Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.4, 2015

Isolation of Entomopathogenic and Opportunistic Fungi from Soil in Duhok Province,Kurdistan Region of Iraq by Different Selective Isolation Media

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Abstract
Soil is a natural habitat for several important insect pathogenic fungi which play a key role in regulating populations of soil dwelling insect pests. Forty soil samples were collected during 2012-2013 from different agro ecosystems at Duhok governorate were screened for the presence of soil dwelling entomopathogenic fungi using four different selective isolation media. The four isolation media were prepared by modifying previously prepared DOC2 medium and a selective medium based on the use of Cetyletrimethyl ammonium bromide (CTAB) with oatmeal agar (OT) as a basal medium. The percentage occurrence of fungi and number of detected species was significantly affected by the type of isolation medium. The least number of recovered species (5 species) was on DOC2 medium, whereas, the highest number (14 species) was displayed by CTAB+OT medium. The two true entomopathogenic species *Lecanicillium lecanii* and *Metarhizium anisopliae* were successfully recovered only with our new formula by combination of DOC2+CTAB and OT+CTAB media, whereas, DOC2 and DOC2+OT media failed to recover the two species. This result indicated that addition of CTAB to media was a vital factor for the recovery of the two entomopathogenic species. *L. lecanii* and *M. anisopliae* have been recorded for the first time from Iraqi soil. Several other opportunistic pathogens were also detected. These include *Aspergillus flavus, A.parasiticus, Clonostachys rosea and Fusarium* species. The distribution of entomopathogenic and opportunistic fungi is discussed in relation to different agroecosystems and to some physical and chemical characteristics of soil samples.
Key words: Entomopathogenic fungi, soil, Iraq.

1. Introduction

Soil is a natural habitat for several important insect pathogenic fungi such as *Beauveria* spp.,*Metarhizium* spp., *Paecilomyces* spp., and *Lecanicillium* spp.,and is acting as a buffered medium against extreme biotic and abiotic influences (Keller and Zimmerman,1989). These soils inhabiting entomopathogenic fungi play a key role in regulating populations of soil dwelling insect pests (Jacson *et al*., 2000).

Knowledge of the species composition and distribution of indigenous entomopathogenic soil fungi is essential and would help to isolate and identify those species in a particular habitat. Many entomopathogenic fungi of the order Hypocreales (Ascomycetes) inhabit the soil and spent part of their life cycle, when they are outside their insect hosts. Isolation of these indigenous species and test their potential as insect pathogens, would help to select for biological control, the most virulent and most adapted isolates for a particular host in a particular habitat.

A wide range of fungi inhabiting soil can grow on artificial media and therefore, specific media have been developed for isolation of certain groups of microorganisms including media for selective isolation of entomopathogenic fungi (Goettel and Inglis, 1997).

The hypocrlean entomopathogenic fungi grow relatively slowly on isolation media in comparison to the ubiquitous saprophytic soil fungi. Thus the content of media for the isolation of specific entomopathogenic fungus from soil should have both a nutrient source for its growth and antimicrobial agent (antibiotic and fungicides) in appropriate concentrations to inhibit the growth of the non-target saprophytic fungi and allow to target fungus to grow (Luz *et al*., 2007).

The earliest selective medium used for isolation of entomopathogenic fungi was based on one used for the general isolation of soil fungi that contained glucose, Oxgall , peptone as nutrient sources with rosebengal, chloramphenicol and cyclohexamide as antibiotics (Veen and Ferron,1966).

A medium was used to isolate *B.bassiana* and *M.anisopliae* from elm bark and soil based on similar constituents but with crystal violet substituted for rose bengal (Doberski and Tribe, 1980).

Beilharz *et al*., (1982) discovered that the dodine (N-dodecylguanidine monoacetate) selectively encouraged the isolation of *B.bassiana* when introduced to medium at 0.56 g/L and inhibited some other soil fungi. A basal medium consisting of oatmeal agar (OTA) with 0.46 g/L dodine and 0.38 g/L benomyl allowed recovery of both *B.bassiana* and *M.anisopliae* at high frequencies in the absence of other soil fungi (Chase *et al*., 1986

A medium was formulated by Shimazu and Sato (1996) consisting of peptone, Cucl2, crystal violet, agar and water was successful in isolating *Beauvaria* species from soil. A novel dodine-free selective medium (CTC medium) has been developed by Fernandes *et al*., (2010) for isolating naturally occurring *Beauvaria* spp. and *Metarhizium* spp., especially *M.acridium* in soil. *M.acridium* is an Orthoptera-host specific fungus and frequently isolated from hot climate and desert in Africa and Asia (Bridge *et al*., 1997; Milner *et al*., 2002)..More recently, Posados *et al*., (2012) evaluated the quaternary ammonium compound cetyl trimethyl ammonium bromide (CTAB) as an alternative to be chemically related dodecylguanidine (dodine) for the selective isolation of entomopathogenic fungi from soil. Oat meal agar with chloramphenicol supplemented with 0.6 g/L CTAB resulted in an efficient medium to isolate *B.bassiana*, *M.anisopliae* and *P.lilacinus* from soil samples. The latter medium is simple and inexpensive to replace medium incorporated with dodine, since the dodine market as a fungicide showed sharp reduction and therefore, it is difficult to obtain (Luz *et al.,* 2007).

The study aimed to carry a survey on the natural occurrence of entomopathogenic and opportunistic fungi inhabiting soils from different agro-ecosystems in Duhok governorate using different selective isolation media.

2. Materials and methods

2.1 Study sites and collection of Soil Samples

Soil samples were collected from different ecosystems at Duhok governorate that included Alfalfa fields; agricultural soils cultivated with different vegetables, forest of the endemic Pine (Pinus brutia Tern), and soil supporting different fruits such as figs, berry, pear and walnut.

Soil sample about (1000 gm each) was taken from the depth of (0-10cm) with a trowel after removing litter or weed plants, placed in plastic bags and brought to the laboratory. Samples were subjected for fungal isolation within two days of collecting. Before use, samples were thoroughly mixed and passed through a 0.4mm mesh sieve breaking soil lumps and separating litter remain.

2.2 Physical and chemical analysis of soils

Before analysis, soil samples were spread on tray. Soil aggregates were broken by hand or using hammer. The soils sieve out, dried at 105C^{o} for 24 hours until soil moisture was equilibrated with that of the laboratory.

Soil texture, pH, Organic matter and E.C (Electric conductivity) were determined for all soil samples Protocols used for soil analysis were those of Anonymous (1979). One measurement was taken per each physiochemical parameter of each soil sample..

2.3 Isolation of entomopathogenic fungi by selective culture media.

To determine the optimal selective medium for isolation of entomopathogenic fungi from soil, various media were prepared by modifying previously reported DOC2 medium (Shimazu and Sato,1996) in combination with that described recently by Posados *et al*., (2012) by introducing Cetyl trimethyl ammonium bromide (CTAB) and oatmeal agar (OT) as a basal medium(Table 1).

Isolation of fungi from soil samples was performed according to Warcup (1960). About 1g of fine soil was sprinkled on the surface of each of above solidified media in Petri dishes. Plates were incubated at 25 C^{o} in the dark for 1 week.

Pure cultures from growing colonies were obtained and transferred to fresh appropriate media for identification. Fungal isolates were identified depending on their morphological characteristic and their reproductive structures with the aid of several taxonomic references (Domsch *et al*. 1980; Goettel and Inglis, 1997; Tzean *et al*., 1997; Bischoff *et al*., 2009).

Table (1) various selective media for isolation of entomopathogenic fungi used in this study.

Selective Media	Composition
DOC2	3g peptone, 0.2g Cucl2, 2mg crystal Violet, 15 g agar, 0.5 g Chloramphenicol 1L distilled water.
DOC2+ Oatmeal agar	3g peptone, 0.2 Cucl2, 2mg crystal Violet, 15 g agar, 0.5chloramphenicol 50 g oat, 1L distilled water.
DOC2 +CTAB	0.6g CTAB, 0.2 g Cucl2, 2 mg Crystal violet, 15 g agar, 0.5g Chloramphenicol, , 1L distilled water
OTA + CTAB	0.6 g CTAB, 15 g agar, 0.5g Chloramphenicol ,50 g oat, 1L distilled water

3. Results and discussion

Table (2) showed the evaluation of four different media for isolation of entomopathogenic and opportunistic fungi from soil. The various media were prepared by the modifying previously prepared DOC_2 media (Shimazu and Sato, 1996) and a selective medium based on the use of cetyle tri methyl ammonium bromide (CTAB) as introduced by Posados *et al*., (2012).

The result of the present study showed that we were able to isolate native strains of entomopathogenic fungi from soil in Duhok province, Kurdistan region of Iraq. The percentage of occurrence and number of detected species was significantly affected by isolation medium. The least number of recovered species (5 species) was on DOC2 medium, followed by DOC2 +OT medium (9 species) (Table 2).

The two true entomopathogenic species *Leccacillium lecanii* and *Metarhizium anisopliae* successfully recovered only with DOC2 +CTAB and OT + CTAB media, whereas, DOC2 alone and DOC2 + OT medium failed to recover the two species. This result indicated that addition of CTAB to media was vital factor for the recovery of the two entomopathogenic fungi. Our result supported the finding of Posados *et al*. (2012) who found that OT medium amended with 0.6 g/L CTAB allows the recovery of *M. anisopliae* and other entomopathogenic fungi. However, in our study, *B. bassian* was not detected by any of those four media.

Our new formula by combination of CTAB (0.6g/L) with the previously described DOC2 medium resulted in the detection of the two entomopathogenic species *L. leccanii* and *M. anisopliae*.

The two media also showed a selective recovery for several opportunistic entomopathogenic fungi. These include *Alternaria alternata*, *Aspergillus flavus, A. niger, A. parasticus, Clonastachys rosea, Fusarium spp., Mocur spp., Penicillium spp*. and *Trichoderma* spp.

Clonastachys rosea and *Fusarium* spp., the two well known opportunistic entomopathogens were detected by all media used. *C. rosea* has been reported to be pathogenic to *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cidadelidae) (Toledo *et al*., 2006).

The genus *Fusarium* showed high percentage of occurrence varying between 75.0 - 100 % and was detected by all media. Several species in *Fusarium* have been reported as weak to virulent insect pathogens (Claydon and Grove, 1984; Ali- Shtayeh *et al.*, 2002; Ameen, 2012).

Data on the distribution of entomopathogenic and opportunistic fungi in soil in different agroecosystems are presented in Table (3).

Metarhizium anisopliae was isolated from soil in alfalfa, onion and berry fields. This result agreed with reports by several authors that *M. anisopliae* preferred cultivated soils (Mietkiewski *et al*., 1991; Rath *et al*., 1992; Vanninen, 1996; Bidochka *et al*. 1998). None of the true entomopathogenic fungi were detected from soils in, fig, walnut and roses fields. However, the true entomopathogen, either alone or both were detected from soil in fields of alfalfa, pine forest and pear.

L. leccanii was isolated from soils under fruit trees and pine forest. Several studies indicated that permanent habitats support more diverse and stable insect communities. Plough the cultivated soils may also prevent the buildup of high population of insect pathogens by disrupting infection foci, exposing pathogen to adverse environmental conditions on the surface of soil or by burying them away from potential hosts (Chandler *et al*., 1998).

Table (2). Percentage occurrence of entomopathogenic and opportunistic fungi from soil using different selective media.

Fungal species		Media			
		DOC_2	DOC2 + OT	DOC2+ CTAB	OT+ CTAB
1	*Alternaria altarnata*	0	0	12.5	12.5
2	*Aspergillus flavus*	0	37.5	12.5	25.0
3	*Aspergillus niger*	0	75.5	87.5	75.0
4	*Aspergillus parasticus*	0	0	12.5	12.5
5	*Aspergillus terreus*	0	12.5	0	0
6	*Clonastachys rosea*	12.5	25.0	37.5	37.5
7	*Cunninghamella echinulata*	25.0	25.0	12.5	0
8	*Emericella nidulans*	0	0	0	12.5
9	*Fusarium* spp.	100	75.0	87.5	87.5
10	*Lecanicillium lecanii*	0	0	37.5	12.5
11	*Metarhizium anisoplaie*	0	0	12.5	37.5
12	*Mucor* spp.	12.5	62.5	75.5	75.5
13	*Peacilomyces* sp.	0	0	12.5	0
14	*Penicillium* sp.	0	12.5	12.5	37.5
15	*Rhizopus stolonifer*	0	25.0	0	12.5
16	*Trichoderma* sp.	12.5	0	25.0	25.0
17	*Ulocladium atrum*	0	0	0	12.5
Total no. of isolated species		5	9	12	14
Total no. of strictly entomopathogenic fungi		0	0	2	2

Results in Table (4) showed the physical and chemical characteristics of soil samples used for isolation of entomopathogenic fungi using different selective media.The PH value was ranging between 6.9 in alfalfa soil to 8.01 in figs soil. The fact that PH value is higher than 8.5 did not favor the isolation of *M. anisopliae* is in agreement with that fungi is in general more turned to acidity rather than alkalinity. The studies of Quesada-Moraga *et al.,* (2007) indicated that *M. anisopliae* was detected more frequently from soil samples with PH ranging from 7 to 8.

The percentage organic matter contents in our sample are ranging between1.27 to 4.55. The occurrence of entomopathogenic fungi was frequently found in soils with large organic matter contents (Inglis *et al.,* 2001). Such soil may support a greater diversity and density of insect hosts in which the fungi can multiply (Klingen and Haukeland, 2006). The higher percentage of organic matter contents in our study favored the isolation of entomopathogenic fungi and this result is in agreement with the study of Questada-Moraga *et al*., (2007), who reported that soils with higher than 3 % organic matter contents enhanced recovery of *M. anisopliae.* Soil texture in our soil samples showed different characters. However, the majority of soil samples showed high clay contents. Inglis *et al.,* (2001), suggested that high clay content in soil would enhance the abundance of entomopathogenic fungi because conidia adsorbed on to clay particles.

Table (3). Distribution of entomopatjhogenic and opportunistic fungi in soil of different field crops.

Field	Fungal species	% Occurrence
Alfalfa	*Alternaria altarnata*	50
	Aspergillus flavus	50
	Aspergillus niger	50
	Clonastachys rosea	50
	Fusarium spp.	100
	Metarhizium anisoplaie	25
	Mucor spp.	25
	Rhizopus stolonifer	25
Figs	*Aspergillus niger*	75
	Clonastachys rosea	25
	Cunninghamella echinulata	25
	Fusarium spp.	100
	Mucor spp.	25
	Penicillium sp.	50
	Trichoderma sp.	25
Pine	*Alternaria altarnata*	25
	Aspergillus niger	75
	Aspergillus terreus	25
	Clonastachys rosea	25
	Fusarium spp.	100
	Lecanicillium lecanii	25
	Mucor spp.	75
	Ulocladium atrum	25
Pear	*Aspergillus niger*	50
	Clonastachys rosea	25
	Cunninghamella echinulata	25
	Fusarium spp.	100
	Lecanicillium lecanii	25
	Mucor spp.	25
	Rhizopus stolonifer	25
	Trichoderma sp.	25
Walnut	*Aspergillus flavus*	75
	Aspergillus niger	75
	Aspergillus parasticus	50
	Fusarium spp.	50
	Mucor spp..	50
	Rhizopus stolonifer	25
	Trichoderma sp.	50
Onion	*Aspergillus flavus*	25
	Aspergillus niger	75
	Cunninghamella echinulata	50
	Emericella nidulans	25
	Fusarium spp.	100
	Lecanicillium lecanii	25
	Metarhizium anisoplaie	25
	Mucor spp.	75
	Penicillium sp.	25
	Rhizopus stolonifer	25
	Trichoderma sp.	25
Berry	*Aspergillus niger*	25
	Clonastachys rosea	50
	Fusarium spp.	75
	Metarhizium anisoplaie	50
	Mucor spp.	25
	Rhizopus stolonifer	25
	Trichoderma sp.	25
Roses	*Aspergillus niger*	75
	Clonastachys rosea	25
	Cunninghamella echinulata	25
	Fusarium spp.	100
	Mucor spp.	75
	Peacilomyces sp.	25
	Penicillium sp.	50

Table: (4). some physical and chemical characteristics of soil samples used to isolation by different selective media.

Type of soil	PH	E.C.	C°	O. M %	%Clay	%Silt	%Sand	Texture
Alfalfa	6.94	4.14	11.9	1.8583	19.904	31.847	48.248	Loam
Berry	7.74	4.26	11.9	4.5529	47.29	37.83	14.86	Silty Clay
Fig	8.01	0.441	11.7	1.2698	28.80	30.32	40.873	Loam
Forestry	7.25	1.275	11.7	3.685	45.50	23.48	31.00	Clay Loam
Pear	7.69	4.17	11.6	2.756	61.56	34.36	4.066	Clay
Onion	7.46	1.88	11	2.137	40.59	52.38	7.0193	Silty Clay
Ornamental	7.87	0.732	11.9	1.6105	36.86	54.61	8.519	Silty Clay Loam
Wallnut	7.78	1.319	11.3	4.243	31.40	43.69	24.90	Clay Loam

4. Conclusion

Two selective medium based on the use of cetyletrimethyl ammonium bromide (CTAB) with oat meal agar and (CTAB) with previously established DOC medium were successfully recovered the two true entomopathogenic fungi *Metarhizium anisopliae* and *Lecanicillium lecanii* along with other opportunistic pathogens from soil in different agro ecosystems in Duhok governorate, North Iraq.

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