

Original Research Article

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Isolation and Identification of Fungal Communities in Organic and Conventional Soils

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ABSTRACT

Keywords

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Mycoflora population from soil of organic and conventional fields was investigated at different plots. The fungi were isolated by using soil dilution method. Total of six genera were obtained from 10 soil samples. On the basis of cultural and microscopic characteristics, the isolated strains were identified as *Aspergillus sp.*, *Penicillium sp.*, *Alternaria sp.*, *Fusarium sp.*, *Cladosporium sp.* and *Gliocladium sp.* *Aspergillus* species showed the highest percentage among the six genera. This work aims to search different potential bio-agents fungi from soil's microflora sampled from selective organic plots. *In vitro*, direct confrontation between pathogens and antagonists showed that *Gliocladium roseum*, *Aspergillus pseudoelegans*, *A. niger*, *A. terreus*, *A. nidulans* and *A. fumigatus* inhibited the mycelial growth of *Fusarium solani* and *Alternaria alternata*, with varying efficiencies. The obtained inhibition rates reach up above 44%. The mycoflora analysis proved that these soils are rich in fungi that could play a crucial role in the biological treatment as antagonists of pathogen fungus.

Introduction

The soil is a highly complex system with many components playing diverse functions and could be due mainly to the activity of soil organisms (Chiang and Soudi, 1994). Soil microflora plays a pivotal role in evaluation of soil conditions and in stimulating plant growth (Nagamani *et al.*, 2006). Microorganisms are beneficial in increasing the soil fertility and plant growth as they are involved in several biochemical transformation and mineralization activities in

soils. Type of cultivation and crop management practices are found to have greater influence on the activity of soil microflora (Mc. Gill *et al.*, 1980). Continuous use of chemical fertilizers over a long period may cause imbalance in soil microflora, as result affecting indirectly biological properties of soil leading to soil degradation (Manickam and Venkataraman, 1972). Fungi are fundamental for soil ecosystem functioning (Warcup, 1951). Fungi are an

important component of the soil micro biota (Christensen, 1989; Ainsworth and Bisby, 1995). Micro fungi play a focal role in nutrient cycling by regulating soil biological activity (Arunachalam *et al.*, 1997). The prevalence of organic and inorganic materials present in the soil has a direct effect on the fungal population of the soil. Biological control of plant disease especially soil-borne pathogens by microorganisms has been considered as a good environmentally alternative to the chemical treatment methods (Freeman *et al.*, 2002; Eziashi *et al.*, 2007). Many antagonistic microorganisms have been proved to be active *in vitro* and *in vivo*.

The aim of the present investigation is to detect and identify mycoflora strains from different crop fields, and to determine the potential of isolated fungi from organic field to act as biocontrol agents.

Materials and Methods

Presentation of experimental site

The study described here is part of long-term trial, which began in 2000. The research station is located at the experimental field located in the Technical Center of Organic Agriculture and the High Agronomic Institute of Chott Meriem (Sousse, region of the Center East of Tunisia, 35 m above the sea level latitude 35°51'32" North and longitude 10°35'38" East Greenwich). This region is characterized by a humid mild winter and hot dry summer. The precipitations vary from 350 to 400 mm and are mainly concentrated between October and April. The annual average temperature ranges from 16 to 19°C, with a maximum recorded in July and a minimum one recorded in January.

Soil sample analysis

Soils were collected randomly at different site from ten fields, eight organic and two

conventional. In organic cropping area, rotations are longer and presenting diversity of cropping such as legumes, cereals, medicinal and aromatics plants, fruit bearing, root and tuberous and leafy vegetables. These rotations also include fallow and green manure. Conventional fields use only synthetic fertilizers and pesticides without legumes and green manure (Table 1).

At each sampling point, four representative sub-samples were taken from a depth of 5 to 20 cm. The first 0–5 cm soil layer was discarded to reduce spatial variability and also possible point contamination. The four sub-samples were taken with a soil auger, mixed, pooled, giving forty independent composite samples, and transferred in sealed plastic bags to the laboratory. The composite samples were sieved to <2 mm, homogenized and stored at 4°C until the analysis. Soil samples were air dried and sieved at 2 mm. On these samples the following analysis were carried out: electrical conductivity and pH, measured on a mixture of soil (10 g dry weight) and distilled water (25 ml) and shaken for at least 2 hours on a shaker at 40 rpm; soil organic matter by the dichromate oxidation method; total nitrogen by the Kjeldahl method; available phosphorus by the Olsen method. Exchangeable bases (Na⁺, K⁺ and Ca⁺⁺) were determined after acid digestion with HF/HNO₃ by a Beckman single beam flame emission spectrophotometer.

Soil-borne fungi analysis

Fungi flora inventory in soils under different agro-systems was accomplished by soil dilution method on PDA. For this, 1g of soil sample was suspended in 10 ml of double distilled water to make microbial suspensions (10⁻¹ to 10⁻⁵). One ml of microbial suspension of each concentration were added to sterile Petri dishes (triplicate of each dilution) containing PDA media (15 ml). One percent streptomycin solution was added to the

medium before pouring into Petri plates for preventing bacterial growth. The Petri dishes were then incubated at 28°C in dark and were observed daily. After 2-7 days, fungal colonies appeared on the PDA media were transferred to other plates for purification and identification. Fungal morphology were studied macroscopically by observing colony features (colour and texture) and microscopically by staining with lacto phenol cotton blue and observe under compound microscope for the conidia, conidiophores and arrangement of spores (Aneja, 2003). The fungi were identified using literature identification keys. *Aspergillus* species were identified using manual about the genus aspergilli (Raper and Fennell, 1965; McClenny, 2005; Diba *et al.*, 2007; Domsch, 1980; Samson and Pitt, 2000). *Penicillium* species were identified as described by (Raper and Thom, 1949; Raper, 1957; Samson and Pitt, 1985; Houbraken and Samson, 2011; Houbraken *et al.*, 2014a; 2014b). *Fusarium* Species identification was based on the morphological characteristics of isolates as described by (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson *et al.*, 1983; Burgess *et al.*, 1994; Leslie and Summerell, 2006). *Alternaria alternata* was identified using literature identification keys of (Simmons, 1992; Simmons, 2007). *Cladosporium* species were identified using manual about the genus *Cladosporium* (Schubert and Braun, 2007; Braun and Schubert, 2007; Braun *et al.*, 2008). Every fungi species was enumerated according to the colonies number in each petri dish.

***In vitro* potential antimicrobial activity**

Pathogens were isolated from both organic and conventional plots and antagonists only from organic plots. Mycelial disc (5 mm diameter) was obtained from the peripheral of five days old mycelial antagonist isolate was placed in front of the pathogen in 4:1 ratio from the opposite edge of the plate. For

the control, a mycelial plug of pathogen was placed in the center of the plate. Three replicates were prepared per treatment (pathogen/ antagonist). The plates were incubated at 25°C (Benhamou and Cheti, 1996) and the diameter growth of colonies of each pathogen was measured. The inhibition rate is calculated according to the following formula: % Inhibition = $(D_o - D_x)/D_o \times 100$; D_o = diameter of the control; D_x = Diameter of the pathogen in treatment. This technique is inspired by the opposed culture technique, recommended by Patel (1974).

Data analysis

Data were subjected to analysis of variance (ANOVA) using SAS. Significance of mean differences was determined using the Duncan's test, and responses were judged significant at the 5% level (P=0.05).

Results and Discussion

Soil chemical and physical properties

The soils selected for this study had different physical structures and contained different plant species, nutrient concentrations and organic matter (Table 2). The rotation program significantly influenced soil pH and EC. These soils were generally alkaline and the pH ranged from 8.08 to 8.84. The addition of cations via manure application has resulted in higher pH levels in the organic systems. Soil organic matter (SOM) is a fertility parameter that responds to changes in soil management in the long term (Feller *et al.*, 2012). SOM of the organic fields was significantly higher than that found in the conventional treatments attained highest value at org 3 plot (4.95%) and lowest in conv 1 plot (2.02%). This increase can be attributed mainly to the application of compost and higher amounts of diversified crop residues remaining on the organic fields than in soil under conventional systems. Total nitrogen

was the lowest in conventional treatments (1500 ppm in conv 1 plot and 1600 ppm in conv 2 plot). Application of compost and green manure in organic treatments provides nitrogen and other nutrition elements. Moreover, the use of organic fertilizers increased soil nitrogen availability for plants (Hatch, 2000). Rotation in org 3 plot was the highest in nitrogen contents (2200 ppm). This can be justified by the fact that this plot has received a double quantity of compost and a triple quantity of manure compared to other organic plots. It has the highest number of legumes (4 legumes in 9 years) and crops have absorbed nutrients from different forms and depths. Organic treatments with a monoculture of aromatic and medicinal plants for five years recorded lower level than other organic treatments (1700 ppm).

Soil microflora identification

A total of 230 fungal isolates were obtained and identified from 10 soil samples taken from organic and conventional soils through soil dilution agar plating. All fungal isolates were obtained in pure cultures by using standard techniques (Fig. 1). The isolates from agricultural soils were identified as filamentous fungi belonging to the phyla Ascomycota. The results obtained, clearly indicates that *Aspergillus* sp. (about 155 individual colony formed in bio fields and 5 in conv) (Fig. 1k, m, n) and *Penicillium* sp. (Fig. 1e-f) presented a high occurrence in all crop fields due to increased sporulation level and some other fungi genera like *Fusarium* (none in bio fields, 7 and 8 in conv), *Alternaria* (two in bio fields) (Fig. 1a) and *Cladosporium* (five in bio fields) (Fig. 1G-h) were less frequent (Table 3). The remaining strains were unidentified owing to the lack of sporulating structures under presently used incubation conditions. *Penicillium* were producing fungal and bacterial antibiotics and *Aspergillus* producing different kinds of

toxins such as aflatoxins, achrotoxins etc. These toxins may prevent the growth of other fungal species.

***In vitro* potential antimicrobial activity**

Results from the dual culture assay showed that all antagonistic microorganisms (*Gliocladium roseum*, *Aspergillus pseudoelegans*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus nidulans* and *Aspergillus fumigatus*) inhibited the mycelial growth of *Fusarium solani* and *Alternaria alternata*, with varying efficiencies (Figures 2 and 3). In culture, all the five *Aspergillus* species and *Gliocladium roseum* screened grew faster than the pathogen. The tested antagonists formed a zone of inhibition in dual culture and hindered the growth of the pathogen. The inhibition rates varied according to the isolates and reach up to more than 44 %. *Aspergillus niger* turns out to be the most efficient against the two pathogens, since it reaches an inhibition rate of 44.97% against *Alternaria alternata* and 26.80% against *Fusarium solani*. However, *Gliocladium roseum*, *Aspergillus pseudoelegans*, *Aspergillus terreus*, *Aspergillus nidulans* and *Aspergillus fumigatus* have displayed a moderate inhibition capacity not exceed 22% against *F. solani* and 40% against *A. alternata* (Figures 2 and 3).

Obtained results of the present investigation revealed, with various degrees, a similarity with other researchs. All of these genera have been reported as common in soil by Deming (2002) and Onofri *et al.*, (2007). Our results were supported by Akpoveta *et al.*, (2011), which they isolated *Penicillium* sp. and *Aspergillus* sp. from soil. Study supported by Obire and Anyanwu (2009), they isolated fourteen fungal genera from soil including the genera of *Alternaria*, *Aspergillus*, *Cladosporium* and *Fusarium*.

Table.1 Details of the different analyzed plots, such as crop rotations of 8 organic (org 1-org 8) and two conventional (conv 1-conv 2)

Plots	Year 2000\01	Year 2001\02	Year 2002\03	Year 2003\04	Year 2004\05	Year 2005\06	Year 2006\07	Year 2007\08	Year 2008\09
Org 1	Fennel Potato	Garlic	Potato	Fallow	Onion Garlic	Potato	Faba bean	Fennel	Bean
Org 2	Potato	Onion	Potato	Vegetables	Potato	Faba bean	Fennel Leek	Artichoke	Artichoke
Org 3	Potato	Faba-bean	Pea Cauliflower Fennel	Potato	Fallow	Green bean Zucchini Cucumber (greenhouse)	Melon Tomato (greenhouse)	Tomato Pepper Eggplant (greenhouse)	Green-bean Zucchini (greenhouse)
Org 4	Fallow Potato	Potato	Tomato	Clover	Aromatic and medicinal plants	Aromatic and medicinal plants	Aromatic and medicinal plants	Aromatic and medicinal plants	Aromatic and medicinal plants
Org 5	Potato	Potato	Tomato Potato	Clover	Fennel	Cauliflower Cabbage	Potato	Leek Garlic	Faba bean
Org 6	Potato	Potato	Potato	Barley	Cauliflower Cabbage	Potato	Pea	Faba bean	Fennel Maize
Org 7	Pea	Potato	Tomato Potato	Clover	Artichoke	Artichoke	Artichoke	Cauliflower Cabbage Potato	Faba bean
Org 8	Fennel Cabbage	Artichoke	Artichoke	Artichoke	Wheat	Potato	Cauliflower Cabbage	Faba bean Maize	Pea Potato
Conv 1	Maize (OF)	Potato (OF)	Fallow (OF)	Potato (OF)	Pepper (OF)	Maize (OF)	Chickpea (OF)	Fallow (OF)	Potato (OF)
Conv 2	Artichoke (OF)	Fallow (OF)	Potato (OF)	Potato (OF)	Oat (OF)	Fallow (OF)	Melon (GH)	Pepper (GH)	Tomato (GH)

Org: organic, Conv: conventional, OF: open field, GH: greenhouse

Table.2 Chemical and physical properties of selected soils samples

	Org 1	Org 2	Org 3	Org 4	Org 5	Org 6	Org 7	Org 8	Conv 1	Conv 2
pH	8.53	8.66bc	8.26 e	8.82 a	8.74ab	8.53d	8.59cd	8.84a	8.08f	8.22e
EC(mmho/cm)	3.74ab	3.7ab	3.98a	2.38d	3.46bc	3.21c	3.16c	3.34c	1.92e	2.66d
Organic matter (%)	3.28c	3.8b	4.95a	2.75d	2.71d	2.76d	3.28c	2.83d	2.02f	2.37e
Clay (%)	15.5	16.0	17.5	12.5	12.0	15.0	14.5	10.5	8.0	10.0
Silt (%)	31.0	25.0	20,5	22.0	24	21.0	23.5	24.0	30	27.0
Sand (%)	53.5	59.0	62.0	65.5	64.0	64.0	62.0	65.5	62.0	63.0
P (ppm)	45 b	46b	49a	24d	21e	49a	38c	39c	26f	30 e
K ⁺ (ppm)	750b	610c	910a	390hg	550cd	460fe	530de	360h	360h	440fg
Na ⁺ (ppm)	210b	190bc	340a	160c	160c	230b	220b	220b	150c	220b
Ca ⁺⁺ (ppm)	2500a	2400ab	2300ab	2100d	2100d	2300cb	2500a	2000d	2100cd	2100d
N _{tot} (ppm)	2000b	1900c	2200a	1700e	1800de	1850cd	1800cd	1750de	1500g	1600f
C _{mic}	240	295	594	202	200	209	205	200	150	190
N _{mic}	30	40	51	25	33	34	31	24	20	22

*Duncan's Multiple Range Test, values followed by different superscripts are significantly different at p≤0.05.

Table.3 Average number of individual fungus identified colonies in organic and conventional soils analysed with dilution plate methods

Plot	Average number of individual colonies*					
	<i>Aspergillus</i> sp.	<i>Penicillium</i> sp.	<i>Fusarium</i> <i>solani</i>	<i>Alternaria</i> <i>alternata</i>	<i>Cladosporium</i> sp.	<i>Gliocladium</i> <i>roseum</i>
Bio 1	20	7	-	1	1	-
Bio 2	17	-	-	-	-	-
Bio 3	21	3	-	-	-	-
Bio 4	28	-	-	-	-	-
Bio 5	30	-	-	-	-	-
Bio 6	15	6	-	1	-	3
Bio 7	12	10	-	-	2	-
Bio 8	5	5	-	-	2	-
Bio 9	17	-	-	-	-	4
Conv 1	5	-	7	-	-	-
Conv 2	-	-	8	-	-	-

*Number of colonies of each fungus.

Fig.1 Morphological aspect of seven fungi at 25°C after 5 days of incubation: colonie aspect and reproductive structures: a-b- *Alternaria alternata*: colonies are fast growing, olivaceous-black, floccose. Microscopically, branched acropetal chains of multicellular conidia (dictyoconidia). Conidia are obclavate, obpyriform with a short conical beak, pale brown, smooth-walled. c-d- *Gliocladium roseum*: grow rapidly in culture producing spreading colonies with a cotton-like texture, covering a Petri dish in one week. The colonies are initially white and cream and become reddish. The conidiophores are erect, dense, and have a brush structure which produce tapering, slimy phialides. Conidia are singlecelled and cylindrical, accumulating in slime droplets at the tips of phialides that become confluent across the apex of the entire conidiophore. E-f- *Penicillium* spp.: colonies velvety, pale grayish green on the surface: reverse pale yellowish. Conidiophores hyaline, erect, branched penicillately at the apexes with verticillate metula, terminal phialides and catenulate conidia oneach phialide, forming rather divergent conidial heads. G-h-*Cladosporium* spp.: conidiophores pale brown, erect, branched 2–3 times at the apical parts. Conidia not well differentiated from branches, ovate, ellipsoidal, cylindrical, subglobose, irregular in shape, apiculate at one end, often truncate at another end. Conidial chains are acropetal, sympodial and profusely branched. K-l – *Aspergillus* and *Emericella nidulans*: colonies rapidly growing, green. Dark green in areas with Cleistothecal production (emericella). Colonies dense, velutinous with floccose surface. Phialides biseriate, limited to upper surface of the vesicle. conidia round, smooth to rugulose in short chains. Cleistothecia numerous, reddish-brown in colour. Smooth Round Hülle Cells. Split Cleistothecium Releasing Ascospores. M-n- *Aspergillus terreus*: conidiophore hyaline, smooth. Conidia globose. Colony color dark orange yellow, reverse light yellow, exudates transparent

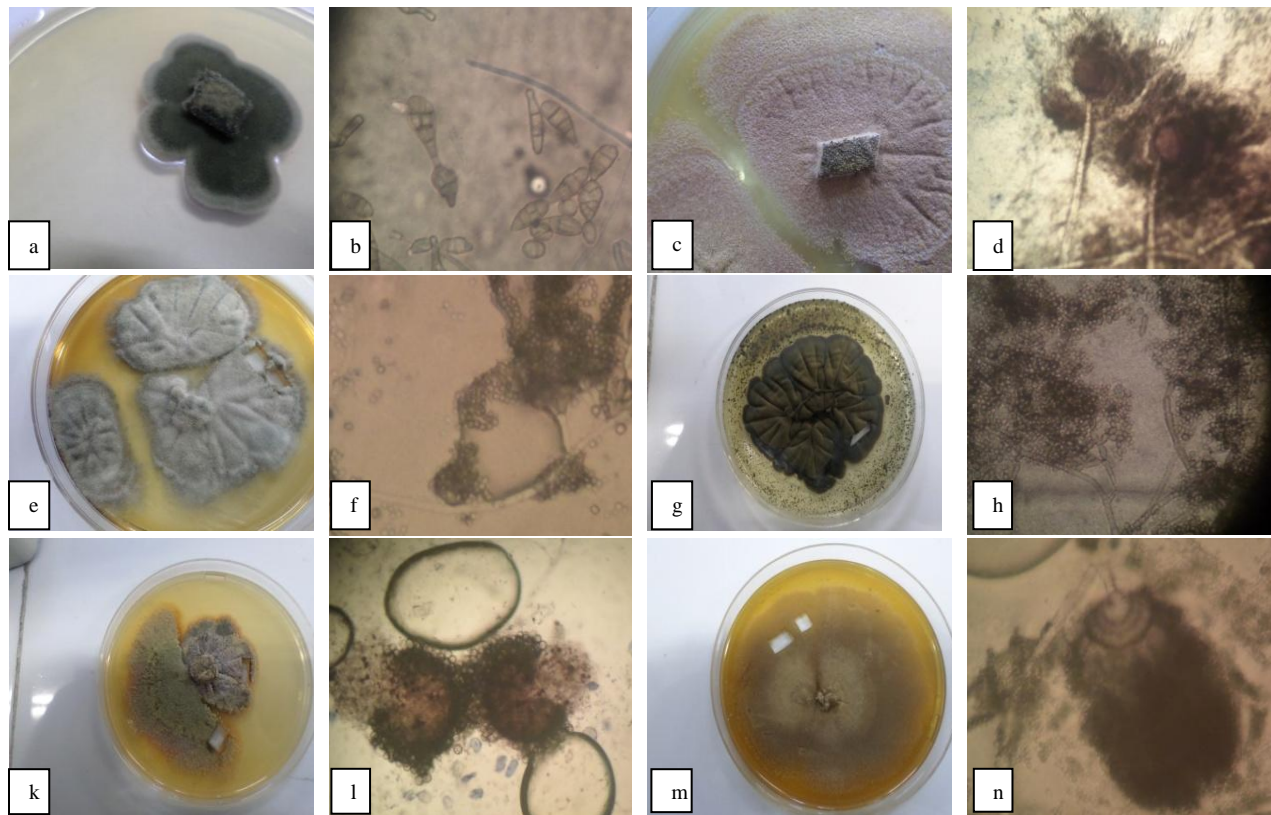


Fig.2 Antifungal activity of six potential bio-agents extracted from soil against *Alternaria alternata* after 6 days of incubation at 25°C according to different confrontation: Alt fum: *Alternaria alternata*/*Aspergillus fumigatus*; Alt pseu: *A. alternata*/*A. pseudoalegans*; Alt Glio: *A. alternata*/*Gliocladium roseum*; Alt ter: *A. alternata*/*A. terreus*; Alt nid: *A. alternata*/*A. nidulans*; A. niger: *A. alternata*/*A. niger*

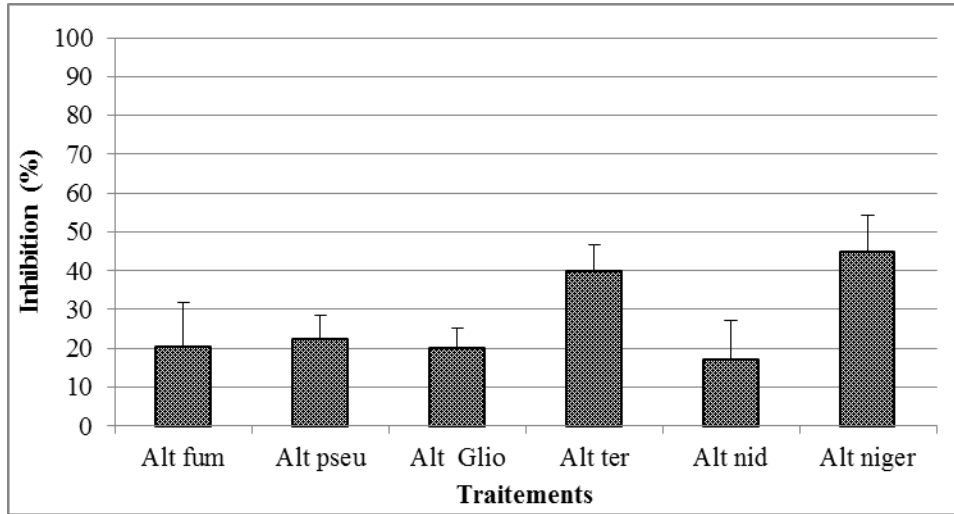
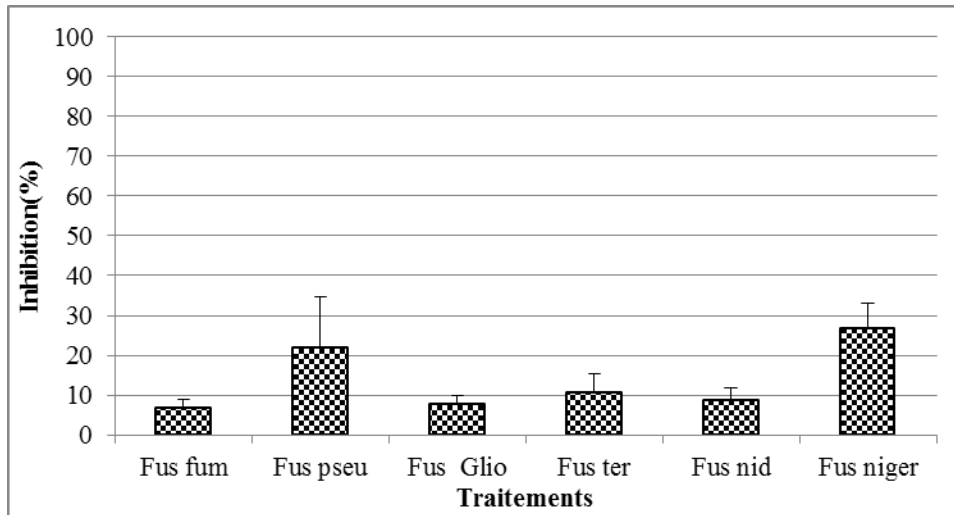


Fig.3 Antifungal activity of potential bio-agents species extracted from soil against *Fusarium solani* after 6 days of incubation at 25°C according to different confrontation: Fus fum: *Fusarium solani*/*Aspergillus fumigatus*; Fus pseu: *F. solani* / *A. pseudoalegans*; Fus Glio: *F. solani* / *Gliocladium roseum*; Fus ter: *F. solani* / *A. terreus*; Fus nid: *F. solani* / *A. nidulans*; Fus niger: *F. solani* / *A. niger*



Furthermore, Gomez *et al.*, (2006) demonstrated a positive correlation between microbial diversity and soil organic carbon. Increased microbial biomass and diversity are beneficial for soil quality because soil microorganisms play a key role in soil nutrient cycling. They accelerate the breakdown of organic substances and mineralize the organic nitrogen and phosphorus contained in manures into plant available inorganic forms. The environmental factors such as the soil pH, moisture, temperature, organic carbon and nitrogen play an important role in the mycoflora distribution (Kumar *et al.*, 2015). These are the main factors affecting the fungal population that was very high in the analyzed soil samples. The mycofloral analysis was in agreement with other studies such as Ratnasri *et al.*, (2014) and Megha Bhutt *et al.*, (2015). Serial dilution method adopted in this study displays the highest number of fungal species isolates belongs to different fungal groups (Kumar *et al.*, 2014). This finding indicated that a reservoir of thermophilic and thermotolerant fungi always coexists (Kumar *et al.*, 2015). In relation to genus detected, the prevalence of *Aspergillus* and *Penicillium* in the soil samples is consistent with the reports of Soderlund (2009) and Onyimba *et al.*, (2014). *Aspergillus* species, when paired with *Alternaria alternata* and *Fusarium solani* produced a zone of inhibition. The zones of inhibition produced were made by the production of antifungal metabolites (Adejumo *et al.*, 1999) or an indication for the production of antibiotic substances either by the pathogen against antagonistic fungi or vice versa (Gomathi and Ambikapathy, 2011). The production of antifungal metabolites or antibiotics of fungal organism which can inhibit the other will be very important in the discovery of biocontrol against pathogenic organisms. *Aspergillus* spp. had been reported an inhibitory effect against several plant pathogens (Getha *et al.*, 2005; Gachomo and

Kotchoni, 2008). Subsequently many works had been reported that *Aspergillus* produces a wide variety of enzymes which may be involved in antifungal activity (Simoes and Tornisielo, 2006). Biological control of soil borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proved to be harmful to the environment. There is a growing demand for sound, biologically-based pest management practices. Recent surveys of both conventional and organic growers indicated an interest in using biocontrol products (Rzewnicki, 2000; Van Arsdall and Frantz, 2001).

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Conflict of Interest

The authors declare that they have no personal or financial relationship (s) which may have inappropriately influenced them in writing this manuscript.

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