INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2004/06–2–310–316
http://www.ijab.org

The Rhizosphere Yeast Fungi as Biocontrol Agents for Wilt Disease of Kidney Bean caused by *Fusarium oxysporum*

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ABSTRACT

The investigation was conducted to study the potential of rhizosphere yeast fungi for biocontrol of plant pathogenic fungi and to find alternatives to the use of chemicals for disease control in order to avoid environmental pollution. Two species of rhizosphere yeast fungi; *Saccharomyces unispora* and *Candida steatolytica* have antagonistic and inhibitory effects on the growth of the fungal pathogen *Fusarium oxysporum*. Addition of these two species to the soil seeded with kidney bean and infested with the pathogen increased the percentage of control plants. It was noticed that the two tested species of yeast fungi increased kidney bean growth measurements (fresh and dry weights of root and shoot, length of root and height of shoot) compared to infected and non-infected plants. Four active components were detected in the culture filtrates of both *S. unispora* and *C. steatolytica* which were found to be inhibitory to *F. oxysporum*, and the active component of R_f 0.1 was common. The chemical analysis of the culture filtrates of both *S. unispora* and *C. steatolytica* revealed the presence of 10 chemical compounds which were arranged descendingly according to their molecular weights.

Key Words: Yeast fungi; Biological control; Kidney bean; *Fusarium oxysporum*

INRODUCTION

The increased growth response of plants by rhizosphere microorganisms depends mainly on the ability of these microorganisms to survive and develop in the rhizosphere (Kleifield & Chet, 1992). The rhizosphere yeast fungi have been used to control many plant pathogens under green house and field conditions (Stangerlin & Pasdcholati, 1994). The mechanisms by which yeast organisms are reported to have reduced mildew development included mycoparasitism of pathogen structures by *Ampelomyces quisqualis* (Sundhein, 1984), secretion of antibiotics such as heptadecanoic and methyl heptadecanoic acids by *Sporothrix* (Hajlaoui *et al.,* 1994; Benyagoub *et al.,* 1996) and the production of lytic enzymes such as β-1,3-glucanase by *Tilletiopsis* sp. (Urquhart & Punja, 1997). In addition, competition for nutrients was postulated to have reduced infection by foliar pathogens such as *Botrytis cinerea* (Elad *et al.,* 1994).

Indeed, for some biocontrol agents, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant disease such as inhibition of the pathogen by antimicrobial compounds (antibiosis); competition for colonization sites and nutrients supplied by seeds and roots; induction of plant resistant mechanisms; inactivation of pathogen germination factors present in seed and root exudates and degradation of pathogenicity factors of the pathogen such as toxins; parasitism that may involve production of extracellular cell wall-degrading enzymes, for example, chitinase that can lyse pathogen cell walls (Arras, 1996).

The potential applications for use of bacteria, fungi and yeasts as biological control agents of fungal disease on vegetable crops are reviewed. The yeast organisms have shown great potential for reducing for foliar diseases, especially those diseases caused by mildew fungi. Their use for post harvest disease control has not been investigated extensively and is worthy of research (Punja, 1997).

Most of the research on evaluation of yeasts for control of vegetable diseases has emphasized powdery mildew diseases on cucumber (Jarvis *et al.,* 1989), although reduction of *Botrytis cinerea* on tomato has also been reported (Elad *et al.,* 1994). The most widely used organisms which have been evaluated for their potential are *Ampelomyces quisqualis* ces (McGrath & Shishkoff, 1996), *Sporothrix* (*Pseudozyma*) spp. (Jarvis *et al.,* 1989) and *Tilletiopsis* spp. (Hijwegen, 1992).

Of 40 yeast fungi isolates obtained from the rhizosphere of maize plant (*zea mays* L.), five yeast fungi were found to be strongly antagonistic to *Cephalosporium maydis*, the causal agent of late wilt disease. These isolates were identified as *Candida glabrata*, *C. maltosa*, *C. slooffii*, *Rhodotorula rubura* and *Trichosporon cutanum*. All these isolates when tested produced non-volatile antifungal metabolites that significantly reduced the growth of the pathogen *in vitro*. These five yeast fungi significantly reduced the late wilt disease incidence compared to the treatment which included the pathogen alone. These yeast fungi have considerable potential for further use as biocontrol agents of late wilt disease under natural conditions (Hassanein *et al.,* 2003).

MATERIALS AND METHODS

Pathogen. One month-old *Phaseolus vulgaris* seedlings, exhibiting wilt disease symptoms were collected and isolation was made within 24 h by placing the whole seedlings under running water for 6 h to remove soil particles and surface sterilization for 3 min in 0.5% NaOCl. They were then rinsed several times in sterile filter paper in laminar flow cabinet to dry for 30 min (Demain *et al.,* 1993). Once dry, the root and the stem bases were excised aseptically and placed on media: I) Potato-dextrose agar supplemented with yeast extract and streptomycin; II) Sabouraud's agar supplemented with yeast extract and streptomycin. The plates were then incubated in the dark at 30 ±2˚C for 8 days and examined daily for fungal growth.

Biocontrol agents. The two yeast fungi isolates were selected from the rhizosphere of healthy kidney bean plant and were tested both *in vitro* and *in vivo* for their antagonistic activity to the pathogen. The two yeast fungi species were *Saccharomyces unispora* and *Candida steatolytica*.

Identification of yeast fungi and the pathogenic fungus. The pathogen *Fusarium oxysporum* was identified following the universal references of Ellis (1971) and Booth (1971). Rhizosphere yeast fungi were isolated on I) Sabouraud's agar, and II) Wickerham's agar medium. These two media contained the antibiotic nystatin (50 mg mL^{-1}) which was added to cooled (45˚C) sterile molten agar media immediately prior to pouring plates. Nine plates were used per dilution, and all plates were incubated at 28±2˚C in the dark for seven days. Colonies of yeast fungi were expressed as (cfu) dry^{-1} soil. The isolates of rhizosphere yeast fungi were identified using the following references (Wickerham, 1951; Barnett, 1966; Lodder, 1970; Arex *et al.,* 1977; Barnett *et al.,* 1983, Kreger, 1984; Barnett *et al.,* 1990).

Green House Pathogenicity Test

Inoculum production and soil infestation. Isolates of *Fusarium oxysporum* were grown on SDYA (Sabouraud's yeast extract agar) at 30±2˚C for seven days in dark. Millet (*Panicum miliaceum* L.) seed-based inoculum was prepared by mixing 25 g of seeds with distilled water into 250 mL conical flasks and kept overnight. The flasks were then autoclaved at 121˚C for 30 min on three consecutive days (Wong *et al.,* 1984). Under aseptic conditions, the millet seeds were then inoculated with eight agar plugs (5 mM diameter) from the actively growing margins of *F. oxysporum.* The flasks were incubated at 30±2˚C in the dark for two weeks and were occasionally shaken to ensure uniformity of colonization. Colonized millet seeds which had been autoclaved twice served as the control. Prior to use, small amounts of the colonized and control millet seeds were plated onto SDYA, to confirm the presence of the isolate under green house conditions. Pathogen inoculum was prepared as previously described by Wong *et al.* (1984). *F. oxysporum* (1% weight of millet seed-based inoculum/weight of steam-pasteurized soil was thoroughly dispersed through the steamed soil contained in two sets of pots in which each pot was filled with 3 kg of clay soil. One set of pots contained the pathogen inoculum and the other received the same quantities of subsequently autoclaved millet seeds to serve as the control. Kidney bean seeds were disinfected by immersing first in 70% ethanol for 2 min then in 0.5% NaOCl for 3 min, those were then washed for several times with sterile distilled water. Seeds were sown at 3 cm depth and when emergence was complete (ca. 10 days), the seedling density was reduced to 5 seedlings per pot. Each treatment was replicated six times with five plants/ replicate. The pots were watered every other day with equal amounts of water. The growing two kidney bean seedlings were observed daily for six weeks and disease index was evaluated for severity of the wilt and foliar symptoms 4 weeks after inoculation (Leath *et al.,* 1989).

***In vitro* detection of antifungal activity of yeast fungi.** The two yeast fungi isolates which were found to be strongly antagonistic to *F. oxysporum* were examined to produce inhibitory compounds active against the pathogen. These isolates were streake-inoculated to one side of Hussien's fish meal extract agar (HFMEA) (Hussein, personal communication; El-Nomany, 1984; El-Tarabily, 1992; El-Tarabily *et al.,* 1996). The plates were then incubated for eight days to allow the production and diffusion of metabolites into the agar. An agar disc (6 cm diameter) containing *F. oxysporum* mycelial plugs was placed on uninoculated HFMEA separately as uninhibited controls. Cultures were incubated in the dark as described by Yuan and Crawford (1995). The level of inhibition was defined as the substraction of the fungal growth radius [γ_o (cm)] of a control culture from the distance of the growth in the direction of the yeast colony [γ (in cm)], where $\Delta\gamma = \gamma_o - \gamma$. Inhibition was indicated when mycelial growth of *F. oxysporum* in the direction of the yeast colony was retarded. The inhibition rating system of (+++, $\Delta\gamma > 3$ cm; ++, 3 cm > $\Delta\gamma > 2$ cm; +, 2 cm > $\Delta\gamma > 0.5$ cm; -, $\Delta\gamma = 0$) was used.

Extraction and chemical characterization of the active components. The broth of each yeast was filtered and extracted with a mixture of chloroform–ethyl acetate (1:1 v/v). The organic layer containing antibiotic was evaporated at 34˚C under vacuum till dryness. The residue was dissolved in the least amount of chloroform (3 mL). The active components in the culture filtrates of the two yeast fungi were investigated by means of descending paper strip chromatography. The solvent system was a 6:4:3 mixture of 1-nbutanol, pyridine and water. After development, the paper strips (Whatman No. 1) were air-dried and placed on agar plates seeded with *F. oxysorum* (Pridham *et al.,* 1956).

Chemical analysis of the culture filtrate. This experiment was carried out according to El-Mehalawy (1999), using mass spectroscopy. Varian gas chromatography coupled with a mass selective detector. Finnigan mat SSQ 700 and equipped with Chem-Station software and NIST spectral

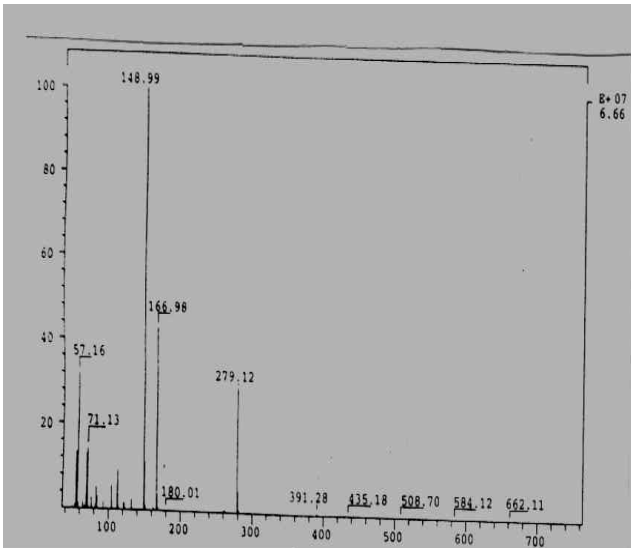
data was used with DB-5 fused silica capillary column (30 x 0.25 μm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: column temperature 60˚C (30 min), raised from 60 to 260˚C (5˚C/ min) and maintained at 260˚C for 10 min, interface, 260˚C; injector temperature 250˚C, ionization energy 70 ev; mass range 50-750; volume injected 1 UL. This experiment was performed at the Mass Spectroscopy Unit, Central Scientific Services Laboratory at the National Research Center, Cairo.

RESULTS

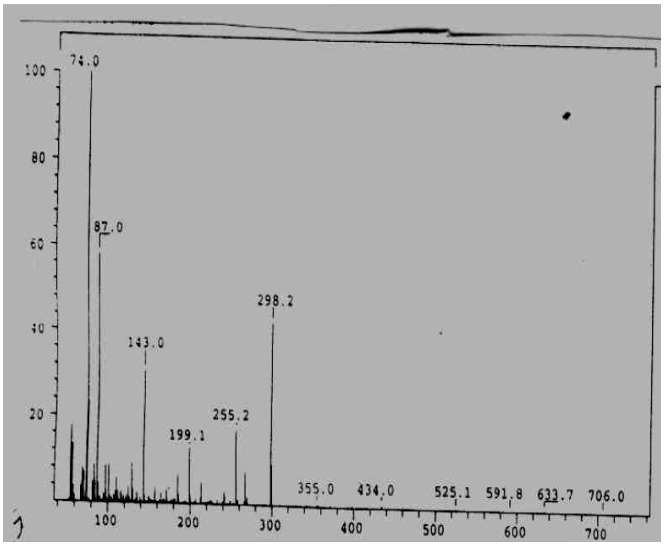
The two rhizosphere yeast fungi species *S. unispora* and *C. steatolytica* were tested for their antagonistic activity against the fungal pathogen *F. oxysprum*, the causal agent of wilt disease (Table I). It is shown from the table that both the two yeast fungi species have antagonistic and inhibitory effects on the fungal pathogen (as % of inhibition). The latter being more effective than the former.

The greenhouse pathogenicity test was carried out to determine the percentage of seed germination, diseased plant and control of both treated and untreated (control) of kidney bean seeds (Table II). Data revealed that infestation of soil with the pathogen (*F. oxysporum)* led to a reduction in the percentage of seed germination of kidney bean compared to the non-infested soil (control), indicating that the percentage of diseased plants was increased. On the other hand, addition of the two yeast fungi species as biocontrol agents led to a remarkable reduction in diseased plants (% of control). It is clear from the table that *C. steatolytica* was the most potent antagonistic species, which antagonized *F. oxysporum* leading to an inhibition of

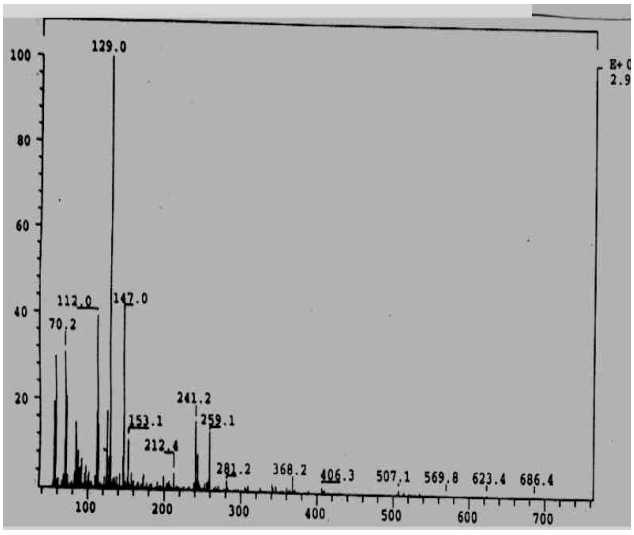
Fig. 1. Active components of culture filtrate of *S. unispora*



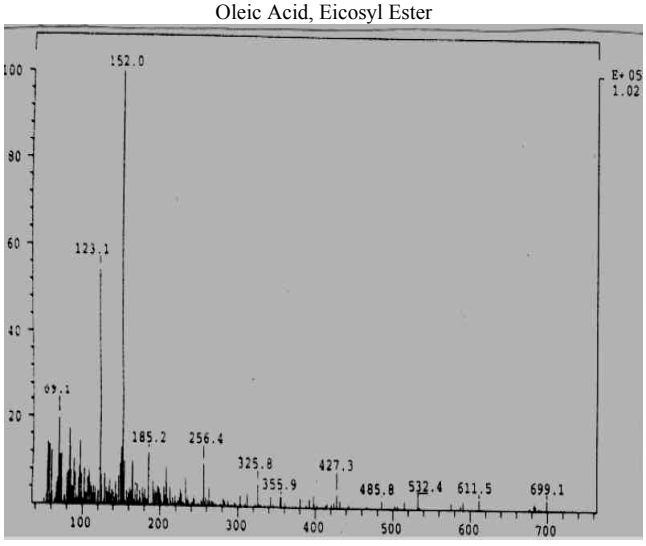
Tetratetracontane



Oleic Acid, Eicosyl Ester

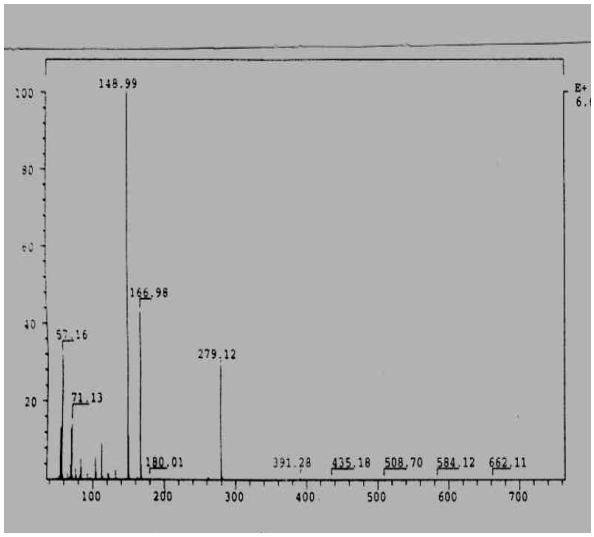


9-Octadecanoic Acid (Z)-Tetradecyl Ester

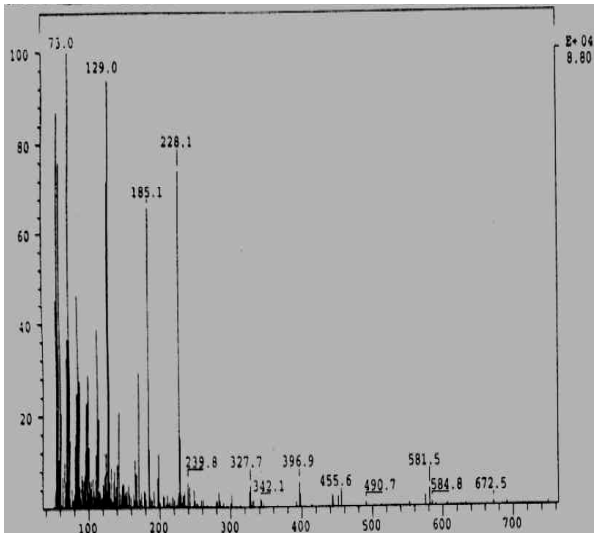


2-Iodoethyl Myristate

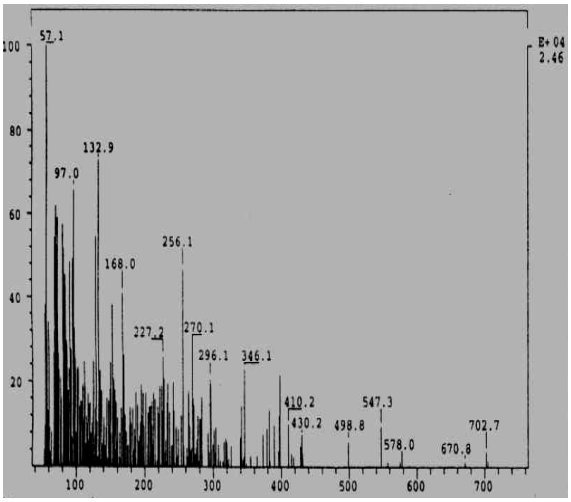
Fig. 2. Active components of culture filtrates of *C. Steatolytica*



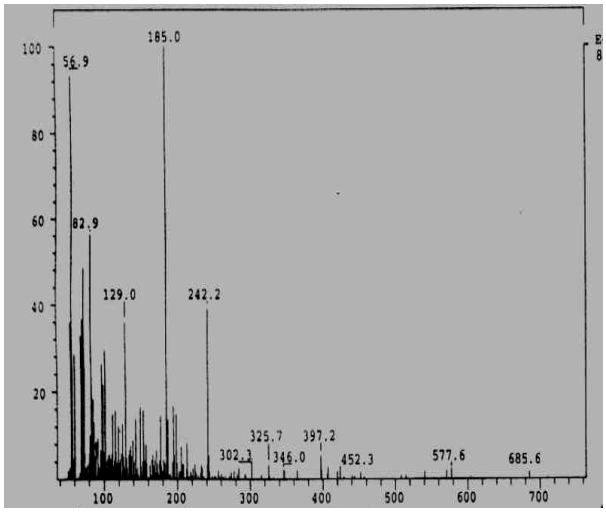
Tetratetracontane



Hentetetracontanoic ACID



Hexadecanoic Acid, Hexadecyl Ester



Nonanedioic Acid, bis-(2-Methyl Propyl) Ester

diseased plant with a percentage higher than that of *S. unispora*.

The two species of yeast fungi which were isolated from the rhizosphere of kidney bean plant with known biocontrol activities were assessed for their roles in antagonizing the effect of the pathogen upon kidney bean growth and development (Table III). The presence of the pathogen significantly decreased all kidney bean growth measurements compared to control. The addition of these rhizosphere antagonists significantly increase kidney bean

Table I. Antagonistic activity of yeast fungi against *Fusarium oxysporum*

Yeast fungi	*Fusarium oxysporum*			
	γ	γ_o	Δγ	% of inhibition
Saccharomyces unispora	4	1.4	2.6	65
Candida steatolytica	4	1.2	2.8	70

γ = radius of control; γ_o = radius of treated; Δγ = Difference in radii

Table II. Effect of yeast fungi on both the germination of kidney bean seeds and Pathogenicity of tested pathogenic fungus

Treatment	Kidney bean		
	% of seed germination	% of diseased plants	% of control
Control	90		
Pathogen only	22.5	77.5	
S. unispora + pathogen	70	22.5	77.5
C. steatolytica + pathogen	80	11.5	88.5

Table III. Effect of the two yeast fungi on the growth measurements of the infected kidney bean

Treatment	Growth measurements					
	Length of root	Height of shoot	Fresh weight of root	Fresh weight of shoot	Dry weight of root	Dry weight of shoot
Control	10.14±(0.33)	22.2±(0.87)	0.44±(0.33)	4.23±(0.11)	0.17±(0.07)[d]	0.53±(0.02)[f]
Pathogen only	5.22±(0.22)	9.93±(.87)	0.23±(0.09)	2.04±(0.08)	0.04±(0.04)[d]	0.28±(0.01)[f]
pathogen and *S. unispora*	17.6±(0.43)[a]	25.77±(1.1)[b]	1.55±(0.05)[c]	7.59±(0.1)	0.4±(0.02)[e]	7.71±(0.01)[f]
pathogen and *C. steatolytica*	18.14±(0.36)[a]	27.5±(0.34)[b]	1.64±(0.03)[c]	9.38±(0.07)	0.53±(0.02)[e]	4.32±(0.02)[f]

Values having the same letter are not significantly different

growth measurements compared to the infected kidney bean.

Table IV. Chemical characterization of the active components produced by the tested two yeast fungi

R_f of the active components	Effect on *F. oxysporum*
S. unispora R_f: 0.1	+
R_f: 0.21	+
R_f: 0.32	+
R_f: 0.72	+
C. steatolytica R_f: 0.1	+
R_f: 0.17	+
R_f: 0.3	+
R_f: 0.83	+

The two yeast fungal species which were found to be strongly antagonistic to *F. oxysporum* were examined for their ability to produce inhibitory active compounds (Table IV). Data in the table showed that four active components were detected in the culture filtrates of both *S. unispora* and *C. steatolytica*. The active component of R_f 0.1 was common in the culture filtrates of both the two yeast species.

The chemical analysis of the culture filtrates of *S. unispora* and *C. steatolytica* revealed the presence of 10 chemical compounds in the culture filtrate of each. These chemical compounds were arranged descending according to their molecular weights (Tables V, VI). Tetratetracontane of high molecular weight (618) was detected in the culture filtrates of both the two yeast fungi species, in addition to iron, tricarbonyl [(2,3,4,5-eta.)-2,4-cycloheptadien-1-0l]- of molecular weight 250.

It is well known that a compound of high molecular weight has a low R_f and *vice versa*. Therefore, the active component of R_f 0.1 may be tetratetracontane of molecular weight 618, the active component of R_f 0.17 may be hentetetracontanoic acid of molecular weight 606, the active component of R_f 0.21 may be oleic acid eicosyl ester of molecular weight 562, the active component of R_f 0.3 may be hexadecanoic acid, hexadecyl ester of molecular weight 480, the active component of R_f 0.32 may be 9-octadecanoic(Z)-tetradecyl ester of molecular weight 506, the active component of R_f 0.72 may be 2-iodoethyl myristate of molecular weight 382 and finally, the active component of R_f 0.83 may be nonanedioic acid, bis (2-methyl propyl) ester of molecular weight 300.

DISCUSSION

Table V. Chemical analysis of culture filtrate of *S. unispora*

Chemical name	Chemical formula	Molecular weight
Tetratetracontane	$C_{44}.H_{90}$	618
Oleic acid, eicosyl ester	$C_{38}.H_{74}.O_2$	562
9-Octadecenoic acid(z)-tetradecyl ester	$C_{34}.H_{66}.O_2$	506
2-Iodoethyl myristate	$C_{16}.H_{31}.O_2.I$	382
1,3(2H,4H)-Isoquinolinedione,4-[2-(3,4- dimethoxyphenyl)ethyl]-	$C_{19}.H_{19}.N.O_4$	325
Heptadecane,2,2,10,15-tetratmethyl-	$C_{21}.H_{44}$	296
Iron,tricarbonyl[(2,3,4,5-eta.)-2,4- cycloheptadien-1-0l]-	$C_{10}.H_{10}.O_4.Fe$	250
3,4-Di(ethoxymethyl)thiophene	$C_{10}.H_{16}.O_2.S$	200
Heptanoic acid, ethyl ester	$C_9.H_{29}.O_2$	158
Hexanoic acid	$C_6.H_{12}.O_2$	116

Table VI. Chemical analysis of the culture filtrate of *C. steatolytica*

Chemical name	Chemical formula	Molecular weight
Tetratetracontane	$C_{44}.H_{90}$	618
Henttetracontanoic acid	$C_{41}.H_{82}.O_2$	606
Hexadecanoic acid, hexadecyl ester	$C_{32}.H_{64}.O_2$	480
Nonanedioic acid, bis (2-methyl propyl)ester	$C_{17}.H_{32}.O_2$	300
Iron,tricarbony[2,3,4,5-eta.)-2,4- cycloheptadien-1-0l]-	$C_{10}.H_{10}.O_4.Fe$	250
Phenol,chloro-4-(1,1-dimethylethyl)-acetate	$C_{12}.H_{15}.O_2.Cl$	226
Phenyl,5-methyl-2-(1-methylethyl)-acetate	$C_{12}.H_{16}.O_2$	192
3-methylbutyl hexanoate	$C_{11}.H_{22}.O_2$	186
Hexanoic acid,2,4-dimethyl-,methyl ester, (2DL,4DL)-	$C_9.H_{18}.O_2$	158
Benzene,1-ethyl-3-methyl	$C_9.H_{12}$	120

The two yeast fungi species showed an antagonistic activity against the pathogenic fungus *F. oxysporum*. This antagonistic activity may be due to the production of water soluble antifungal metabolites diffused into the medium as indicated by the large areas of inhibition zones on the agar plates (Crawford *et al.,* 1993). These antifungals may interact with sensitive cells involving the cellular membrane. As a result of this interaction, the membrane no larger serve as a selective restraining barrier and the specific permeability of the cell wall will be loosed. After permeability was disrupted, the essential cytoplasmic constituents were lost from the cell. This finding is in accordance with those reported by Liu *et al.* (1987), and Abd-Allah and El-Mehalawy (2002). The above result may also be due to the production of toxins by the yeast fungi (killer toxins). This finding agrees with that reported by Hodgson *et al.* (1995).

In the present investigation, the two yeast fungi species significantly increased the percentage of inhibition of disease caused by the pathogen (*F. oxysporum*). This may be due to the antifungal production which may play a significant role in antagonism at the microhabitat level. This finding runs parallel with that reported by Rothrock and Gottlied (1981). The reduced incidence of wilt disease caused by the addition of yeast fungi may also be due to the incubation period in the soil which may aid in the establishment of the introduced yeast fungi and enable them to multiply in the soil or to activate the mechanism(s) of antagonism. This finding agrees with that reported by Rothrock and Gottlied (1984). On the other hand, the increased percentage of diseased plants may be due to the role of the pathogen which may act as a causal agent of the disease and was able to overcome the defense mechanisms of kidney bean. This finding is in accordance with that reported by Begum *et al.* (1989).

The increased response of plants by rhizosphere microorganisms depends on the ability of these microorganisms to survive and develop in the rhizosphere (Kleified & Chet, 1992). From the possible mechanisms for increase plant growth, the production of active metabolites particularly plant growth regulators by rhizosphere microbiota. Thus the ability of plant growth promoting microorganisms to induce increased plant growth is related in part to antibiosis that occurs in root zones and the subsequent displacement of certain colonizing microorganisms (Kloepper & Schroth, 1981).

Each of the two yeast fungi produces four active components. This may be due to that yeast fungi cell metabolism under conditions of nutritional excess is directed towards the generation of cell mass rather than the production of secondary metabolites and when depletion of key nutrients occurs, it shifts the cell cycle to the stationary phase and signals the transition from primary to secondary metabolism in which the active components are produced. This finding agrees with those reported by Abbanat *et al.* (1999), and Abd-Allah and El-Mehalawy (2002).

The culture filtrate of both *S. unisora* and *C. steatolytica* revealed the presence of 10 chemical compounds of different molecular weights. Some of these compounds have an inhibitory effect on the growth of the pathogen, *F. oxysporum*. It was suggested that the organism may divert its metabolic effort to making antibiotics only when it is in competition for a substrate which it requires for growth. This explanation runs parallel with those reported by Bushell (1989) and El-Mehalawy (1999).

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(Received 10 November 2003; Accepted 10 February 2004)