



## Full Length Article

# Antifungal Properties of Extracts of Sesame (*Sesamum indicum*)

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## Abstract

Plant secondary metabolites have important ecological function not only as attractant for pollinators and seed dispersing animals but also as resistance factor against pests and pathogens. Accessions that produce high levels of metabolites involved in resistance are valuable source for sesame breeding. With the aim of identifying genotypes with high content of metabolites potentially involved in resistance against fungal pathogens, 32 sesame accessions were investigated. Metabolites were extracted from leaves, stems and roots and tested against plant pathogenic fungi *Macrophomina phaseolina*, *Alternaria sesami* and *Fusarium oxysporum*. Extracts obtained with 80% ethanol were assayed in microtiter plates and changes in the optical density of the growing fungal cultures were recorded at 550 nm. Most of the extracts had inhibitory effect on all the tested fungal species. Some of the root extracts supported the growth of *A. sesami* whereas some leaf extracts enhanced the growth of *F. oxysporum* as compared to the control. The variations observed amongst different sesame accessions lead to the assumption that there is potential to improve plant disease resistance in sesame using metabolic pathway engineering. Crude ethanol extracts of one the accession UCLA1 was fractionated with organic solvents. The inhibition effects of diethylether, chloroform and hexane fractions were higher than of ethanol fraction remaining after extraction with the other solvents. To test whether the synthesis of antifungal metabolites was inducible, plants were exposed to UV light, inoculation with *M. phaseolina* or *F. oxysporum* or spraying with  $\text{CuCl}_2$ . The extract of the plants sprayed with  $\text{CuCl}_2$  were the most inhibitory against *M. phaseolina* and *F. oxysporum* in case of leaves extract and against *F. oxysporum* in case of stem extract. © 2015 Friends Science Publishers

**Keywords:** Sesame; Antifungal; *Fusarium oxysporum*; *Alternaria sesami*; *Macrophomona phaseolina*

## Introduction

In organic farming the use of natural products for crop disease management is instrumental. Because organic farmers are not allowed to use modern synthetic plant protection chemicals optimized regarding efficacy and safety, they rely on a limited set of available natural compounds, some of which are toxic, accumulate in soil and potentially damage the environment. Extension of this repertoire by new metabolites of plant origin is highly desirable. Examples of antimicrobial compounds derived from plants that potentially could be used in diseases management include phenolics, terpenoids, alkaloids, lectins, polypeptides and polyacetylenes (Cowan, 1999). Sesame (*Sesamum indicum*), also known as the queen of oil seed crops, is mainly grown for its oil which has a pleasant taste and antioxidant properties (Bedigian, 2000), accounted for by the presence of secondary metabolites. Secondary metabolites purified from seeds and roots of sesame include sesamol, sesaminol, sesamin, sesamolol (Shukla *et al.*,

1997), naphthoquinone (Ogasawara *et al.*, 1993; Hasan *et al.*, 2000), hydroxysesamone and 2,3-epoxysesamone (Hasan *et al.*, 2001), and anthrasesamones A-F (Furumoto *et al.*, 2003, 2006; Kwan *et al.*, 2008). Some of these chemicals are reported to have antimicrobial properties.

Sesame crop is infected by a number of fungal, bacterial, viral, mycoplasma and non-parasitic diseases around the world. Among the most important fungal pathogens, *Fusarium oxysporum* f. sp. *sesami* causes seedling blight and Fusarium wilt of sesame (Cho and Choi, 1987) by blocking the root xylem vessels. *Macrophomina phaseolina* is a virulent fungus causing charcoal rot or stem rot disease in sesame, which leads to heavy economic losses to the grower by significantly reducing the oil content if the crop is attacked at maturity stage (Javed *et al.*, 1995). *Alternaria sesami* causes damage by reducing photosynthesis through leaf damage and premature defoliation (Kolte, 1985) and by producing elongated lesion on capsules and reducing yield. Additionally fungi such as *Aspergillus* sp. *Penicillium citrinum* and *Fusarium* sp. might

also infect sesame seeds and cause immediate damage as the seeds germinate (Jonsyn, 1988).

Most plant disease control strategies require integration of different approaches. Use of plant resistant cultivars is one of the most efficient methods for disease control. Host plant resistance eliminates or minimizes crop losses due to diseases and reduces the need for and cost of other control strategies. To develop disease resistant cultivars, desired traits from several sources of resistance must often be combined in a single cultivar (Pataky and Carson, 2004). Metabolic characterization is one of the tools that breeders may use for the identification of potential sources of resistance based on antimicrobial metabolites and that ultimately may lead to the discovery of new biologically active compounds.

While genetic diversity in sesame has been studied extensively, data on the metabolic diversity in sesame are limited (Laurentin *et al.*, 2008). In the present study we selected a set of sesame accessions well characterized regarding their genetic diversity (Laurentin and Karlovsky, 2006, 2007) to explore natural variation of sesame in the content of antifungal metabolites and their distribution among plant organs.

## Materials and Methods

All chemicals and growth media including ethanol, diethyl ether, chloroform, hexane, PDA (potato dextrose agar) and PDB (potato dextrose broth) were purchased from Carl Roth GmbH, Germany. Fungal strains *M. phaseolina* (Maubl.) Ashby DSMZ 62743, *A. sesami* (Kawamura) Mohanty and Behera DSMZ 62027, and *F. oxysporum* f. sp. *sesami* CABI 141118 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and CABI Genetic Resource Collection (CABI Biosciences, Egham, UK).

## Growth of Plants

Sesame seeds from 32 accessions or cultivars (Table 1 and 2) were grown in pots containing 1:1 mixture of compost and sand with eight plants per pot. The pots were irrigated every day and kept under controlled environmental conditions of 14 h light (20 Lux), 26°C and 60% relative humidity. Seventeen days after germination, plants were uprooted and washed with tap water. Leaves, roots and stems were collected and immediately frozen with liquid nitrogen. Samples were stored at -20°C until use.

For the experiment with inductive metabolites, plants were grown exactly under the same conditions described above with the exception that five plants were used per pot. Plants were treated 25 days after germination with either *M. phaseolina*, *F. oxysporum* UV light or CuCl<sub>2</sub>. Microsclerotia of *M. phaseolina* and spores of *F. oxysporum* separately were drenched around the stems of plants. For the UV light treatment, plants were simply placed daily under UV at

20°C for 10 min and for 10 days. For the treatment with CuCl<sub>2</sub>, a solution of 2 mM CuCl<sub>2</sub> was sprayed three times, once on 25 days old plants leaves. Necrotic symptoms were developed next day on leaves and further sprays were done on the stem 29 and 32 days of germination. After 35 days of germination all plants were harvested, washed, separated into root, stem and leaves, immediately frozen into liquid N<sub>2</sub> and kept in -20°C freezer in the same way as described above. Control plant (without any treatment) and CuCl<sub>2</sub> control (plants sprayed with CuCl<sub>2</sub> just before harvesting) were also raised parallel with treatment plant, harvested, processed in the same age as treatment one.

## Extraction and Fractionation for Anti-Fungal Assay

Extraction of metabolites from sesame leaves, stems and roots were carried out as described by Laurentin *et al.* (2008). Briefly, 1 g of frozen plant tissues was ground with pestle and mortar in the presence of liquid nitrogen. Ground tissue was extracted with 80% ethanol (5 mL per 1 g plant material) overnight on a rotary shaker (100 rpm) and filtered (25 µm sieve). Extracts were stored at -20°C.

The ethanol extract of one accession (UCLA-1) was partitioned into fractions using organic solvents of different polarities. Two different strategies were used. In the first case, ethanolic extracts were mixed with equal volume of distilled water and extracted on mechanical shaker for 2 h twice with an equal volume of diethyl ether. The diethyl ether and ethanol phases were separated by centrifugation, evaporated to dryness and re-suspended in 80% of ethanol keeping the same concentration as with the initial ethanolic extract (1 g fresh weight of tissues/5 mL of 80% ethanol). In the second strategy, the ethanolic extract was first extracted with an equal volume of hexane (twice) and separated into two phases of (ethanol and hexane fractions). The ethanol fraction was re-extracted with equal volume of chloroform (twice), and separated into two phases (chloroform and ethanol), evaporated to dryness and re-suspended in 80% ethanol as described above.

## Inoculums Preparation

Sclerotial suspension of *M. phaseolina* was prepared with slight modification of Laurentin (2007). Ten day old fungus growing at 32°C on PDA medium (modified with 4% agar w/v) was blended for 2 min with the addition of sterilized water with a kitchen blender, and then strained on a 200 µm sieves. The strained mixture was then centrifuged at 4500 rpm for 5 min and pellets of sclerotia were re-dissolved in PDB medium. PDA plates without fungus were also treated in the same fashion to serve as control. Concentration of sclerotia was determined under the microscope by placing 50 µL of aliquots on a filter paper. Final concentration was adjusted to 2 sclerotia/µL. *F. oxysporum* spores were produced on mung bean medium as described by Bai and Shaner (1996) with modification by Ahmed (2010). 200 mL mung bean medium were inoculated with

**Table 1:** Accessions from GENAP Germplasm Bank (Venezuela) and their origin

Working code	Country of Origin	Accessions	Diversity Centre
India 1	India	93-2223	India
India 2	India	95-465	India
India 3	India	95-469	India
India 7	India	95-464	India
India 8	India	92-2918	India
Korea 1	Korea	92-3091	China-Japan-Korea
Korea 2	Korea	92-3093	China-Japan-Korea
Turkey	Turkey	92-2922	Western Asia
Greece	Greece	92-3125	Western Asia
Syria	Syria	93-2022	Western Asia
Sudan 1	Sudan	93-2113	Africa
Sudan 2	Sudan	92-310	Africa
Sudan 4	Sudan	93-2105	Africa
Ethiopia	Ethiopia	93-2010	Africa
Africa 2	Unknown	95-234	Africa
Africa 3	Unknown	95-223	Africa
Japan 1	Japan	92-2856	China-Japan-Korea
Japan 2	Japan	92-3030	China-Japan-Korea
Japan 3	Japan	92-3031	China-Japan-Korea
China 1	China	92-3108	China-Japan-Korea
China 2	China	95-383	China-Japan-Korea
Turkmenistan 1	Turkmenistan	92-2952	Central Asia
Tadjikistan 2	Tadjikistan	92-2950	Central Asia
Tadjikistan 3	Tadjikistan	92-2917	Central Asia

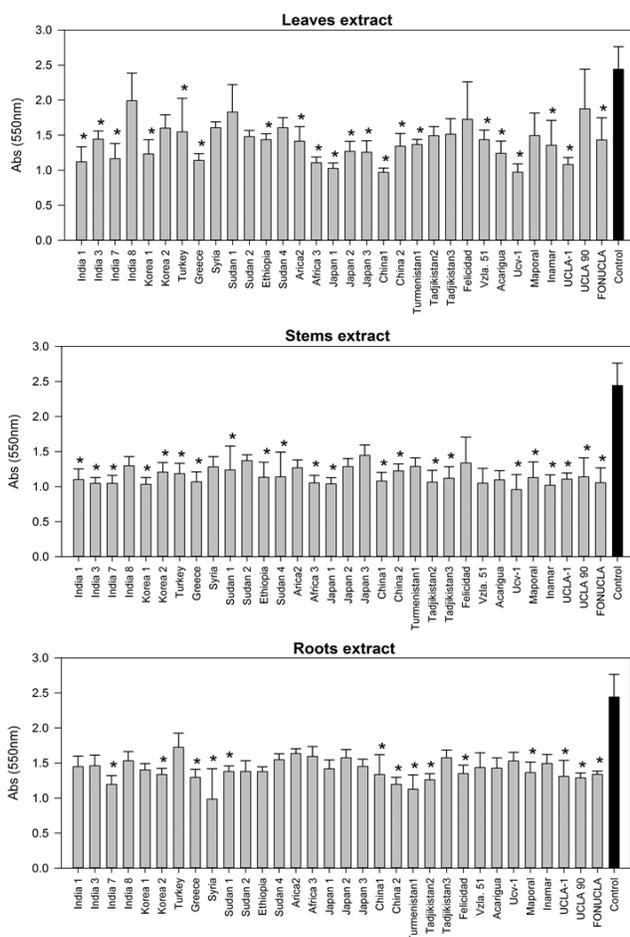
**Table 2:** Commercial cultivars and experimental lines used as source of root, stem and leaf extracts

Commercial cultivars or experimental lines	Country of Origin
Felicidad	Introduced from Mexico. Unknown origin
Venezuela 51	Originated by individual selection from the offspring of a Chinese accession (Langham and Rodriguez, 1946).
Acarigua	A high performance F2 plant obtained by the cross between a cultivar from Nicaragua and a cultivar from China, was crossed with the cultivar Venezuela 51, its offspring was selected for three seasons, resulting in "Acarigua" (Mazzani, 1952)
UCV-1	Elite line selected from first cycle of recurrent selection toward high yield. The original population was obtained by cross, one to one, among 50 exotic accessions (Laurentin <i>et al.</i> , 2000).
Maporal	Selected from cultivar Arapatol, from Ethiopia (Mazzani <i>et al.</i> , 1973).
Inamar	Individual selection from the offspring from the same Acarigua's parents (Mazzani, 1953).
UCLA1	Individual selection from a USA accession (Montilla and Teran, 1996). Unknown origin
Fonucla	Selection from cultivar Arawaca (Montilla and Cedeno, 1991). Arawaca was obtained by selection of the mixture of 496 F1 plants obtained from crosses among 32 cultivars without reciprocal. The origin of these cultivars is unknown.
UCLA90	Elite lines from Universidad Centrooccidental Lisandro Alvarado Sesame Breeding Program. Unknown origin

two plugs of actively growing *F. oxysporum* on PDA. Inoculated and uninoculated (control) mung bean liquid media were incubated at 28°C with shaking at 200 rpm. PDA plugs were separated by straining with mesh cloth directly at the top of the Erlenmeyer flask, medium was centrifuged for 10 min at 4500 rpm and pellet of fungal spores was re-suspended in PDB. The concentration of spores was determined in a Thoma counting chamber and adjusted to  $1.5 \times 10^5$ /mL. For spores of *A. sesami*, 30 mL PDB were poured on the surface of V8-A medium solidified with agar with a seven days old fungal culture. The fungal colony was scratched with an inoculating needle, PDB was removed from the plate, mycelium dislodged from agar was removed by straining with mesh cloth directly on top of the Erlenmeyer flask, the concentrations of spores was determined under microscope by placing 20  $\mu$ L aliquots on filter paper and the final concentration was adjusted to 2 spores/ $\mu$ L.

### Bioassay

To determine the effect of extracts on fungal growth, bioassays were conducted in 96-well microplates. Two hundred microliters of 80% ethanolic extracts were added in the wells of microtiter plate while 80% ethanol was added in other wells as controls. After air drying of extracts in a sterile bench, 40  $\mu$ L PDB containing 20 sclerotia of *M. phaseolina*, 20 spores of *A. sesami* or  $1.5 \times 10^5$ /mL spores of *F. oxysporum* and PDB were added per well. Plates were enclosed in plastic box (containing sterile water at bottom of sieves to avoid the evaporation in the plate) and incubated for *M. phaseolina* at 32°C and 25°C for *A. sesami* and *F. oxysporum*. Fungal growth in the wells was monitored every 24 h taking readings of optical density at 550 nm for five days with a microplate spectrophotometer  $\mu$ Quant Universal (BioTekR Instruments, Inc. USA) and visualized by the software KC4 v.3.4 (BioTekR Instruments, Inc. USA).

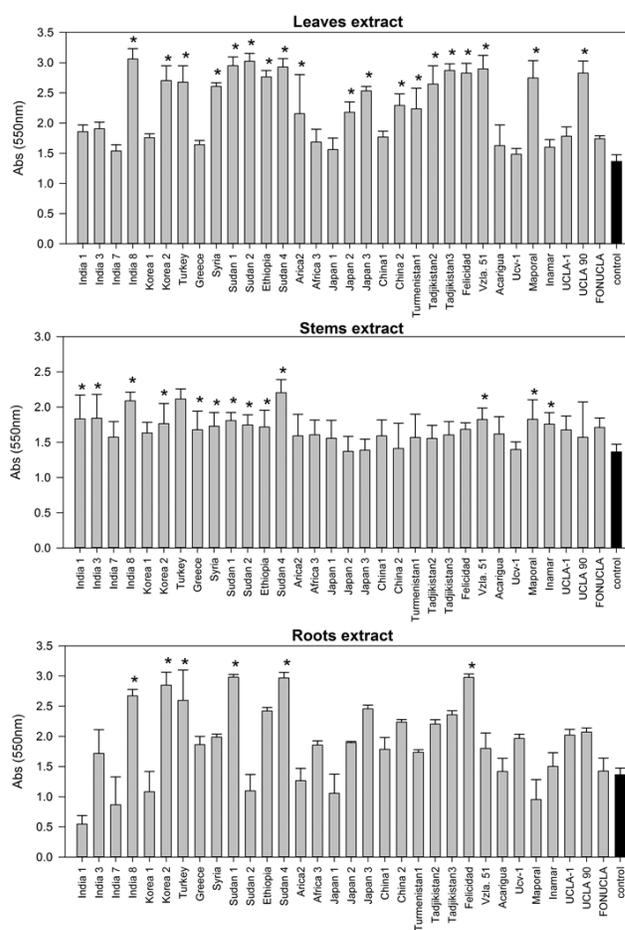


**Fig. 1:** Effect of extracts from leaves, stem, and root of sesame on *M. phaseolina*. Accessions and cultivars of sesame (32 in total) are listed on the horizontal axis. Error bars indicate standard deviation of the mean of three replications. Asterisks mark significant difference from the control ( $P = <0.005$ ) based on Dunnett's multiple comparisons test

There were three independent replication for each extract (plants were grown three different time, extracted, treated/extracted and bioassay were carried out separately with 8 replication (twenty four replication in total).

## Results

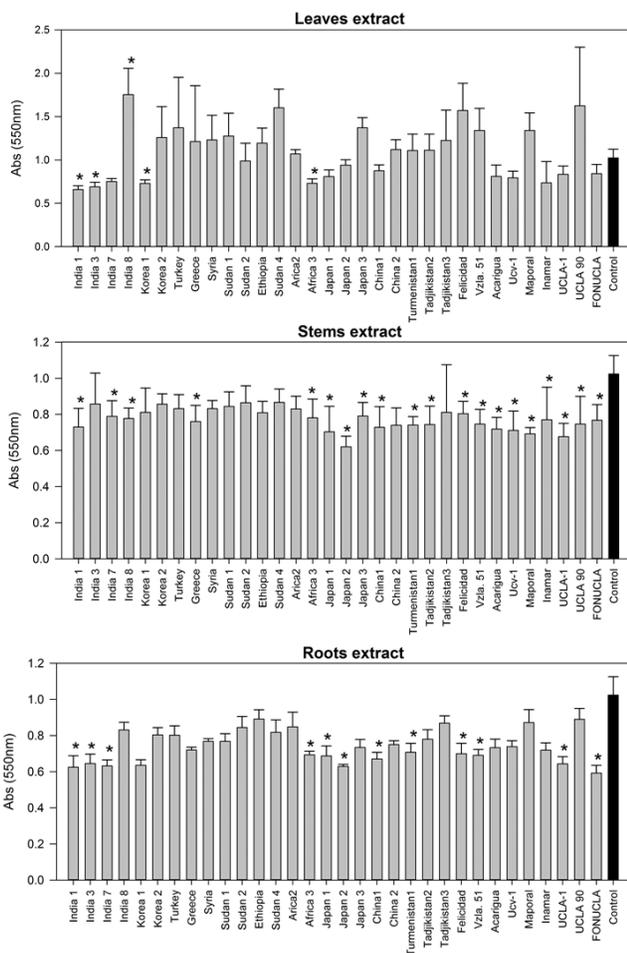
Antifungal activity of leaf, stem and root extracts of 32 accessions and cultivars of sesame were assayed for growth-inhibiting activities by adding the extracts to cultures of three phytopathogenic fungi and monitoring fungal growth by light absorption (Fig. 1 to 3). Large differences of antifungal activity among accessions were observed. Leaf, stem and root extracts of all accessions inhibited the growth of *M. phaseolina* (Fig. 1). Surprisingly, extracts of leaf, stem and root stimulated the growth of *A. sesami* (Fig. 2). The growth of *F. oxysporum* was stimulated



**Fig. 2:** Effect of 80% ethanolic extract from leaves, stem and root of 32 sesame accessions and cultivars on growth of *A. sesami*. Error bars indicate the standard deviation of the mean of three replications 24 measurement in total. Bars that contain sign of asterisk are significantly different ( $P = <0.005$ ) with multiple comparisons procedure versus Control (Dunnett's method)

by leaf extracts of some accessions; the remaining leaf extracts and all stem and root extracts exerted moderate inhibitory effects on *F. oxysporum* (Fig. 3).

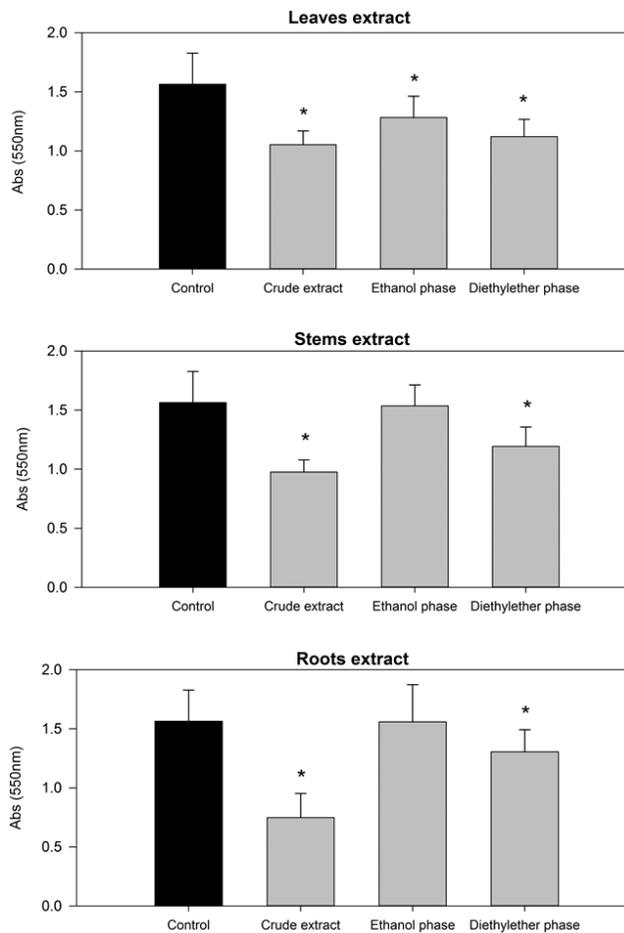
To gain insights into the nature of the metabolites inhibiting fungal growth, ethanolic extract of accession UCLA-1 was fractionated using organic solvents of different polarities and each fraction was tested for its antifungal activity against *M. phaseolina*. Two strategies were used. The first one consisted of extracting the ethanolic extract with diethyl ether; the second used hexane followed by chloroform. The inhibition of a complete crude extract was slightly higher than the effect of diethyl ether fraction, followed by the effect of ethanol fraction remaining after diethyl ether extraction (Fig. 4). Strong antifungal activity was observed in both chloroform and hexane fractions (Fig. 5). In both extraction protocols, metabolites remaining in the ethanol phase inhibited fungal growth the lowest antifungal



**Fig. 3:** Effect of 80% ethanolic extract from leaves, stem and root of 32 sesame accession and cultivars on growth of *Fusarium oxysporum*. Error bars indicate the standard deviation of the mean of three replications 24 measurement in total. Bars that contain sign of asterisk are significantly different ( $P = <0.005$ ) with multiple comparisons procedure versus Control (Dunnett's method)

activity, indicating that metabolites responsible for antifungal effects dissolved in nonpolar organic phase.

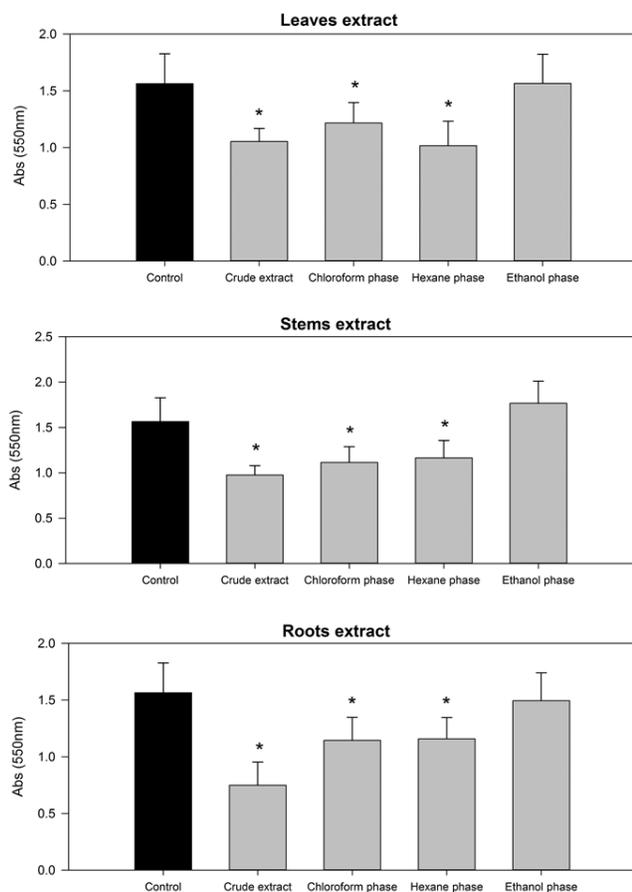
The inducibility of the synthesis of antifungal compounds by abiotic and biotic stresses was studied by exposing the plants to UV-light, treating them with  $CuCl_2$  and inoculating them with pathogens *M. phaseolina* and *F. oxysporum* and testing ethanolic plant extracts for their effect on *M. phaseolina* and *F. oxysporum*. The results varied with plant tissue used for the extraction as well as with the assay organism (Fig. 6). For instance, extracts of stems treated with  $CuCl_2$  inhibited *F. oxysporum* much stronger than extract of control plants and from all other treatment but the effects of these extracts on *M. phaseolina* did not differ (Fig. 6). Treatments other than copper chloride did not or only negligibly induced antifungal activities (Fig. 6).



**Fig. 4:** Effect of crude fractionating ethanolic extract of a sesame accession "UCLA1" leaves, stems and roots on growth of *Macrophomina phaseolina*. Leaves stems and roots were extracted with 80% ethanol (crude extract) and partitioned between diethylether and ethanol. Growth was recorded as optical density 120 h. Error bars indicate the standard deviation of the mean of three replications 24 measurement in total. Asterisk sign on bars indicating significant differences between control and treatments based on one way ANOVA with multiple comparisons versus control group (Dunnett's method) with at ( $P < 0.05$ )

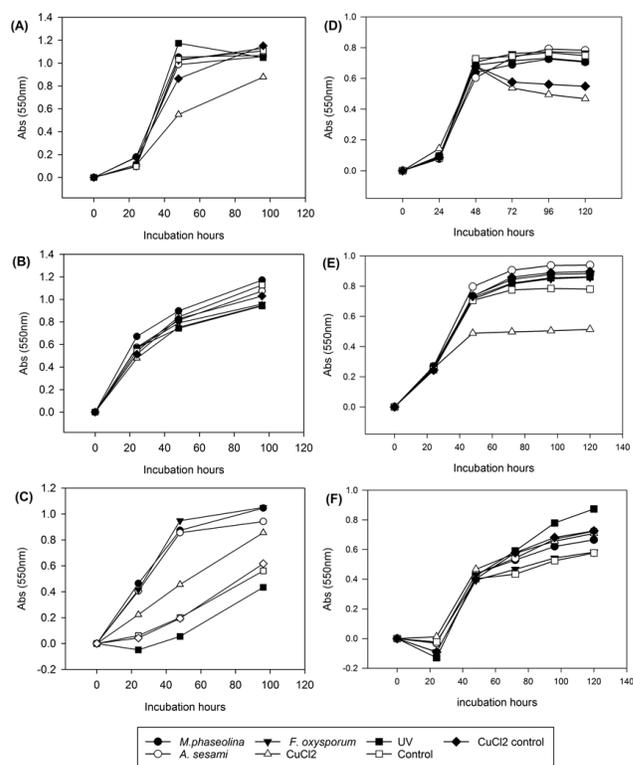
## Discussion

While antifungal properties of leaf extracts of sesame have been extensively studied, limited data are available on antifungal activities of stem and root extracts. Sesame leaves extracts were found to have inhibitory effects on *Streptococcus pneumoniae*, *Candida albicans* (Shittu *et al.*, 2006; Ahmed *et al.*, 2009), *Staphylococcus aureus* (Ahmed *et al.*, 2009), and symbiotic fungus of leafcutter ants (Stelamaris *et al.*, 1998). Sesame leaves, stem and root extracts showed inhibitory activity against *M. phaseolina* and *F. oxysporum* (Laurentin, 2007). Sesamol, which is major lignan of sesame supposed to be involved in



**Fig. 5:** Effect of crude ethanolic extract of a sesame accession ("UCLA1") and its fractionation on growth of *Macrophomina phaseolina*. Leaves, stems and roots were extracted with 80% ethanol (crude extract) and fractionated with hexane (hexane phase), remaining ethanolic extract after hexane extraction were fractionated with chloroform (chloroform phase) and remaining ethanol phase after chloroform extraction (Ethanol phase). Growth was recorded as optical density 120 h. Error bars indicate the standard deviation of the mean of three replications 24 measurement in total. Asterisk sign on bars indicating significant differences between control and treatments based on one way ANOVA with multiple comparisons versus control group (Dunnnett's Method) with at (  $P < 0.05$  )

the inhibition of the growth of invading pathogens, significantly decreasing the charcoal rot (*M. phaseolina*) symptoms of soybean in *in vivo* experiment (Brooker et al., 2000) and inhibited growth of *Mucor circinelloides* (Wynn et al., 1997). Fungal toxicant compounds were present in all the accessions of sesame but the effect differed; the author suggested that the balance of different compound was responsible for the effects (Laurentin, 2007). The variation in effects observed among the different accessions in our study corroborated results of comparative metabolic profiling (Laurentin et al., 2008), which demonstrated that



**Fig. 6:** Growth of *Macrophomina phaseolina* (left figures) and *Fusarium oxysporum* (right figures) in the presence of sesame accession(UCLA1) leaves (A and D), stems (B and E) and roots (C and F) extracts. For the inducibility of anti fungal effect plant were kept in UV or inoculated with fungus or sprayed with  $CuCl_2$ . Control plants were without treatment. Each data point is the average of two replications with 10 plants each

chemical diversity in sesame is large. These results indicate that selection for antifungal activities and metabolic pathway engineering has a potential to improve disease resistance in sesame. The variation among the effects of extracts from leaves, stems and roots and the presence of inhibitory activities in fractions of different polarities suggest that many secondary metabolites participated on the observed antifungal effect of plant tissue extracts.

Constitutive and inducible metabolites with antifungal properties are part of plant chemical defense against pathogens. Antimicrobial metabolites induced by infection are called phytoalexins (Müller and Borger, 1940). Although phytoalexins are defined by antibiotic effects and the response of their synthesis to microbial colonization, the synthesis of many phytoalexins is known to be by abiotic stress such as exposure to UV or heavy metal ions (Grayer and Kokubun, 2001). In our experiments treatment with  $CuCl_2$  induced stronger antifungal activity than infection with pathogens but the effect on two fungal species used in the bioassay differed. We also found that plants sprayed with  $CuCl_2$  had a higher content of salicylic acid (unpublished

data) which is an important signal involved in plant defense against biotrophic pathogens. The cross-talk between defense pathways in plants against pathogens have been documented extensively but our knowledge of interactions between signal pathways induced by biotic and abiotic stresses is limited. The results of our study indicate that abiotic stress may affect plant responses in a specific ways, affecting the defense against some pathogens, while other pathogens remain unaffected.

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