [a]	5.3±1.1 [a]	10.1±3.2 [a]	22.1±0.3 [b]
Neem (ethanol)	159.7±19.4 [b]	371.2±44.9 [c]	5.5±0.9 [a]	5.4±1.6 [a]	15.9±2.4 [ab]	32.4±9.7 [bc]
Neem (EA)	69.5±18.6 [a]	98.5±12.8 [ab]	5.2±0.6 [a]	5.2±0.4 [a]	11.4±3.1 [a]	19.8±1.2 [b]

[a] All the means within a column followed by the same letter are not significantly different at the $P<0.05$ level of confidence according to Fisher's test. The PPO activity was expressed as U398, where U398 = 0.01ΔOD398/mg protein/min. These experiments were repeated two times and there were three replicates in each treatment. There were three sets of controls: plant leaves sprayed with sterile deionized water (Control 1), plant leaves sprayed with 100 mL autoclaved deionized water containing 2.5 ml of 10 N acetic acid and adjusted to pH 5.6 using 1 N NaOH (Control 2) and plant leaves inoculated with the pathogen (isolate 1) (Control 3). The activity of POD was expressed as U460, where U460 = 0.01ΔOD460/mg protein/min. The PAL activity was expressed as U290, where U290 = 0.01ΔOD290/mg protein/min.

DISCUSSION

S. sclerotiorum is one of the most important diseases on a large number of crops including tuber mustard. There are a large number of studies showing that bioactive components derived from aromatic plants can be considered as non-phytotoxic, easily biodegradable and systemic antimicrobial agents. Although efficacy of essential oils derived from fennel and oregano against *S. sclerotiorum* has been reported **(**Soylu *et al.,* 2007), this is the first study showing that crude extracts of **g**inger rhizomes and neem leaves have inhibitory activity mustard white mold. Moreover, the change of resistance-related enzymes in mustard leaves treated with crude extracts was assessed.

It is well known that antifungal compounds with different properties may be solved in different solvents (Wojcikowski *et al.,* 2007). Therefore, ethyl acetate and ethanol were used as solvents in this research. This study showed that crude extracts were able to significantly decrease mycelial growth of the pathogen. In this study, ethanol extract of neem at the concentration of 2 g/l reduced mycelial growth of the isolate 2 by 63.1% in contact phase. There are several researches showing that neem extracts contain triterpenoidal compounds which are known to have inhibitory efficacy against different plant pathogenic fungi (Coventry and Allan, 1996; Govindachari *et al.,* 1999; Wang et al., 2010). Antifungal activity of neem has been attributed to some bioactive compounds such as nimbidin (Ogbuewu *et al.,* 2011), gedunin (Nazma et al., 1977), cyclic trisulphide and cyclic tetrasulphide (Pant et al., 1986). In addition, isolate 2 was inhibited by ethanol extract of ginger (contact phase) at the concentration 2 g/l by 55.7%. Confirming previous researches over antifungal activity of ginger (Suleiman and Emua, 2009; Islam and Faruq, 2012), this study showed that ginger extracts have inhibitory potential against *S. sclerotiorum*. Although Soylu *et al.* (2007) reported a relatively similar antifungal efficacy for volatile and contact phase of essential oils from oregano and fennel against *S. sclerotiorum*, this study showed that contact phase of ginger and neem crude extracts are more effective against mycelial growth of the pathogen. The results of *in vitro* tests showed that isolate 2 was more inhibited than other two isolates. According to previous studies, different isolates of *S. sclerotiorum* show various aggressiveness (Kull et al., 2004) and different reactions to environmental or biocontrol conditions (Wu and Subbarao, 2008; Ojaghian *et al.*, 2013). It has been reported that systemic resistance against plant pathogens are associated with the changes of activity of three enzymes which have been evaluated in this study. The PPOs are involved in the oxidation of polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during the microbial invasion. The PODs are oxido-reductive enzymes that

participate in the wall building processes such as oxidation of phenols, suberization, and lignification of host plant cells during the defense reaction against pathogenic agents. Furthermore, PAL is an essential enzyme in the phenyl propanoid biosynthesis pathway leading to the synthesis of phytoalexins or phenols, which have defense functions in plants, such as fortification of the plant cell wall (Ojaghian *et al.,* 2014). There are many studies showing a close association between the resistance against plant pathogens and expression of the resistance related genes (Métraux *et al*., 1991; McDowell and Woffenden, 2003; Ojaghian *et al.,* 2018). This study showed that application of ethanol extracts of neem and ginger are able to significantly increase resistance related enzymes over time indicating that these extracts can be considered as resistance inducers against mustard white mold.

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