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Distribution and Occurrences of Entomopathogenic Fungi in Southern and Western Zones of Ethiopia

Solomon Yilma*, Denberu Kebede and Tsegaye Mihrete

Ethiopian Institute of Agricultural Research, Ambo Agricultural Research Center, P.O.box 37, Ambo, Ethiopia

**Corresponding author*

Abstract

Entomopathogenic fungi have a high potential for biological control of insect pests. Natural habitats are the most important resources for these micro-organisms**.** The aim of this study was to identify and assess occurrence and distribution of entomopathogenic fungi in the soil from different part of the country. Soil samples was taken from Wolega, Horo Gudru, Jimma zone, Gamo Gofa, Dawuro, Kembata, Wolayta Sodo, Gurage Silte, Wonji- Showa, Gedeo, Sidama, Hadya, Metahara and Finchaa between October and November of each study year of 2016-2018. Entomopathogenic fungi were isolated from soil using *Galleria mellonella* as bait trap (insect bait trap method). From the total 306 soil sample collected 96 fungal isolates were identified from which 50(52.1%) of them were *B. bassiana, and* 46(47.9%) were *M. anisopliae.* 64.58% of the identified Entomopathogenic fungi were obtained from forest followed by farm land(28.08%). The abundance of *Metharihizium anisopliae* and *Beauveria bassinia* in the forest and farm land are vice versal. The availability of *Metharihizium anisopliae* in the forest are higher whereas *Beauveria bassinia* in the farmland are more abundant. Evaluation of biodiversity and pathogenicity of these isolates can help to develop effective biological agents to control insect pest

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Entomo-pathogenic fungi, Soil, *Galleria melonella*

Introduction

Use of entomopathogenic fungi as biological control agents for insect species has increased the global attention during the last few decades. The myco-insecticide based on *Beauveria bassiana Metarizihium anisopliae, Vaillemin* (Babu *et al.*, 2001; Sharma, 2004), *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Alter and Vandenberg, 2000; Avery *et al.*, 2004) and *Verticillium lecanii* (Zimm.) Viegas (Butt *et al.*, 2001) have been used to control various insect pests.

There is an increasing interest in the exploitation of Hypomycetous fungi for the control of invertebrate pests and diseases. Fungal biological control is an exciting and rapidly developing research area with implications for plant productivity, human health and food production. Entomopathogenic fungi (EPF) and several taxa of the other fungi have demonstrated excellent suppression of insect pests in green house conditions (McCoy *et al.*, 1988; Ferron *et al.*, 1991; Tanada and Kaya, 1993 and Inglis *et al.*, 2001). *Beauveria bassiana* has recently been registered against array of greenhouse pests, including stem borers, aphids, thrips, white flies and spider mites (Shah and Goettel, 1999).

In Ethiopia, research on entomopathogenic fungi that control of insect pests at different commodity has been carried out during the past several years. According to (Tesfaye and Emiru Seyoum, 2010; Seneshaw Aysheshim *et al.*, 2003; Tesfaye Hailu *et al.*, 2012) the *Metarihizium* fungus was isolated and identified from parts of different insects and soil from various parts of Ethiopia including Alamata, Arba Minch, Metahara, Wonji-Shoa and Finchaa. The ubiquitous fungus, *Beauveria bassiana* was also isolated and identified from parts of different insects from many parts of Ethiopia including Fura, Sekota, Wikro, Erer, Gusquam, Debremarkos, Ashengie, Tikurinchini, Metahara, Wonji-Shoa and Finchaa (Tesfaye and Emiru Seyoum, 2010; Seneshaw Aysheshim *et al.*, 2003; Tesfaye Hailu *et al.*, 2012). However, the potential of these bio-agents is not exploited well.

Under laboratory based experiments and small-scale applications various studies in Ethiopia have demonstrated the effectiveness of fungal pathogens for the control of spotted stem borer, pink stem borer (*Sesamia calamistis* Hampson *Chilo partellus* in Maize (Tadele and Pringle, 2004), *Acanthoscelides Obtectus* (Julia and Lina, 2010), wax moth, *Galleria mellonella* (Namusana and Emiru, 2010), Desert Locust (Seneshaw Aysheshim *et al.,* 2003), cotton/melon aphid (Tesfaye and Emiru Seyoum, 2010), pink stem borer (Tesfaye Hailu *et al.,* 2012), cotton/melon aphid (Tesfaye and Emiru Seyoum, 2010).

Almost all of this research has been undertaken with *B. bassiana* and *Metarhizium anisopliae* of Ambo Agricultural Research Center collections. Not only their small number of isolate in the center but also their weaken virulence showed in different experiment limits to apply in the field and engage them in formulation, mass production, shelf life determination and delivery system. Hence, now a day in Ethiopia, the locally isolated Entomopathogenic fungi are not extensively applied as a bio pesticide. However, the demand for entomopathogenic fungi increase among research center and universities Therefore, this study was undertaken to assess the distribution and occurrences of potential entomopathogenic fungi from different agro-ecological zone of Ethiopia.

Materials and Methods

Collection of soil samples

Soil sample collection were carried out over the previously known area of Ethiopia where Entomopathogenic fungi ecology are recorded namely, Wolega, Horo Gudru, Jimma zone, Gamo Gofa, Dawuro, Kembata, Wolayta Sodo, Gurage Silte, Wonji- Showa, Sidama, Gedeo,Hadya, Metahara and Finchaa between October and November of each study year (2016-2018). From each surveyed area 600g soil samples were taken at a depth of 5-10 cm by removing the most top surface soil (Tsay *et al.*, 2006). A total of 306 soil samples were collected in W shape pattern in every 1km intervals. The collected sample were packed in plastic bags and transported to Ambo Agricultural Research Center Bio-control Laboratory.

Isolation of EPF

Adults of *G. mellonella* were used from Ambo Agricultural Research Center, Ethiopia. Rearing were performed in plastic boxes incubated in dark inside incubator at 20^{o}C. In a flask, adult moths (in 1:1female to male ratio) were kept by providing honey and water. In addition, folded tissue paper was placed for oviposition. The paper was removed with the eggs attached and placed in new plastic jar with feed ingredient (Meyling, 2007). The feed composition was 180 g honey, 180 g glycerine and 50 g wheat bran. First honey and glycerine were melted in a cooking pot and mixed thoroughly with wheat bran after 15 minutes. The feed and egg attached tissue papers were transferred in a bigger jar for rearing inside incubator at 20oC. The feed was changed periodically based on the larval growth stage of *G. mellonella*. Larvae of approximately 2.5-3 cm in length (4 weeks after hatching) were used for baiting soil samples (Meyling, 2007).

Before inoculating in the soil, larvae were immersed in boiled water (at 56^{o}C) for 10 seconds and transferred for cooling in a running bath water for 30 seconds in order to prevent webbing. Ten third instar larvae of *G. mellonela* were placed into small glass jars of about 500 ml (on average 300 g soil). Soils were placed on top of the larvae until approximately 2/3 of the containers are filled and incubated at 22 o C. Every day, the containers were inverted, so that the larvae continually have a chance to move through the soil and repeatedly exposed to infective conidia. In the course of the experiment data on mortality were recorded daily for ten days. The dead larvae were collected and submerged into 1% sodium hypo chlorate, 70% ethanol for one minute respectively and washed in sterile distilled water for three minutes to remove saprophytes and all conidia found the outer surface of the larvae body (Odindo, 1994). The disinfected cadavers (dead larvae) were allowed on filter paper to dry for three minutes. This step were added to ensure that, mycosis observed on the surface of the cadavers would not be attributed to spores used during the treatment but rather to growth from the interior to the exterior of the insect after colonization of internal organs. Cadavers were held under high humidity on Petri dishes containing damp filter paper to provide sufficient humid conditions to promote fungal outgrowth. Petri dishes were sealed with Para film to maintain greater than 95% RH and incubated in the dark at 27°C. Larvae considered mycosis when growth of the fungus are visible on the external surface and those which showed hyphal growth characteristics of the EPF were recorded as infected.

Purification of Isolated EPF

Larvae of *G. mellonela* mortality were checked continuously for 10 successive days after inoculating in the soil sample. Fungi samples outgrowing and sporulating on the cadaver were cultured on artificial media (Sabouraud's Dextrose Yeast Agar (SDYA) and pure cultures were obtained through successive transfer for identification. Tentative identification were done based on macro and micro growth and morphological characters of fungi (Subramanian, 1971; Lacey, 1997; Sinishaw, 2002). Pure cultures of EPF were sub-cultured on to Sabouraud's dextrose agar with yeast extract (SDAY) and incubated at 27^{o}C, 75% RH and photoperiod of 12:12h light and dark for ten successive days till they were full culture development and sporulation.

Confirmatory test against *G. melonella*

The surface of ten-day old cultures were scrapped with a sterile scalpel and suspended in aqueous solution of 0.01% Tween 80. The fungal suspension were vortexed for one minute to break up the conidial chains or clumps and filtered through several layers of sterile cheesecloth to remove mycelia. The dose of conidia in the filtrate were estimated using haemocytometer under a light microscope (40 x magnifications). Conidial suspension ($1x10^7$, $1x10^8$ and $1x10^9$ conidia per ml) were prepared for each isolate and applied on 2.5-3cm larvae of *Galleria melonella*. Ten larvae inoculated with $1x10^8$ ml-1 conidia were introduced in each Petridish with filter paper. Sterile distilled water with 0.01% Tween 80 was used as free control treatment. The treated larval insects were incubated at 27 °C and 70 ± 5% RH and maintained for 10 days. The dead larvae were collected and submerged into 70% ethanol for three seconds and 1% sodium hypochlorite for three minutes (Odindo, 1994) and washed in sterile distilled water for three minutes to remove saprophytes and all conidia found on the outer surface of cadavers. The disinfected cadavers (dead larvae) were allowed to dry for ten minutes on Watmann No.1 filter paper. Cadavers were held under high humidity on Petridishes containing damp filter paper to provide sufficient humid conditions to promote fungal outgrowth and sealed with Para film to maintain greater than 95% RH and incubated in the dark at 27°C. A larvae were considered mycosis when growth of the fungus are visible on the external surface and those which showed hyphal growth characteristics of the EPF were recorded as infected.

Results and Discussions

Soil sample collection

Three hundred six soil samples were collected from different geographical sites of the major area of the country where forest, farm land and grazing land are common in Southern, Western and Central area of Ethiopia. Of which 96 sample were positive to the test and recorded as Entomopathogenic fungi (EPF). The locations and altitudes of the positive sampled soils were recorded using global positioning system (GPS) equipment and presented in Table 1. For each sample, soil type was recorded.

Entomo-pathogenic fungi isolate obtained from different part of the country are presented in Table 2. 306 soil sample were collected from Wolega, Horo Gudru, Jimma zone, Gamo Gofa, Dawuro, Kembata, Wolayta Sodo, Gurage, Silte, Wonji- Showa, Sidama, Gedeo, Hadya, Metahara and Finchaa between October and November of each study year of 2016-2018. A total of 96 entomopathogenic fungi were isolated from the soil collected from 13 zones using *G. mellonella* larvae as a baiting trap: From the different agro-ecology of the country forest showed relatively richer in entomopathogenic fungi composition followed by grazing land. Similar finding from Syria by Almanoufi *et al.,* (2012) also confirmed that EPF isolates were obtained from most locations, the highest percentage of EPFs presence was reported in soil collected from forest. In addition, apart from typical insect-pathogenic fungi, some accompanying fungal species with unproved entomopathogenic abilities were isolated from investigated soils by means of *Galleria* bait method.

Based on our observations, the most abundant EPF sites were under forest, for this reason, the most infected cadavers of *Galleria melonella* to fungi were also obtained from these habitats and this finding is concedes with the study done in Iran by Maryam *et al.*, (2014) which claims that the abundance of entomopathogenic fungi greater in under oak tree.

Distribution of Entomopathogenic fungi in different location

Following isolation of EPF using the Galleria bait method identification was done using mycelia growth pattern, color and spore shape with the aid microscope. Their distribution in different geographical location are presented in Figure 1. Their distribution seems more greater in Jimma zone, East Showa, Horo Gudru and Wolayta Sodo than the rest assessed area. The abundance of *Beauveria bassinia* in Jimma zone are higher than the other site however, its availability in Gamo Gofa, wolayta, Dawuro, Hadya, Kembata, Sidama, Gedeo, Gurage and Silte are highly reduced.

Table.1 Geographical location of entomopathogenic fungi isolates and soils type

Sr.No	EPF Isolate code	Geographical Location			Soil type
		Latitude	Longitude	Altitude	
1	BC-4	36.94486667	7.894533333	2128	Loam, humus
2	BC-7	36.95388333	7.89755	2126	Loam, humus
3	BC-9	36.97271667	7.909166667	2171	Loam, Vertisol
4	BC-11	36.97625	7.917066667	2181	Loam humus
5	BC-15	36.99725	7.930516667	2057	Loam , humus
6	ye-20	37.21593333	8.27675	1941	Loam , humus
7	ge-23	37.22796667	8.252616667	2091	Loam , humus
8	ji-27	37.25745	8.22065	2061	Loam , Vertisol
9	ji-28	37.25745	8.22065	2061	Loam, Vertisol
10	gum-30	37.2759	8.221966667	2048	Loam , humus
11	gm-31	37.28473333	8.220566667	2028	Loam , humus
12	gm-34	37.29265	8.217566667	2029	Loam , Vertisol
13	GB-38	37.32373333	8.2118	1959	Loam , humus
14	GB-39	37.34266667	8.200583333	1926	Loam , humus
15	JARC-40	37.32985	8.111516667	1737	Loam , Vertisol
16	JAP-44	37.3546	8.105666667	1721	Loam , humus
17	JAR-45	37.4773	8.104166667	1788	Loam , humus
18	SWB-46	37.48803333	8.1195	1823	Loam , humus
19	M-51	37.51861667	8.148266667	1775	Loam, clay
20	M-52	37.52725	8.157816667	1816	Loam, humus
21	M-53	37.53766667	8.16055	1820	Loam , humus
22	M-54	37.54908333	8.161933333	1834	Loam, clay
23	Ser-55	37.5613	8.161616667	1839	Loam, humus

24	Ser-56	37.56495	8.162483333	1821	Loam, humus
25	Bab-57	37.59285	8.1743	1817	Loam, clay
26	Omt-58	37.60396667	8.17845	1827	Loam, humus
27	YL-60	36.73631667	8.6004	1891	Loam , Vertisol
28	Gbe-59	36.72063333	8.55475	1786	Loam, humus
29	YL-60	36.73631667	8.6004	1891	Loam , humus
30	Wj-6	35.70148333	9.2323	1882	Loam , humus
31	Hh-7	35.69503333	9.240366667	1879	Loam, clay
32	Hh-9	35.6966	9.2497	1862	Loam , humus
33	G-10	35.68178333	9.2709	1792	Loam, clay
34	Le-12	35.73025	9.19995	1857	Loam , humus
35	Tn-16	35.6587	9.173183333	1814	Loam, Vertisol
36	Tn-16	35.6587	9.173183333	1814	Loam, clay
37	N-20	35.94156667	9.048666667	1695	Loam, humus
38	Ab-21	35.97	9.031616667	1644	Loam, Vertisol
39	Nf-22	36.00231667	9.052033333	1458	Loam , humus
40	B-26	36.35361667	9.703806667	1561	Loam, clay
42	J-28	36.47181667	9.20793	2258	Loam , humus
43	Do-30	37.14008333	9.577666667	2404	Loam , humus
44	Ho-34	37.24826667	9.62815	2328	Loam, Vertisol
45	s-22	37.63058333	6.9065	2111	Loam , humus
46	s-24	37.48811667	6.874616667	1250	Loam, clay
47	s-30	37.18701667	7.799333333	2053	Loam, Vertisol
48	s-39	37.25406667	7.049333333	2313	Loam, humus
49	s-40	37.28146667	7.090833333	2117	Loam, Vertisol
50	s-54	37.89033333	7.0535	1907	Loam, humus
51	s-60	37.93616667	7.211066667	2048	Loam, Vertisol
52	s-65	37.759	7.20865	1789	Loam , humus
53	s-68	37.735	7.258133333	2289	Loam, Vertisol
54	s-72	37.73693333	6.997733333	1864	Loam , humus
55	s-74	37.71045	7.967166667	1711	Loam, humus
56	s-77	37.77868333	7.330666667	2716	Loam , humus
57	s-81	37.87473333	7.60585	2340	Loam, Vertisol
58	s-85	37.90356667	7.766616667	2638	Loam, humus
59	s-89	37.89735	7.79515	2595	Loam, Vertisol

60	s-96	38.461	8.212333333	2802	Loam , humus
61	s-98	37.95323333	9.0445	2157	Loam, Vertisol
62	Geshe	38· 15 16.81726	6.216.45361	2467	Loam , humus
63	Geshe	38. 14 46.8970	6 .3 9.59738	2541	Loam , clay
64	kurumi	38 .14 42.07944	6 .3 51.10531	2418	Loam ,humus
65	kurumi	38 .14 32.44848	6 .4 46.14557	2300	Loam, humus
66	kishe	38 .12 44.14205	6 .3 59.29714	1872	Loam , clay
67	konga	38 .12 8.29532	6 .7 47.30545	1826	Loam, humus
68	domerso	38 .12 38.99198	6 .13 32.28456	1852	Loam, clay
69	chelba	38 .12 32.15308	6 .15 23.85572	1870	Loam , humus
70	digcha	38 .12 44.14964	6 .16 25.39502	1931	Loam , clay
71	qote	38 .13 9.72072	6 .17 43.5574	1851	Loam, clay
72	korasodity	38 .14 8.72538	6 .18 31.85165	1625	Loam, humus
73	tumata	38 .16 8.76619	6 .20 30.26802	1537	Loam, clay
74	bunata	38 .16 33.99804	6 .20 39.52687	2307	Loam , humus
75	basoda	38 .22 7.95178	6 .19 2.92804	2307	Loam , Vertisol
76	amba	38 .21 29.61749	6 .19 3.55735	2292	Loam, Vertisol
77	amba	38 .21 25. 30634	6 .19 11.76798	2294	Loam , humus
78	amba	38 .20 0.58031	6 .19 17.00944	2119	Loam Vertisol
79	tumiticha	38 .19 58.33088	6 .19 20.35196	2119	Loam, humus
80	tumiticha	38 .19 58.33088	6 .19 20.35196	2062	Loam , Vertisol
81	tumiticha	38 .19 56.03376	6 .19 31.51009	1986	Loam , humus
82	tumiticha	38 .19 26.8513	6 .20 18.75707	1588	Loam , humus
83	chichu	38 .18 13.0752	6 .21 32.66732	1679	Loam, humus
84	kebedo	38 .19 15.92091	6 .28 10.6828	1644	Loam, Vertisol
85	kumato	38 .19 29.68476	6 .28 56.79588	1660	Loam, Vertisol
86	dibcha	38 .19 31.26137	6 .30 10.86797	1733	Loam, humus
87	teso	38 .19 47.90878	6 .31 53.0662	1748	Loam, humus
88	babelo	38 .19 15.60954	6 .32 15.34571	1775	Loam, clay
89	gambel	38 .19 26.59253	6 .33 5.3869	1907	Loam, humus
90	shefna	38 .28 39.02329	6 .42 15.45724	1887	Loam, clay
91	shefna	38 .28 30.86839	6 .42 55.9592	1887	Loam, clay
92	shefna	38 .28 30.86839	6 .42 55.9592	1885	Loam, humus
93	tedicho	38 .25 19.50182	6 .42 5.5794	1777	Loam, humus
94	tedicho	38 .24 2.06406	6 .41 50.39286	1765	Loam, Vertisol

95	omuracho	38 .26 56.04205	6 .54 48.84066	1900	Loam, Vertisol
96	Murarech	38 .27 28.7266	6 .55 24.59543	2329	Loam , Vertisol

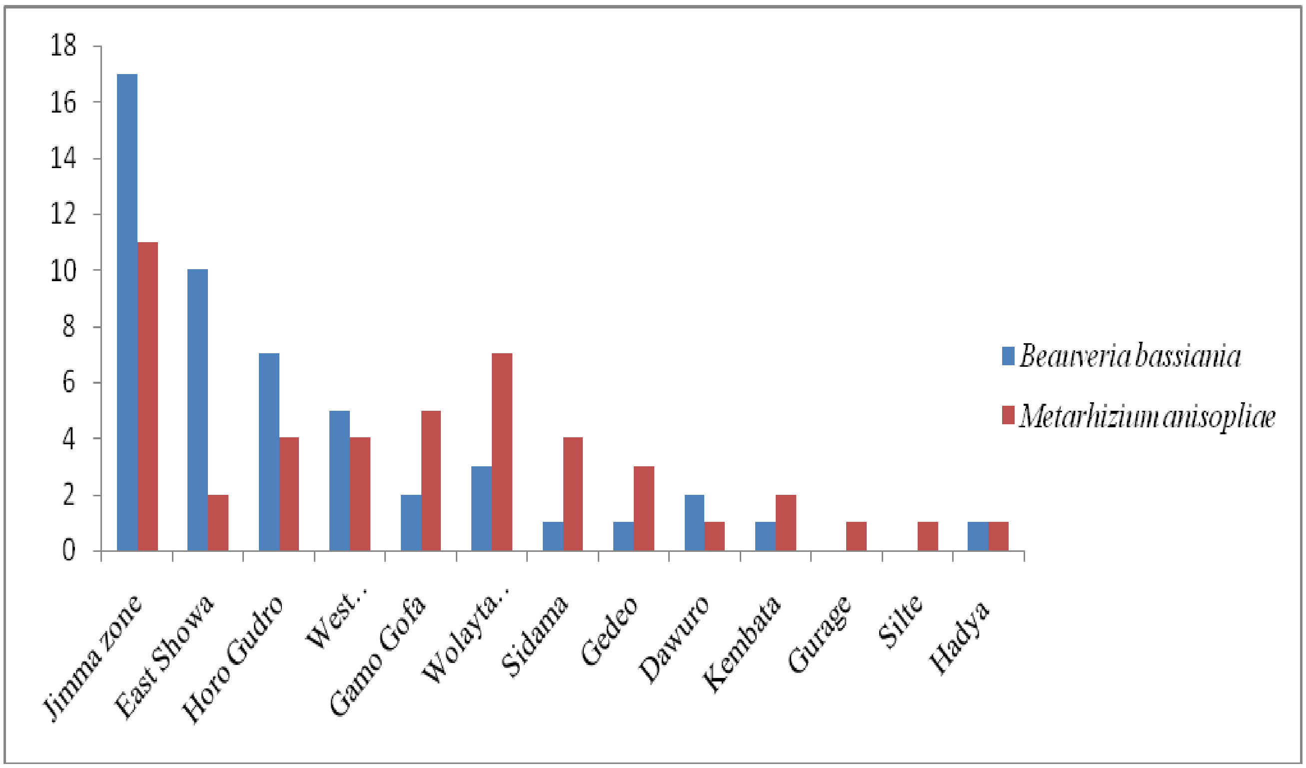
Table.2 Ecology of Entomopathogenic fungi

Site	Ecology	No of Sample	No of dead Galleria /positive sample/	No of EPF isolate recorded
West Showa	Semi-forest	2	2	1
	Sugarcane farm	18	16	2
	Semi forest	10	9	3
	Acacia	9	6	2
	Maize farm	1	1	1
	Semi forest	3	3	1
	Acacia	6	5	2
	Grazing land	1	1	1
Jimma zone	Forest	19	13	11
	Farm land	38	21	17
	Grazing land	3	1	1
Horo Gudru	Rod side	8	2	1
	Grazing land	1	1	1
	Farmland	2	1	1
	Forest	6	2	2
	Coffee farm	3	3	2
	Banana farm	1	1	1
	Mango farm	1	1	1
	Shrub	1	1	1
	Wooden land	4	2	1
West Wolega	Rod side	6	2	1
	Grazing land	4	2	1
	Farmland	5	3	2
	Forest	1	1	1
	Chat farm	1	1	1
	Niger farm	1	1	1
	Wood land	7	3	2
Gamo Gofa	Enset	19	9	7
Wolayta	Enset	22	13	10
Dawuro	Enset	12	7	3
Hadya	Enset	10	4	1
Kembata	Enset	6	2	1
Gurage	Enset	5	3	1
Silte	Enset	4	1	1
Gedeo	Coffee	30	10	4
Sidama	Coffee	25	8	3
Total		306	167	96

Table.3 Distribution of entomopathogenic fungi isolated from major ecological habitat(n=96)

EPF isolates	Habitat				
	Forest	Grazing land	Wood land	Farm land	Road side
Beauveria bassiana	25(29.16%)	3(3.12%)	1(1.04%)	19(19.79%)	2(2.1%)
Metarhizium anisopliae	37(38.54%)	1(1.04%)	2(2.1%)	6(6.25%)	0
Total	62(64.58%)	4(4.16%)	3(3.12%)	25(28.08%)	2(2.1%)

Fig.1 Distribution of Entomopathogenic fungi isolated from different site of the country



Entomopathogenic fungi occurrence in major study habitat

In these samples only *Beauveria bassiana* and *Metarhizium anisopliae* were isolated and *B. bassiana* was the most common. 64.58 % isolated entomopathogenic fungi were from forest followed by farm land (28.08%). The agricultural field soil(farm land) most frequently harbored *Beauveria bassiana* (19.79%) while soil from the forest most often contained *Metarhizium anisopliae* (38.54 %) (Table 3). This finding is in line with the report by Meyling and Eilenberg (2005) which states the agricultural field soil most frequently harboured *B. bassiana* while soil from the hedgerow most often contained *Paecilomyces fumosoroseus* . However, *B. bassiana* was also common in forest soil (29.16 %). In the farm land soil, *Metarhizium anisopliae* was less frequently isolated than *Beauveria bassiana*. *M. anisopliae* was not found in the soil of the road side. The species detected in the present study were within the expected range based on previous studies performed at similar ecology using bait insects Meyling and Eilenberg (2005), Steenberg (1995) and Chandler *et al.*, (1997). Grazing land and road side are relatively a higher composition of *Beauveria bassiana* than *Metarhizium anisopliae* but very little EPF isolates were obtained from uncultivated land like road side.

Similar studies from Syria confirmed that no isolates were obtained from uncultivated lands Almanoufi *et al.*, (2012).

In conclusion, the study showed that the soil from forest and farm land is characterized by a richer species composition of entomopathogenic fungi than of adjacent grazing and road side fields. Natural habitats are a source for the occurrences of entomopathogenic fungi. Future research works in techniques for mass production, appropriate formulation to keep the quality, large scale application are needed. These organisms may play an important role for the biological control and therefore the study of these native entities should be evaluated more in detail. Molecular markers to assess genetic diversity are important tools in future studies of indigenous populations of *B. bassiana* and *M. anisopliae*.

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