EFFICACY OF SOME PLANT EXTRACTS AGAINST *RHIZOCTONIA SOLANI* ON PEA

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Abstract: Antifungal activity of ethanol-water extracts of four medicinal plants, cinnamon (*Cinnamomum verum* Presl.), anise (*Pimpinella anisum* L.), black seed (*Nigella sativa* L.) and clove (*Syzygium aromaticum* L. Merr. & Perry.) was investigated against pea (*Pisum sativum* L.) root-rot fungus *Rhizoctonia solani*. *In vitro* antifungal activity test shown a high growth inhibition at concentration (4%) of each plant extract. The highest antifungal activity was recorded for clove extract which causes complete growth inhibition at concentration of 1%. Efficacy of clove extract on disease incidence of *Rhizoctonia* root-rot of pea was investigated in the greenhouse pot experiment. Clove extract at concentration 4% as well as the chemical fungicide recorded highly significant increase in the percentage of survived plants (40 and 48%, respectively) and highly significant decrease in disease incidence.

Key words: control, essential oil, clove, cinnamon, anise, black seed, *Rhizoctonia solani*

INTRODUCTION

Pea (*Pisum sativum*) is one of the most important leguminous crops in many countries including Saudi Arabia. Root rot is a major soil borne disease of pea and is often considered to be the major limiting factor in production of this plant (Grünwald *et al*. 2004). The most common pathogens causing this disease are *Rhizoctonia solani, Aphanomyces euteiches, Pythium ultimum* and *Fusarium solani* f. sp. *pisi* (Kraft and Pfleger 2001). *Rhizoctonia* root-rot caused by *Rhizoctonia solani* Kühn, is one of the most serious diseases of pea in Saudi Arabia causing heavy damage in plant production. Seed may fail to germinate or young seedlings may fall over at soil line. Seedlings as well as mature plants exhibit a reddish-brown lesion or canker on the lower stem that enlarge to a point of girdling the plant causing plant death (Grünwald *et al*. 2004).

Chemical fungicides are commonly used successfully for control of *Rhizoctonia* root-rot of pea (Khan *et al*. 1998). However, their field application may not always be desirable. The persistent, injudicious use of chemicals was discouraged owing to their toxic effects on non-target organisms, the undesirable changes they inflict upon the environment (Arcury and Quandt 2003) and due to the development of resistant strains of pathogens against various chemical fungicides (Deising *et al*. 2008). Keeping in view the drawback of chemical control of plant diseases, the use of plant extracts in the control of plant diseases is gaining importance. Various plant products like plant extracts, essential oils, gum, resins… etc. were shown to exert biological activity *in vitro* and *in vivo* and are used as bio-fungicidal compounds (Pawar and Thaker 2006; El-Mougy and Alhabeb 2009; Fawzi *et al*. 2009). The main reasons for using essential oils as antifungal agents is their natural origin and low chance of pathogens developing resistance. They may have a minimum adverse effect on physiological processes of plants and less environmental hazards compared to their synthetic alternatives, being plant products are easily convertible into a common organic material (eco-friend) (Gnanamanickam 2002).

The objective of the performed research work was to investigate the antifungal activity of ethanol-water extracts of cinnamon (*Cinnamomum verum*), anise (*Pimpinella anisum*), black seed (*Nigella sativa*) and clove (*Syzygium aromaticum*) *in vitro* on growth of *R. solani* as well as evaluate the protective effects of the most effective one against the fungal invasion of pea seeds and seedlings under greenhouse conditions.

MATERIALS AND METHODS

Isolation of the pathogen

R. solani was isolated from naturally diseased pea plant exhibiting typical symptoms of root-rot disease. Purification of the isolated fungi was done using the hyphal tip technique. The pathogen was identified as *R. solani* based on the cultural properties, morphological and microscopical characteristics as described by Sneh *et al*. (1991).

Pathogenicity test

It was carried out to determine the pathogenic potential of different isolates of *R. solani*, and the most aggressive isolate was used for further investigations.

Sterilized pots were filled with disinfested soil. Inocula of *R. solani* isolates were prepared by growing each

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isolate in bottles containing sterilized sorghum grain medium and incubated at 25±2°C for 15 days. Soil infestation was achieved by mixing the inoculum of each isolate with the upper layer of the soil at the rate of 2% (w/w) potential inoculum. The disease severity was determined by recording the percentage of unemerged seeds 15 days after sowing as well as percentage of dead seedlings 45 days after sowing.

Anastomosis grouping (AG)

The selected isolate of *R. solani* was assigned to AG according to hyphal anastomosis with tester isolates from AG 1 to AG 10 using the slide technique of Kronland and Stanghellini (1988). The tester isolates used in this study were kindly provided by Dr. Shiro Kuninaga (Health Sciences University of Hokkaido, Japan).

***In vitro* antifungal activity of the plant extracts**

The plant materials (cinnamon, anise, black seed and clove) were washed with distilled water and dried in shade. They were then finely grinded to powder. Fifty grams of each plant material in powder form was homogenized by laboratory blender in 200 ml of ethanol (96%) and distilled water (20 : 80 v/v) for 10 min, and then left in dark glass bottles for 72 h for complete extraction. The extracts were filtered through thin cheesecloth sheets. The final extracts were collected separately in other dark glass bottles and exposed to 60°C in water bath for 30 min for ethanol evaporation. The collected extracts were then stored in a refrigerator at 5°C until needed. The plant extracts were added to conical flasks containing sterilized PDA before solidification to obtain the proposed concentrations of 0.5, 1, 2, 3 and 4% (v/v). 20 ml of amended media were poured into 9 cm diameter Petri dishes, and another set of untreated PDA plates was used as control. For each treatment, 3 replicates (plates) were used. All plates were inoculated individually with 0.5 cm diameter discs of the tested fungal cultures, and then incubated in the dark at 25±2°C, until the control plates reached full growth.

Evaluation of the antifungal activity under greenhouse conditions

The most potent plant extract was selected from the *in vitro* test and evaluated under greenhouse conditions against *Rhizoctonia* root-rot disease. Pots were filled with disinfested soil. Soil infestation was achieved by mixing the inoculum with the upper layer of the soil at the rate of 2% (w/w) potential inoculum. Pea seeds were surface disinfected by immersing in sodium hypochlorite (2%) for 2 min, and washed several times with sterilized distilled water, then dried between two sterilized layers of filter paper. The treated pea seeds were soaked in previously prepared clove extract at concentrations of 2, 3 and 4%. Seeds were soaked for 12 h, then picked up and left for air drying onto plastic tray. A set of disinfected pea seeds were dressed with the fungicide Rizolex-T (50% of tolclofos-methyl) at the recommended dose (3 g/kg) and used as a positive control. Another set of disinfected seeds were used for control treatment. Five seeds of pea were sown in each of five replicated pots for a particular treatment. The experiment was arranged in a completely randomized block design. All pots were kept under natural conditions (day temperature 25°C, night temperature 20°C, 16 h photoperiod) and watered when necessary. Disease incidence was determined by recording the percentage of unemerged seeds 15 days after sowing as well as percentage of dead seedlings 45 days after sowing. The percentage of survived plants was also recorded 60 days after sowing.

Statistical analysis

Data were analyzed with the statistical analysis system (CoStat 2005). All multiple comparisons were first subjected to analysis of variance (ANOVA), comparisons among means were made using Duncan's multiple range test (Duncan 1955).

RESULTS

Pathogenicity test

By the end of the isolation of *Rhizoctonia* root-rot pathogen from diseased pea roots, 10 fungal isolates were obtained and identified as *R. solani*. The isolate 102 showed the highest disease incidence on pea plants and was used for subsequent studies.

Anastomosis grouping (AG)

The selected isolate of *R. solani* was assigned to AG according to hyphal anastomosis with tester isolates from AG 1 to AG 10 using the slide technique. Results obtained indicated that the selected isolate of *R. solani*102 belonged to AG 4 HG-I.

***In vitro* antifungal activity of the plant extracts**

The growth reduction of *R. solani* in response to the tested plant extracts is presented in table 1. All tested

Table 1. *In vitro* growth reduction [%] of *R. solani* in response to the tested plant extracts

Plant extract	Plant extracts concentration*				
	0.5%	1%	2%	3%	4%
Cinnamon	23.4 b	36.7 b	50.6 b	78.2 b	95.8 b
Anise	18.8 c	23.3 c	48.1 c	70.5 c	90.8 c
Black seed	16.6 d	20.0 d	45.5 d	68.5 c	90.5 c
Clove	98.8 a	100 a	100 a	100 a	100 a

*each value represents the mean of 3 replicates
Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test (p = 0.01)

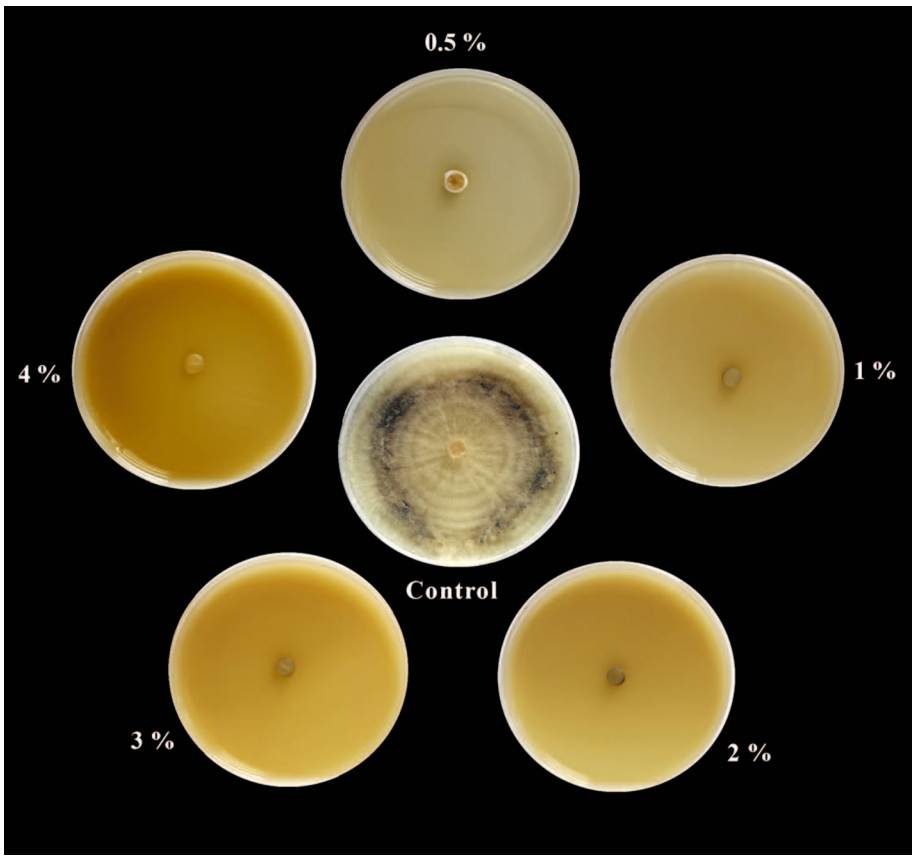

Fig. 1. Effect of different concentrations of clove extract on the radial growth of *R. solani*

plant extracts had an antifungal activity against *R. solani*. Radial growth of *R. solani* decreased significantly with increasing the concentration of plant extracts. High growth inhibition was observed at concentrations 4% of each plant extract (from 90.5 to 100%). The highest antifungal activity was recorded for clove extract which causes complete inhibition at concentration of 1% (Fig. 1). So, it was selected to the application in pot experiment under greenhouse conditions.

Evaluation of the antifungal activity under greenhouse conditions

Clove extract at different concentrations significantly reduced the percentage of disease incidence of *Rhizoctonia* root-rot of pea (Table 2). Clove extract at concentration of 4% as well as Rizolex-T recorded high significant increase in the percentage of survived plants (40 and 48%, respectively) and high significant decrease in disease incidence percentage (seed rot, pre-emergence, post-emergence and root-rot) when compared with the pathogen treatment.

DISCUSSION

The obtained results of anastomosis grouping agree with those of Hwang *et al*. (2007) who identified AGs of *R. solani* from rotted roots of pea plants as AG 4. Of the 94 isolates of *R. solani* isolated from 37 pea fields, 60 were identified as AG-4. Almost all of highly pathogenic isolates were of AG-4.

Our results indicated that all tested plant extracts showed an antifungal activity against *R. solani* with the highest effect of clove extract. This finding is in agreement with that of Beg and Ahmad (2002). The *in vitro* efficacy of cinnamon**,** anise, black seed and clove oil was

Table 2. Effect of clove extract on incidence of *Rhizoctonia* root-rot disease of pea*

Treatment		Seed rot [%]	Pre-emergence [%]	Post-emergence [%]	Root-rot [%]	Survival plants [%]
Control		4 e	4 e	0 d	4 d	88 a
Pathogen		28 a	28 a	32 a	12 c	0 e
Rizolex-T		8 de	12 d	16 c	16 b	48 b
clove	2%	16 bc	20 b	24 b	20 a	20 d
	3%	20 b	20 b	16 c	16 b	28 cd
	4%	12 cd	16 c	16 c	16 b	40 bc

*each value represents the mean of 5 pot replicates
Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test (p = 0.01)

reported against different pathogens by Ozcan and Chalchat (2006), Sukatta *et al*. (2008) and Sitara *et al*. (2008). The antifungal effect of these plant extracts is related to their chemical composition. The main constituent of cinnamon oil is cinnamaldehyde, which is the compound containing an aldehyde group and conjugated double bond outside the ring. This compound possesses much stronger antifungal activity (Wang *et al*. 2005) and it may be a potential lead compound for the development of antifungal drugs through the control β-(1,3)-glucan and chitin synthesis in fungi (Bang *et al*. 2000). The main constituents of anise oil are trans-anethole (93.9%) and estragole, (E)-methyeugenol, α-cuparene, α-himachalene, β-bisabolene, p-anisaldehyde and cis-anethole. The antifungal activity of anise extract is attributed mainly to the chemical constituent anethole which has potent antimicrobial properties (Ozcan and Chalchat 2006). The presence of biological active compounds such as α-thujene, 2(1H)-naphthalenone, α-pinene, α-phellandrene, limonene, thymoquinone, myristicin etc. in black seed volatile oil contributed the antimicrobial activity (Gerige *et al*. 2009). Eugenol is the main component of clove oil. Pepeljnjak *et al*. (2003) pointed out that eugenol is one of the strongest inhibitors of enzyme processes and related compounds as methyle- or acetyleugenol could change this property. Antimicrobial activity of this oil can be attributed to the presence of an aromatic nucleus and a phenolic OH group that are known to be reactive and can form hydrogen bonds with –SH groups in the active sites of target enzymes, resulting in deactivation of enzymes in fungi (Velluti *et al*. 2003; Alma *et al*. 2007). The site(s) and number of hydroxyl groups on the phenol group are thought to be related to the irrelative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, Cowan (1999) found that more highly oxidized phenols are more antimicrobial.

Data reveal that clove extract at different concentrations significantly reduced the percentage of the disease incidence in greenhouse trials. Results obtained are in agreement with those of Suwitchayanon and Kunasakdakul (2009) who tested clove and turmeric extracts against crucifer pathogens using soaking method. They found that the occurrence of infected seedlings treated with clove extract was lower in *Alternaria brassicicola* and *Fusarium oxysporum* than that treated of turmeric extract at rate of 10%, 6% and 75%, 13% respectively compared to 33% infected seedling in control treatment. In general, the mechanisms thought to be responsible for phenolic toxicity of plant extracts to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins. Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and are often cited as antimicrobial as well (Gutierrez *et al*. 2008). Many authors emphasized that antimicrobial effects of essential oil constituents are dependent on their hydrophobicity and partition in the microbial plasmatic membrane. The effect of specific ions due to their addition in/on plasmatic membrane had a great effect on the proton motive force, intracellular ATP content and overall activity of microbial cells including turgor pressure control, solute transport and metabolism regulation (Lanciotti *et al*. 2004). The fungicidal effect of eugenol (clove oil) resulted from an extensive lesion of the cell membrane. Clove oil and eugenol also caused a considerable reduction in the quantity of ergosterol, a specific fungal cell membrane component (Pinto *et al*. 2009). Hence, the objective of this study was to determine if plant extracts could provide protective effect against invasion by *R. solani.* Considering their attribute and broad-spectrum activities, successful development of such compounds as antifungal would not only provide a potent tool for control of pea root-rot, but also could promise success in multipurpose biorational alternatives to conventional fungicides for the management of other plant diseases.

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POLISH SUMMARY

SKUTECZNOŚĆ NIEKTÓRYCH WYCIĄGÓW ROŚLINNYCH PRZECIWKO *RHIZOCTONIA SOLANI* NA GROCHU

Badano aktywność przeciwgrzybową etanolowowodnych wyciągów z czterech roślin lekarskich: cynamonu (*Cinnamomum verum*), anyżu (*Pimpinella anisum*), czarnuszki (*Niella sativa*) oraz goździkowca (*Syzygiom aromaticum*) przeciwko zgniliźnie korzeni grochu (*Pisum sativum*) wywoływanej przez grzyb *Rhizoctonia solani*. Test *in vitro* na przeciwgrzybową aktywność, wykazał wysoką inhibicję wzrostu przy stężeniu 4% każdego z wyciągów roślinnych. Najwyższą przeciwgrzybową aktywność stwierdzono dla wyciągu z goździków, który wywołuje całkowitą inhibicję wzrostu w stężeniu 1%. Skuteczność wyciągu z goździków na występowanie powyższej choroby badano w warunkach szklarniowych. W stężeniu 4%, tak samo jak fungicyd chemiczny, powodował on istotny wzrost procentu przeżywalności roślin (odpowiednio 40 i 48%) oraz wysoce istotny spadek występowania choroby.