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Evaluation of Essential Oils and Their Components for Broad-Spectrum Antifungal Activity and Control of Late Leaf Spot and Crown Rot Diseases in Peanut

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ABSTRACT

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Clove oil, cinnamon oil, and five essential oil components (citral, eugenol, geraniol, limonene, and linalool) were tested for growth inhibition of 14 phytopathogenic fungi. Citral completely inhibited the growth of *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium moniliforme*, *F. pallidoroseum*, and *Phoma sorghina* in paper disc agar diffusion assays. Cinnamon oil, citral, and clove oil as low as 0.01% (vol/vol) inhibited the spore germination of *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis* by >90% in vitro. Limonene and linalool were observed to be the least antifungal against the test fungi and were not used in further studies. Clove oil (1% vol/vol) applied as a foliar spray 10 min before *Phaeoisariopsis personata* inoculation reduced the severity of late leaf spot of peanut up to 58% when challenge inoculated with 10^4 conidia ml⁻¹. This treatment was more effective ($P = 0.01$) than 0.5% (vol/vol) citral, cinnamon oil, or clove oil and 1% (vol/vol) eugenol or geraniol. Seed treatment with the test compounds had no effect on the incidence of crown rot in peanut in *Aspergillus niger*-infested soil. However, soil amendment with 0.25% (vol/wt) clove oil and cinnamon oil reduced the preemergence rotting by 71 and 67% and postemergence wilting by 58 and 55%, respectively, compared with the nontreated control. These two treatments were more effective ($P < 0.01$) than geraniol on preemergence rotting, and more effective than citral, eugenol, and geraniol on postemergence wilting. All treatments significantly outperformed the nontreated control but none were as effective as thiram treatment.

Additional keywords: antimicrobial, groundnut

Synthetic fungicides that combat phytopathogenic fungi can increase crop yields and provide stability of crop production and market quality. However, exponential increase in the use of fungicides has resulted in the development of fungicide-tolerant pathogen strains (31) and accumulation of fungicide residues in the food chain above safe limits (11). There is a need for better fungal disease management based on alternatives to synthetic fungicides. Among the various alternatives are plant-derived compounds (17). Essential oils, a group of plant-derived compounds, are a mixture of different terpenoid compounds and their oxygenated derivatives (34). These oils are known for their broad-spectrum antifungal activity against both human and plant pathogens (7,16,24). The antifungal essential oils reduce hyphal growth and also induce lysis and cytoplasmic evacuation in fungi (14). Growth

inhibition by essential oils often involves induction of changes in cell wall composition (15), plasma membrane disruption, mitochondrial structure disorganization (8), and interference with enzymatic reactions of the mitochondrial membrane, such as respiratory electron transport, proton transport, and coupled phosphorylation steps (19).

Essential oils of *Cinnamomum zeylanicum* and *Syzygium aromaticum* (clove) consisting of cinnamaldehyde and eugenol, respectively, as major components (25) are known to be potent antifungal materials (4,28). Citral and geraniol are the major components in essential oils of *Cymbopogon citratus* (lemongrass) and *C. martinii* (palmarosa), respectively, which are antifungal compounds (26,33). Linalool is a major component in essential oil of *Thymus mastichina*, with antimicrobial activity (13), and both limonene and linalool are the minor components in essential oils derived from different plants (2,12,30). The majority of these essential oils and their components have proved valuable in protection against postharvest fungal diseases which cause buildup of toxic fungal metabolites in stored foods (17). Few essential oils (for example, oil from seeds of *Azadirachta indica*) have been shown to be

equally effective as chemical fungicides in control of foliar fungal diseases (1).

Late leaf spot caused by *Phaeoisariopsis personata* (Berk. & M.A. Curtis) Arx (= *Cercosporidium personatum* (Berk. & M.A. Curtis) Deighton) is an important foliar disease of peanut or groundnut (*Arachis hypogaea* L.). The disease is globally important and can cause yield losses of up to 50% (22). The soilborne disease crown rot, caused by *Aspergillus niger* van Tieghem, can cause yield losses of >10% (23) and is more prevalent in soils with low moisture content and high temperature (approximately 30°C). In the present study, we evaluated two essential oils and five essential oil components for their broad-spectrum antifungal activity against 14 phytopathogenic fungi, including *P. personata* and *A. niger*. The 14 selected fungi cause significant quantitative and qualitative yield losses of chickpea, peanut, pigeonpea, and sorghum, commonly grown in the semi-arid tropics. Selected essential oils or components were evaluated further for control of late leaf spot and crown rot diseases of peanut following their application as a foliar spray, seed treatment, or soil amendment, with a broader objective of identification of ecofriendly tactics for management of these two diseases.

MATERIALS AND METHODS

Fungal strains. Single spore isolates of 11 culturable phytopathogenic fungi, *Alternaria alternata*, *Ascochyta rabiei*, *Aspergillus niger*, *A. flavus*, *Curvularia lunata*, *Fusarium moniliforme*, *F. oxysporum* f. sp. *ciceri*, *F. pallidoroseum*, *F. udum*, *Phoma sorghina*, and *Rhizoctonia bataticola* were obtained from the culture collection of the Department of Pathology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Three obligate parasites, *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis* were harvested from early leaf spot-, late leaf spot-, and rust-infected peanut leaves, respectively, in the farm fields of ICRISAT. Single lesion or pustule isolates of these fungi were multiplied on peanut cv. TMV 2 using the detached leaf technique (32).

Determination of in vitro antifungal activity. Two essential oils (cinnamon oil and clove oil) and five essential oil components (citral, eugenol, geraniol, limonene, and linalool) were purchased from

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Sigma-Aldrich (St. Louis). Antifungal activity of these essential oils and essential oil components against 11 culturable fungi was determined by paper disc agar diffusion assay (3). A spore germination assay (18) was used for evaluating activity of these oils and oil components against the three obligate parasites, which cannot be readily cultured.

Paper disc agar diffusion assay. Autoclaved potato dextrose agar (PDA, 1.5% agar) was dispensed into 90-mm-diameter petri plates and incubated at room temperature for 24 h. A 5-mm diameter sterilized filter paper disc (Whatman no. 1) was placed at the center of each petri plate and loaded separately with 2 or 4 µl of each of the individual test compounds. Paper discs loaded with sterile distilled water (SDW) in place of a test compound served as controls. After 30 min, the plates were overlaid with a 10-ml suspension of fungal spores in PDA (0.5% agar; approximately 10⁴ spores ml⁻¹) and sealed with Parafilm. Plates inoculated with *Ascochyta rabiei* were incubated at 20°C and all other fungi at 28°C. The inhibition zone around the paper disc in each treatment was measured in millimeters 72 h after incubation. Three plates were maintained as replicates of each treatment and the experiment was repeated twice.

Spore germination assay. Fresh spores of *C. arachidicola*, *Phaeoisariopsis Personata*, and *Puccinia arachidis* were harvested from sporulating lesions or pustules on peanut leaves using a cyclone spore collector. The spores were suspended in SDW with 0.01% Tween 20 at 10⁵ spores ml⁻¹. The test compounds were diluted to three concentrations (0.01, 0.1, and 1% [vol/vol]) with 1% Tween 20 for uniform distribution of the emulsion, and tested for inhibition of spore germination. The spore suspension and individual test compounds

(50 µl each) were mixed well on a cavity slide, with 1% Tween 20 as the control. The slides were placed in humid chambers and incubated in the dark at 24 ± 1°C. A drop of lactophenol-cotton blue (40 ml of glycerol, 20 ml of lactic acid, 20 g of phenol, and 5 ml of 1% aqueous cotton blue) was added to conidia of *C. arachidicola* and *Phaeoisariopsis personata* after 24 h, and urediniospores of *Puccinia arachidis* after 8 h, and spores observed for germination under a microscope. In each treatment, 100 spores were observed and the percent germination inhibition with respect to the control was calculated. The conidia were considered germinated if the length of the germ tube was twice the width of the conidium. The experiment was conducted three times with three replications of each treatment.

Determination of in vivo disease control. Two essential oils, cinnamon oil and clove oil, and three essential oil components, citral, eugenol, and geraniol, were tested for control of late leaf spot and crown rot diseases in peanut in a controlled environment.

Late leaf spot in peanut. Thirty-day-old peanut plants of cv. TMV 2, which are highly susceptible to late leaf spot (9), were used in the experiment. The seedlings were grown in a potting mixture consisting of red alfisol, farm yard manure, and sand (3:1:1) in a greenhouse at 28 ± 3°C. Plants in 15-cm-diameter pots (four per pot) were evenly sprayed with suspensions of essential oils or components in 1% Tween 20 at concentrations of 0.1, 0.5, and 1% (vol/vol). Plants treated with 1% Tween 20 and chlorothalonil (Kavach; Syngenta India Ltd., Mumbai, India) at 2 g liter⁻¹ served as untreated control and fungicide control, respectively. The test compounds were applied as a prophylactic application at 96, 48, and 24 h and 10 min before the

pathogen inoculation. The plants were challenge inoculated with conidial suspension of *P. personata* (conidia at 10⁴ ml⁻¹) in 0.01% Tween 20 applied as a uniform foliar spray.

Inoculated plants were provided with alternate wet (16 h) and dry (8 h) periods of leaf wetness for 8 days after inoculation (DAI) by shifting between the dew chambers and greenhouse at 24 ± 2°C. Subsequently, the plants were maintained in a greenhouse at 25 ± 3°C. Severity of late leaf spot in different treatments was measured 15 DAI as lesion frequency (i.e., number of lesions per square centimeter of leaf area) using the third quadrifoliate leaf from the top tagged before the inoculation. Two pots of each treatment were considered as one replication and the experiment was conducted three times with three replications of each treatment.

Crown rot in peanut. For evaluation of control of crown rot of peanut, selected compounds were applied as both a seed treatment and soil amendment. *Aspergillus niger* was multiplied on autoclaved sorghum seed for 8 days at 30°C. The profusely sporulating culture was mixed with a potting mixture consisting of red alfisol, farm yard manure, and sand (2:1:2) at 40 g kg⁻¹. *A. niger*-infested potting mixture was filled in the top 6 cm of the 15-cm-diameter pots. The pots were watered and left in the greenhouse for 48 h.

For the seed treatment test, peanut seed (cv. TMV 2) were surface sterilized with 0.02% HgCl₂ and then suspended for 30 min in solutions of either cinnamon oil, citral, clove oil, eugenol, or geraniol. The solutions consisted of each oil or oil component at three different concentrations (0.1, 0.5, and 1% [vol/vol]) with 1% Tween 20 in water. Eight treated seed were sown in each of the 15-cm-diameter pots with *A. niger*-infested soil. Peanut seed

Table 1. Antifungal activity of selected essential oils and their components against phytopathogenic fungi, tested by paper disc agar diffusion assay in vitro

Treatment ^b	Conc. (µl)	Diameter of inhibition zone (mm) ^a										
		AA	AR	AN	AF	CL	FM	FO	FP	FU	PS	RB
Cinnamon oil	2	43.6	26	33.6	36.6	47	35	*	40	30	54.6	17
	4	45	37.6	41.8	40	57	40	*	50.6	33.6	*	25.6
Citral	2	*	47.6	30.6	*	*	*	12.6	*	20.6	*	12
	4	*	*	35.2	*	*	*	23	*	*	*	*
Clove oil	2	36.6	17	20	19.6	36.6	25.6	13	28	36	46	18
	4	46	21	27.2	25	43	28	37	39	36	55	23.6
Eugenol	2	37.6	23.6	24.4	25	36.6	30	17	39.6	35.6	42.6	29.6
	4	46.6	26.6	29.2	28.6	46.6	32.6	46.6	47	36	47.6	29
Geraniol	2	42.6	24	14	15.6	23	21.6	9.6	15.6	18	41.6	13
	4	34.6	28	22	23	31.6	19	29.6	24.6	20	59	16.6
Limonene	2	0	0	0	0	0	5	0	0	0	0	0
	4	0	0	0	0	0	8	0	0	0	0	0
Linalool	2	7.8	0	0	0	5.1	9	0	7.6	8	0	0
	4	*	0	0	0	*	*	10	10	13.6	0	8.6
Control	2	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0
LSD (<i>P</i> = 0.01)	...	3.2	2.9	2.8	2.9	3.4	2.9	2.7	3.7	2.7	4.0	2.1

^a Values presented are the mean of three repetitions, each with three replications of the experiment; * = no fungal growth was observed in the petri plate. AA = *Alternaria alternata*, AR = *Ascochyta rabiei*, AN = *Aspergillus niger*, AF = *A. flavus*, CL = *Curvularia lunata*, FM = *Fusarium moniliforme*, FO = *F. oxysporum* f. sp. *ciceri*, FP = *F. pallidoroseum*, FU = *F. udum*, PS = *Phoma sorghina*, and RB = *Rhizoctonia bataticola*.

^b Essential oil or component. LSD = least significant difference (essential oil × concentration [Conc.]).

treated with 1% Tween 20 and thiram at 2 g kg⁻¹ of seed served as the untreated control and fungicide control, respectively.

For the soil amendment test, 100 ml of a 2.5% (vol/vol) suspension of essential oils or essential oil components in 1% Tween 20 was thoroughly mixed with 1 kg of *A. niger*-infested potting mixture and placed in the top 6 cm of the 15-cm-diameter pots 24 h before planting. Pots mixed with 1% Tween 20 served as the untreated control. For the fungicide comparison, *A. niger*-infested soil was treated with thiram at 2 g kg⁻¹ of soil. Eight seed were planted in each pot followed by the addition of 200 ml of SDW. Planted pots for seed treatment and soil amendment tests were maintained in a greenhouse at a temperature of 30 ± 2°C and watered with 100 ml of SDW on alternate days to maintain minimum soil moisture. Incidence of crown rot was measured as preemergence rotting 7 days after sowing (DAS) based on the number of seed germinated (ungerminated seed were considered as rotted) and postemergence wilting 20 DAS based on the number of wilted seedlings with respect to the number of emerged seedlings. The experiment consisted of three replications and was conducted three times.

Data analysis. All the experiments were conducted with three replications per treatment and arranged in a randomized complete block design, and each test was repeated twice. Based on Bartlett's test, it was observed that the data from different repetitions were not significantly different ($P \leq 0.05$) from each other. Hence, data from individual repetitions were pooled and subjected to analysis of variance (ANOVA) using Genstat 6.0 statistical package (Lawes Agricultural Trust, Rothamsted, UK) to determine the attained level of significance and separate the treatment means.

RESULTS

Determination of in vitro antifungal activity. *Paper disc agar diffusion assay.* Cinnamon oil, citral, clove oil, eugenol, and geraniol inhibited the growth of 11 culturable fungi and, generally, greater inhibition was observed with 4 µl of test compound rather than 2 µl (Table 1). Citral was the most effective antifungal compound in the test. It completely inhibited the growth of *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *F. moniliforme*, *F. pallidroseum*, and *Phoma sorghina* at a concentration of 2 µl disc⁻¹. *F. oxysporum* and *R. bataticola* were the least sensitive to citral at the same concentration. Limonene was the least effective among all the test compounds, and was slightly inhibitory only to *F. moniliforme*. The antifungal activity of linalool was fungus specific, because it completely inhibited the growth of *Alternaria alternata*, *C. lunata*, and *F. moniliforme* while being ineffective against *Ascochyta rabiei*,

Aspergillus niger, *A. flavus*, and *Phoma sorghina* at a concentration of 4 µl disc⁻¹ (Table 1).

Spore germination assay. For all control treatments, which contained only 1% Tween 20 in SDW, conidial germination was >90%. Cinnamon oil, citral, clove oil,

and eugenol at 1 and 0.1% concentrations and geraniol at 1% concentration completely inhibited the in vitro germination of spores of *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis* (Fig. 1). Cinnamon oil, citral, and clove oil at 0.01% (vol/vol) inhibited >90%

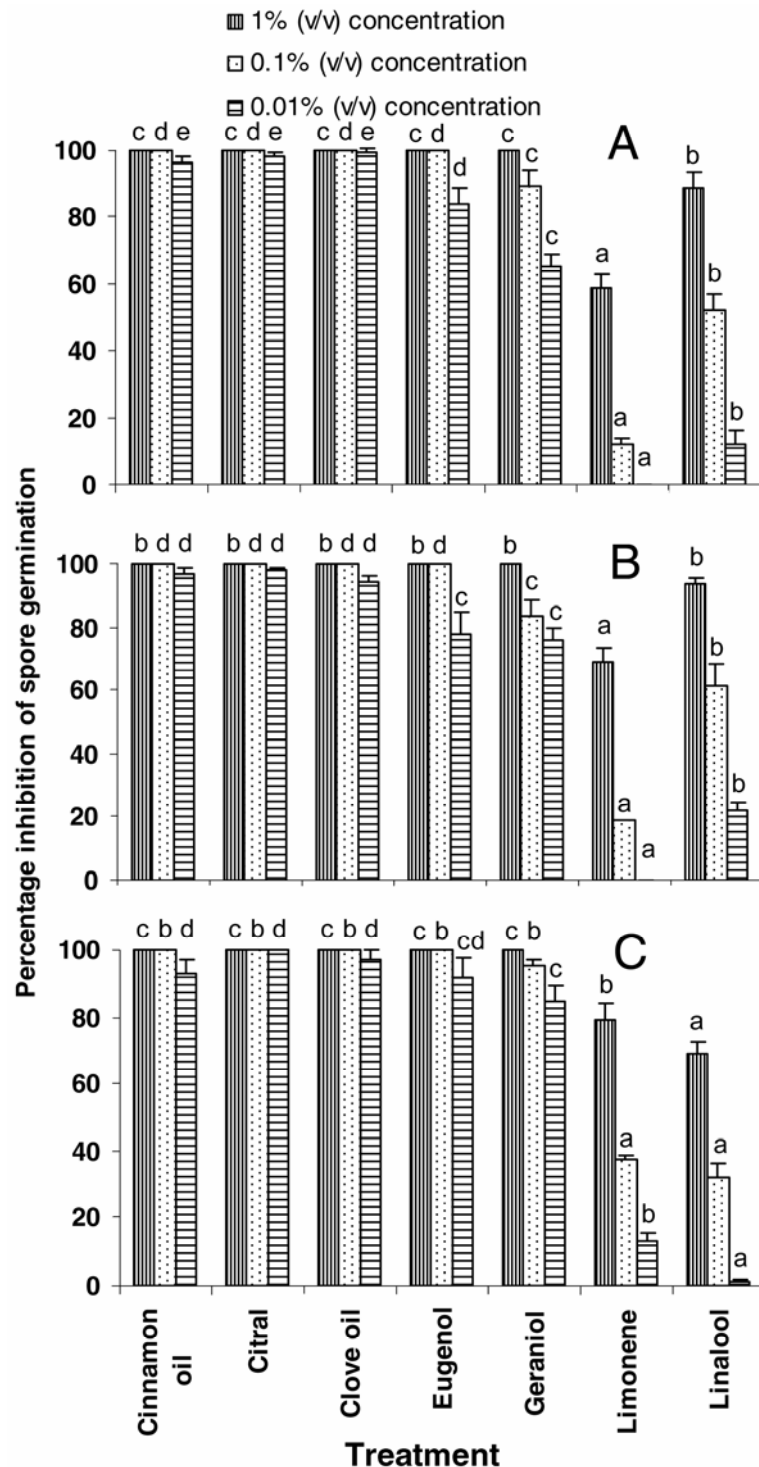


Fig. 1. In vitro antifungal activity of essential oils and their components against obligate phytoparasitic fungi **A**, *Cercospora arachidicola*, **B**, *Phaeoisariopsis personata*, and **C**, *Puccinia arachidis* tested by spore germination assay. Germination of conidia of *C. arachidicola* and *Phaeoisariopsis personata* was recorded 24 h after incubation, and urediniospores of *Puccinia arachidis* 8 h after incubation. The percent inhibition of conidial or urediniospore germination was calculated with respect to germination in water control, and the values are the mean of three repetitions, each with three replications of the experiment. The horizontal bars above each bar represent the standard error of mean. Individual bars in each series represented by the same letter do not differ significantly at $P = 0.01$.

of spore germination for the three fungi. Limonene was the least effective compound against germination of *C. arachidicola* and *Phaeoisariopsis personata*, and linalool was least effective against *Puccinia arachidis* spore germination (Fig. 1).

Determination of in vivo disease control. Late leaf spot in peanut. The essential oils and essential oil components applied as a prophylactic spray at 96, 48, and 24 h before the pathogen inoculation had lesion frequencies similar to the unprotected control (*data not shown*). The essential oil or components applied 10 min before pathogen inoculation significantly ($P < 0.001$) reduced the lesion frequency compared with the untreated control (Table 2). Lesion frequency was minimum with 1% (vol/vol) clove oil treatment (58% less compared with the untreated control) but similar to that with cinnamon oil and citral at 1%. Clove and cinnamon oils at 1% were significantly ($P = 0.01$) more effective than 0.5% (vol/vol) clove oil or either rate of geraniol or eugenol. However, lesion frequency with clove oil at 0.5% (vol/vol) concentration did not differ from other essential oil treatments. Treatment with chlorothalonil was more effective than any of the essential oil treatments because there was no lesion development with this treatment.

Crown rot in peanut. Seed treatment with selected essential oils or components at the three concentrations of 0.1, 0.5, and 1% (vol/vol) had no significant ($P = 0.05$) effect on incidence of preemergence and postemergence infection by *Aspergillus niger* in peanut seedlings (*data not shown*). Seed treatment with the chemical fungicide thiram reduced both the preemergence rotting and postemergence wilting by approximately 75%.

Soil amendment with cinnamon oil, citral, clove oil, eugenol, and geraniol at

Table 2. Effect of essential oils and their components, applied as prophylactic sprays 10 min prior to inoculation, on the severity of late leaf spot in peanut^a

Treatment	Conc. (%)	Lesion frequency
Cinnamon oil	0.5	1.2
	1.0	0.9
Citral	0.5	1.2
	1.0	1.0
Clove oil	0.5	1.1
	1.0	0.8
Eugenol	0.5	1.3
	1.0	1.2
Geraniol	0.5	1.3
	1.0	1.2
Chlorothalonil	0.2	0.0
Water control	...	1.9
LSD ($P=0.01$)	...	0.21

^a Conc. = concentration (percent vol/vol or wt/vol), lesion frequency data are the number of lesions per square centimeter of leaf area and are the mean of three repetitions, each with three replications of the experiment, and LSD = least significant difference.

0.25% (vol/wt) significantly ($P < 0.001$) reduced the preemergence rotting in peanut seedlings, compared with the untreated control (Fig. 2). Soil amendment with clove oil and cinnamon oil were the most effective treatments for reducing preemergence rotting. For these two treatments, the postemergence wilting was reduced by 58 and 55%, respectively, compared with the untreated control. Also, these two treatments were more effective in reducing postemergence wilting ($P = 0.01$) than citral, eugenol, and geraniol. Soil treatment with citral, eugenol, and geraniol reduced preemergence rotting by 62, 58, and 54% and postemergence wilting by 38, 17, and 14%, respectively. Soil amendment of thiram reduced the preemergence rotting by 90% and postemergence wilting by 70%.

DISCUSSION

Plant-derived compounds as crop protectants represent a vast and rapidly progressing resource. Essential oils are a rich source of broad-spectrum antifungal plant-derived metabolites that inhibit both fungal growth and production of toxic metabolites (17). This study evaluated the broad-spectrum antifungal activity of selected essential oils and their components and their use as fungicides for management of fungal diseases of peanut. Compounds with broad-spectrum activity are expected to provide protection against a range of pathogenic fungi that attack the plant at the same or subsequent growth stages following their application. The essential oils and

essential oil components selected for this study are known for antifungal activity and also are commercially available. A paper disc agar diffusion assay was selected to evaluate the antifungal activity of test compounds against culturable fungi. This assay method provides qualitative information on the efficacy of test compounds and is used routinely in such studies (10,20). We observed large inhibition zones in the paper disc agar diffusion assay which established the antifungal activity of the essential oils and essential oil components.

Cinnamon oil, clove oil, and the essential oil components citral, eugenol, and geraniol were found to have broad-spectrum antifungal activity and inhibited 14 phytopathogenic fungi tested in the disc diffusion assay or spore germination assay. The fungi used in the present study represented pathogens of many different hosts, each of which cause significant economic losses in agricultural yields worldwide. The above-mentioned oil compounds are known to be antifungal against one or more of the test fungi in different research studies (17,21,29). However, the current study provides comparative information on the activity spectrum of these compounds against selected agriculturally important fungi under similar test conditions.

In a controlled environment, all the essential oils and essential oil components tested were ineffective against late leaf spot in vivo when applied at least 24 h before pathogen inoculation. The ineffectiveness of essential oils in reducing the severity of the foliar disease could be due

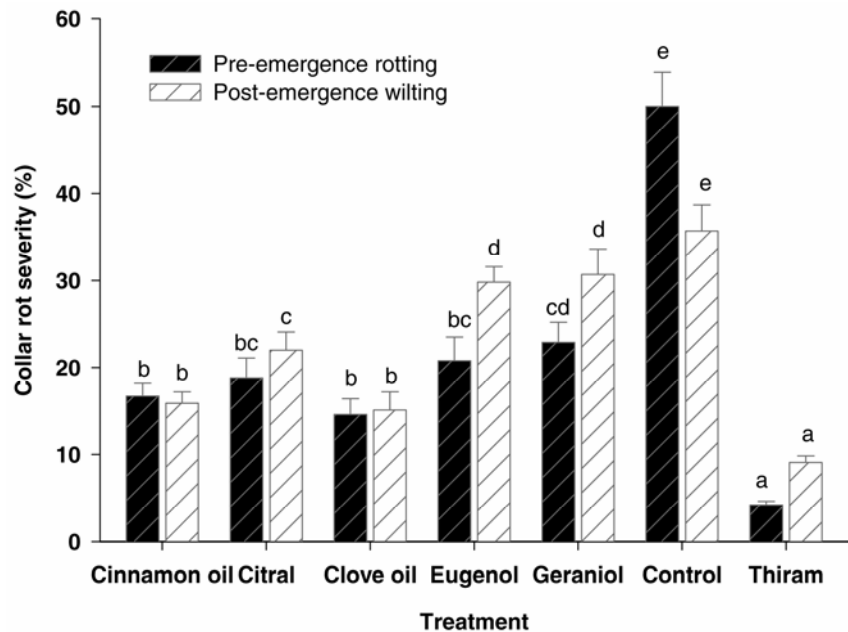


Fig. 2. Effect of soil amendment with essential oils and their components on the severity of crown rot in peanut seedlings. *Aspergillus niger*-infested potting mixture was treated with essential oils or essential oil components at 0.25% (vol/wt) 24 h before planting. Preemergence rotting and postemergence wilting caused by *A. niger* was measured in different treatments at 7 and 20 days after sowing, respectively. Disease incidence values are the mean of three repetitions, each with three replications of the experiment. The horizontal bars above each bar represent the standard error of mean. Individual bars in each series represented by the same letter do not differ significantly at $P = 0.01$.

largely to the volatile nature of essential oils. It is known that germination and penetration of *Phaeoisariopsis personata* conidia on the leaf surface of peanut cv. TMV 2 requires 24 to 72 h (27). The majority of the essential oils are more fungistatic than fungicidal (17) and, thus, their persistence on the phylloplane determines their in vivo efficacy. Our results indicate that it is impractical to use essential oils as foliar sprays for control of late leaf spot.

There have been limited reports on the potential role of essential oils and essential components as protectants against soil-borne fungi when applied as seed treatment (17). However, all the essential oils and essential oil components applied as seed treatment failed to protect peanut from *A. niger* infection. In contrast, soil amendment with selected essential oils and essential oil components effectively reduced the incidence of both preemergence rotting and postemergence wilting of peanut seedlings in *A. niger*-infested soil. Cinnamon oil and clove oil were more effective than the essential oil components in control of postemergence wilting. Decrease in plant disease incidence through reduction in soil fungal populations with use of oils has been reported. Bowers and Lock (6), for example, reported that treatment with 5 and 10% clove oil and synthetic cinnamon oil reduced soil populations of *Phytophthora nicotianae* by 98% after 21 days compared with unprotected soil. Clove oil also reduced the population density of *F. oxysporum* f. sp. *chrysanthimi* by 97.5%, measured 3 days after treatment (5).

The broad-spectrum antifungal essential oils and their components tested in the present study do not hold promise for control of late leaf spot and crown rot diseases of peanut. The essential oils applied as a prophylactic foliar spray were not effective against late leaf spot in controlled environment tests. Although soil amendment with essential oils did show some effectiveness in control of crown rot, application of higher concentrations of these compounds may be needed but is impractical in field conditions.

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