CZECH MYCOLOGY 67(1): 1–22, APRIL 10, 2015 (ONLINE VERSION, ISSN 1805-1421)

Leaf surface and endophytic fungi associated with onion leaves and their antagonistic activity against *Alternaria porri*

SOBHY I. I. ABDEL-HAFEZ[1], KAMAL A. M. ABO-ELYOUSR[2], ISMAIL R. ABDEL-RAHIM[1]*

[1] Botany and Microbiology Department, Faculty of Science, Assiut University, EG-71516 Assiut, Egypt; ismailramadan2008@gmail.com
[2] Plant Pathology Department, Faculty of Agriculture, Assiut University, EG-71526 Assiut, Egypt
*corresponding author

Abdel-Hafez S.I.I., Abo-Elyousr K.A.M., Abdel-Rahim I.R. (2015): Leaf surface and endophytic fungi associated with onion leaves and their antagonistic activity against *Alternaria porri*. – Czech Mycol. 67(1): 1–22.

Sixty-eight fungal species belonging to 29 genera were isolated as leaf surface and endophytic fungi from healthy and purple blotch diseased onion leaves. The fungal populations associated with diseased onion leaves (1.360 × 10^3 CFU/g leaf in the phyllosphere, 2.614 CFU/leaf segment in the phylloplane and 1.324 CFU/leaf segment in the surface-sterilised diseased leaves) were higher than those in healthy samples (0.804 × 10^3 CFU in the phyllosphere, 1.184 CFU in the phylloplane, and 0.35 CFU as endophytes). Endophytic fungi of healthy leaves were represented by 12 genera and 15 species, while fungi of surface-sterilised diseased leaves included 17 species from 13 genera. The mycobiota associated with surface-sterilised diseased leaves were different from the endophytic fungi of healthy samples, whereas the disease may stimulate colonisation of opportunistic fungi causing secondary infections such as *Botrytis cinerea*, *Penicillium aurantiogriseum*, *Alternaria alternata* and *Cladosporium* spp. In contrast, healthy leaves were a source of antagonistic endophytic fungi such as *Trichoderma harzianum* and *T. koningii*. Testing the antagonistic effect of 91 fungal isolates against *Alternaria porri* showed that nine isolates of *Trichoderma* produced the highest suppressive potential (73.1%) depending on competition and mycoparasitism. *Epicoccum nigrum* and *Penicillium oxalicum* exhibited antibiosis against *A. porri* producing a 12 mm broad inhibition zone. In conclusion, the quantitative and qualitative compositions of fungi associated with onion leaves were distinctly influenced by *A. porri* infection. Mycobiota associated with asymptomatic onion leaves such as *Epicoccum nigrum*, *Penicillium oxalicum* and *Trichoderma harzianum* are a natural source of eco-friendly bioagents. They showed an effective antagonistic potential against *A. porri*, and may thus be applied as an alternative to fungicides.

Key words: phyllosphere, phylloplane, endophytes, purple blotch disease, antagonism.

Article history: received 21 November 2014, revised 4 February 2015, accepted 9 March 2015, published online 10 April 2015.

Abdel-Hafez S.I.I., Abo-Elyousr K.A.M., Abdel-Rahim I.R. (2015): Fylosférní, fyloplánní a endofytické houby cibulových listů a jejich antagonistická aktivita proti *Alternaria porri*. – Czech Mycol. 67(1): 1–22.

Celkem 68 druhů hub z 29 rodů bylo izolováno z povrchu a pletiva cibulových listů, zdravých nebo napadených alternariovou skvrnitostí. Početnější populace hub byly zjištěny na napadených listech (1.360 × 10^3 CFU/gram listové hmoty ve fylosféře, 2.614 CFU/listový segment ve fylopláně a 1.324 CFU/listový segment z pletiv povrchově sterilizovaných listů) než ve zdravých vzorcích (0.804 × 10^3 CFU ve fylosféře, 1.184 CFU ve fylopláně a 0.35 CFU endofytických hub). Ve zdravých listech bylo zjištěno 15 endofytických druhů z 12 rodů, zatímco v povrchově sterilizovaných napadených listech bylo 17 druhů z 13 rodů. V pletivech napadených listů jsou odlišné houby než ve zdravých vzorcích, neboť nákaza může stimulovat kolonizaci oportunistickými houbami, způsobujícími sekundární infekci, jako jsou *Botrytis cinerea*, *Penicillium aurantiogriseum*, *Alternaria alternata* a druhy rodu *Cladosporium*. Naproti tomu zdravé listy jsou zdrojem antagonistických endofytů, jako *Trichoderma harzianum* a *T. koningii*. Testování antagonistického efektu 91 izolátů proti *Alternaria porri* ukázalo, že devět izolátů rodu *Trichoderma* vykazuje nejvyšší supresivní potenciál (73.1%), založený na kompetici a mykoparazitismu. *Epicoccum nigrum* a *Penicillium oxalicum* působí antibioticky proti *Alternaria porri* za vzniku 12 mm široké inhibiční zóny. Závěrem lze říci, že infekce *A. porri* má výrazný vliv na zjištěné složení a početnost společenstva hub na/v cibulových listech. Druhy spojené s asymptomatickými listy, jako jsou *Epicoccum nigrum*, *Penicillium oxalicum* a *Trichoderma harzianum*, jsou přirozeným zdrojem šetrných bioagens, které vykazují efektivní antagonistický potenciál proti *A. porri* a mohou tak být aplikovány jako alternativa k fungicidům.

INTRODUCTION

Aerial plant surfaces provide a suitable habitat for epiphytic microorganisms, which are influenced by the nutrients present on the leaf surfaces. The phyllosphere and phylloplane of the surface of plant leaves are a complex terrestrial habitat characterised by a variety of microorganisms including bacteria, filamentous fungi and yeasts, which play a vital role in health of biological systems (Pandey et al. 1993). Saprotrophic leaf surface fungi perform key ecological roles in the plant, mainly related to natural control of plant pathogens (Fokkema & Lorbeer 1974, Tyagi et al. 1990). Endophytic fungi are those which live in the interior of apparently healthy and asymptomatic hosts. Fungi fitting this description appear to be ubiquitous. Indeed, no study has yet shown the existence of a plant species without endophytes (Promputtha et al. 2007).

Endophytes play a major role in physiological activities of host plants influencing (enhancing) disease resistance (Azevedo et al. 2000, Carroll 1988, Rubini et al. 2005). They constitute a valuable source of bioactive secondary metabolites of biotechnological importance in plant disease management programs (Schulz et al. 2002).

Purple blotch disease, which is caused by *Alternaria porri* (Ellis) Cif., is one of the most destructive diseases restricted to the genus *Allium* and is widespread in many regions of the world (Cramer 2000). Purple blotch disease of onion cause significant reduction in foliar production (Utikar & Padule 1980) and bulb yield (Gupta & Pathak 1988). The disease is more severe on seed crop as compared to bulb crop sometimes causing a 100% loss of onion seed production (Schwartz 2004, Singh et al. 1992). Today, there are strict regulations on using chemical fungicides due to their carcinogenic effects, problems of residual toxicity, environmental pollution and development of fungicide-resistant strains (Marín et al. 2003, Rial-Otero et al. 2005).

Therefore, there is a need for an effective alternative strategy to manage foliar plant pathogens using eco-friendly leaf surface and endophytic microorganisms (Abo-Elyousr et al. 2014, Abo-Shady et al. 2007, Alwathnani & Perveen 2012, Soria et al. 2012).

The present study investigated the composition of mycobiota of healthy and purple blotch diseased leaves of onion plants from commercial fields in Assiut Governorate, Egypt, and evaluated the ability of fungal isolates to suppress *Alternaria porri*.

MATERIAL AND METHODS

Sample collection and medium of isolation. Forty samples of onion leaves (20 healthy and 20 showing typical symptoms of purple blotch disease) were collected from agricultural sites in Assiut Governorate, Egypt. Samples were collected monthly during January–April 2011. The collected samples were packed immediately into sterilised polyethylene bags and transferred to a mycological laboratory to assay their phyllosphere, phylloplane and endophytic fungal content. The medium of fungal isolation was potato dextrose agar (PDA) medium supplemented with 66.7 mg/l rose-bengal and 250 mg/l chloramphenicol (Smith & Dawson 1944).

Incubation conditions for isolation and fungal counts. Five replicates were carried out for each experiment of isolation and then the plates were incubated at 25 °C for 6 days. The fungal populations of the phyllosphere were counted as colony forming units (CFU) per gram of healthy or diseased leaves. On the other hand, the counts of the phylloplane and endophytic fungi were calculated as CFU per 25 segments of healthy or diseased leaves.

Phyllosphere fungi. The dilution plate method was used for isolation of phyllosphere fungi from healthy and purple blotch diseased leaves, as described by Abdel-Hafez (1981). An amount of 20 g of onion leaves was added to an Erlenmeyer flask (250 ml) containing 100 ml sterilised distilled water. The flasks were shaken for 20 minutes. The final desired dilution (1/500) was prepared by transferring 10 ml of the suspension into another flask (250 ml) containing 90 ml sterilised distilled water and then the flask was shaken by hand for 5 minutes. The last step was repeated to reach the final desired dilution. One ml of the final dilution was transferred to a sterilised Petri plate on which 15 ml of melted PDA medium was poured.

Phylloplane fungi. The fresh onion leaves were washed several times with sterilised distilled water, dried thoroughly using sterilised filter paper and then cut into segments (1 cm^2). Five leaf segments were placed on the surface of PDA medium in each plate and then all plates were incubated (Abdel-Hafez 1981).

Endophytic fungi and fungi of surface-sterilised diseased onion leaves. Endophytic fungi of healthy leaves as well as fungi associated with surface-sterilised purple blotch diseased leaves were isolated according to Wang & Guo (2007). Leaf segments were washed several times with sterilised distilled water, immersed in 75% ethanol for 1 min., in 3.5% aqueous sodium hypochlorite for 5 min., then in 75% ethanol for 30 s and washed three times with sterilised distilled water. Then the leaf segments were dried thoroughly using sterilised filter paper under a laminar flow. Twenty-five leaf segments were placed on the surface of PDA medium in five plates, which were then incubated.

Purification and identification of leaf surface and endophytic fungi. The fungal colonies were purified using signal spore or hyphal tip techniques suggested by Booth (1971) and Sinclair & Dhingra (1995). Then the purified fungi were identified according to their macroscopic and microscopic characteristics as described by Booth (1977), Domsch et al. (1980), Ellis (1971), Moubasher (1993), Pitt (1979), Raper & Fennell (1965) and Harman & Kubicek (2002). To clearly distinguish anamorphic and teleomorphic stages isolated from the leaves, traditional separate teleomorph and anamorph names are used in the study (e.g. species of anamorphic genus *Aspergillus* and teleomorphic genera *Emericella* or *Eurotium*, although these are currently all classified as *Aspergillus*).

Alternaria porri, pathogen of onion purple blotch disease. *Alternaria porri* was isolated by the authors from onion samples showing typical symptoms of purple blotch on commercial onion farms in Assiut Governorate, Egypt. The isolate was identified based on morphological and microscopic characteristics (Ellis 1971) and deposited in Assiut University Mycological Centre (AUMC) under code number AUMC 9301. The pathogenicity of this isolate was previously tested showing high disease severity (81.25%) as recorded by Abdel-Hafez et al. (2014).

Evaluation of antagonistic effect of leaf surface and endophytic fungi against *Alternaria porri*. Antagonistic potentiality of leaf surface and endophytic fungi against *Alternaria porri* was evaluated using a dual culture method (Li et al. 2003). Prepared sterilised Petri plates (9 cm diameter) containing 15 ml sterilised PDA medium were inoculated with a 7 day old culture of *A. porri* at one plate edge, while the tested fungal isolates were seeded on the opposite edge. A plate inoculated with *A. porri* alone served as control. Three replicates of control and treated plates were used and then all plates were incubated at 28 °C. The inhibition zones and relative growth inhibition were measured after 7 days of incubation. The percentage of mycelial growth inhibition using a bioagent was calculated with the following formula (Hajieghrari et al. 2010):

$$\text{Percentage of growth inhibition of the pathogen} = [(C - T) / C] \times 100$$

where C = radial growth of *A. porri* in control plate, T = radial growth of *A. porri* in treatment plate with bioagent. The radial growth (in millimetres) was recorded by measuring the radius of the colony from the inoculated side of the plate toward the opposite side.

An arbitrary antagonistic scale was applied according to the measured inhibition zone to explain and classify the antagonists as follows: 0 = showing no antagonistic effect, OG = showing overgrowth on pathogen mycelia, N = showing a narrow inhibition zone (less than 3 mm), M = showing a moderate inhibition zone (3–7 mm), B = showing a broad inhibition zone (more than 7 mm).

RESULTS

Sixty-eight species including four varieties belonging to 29 fungal genera were isolated during this investigation. These fungi were recovered as leaf surface and endophytic fungi from healthy and purple blotch diseased onion leaves. These fungi were represented by the following groups: Zygomycota (5 genera and 6 species), teleomorphic Ascomycota (7 genera and 13 species; two varieties distinguished in one species) and anamorphs classified in the artificial class of Hyphomycetes (17 genera and 48 species; two varieties distinguished in one species).

Phyllosphere fungi

Sixty-three fungal species including four varieties belonging to 25 genera were isolated from the phyllosphere of healthy and purple blotch diseased leaves. Phyllosphere fungi associated with purple blotch diseased leaves (2.720×10^4 CFU per 20 g leaf) showed a total fungal count higher than that in healthy ones (1.608×10^4). In contrast, the number of genera and species in healthy leaves (58 species and 25 genera) was more than that in purple blotch diseased leaves (49 species and 19 genera) (Tab. 1).

Alternaria, *Aspergillus*, *Cladosporium* and *Penicillium* were the most prevalent genera recovered from the phyllosphere of healthy and diseased samples comprising 65–100% of total samples. From these genera, *Alternaria alternata*, *Aspergillus niger* and *Penicillium funiculosum* were recovered in high frequency from healthy and diseased leaves. *Aspergillus terreus* and *Cladosporium cladosporioides* were recovered in high frequency only from healthy leaf samples, while *Aspergillus flavus* var. *flavus* and *Stemphylium vesicarium* were frequently recovered from purple blotch diseased samples. Some fungal genera were recovered only with high frequency from one substrate such as *Trichoderma* from healthy samples (60% of the samples comprising 7.34% of total fungi), while

Fusarium and *Stemphylium* from diseased leaves in 70 and 90% of the samples comprising 2.35 and 21.43% of total fungi, respectively.

Some fungal species were only recovered from purple blotch diseased leaves (with frequencies comprising 5 or 10% of the samples), such as *Aspergillus awamori*, *Emericella quadrilineata*, *Eurotium rubrum*, *Penicillium pinophilum* and *P. waksmanii*. On the other hand, *Myrothecium verrucaria*, *Absidia corymbifera*, *Eurotium repens*, *Humicola grisea*, *Aspergillus carbonarius*, *A. flavipes*, *A. tamarii*, *A. wentii*, *Cunninghamella echinulata*, *Epicoccum nigrum*, *Nigrospora sphaerica*, *Penicillium citrinum*, *P. glabrum* and *Scopulariopsis brevicaulis* were only isolated from healthy leaves (with frequencies ranging from 5 to 30% of the samples).

Tab. 1. Phyllosphere fungi recovered from leaves of onion plants cultivated for seed production during January–April 2011 on PDA medium at 25 ± 1 °C.
Abbreviations: TC – total fungal count (CFU / 20 g of healthy or diseased leaves); %TC – percentage of total fungal count; %F – % frequency of the fungal species / 20 collected samples of healthy or diseased leaves.

Fungal species	**Healthy leaves**			**Purple blotch diseased leaves**		
	TC	**%TC**	**%F**	**TC**	**%TC**	**%F**
Absidia corymbifera (Cohn) Sacc. & Trotter	130	0.81	15	0	0.00	0
Acremonium strictum Gams	150	0.93	20	270	0.99	40
Acrophialophora fusispora (S.B. Saksena) Samson	20	0.12	5	80	0.29	15
Alternaria alternata (Fr.) Keissl.	1370	8.52	75	4340	15.96	85
Aspergillus	5190	32.28	100	6180	22.72	100
A. awamori Nakaz.	0	0.00	0	600	2.21	10
A. carbonarius (Bain.) Thom	100	0.62	5	0	0.00	0
A. flavipes (Bain. & Sart.) Thom & Church	20	0.12	5	0	0.00	0
A. flavus Link var. *flavus*	510	3.17	35	1440	5.29	55
A. flavus var. *columnaris* Raper & Fennell	40	0.25	10	40	0.15	10
A. fumigatus Fresen.	780	4.85	30	740	2.72	35
A. melleus Yukawa	50	0.31	5	40	0.15	10
A. niger Tiegh.	2520	15.67	100	2640	9.71	70
A. ochraceus Wilh.	100	0.62	15	20	0.07	5
A. oryzae (Ahlb.) Cohn	80	0.50	10	40	0.15	10
A. sydowii (Bain. & Sart.) Thom & Church	40	0.25	5	60	0.22	10
A. tamarii Kita	20	0.12	5	0	0.00	0
A. terreus Thom	750	4.66	70	460	1.69	45
A. ustus (Bain.) Thom & Church	60	0.37	5	60	0.22	10
A. versicolor (Vuill.) Tirab.	100	0.62	20	40	0.15	5
A. wentii Wehmer	20	0.12	5	0	0.00	0
Chaetomium globosum Kunze	620	3.86	35	140	0.51	20

Fungal species	Healthy leaves			Purple blotch diseased leaves		
	TC	%TC	%F	TC	%TC	%F
Cladosporium	1020	6.34	80	2580	9.49	65
C. cladosporioides (Fresen.) G.A. de Vries	740	4.60	60	1020	3.75	45
C. sphaerospermum Penz.	280	1.74	30	1560	5.74	45
Cochliobolus	90	0.56	15	80	0.29	10
C. lunatus R.R. Nelson & F.A. Haasis	50	0.31	10	20	0.07	5
C. spicifer R.R. Nelson	40	0.25	5	60	0.22	10
Cunninghamella echinulata Thaxt.	110	0.68	15	0	0.00	0
Emericella	250	1.55	45	160	0.59	15
E. nidulans (Eidam) Vuill. var. *nidulans*	120	0.75	20	20	0.07	5
E. nidulans var. *lata* (Thom & Raper) Subram.	20	0.12	5	60	0.22	5
E. quadrilineata (Thom & Raper) C.R. Benj.	0	0.00	0	40	0.15	5
E. rugulosa (Thom & Raper) C.R. Benj.	90	0.56	15	20	0.07	5
E. variecolor Berk. & Broome	20	0.12	5	20	0.07	5
Epicoccum nigrum Link	50	0.31	10	0	0.00	0
Eurotium	110	0.68	20	100	0.37	15
E. amstelodami L. Mangin	20	0.12	5	60	0.22	10
E. repens De Bary	90	0.56	15	0	0.00	0
E. rubrum König	0	0.00	0	40	0.15	5
Fusarium	160	1.00	25	640	2.35	70
F. nygamai L.W. Burgess & Trimboli	40	0.25	5	120	0.44	15
F. oxysporum Schltdl.	40	0.25	10	180	0.66	40
F. solani (Mart.) Sacc.	60	0.37	15	280	1.03	35
F. verticillioides (Sacc.) Nirenberg	20	0.12	5	60	0.22	15
Gliocladium roseum Bainier	160	1.00	25	40	0.15	10
Humicola grisea Traaen	30	0.19	15	0	0.00	0
Mucor	680	4.23	35	130	0.48	20
M. circinelloides Tiegh.	130	0.81	20	40	0.15	5
M. hiemalis Wehmer	550	3.42	15	90	0.33	15
Myrothecium verrucaria (Alb. & Schwein.) Ditmar	270	1.68	30	0	0.00	0
Nigrospora sphaerica (Sacc.) E.W. Mason	110	0.68	15	0	0.00	0
Penicillium	3500	21.77	90	5910	21.73	95
P. aurantiogriseum Dierckx	20	0.12	5	320	1.18	20
P. chrysogenum Thom	80	0.50	20	40	0.15	10
P. citrinum Thom	20	0.12	5	0	0.00	0
P. corylophilum Dierckx	100	0.62	15	20	0.07	5
P. duclauxii Delacr.	500	3.11	50	480	1.76	45
P. funiculosum Thom	2200	13.68	65	4130	15.18	85
P. glabrum (Wehmer) Westling	70	0.44	10	0	0.00	0
P. oxalicum Currie & Thom	310	1.93	35	620	2.28	30
P. pinophilum Hedgc.	0	0.00	0	40	0.15	5
P. purpurogenum Stoll	200	1.24	35	220	0.81	30
P. waksmanii K.M. Zaleski	0	0.00	0	40	0.15	5
Rhizopus stolonifer (Ehrenb.) Vuill.	340	2.11	30	380	1.40	25

Fungal species	Healthy leaves			Purple blotch diseased leaves		
	TC	%TC	%F	TC	%TC	%F
Scopulariopsis	80	0.50	10	20	0.07	5
S. brevicaulis (Sacc.) Bainier	40	0.25	5	0	0.00	0
S. candida (Guég.) Vuill.	40	0.25	5	20	0.07	5
Setosphaeria rostrata K.J. Leonard	80	0.50	15	60	0.22	5
Stachybotrys chartarum (Ehrenb.) S. Hughes	160	1.00	25	40	0.15	10
Stemphylium	220	1.37	35	5830	21.43	90
S. botryosum Wallr.	20	0.12	5	120	0.44	10
S. vesicarium (Wallr.) E.G. Simmons	200	1.24	30	5710	20.99	90
Trichoderma	1180	7.34	60	220	0.81	20
T. harzianum Rifai	780	4.85	40	140	0.51	10
T. longibrachiatum Rifai	400	2.49	20	80	0.29	10
TC (CFU / 20 g)	**16,080**			**27,200**		
Number of genera = 25	**25**			**19**		
Number of species = 63	**58**			**49**		

Phylloplane fungi

Forty fungal species belonging to 21 genera were isolated from phylloplane of both healthy (25 species belonging to 15 genera) and purple blotch diseased leaves (35 species belonging to 21 genera). The total fungal counts of phylloplane were 592 and 1307 CFU/500 leaf segments of healthy and purple blotch diseased leaves, respectively (Tab. 2).

Aspergillus and *Cladosporium* were the most prevalent genera in the phylloplane of both samples, occurring in 60–100% of total samples. Of the above genera, *Aspergillus niger* was the most prevalent species in the two substrates (80 and 85% of the samples comprising 28.38 and 15.68% of total fungi in healthy and diseased leaves, respectively).

Some fungal genera were recovered with high frequency from purple blotch diseased samples and with low frequency from healthy leaves, e.g. *Alternaria* (70% and 35% of the samples comprising 11.94 and 4.22% of total fungi, respectively), *Fusarium* (65% and 20% of the samples constituting 3.14 and 2.03% of total fungi, respectively) and *Stemphylium* (100% and 50% of the samples constituting 32.29 and 9.12% of total fungi, respectively). On the other hand, some species were recovered only from one substrate, but with different counts and frequencies, such as: *Aspergillus tamarii*, *Botrytis cinerea*, *Emericella nidulans* var. *lata*, *E. variecolor*, *Mucor circinelloides*, *Nigrospora sphaerica*, *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. citrinum*, *Periconia byssoides*, *Scopulariopsis candida*, *Setosphaeria rostrata*, *Stachybotrys chartarum*, *Torula graminis* and *Trichoderma harzianum* from purple blotch diseased leaves on the one hand, and *Aspergillus carbonarius*, *A. flavus* var. *columnaris*, *A. sydowii*, *Emericella quadrilineata*, *Scopulariopsis brevicaulis* and *Trichoderma longibrachiatum* from healthy leaves on the other hand.

Tab. 2. Phylloplane fungi recovered from leaves of onion plants cultivated for seed production during January–April 2011 on PDA medium at 25 ± 1 °C.
Abbreviations: TC – total fungal count (CFU / 500 segments of healthy or diseased leaves); %TC – percentage of total fungal count; %F – % frequency of the fungal species / 20 collected samples of healthy or diseased leaves.

Fungal species	Healthy leaves			Purple blotch diseased leaves		
	TC	%TC	%F	TC	%TC	%F
Acremonium strictum Gams	8	1.35	15	24	1.84	35
Alternaria alternata (Fr.) Keissl.	25	4.22	35	156	11.94	70
Aspergillus	242	40.88	90	334	25.55	100
A. carbonarius (Bain.) Thom	9	1.52	15	0	0.00	0
A. flavus Link var. *flavus*	31	5.24	45	25	1.91	55
A. flavus var. *columnaris* Raper & Fennell	2	0.34	5	0	0.00	0
A. fumigatus Fresen.	20	3.38	20	26	1.99	20
A. niger Tiegh.	168	28.38	80	205	15.68	85
A. sydowii (Bain. & Sart.) Thom & Church	5	0.84	15	0	0.00	0
A. tamarii Kita	0	0.00	0	2	0.15	5
A. terreus Thom	7	1.18	15	76	5.81	50
Botrytis cinerea Pers.	0	0.00	0	2	0.15	5
Chaetomium globosum Kunze	49	8.28	30	22	1.68	30
Cladosporium	68	11.49	60	163	12.47	60
C. cladosporioides (Fresen.) G.A. de Vries	29	4.90	40	120	9.18	60
C. sphaerospermum Penz.	34	5.74	25	43	3.29	20
Cunninghamella echinulata Thaxt.	5	0.84	5	5	0.38	5
Emericella	4	0.68	5	4	0.31	10
E. nidulans var. *lata* (Thom & Raper) Subram.	0	0.00	0	2	0.15	5
E. quadrilineata (Thom & Raper) C.R. Benj.	4	0.68	5	0	0.00	0
E. variecolor Berk. & Broome	0	0.00	0	2	0.15	5
Fusarium	12	2.03	20	41	3.14	65
F. nygamai L.W. Burgess & Trimboli	2	0.34	5	2	0.15	5
F. oxysporum Schltdl.	7	1.18	15	17	1.30	35
F. solani (Mart.) Sacc.	3	0.51	10	22	1.68	25
Gliocladium roseum Bainier	6	1.01	10	2	0.15	5
Mucor	25	4.22	25	21	1.61	30
M. circinelloides Tiegh.	0	0.00	0	5	0.38	5
M. hiemalis Wehmer	25	4.22	25	16	1.22	25
Myrothecium verrucaria (Alb. & Schwein.) Ditmar	6	1.01	10	45	3.44	45
Nigrospora sphaerica (Sacc.) Mason	0	0.00	0	15	1.15	10

Fungal species	**Healthy leaves**			**Purple blotch diseased leaves**		
	TC	**%TC**	**%F**	**TC**	**%TC**	**%F**
Penicillium	79	13.34	45	27	2.07	45
P. aurantiogriseum Dierckx	0	0.00	0	10	0.77	15
P. chrysogenum Thom	0	0.00	0	1	0.08	5
P. citrinum Thom	0	0.00	0	2	0.15	5
P. duclauxii Delacr.	7	1.18	30	8	0.61	30
P. pinophilum Hedgc.	72	12.16	15	6	0.46	15
Periconia byssoides Pers. ex Mérat	0	0.00	0	7	0.54	10
Scopulariopsis	2	0.34	5	1	0.08	5
S. brevicaulis (Sacc.) Bainier	2	0.34	5	0	0.00	0
S. candida (Guég.) Vuill.	0	0.00	0	1	0.08	5
Setosphaeria rostrata K.J. Leonard	0	0.00	0	2	0.15	5
Stachybotrys chartarum (Ehrenb.) S. Hughes	0	0.00	0	2	0.15	5
Stemphylium	54	9.12	50	422	32.29	100
S. botryosum Wallr.	1	0.17	5	4	0.31	5
S. vesicarium (Wallr.) E.G. Simmons	53	8.95	45	418	31.98	100
Torula graminis Desm.	0	0.00	0	3	0.23	5
Trichoderma	12	2.03	15	9	0.69	15
T. harzianum Rifai	0	0.00	0	9	0.69	15
T. longibrachiatum Rifai	12	2.03	15	0	0.00	0
TC (CFU / 500 leaf segments)	**592**			**1307**		
Number of genera = 21	**15**			**21**		
Number of species = 40	**25**			**35**		

Endophytic fungi and fungi of surface-sterilised purple blotch diseased leaves

The total fungal counts associated with surface-sterilised purple blotch diseased leaves (662 CFU/500 leaf segments) were higher than the counts of endophytic fungi recovered from healthy leaves (175 CFU/500 leaf segments). These propagules were represented by 15 species and 12 fungal genera as endophytes from healthy leaves and 17 species and 13 genera from surface-sterilised purple blotch diseased leaves (Tab. 3).

Cladosporium (*C. cladosporioides* and *C. sphaerospermum*) was recovered in high frequency from healthy and diseased samples (55 and 70% of the samples comprising 17.14 and 14.05% of total fungi, respectively).

Alternaria, *Penicillium* and *Stemphylium* were recovered in high frequency from surface-sterilised purple blotch diseased samples, but less frequently recovered from healthy samples. They emerged in 65, 60 and 100% of the diseased samples yielding 14.95, 3.93 and 54.83% of total fungi, respectively.

Five fungal species (*Absidia corymbifera*, *Botrytis cinerea*, *Penicillium aurantiogriseum*, *P. glabrum* and *Syncephalastrum racemosum*) were recovered only from surface-sterilised purple blotch diseased onion leaves, while three species (*Fusarium oxysporum*, *Trichoderma harzianum* and *T. koningii*) were isolated only from healthy samples.

Tab. 3. Endophytic fungi and fungi associated with surface-sterilised purple blotch diseased leaves of onion plants cultivated for seed production during January–April 2011 on PDA medium at 25 ± 1 °C. Abbreviations: TC – total fungal count (CFU / 500 segments of healthy or diseased leaves); %TC – percentage of total fungal count; %F – % frequency of the fungal species / 20 collected samples of healthy or diseased leaves.

Fungal species	Endophytic fungi from healthy leaves			Fungi of surface sterilised purple blotch diseased leaves		
	TC	%TC	%F	TC	%TC	%F
Absidia corymbifera (Cohn) Sacc. & Trotter	0	0.00	0	9	1.36	20
Alternaria alternata (Fr.) Keissl.	14	8.00	20	99	14.95	65
Botrytis cinerea Pers.	0	0.00	0	4	0.60	10
Chaetomium globosum Kunze	14	8.00	30	30	4.53	30
Cladosporium	30	17.14	55	93	14.05	70
C. cladosporioides (Fresen.) G.A. de Vries	20	11.43	50	44	6.65	45
C. sphaerospermum Penz.	10	5.71	15	49	7.40	50
Cunninghamella echinulata Thaxt.	5	2.86	5	2	0.30	5
Fusarium oxysporum Schltdl.	1	0.57	5	0	0.00	0
Gliocladium roseum Bainier	4	2.29	5	1	0.15	5
Myrothecium verrucaria (Alb. & Schwein.) Ditmar	11	6.29	15	4	0.60	10
Nigrospora sphaerica (Sacc.) Mason	20	11.43	30	13	1.96	20
Penicillium	20	11.43	30	26	3.93	60
P. aurantiogriseum Dierckx	0	0.00	0	8	1.21	15
P. duclauxii Delacr.	2	1.14	5	15	2.27	30
P. glabrum (Wehmer) Westling	0	0.00	0	1	0.15	5
P. oxalicum Currie & Thom	18	10.29	25	2	0.30	10
Stemphylium vesicarium (Wallr.) E.G. Simmons	28	16.00	35	363	54.83	100
Syncephalastrum racemosum Cohn ex J. Schröt.	0	0.00	0	13	1.96	10
Torula graminis Desm.	5	2.86	5	5	0.76	10
Trichoderma	23	13.14	35	0	0.00	0
T. harzianum Rifai	20	11.43	20	0	0.00	0
T. koningii Oudem.	3	1.71	15	0	0.00	0
TC (CFU / 500 leaf segments)	**175**			**662**		
Number of genera = 15	**12**			**13**		
Number of species = 20	**15**			**17**		

Antagonistic effect of leaf surface and endophytic fungi against *Alternaria porri*

The antagonistic effect of ninety-one fungal isolates was tested, in vitro, against *Alternaria porri* AUMC 9301 on PDA medium using the dual culture technique. Forty-eight isolates (52.75% of tested isolates) showed different mechanisms of antagonism in various degrees against the fungal pathogens (Tab. 4).

Eighteen isolates (37.5% of positive isolates) belonging to five fungal genera showed overgrowth on the pathogenic fungal mycelia, one isolate of them belonging to *Aspergillus flavus* var. *flavus*, three isolates to *Gliocladium roseum*, three isolates to *Myrothecium verrucaria*, two isolates to *Penicillium pinophilum*, four isolates to *Trichoderma harzianum* and five isolates to *T. longibrachiatum*.

Thirty fungal isolates (62.5% of positive isolates) exhibited an antibiosis effect suppressing mycelial growth of pathogens producing inhibition zones. These isolates included three categories according to inhibition zone values as shown in Tab. 4:

i) Narrow inhibition zone (less than 3 mm in diameter): shown by 16 fungal isolates, namely *Acrophialophora fusispora*, *Alternaria alternata* (2 isolates), *Aspergillus fumigatus*, *Cochliobolus spicifer*, *Emericella nidulans* var. *lata*, *E. nidulans* var. *nidulans* (2 isolates), *E. rugulosa*, *E. variecolor*, *Fusarium solani* (2 isolates), *Nigrospora sphaerica*, *Penicillium funiculosum*, *P. purpurogenum* and *Periconia byssoides*.

ii) Moderate inhibition zone (3–7 mm): represented by 11 fungal isolates, namely *Chaetomium globosum* (3 isolates), *Emericella rugulosa*, *Eurotium amstelodami*, *Penicillium duclauxii* (2 isolates), *P. oxalicum* (2 isolates) and *Stachybotrys chartarum* (2 isolates).

iii) Broad inhibition zone (more than 7 mm): detected by 3 fungal isolates, namely *Epicoccum nigrum* No. 3011, *Penicillium oxalicum* No. 1033 and *Stachybotrys chartarum* No. 2031.

According to the ability of the tested isolates to compete for nutrients and space with *Alternaria porri*, 16 isolates (out of 90 tested isolates) showed more than 50% of inhibition of the growth of the pathogen. Four isolates of *Trichoderma harzianum* exhibited the highest rate of *Alternaria porri* inhibition contributing 73.12%. Five isolates of *T. longibrachiatum* showed an inhibition rate of 70%, while *Epicoccum nigrum* No. 3011, *Myrothecium verrucaria* (3 isolates), *Penicillium oxalicum* (3 isolates) and *Stachybotrys chartarum* No. 2031 showed an inhibition rate of 50.34–52.18%.

Tab. 4. Antagonistic effect of fungal isolates against mycelial growth of *Alternaria porri*, in vitro. Arbitrary antagonistic scale: 0 = showing no antagonistic effect; OG = showing overgrowth on pathogen mycelia; N = showing narrow inhibition zone (less than 3 mm wide); M = showing moderate inhibition zone (3–7 mm); B = showing broad inhibition zone (more than 7 mm).

Fungal species	No.	% of mycelial growth inhibition (average)	Inhibition zone width	
			Value in mm	Index
Absidia				
A. corymbifera (Cohn) Sacc. & Trotter	3017	5.12	0	0
A. corymbifera (Cohn) Sacc. & Trotter	3060	5.12	0	0
Acremonium				
A. strictum Gams	3016	0	0	0
A. strictum Gams	3046	0	0	0
Acrophialophora				
A. fusispora (S.B. Saksena) Samson	3009	6.12	2	N
A. fusispora (S.B. Saksena) Samson	3037	6.12	0	0
Alternaria				
A. alternata (Fr.) Keissl.	2001	12.24	2	N
A. alternata (Fr.) Keissl.	2002	9.13	0	0
A. alternata (Fr.) Keissl.	2010	12.86	1	N
Aspergillus				
A. flavus Link var. *flavus*	17	18.57	0	0
A. flavus Link var. *flavus*	29	24.48	0	0
A. flavus Link var. *flavus*	34	24.48	0	OG
A. fumigatus Fresen.	31	24.48	2	N
A. fumigatus Fresen.	43	24.48	0	0
A. niger Tiegh.	16	24.48	0	0
A. niger Tiegh.	19	24.48	0	0
A. niger Tiegh.	27	24.48	0	0
A. ochraceus Wilh.	8	12.42	0	0
A. ochraceus Wilh.	12	15.71	0	0
A. oryzae (Ahlb.) Cohn	21	24.48	0	0
A. sydowii (Bain. & Sart.) Thom & Church	56	0	0	0
A. sydowii (Bain. & Sart.) Thom & Church	70	0	0	0
A. terreus Thom	4	24.48	0	0
A. terreus Thom	9	22.57	0	0
A. terreus Thom	13	22.57	0	0
A. ustus (Bain.) Thom & Church	55	6.17	0	0
A. versicolor (Vuill.) Tirab.	53	8.57	0	0
A. wentii Wehmer	65	6.17	0	0
Chaetomium				
C. globosum Kunze	3003	43.71	6	M
C. globosum Kunze	3038	43.71	7	M
C. globosum Kunze	3045	43.71	6	M
Cladosporium				
C. cladosporioides (Fresen.) G.A. de Vries	2005	0	0	0

Fungal species	**No.**	**% of mycelial growth inhibition (average)**	**Inhibition zone width**	
			Value in mm	**Index**
C. sphaerospermum Penz.	2011	0	0	0
Cochliobolus				
C. lunatus R.R. Nelson & F.A. Haasis	2030	8.57	0	0
C. spicifer R.R. Nelson	2027	8.57	2	N
Cunninghamella echinulata Thaxt.	3044	43.71	0	0
Emericella				
E. nidulans var. *lata* (Thom & Raper) Subram.	3002	34.29	2	N
E. nidulans var. *lata* (Thom & Raper) Subram.	3008	34.29	0	0
E. nidulans (Eidam) Vuill. var. *nidulans*	3015	34.29	2	N
E. nidulans (Eidam) Vuill. var. *nidulans*	3024	34.29	1	N
E. rugulosa (Thom & Raper) C.R. Benj.	3022	34.29	1	N
E. rugulosa (Thom & Raper) C.R. Benj.	3025	34.29	6	M
E. variecolor Berk. & Broome	3031	24.48	1	N
Epicoccum nigrum Link	3011	52.18	12	B
Eurotium				
E. amstelodami L. Mangin	3049	43.71	4	M
E. rubrum König	3053	0	0	0
Fusarium				
F. nygamai L.W. Burgess & Trimboli	5015	34.29	0	0
F. oxysporum Schltdl.	5016	34.29	0	0
F. oxysporum Schltdl.	5018	34.29	0	0
F. solani (Mart.) Sacc.	5017	34.29	2	N
F. solani (Mart.) Sacc.	5019	32.86	2	N
Gliocladium				
G. roseum Bainier	3020	32.86	0	OG
G. roseum Bainier	3033	32.86	0	OG
G. roseum Bainier	3047	32.86	0	OG
Mucor				
M. circinelloides Tiegh.	3050	32.86	0	0
M. circinelloides Tiegh.	3051	32.86	0	0
M. hiemalis Wehmer	3028	32.86	0	0
Myrothecium				
M. verrucaria (Alb. & Schwein.) Ditmar	3014	51.43	0	OG
M. verrucaria (Alb. & Schwein.) Ditmar	3039	51.43	0	OG
M. verrucaria (Alb. & Schwein.) Ditmar	3048	51.43	0	OG
Nigrospora sphaerica (Sacc.) Mason	2021	43.71	2	N
Penicillium				
P. chrysogenum Thom	1005	18.57	0	0
P. chrysogenum Thom	1009	18.57	0	0
P. corylophilum Dierckx	1014	18.57	0	0
P. duclauxii Delacr.	1047	48.57	5	M
P. duclauxii Delacr.	1048	48.57	5	M
P. funiculosum Thom	1060	0	2	N

Fungal species	No.	% of mycelial growth inhibition (average)	Inhibition zone width	
			Value in mm	Index
P. oxalicum Currie & Thom	1033	50.34	10	B
P. oxalicum Currie & Thom	1037	50.34	7	M
P. oxalicum Currie & Thom	1040	50.34	7	M
P. pinophilum Hedgc.	1063	0	0	OG
P. pinophilum Hedgc.	1064	0	0	OG
P. purpurogenum Stoll	1049	11.43	2	N
P. purpurogenum Stoll	1050	0	0	0
Periconia byssoides Pers. ex Mérat	2019	11.43	1	N
Scopulariopsis				
S. brevicaulis (Sacc.) Bainier	3027	0	0	0
S. candida (Guég.) Vuill.	3045	0	0	0
Stachybotrys				
S. chartarum (Ehrenb.) S. Hughes	2031	52.18	12	B
S. chartarum (Ehrenb.) S. Hughes	2032	48.57	7	M
S. chartarum (Ehrenb.) S. Hughes	2033	48.57	6	M
Syncephalastrum racemosum Cohn ex J. Schröt.	3001	11.43	0	0
Torula graminis Desm.		0	0	0
Trichoderma				
T. harzianum Rifai	3013	73.12	0	OG
T. harzianum Rifai	3019	73.12	0	OG
T. harzianum Rifai	3028	73.12	0	OG
T. harzianum Rifai	3032	73.12	0	OG
T. longibrachiatum Rifai	3021	70.30	0	OG
T. longibrachiatum Rifai	3055	70.30	0	OG
T. longibrachiatum Rifai	3056	70.30	0	OG
T. longibrachiatum Rifai	3057	70.30	0	OG
T. longibrachiatum Rifai	3058	70.30	0	OG

DISCUSSION

Diversity of fungi in healthy and diseased leaves

Phyllosphere, phylloplane and endophytic microbiota associated with onion leaves are a potential source of microorganisms having antagonistic activities to plant pathogens and being ecologically adapted to growth and activity in association with the host (Rubini et al. 2005, Schulz et al. 2002, Sutton et al. 1997). Phyllosphere and phylloplane non-pathogenic fungi associated with plant surfaces are often able to suppress growth and sporulation of plant pathogens (Sutton et al. 1997). The possibility of controlling pathogenic fungi by antagonistic microorganisms has been explored in many investigations (Abo-Elyousr et al. 2014, Fokkema & Lorbeer 1974, Tyagi et al. 1990).

In the present investigation, 68 species belonging to 29 fungal genera were recovered and identified as leaf-surface and endophytic fungi from healthy and purple blotch diseased onion leaves. The populations of leaf surface fungi (phyllosphere and phylloplane) in purple blotch diseased leaves were higher than those in healthy onion leaves. These results may be attributed to the diseased leaves being susceptible because the epidermal cells are damaged and the lamellar seta shed, so nutrients easily run off and these stimulate the fungal growth in the diseased lesion. Li et al. (2012) reported that the quantities of epiphytic fungi in diseased leaves of *Bambusa* were more than those of healthy leaves. Raithak & Gachande (2013) reported that host-pathogen interactions in plant diseases are known to bring about considerable changes in the metabolism of the infected plant. These metabolic changes are expected to alter the quality and/or quantity of leaf exudates, which in turn will be reflected in the leaf surface microflora of such plants. They found that the number of leaf surface fungi increased in virus-infected tomato plant leaves as compared to healthy leaves. Sharma & Tiwari (1981) studied the phyllosphere and phylloplane microflora of healthy and diseased (*Phytophthora infestans*) leaves of *Solanum khasianum*. They reported that the largest numbers of fungus species were observed on diseased leaves. In the present study, the phyllosphere mycobiota of healthy leaves (58 species and 25 genera) was richer than that in the phylloplane (25 species and 15 genera). This means that about 56.7% of fungal species are not really inhabitants of the leaf surface but are deposited from air. These results were in agreement with those reported by Mohamed (2001), who examined the leaf surface fungi of onion plants and reported that the fungi of the phyllosphere (20 species and 13 genera) were larger in number than those of the phylloplane (9 species and 7 genera).

In the case of purple blotch diseased samples, the fungal diversity of the phylloplane was higher than that in healthy samples. This means that the infection may cause changes in the nutrient availability on the onion leaf surface which stimulates the already present saprophytic fungi to grow on it and inhabit it. In contrast, the diversity of phyllosphere fungi in purple blotch diseased samples was lower than that of healthy leaves. This could be due to the increase of the superficial mycelial growth of the pathogen and other secondary pathogenic fungi which reduced the number of available leave sites for settling of new spores.

This study showed that *Alternaria alternata*, *Aspergillus niger*, *A. terreus*, *Penicillium funiculosum* and *Cladosporium cladosporioides* were the most common species as leaf surface fungi (phyllosphere and phylloplane) of healthy and purple blotch diseased onion leaves. In this respect, Mohamed (2001) reported that the most predominant fungal species recovered from the leaf surface of onion plants were *Alternaria alternata*, *Aspergillus niger*, *A. sydowii*, *A. versicolor*, *Cladosporium herbarum*, *Cochliobolus lunatus*, *Pleospora herbarum* and *Setosphaeria rostrata*.

In the present study, the total counts of fungi associated with surface-sterilised diseased leaves were higher than those recovered as endophytes from healthy leaves. The mycobiota associated with surface-sterilised purple blotch diseased leaves were different from those isolated as endophytes from healthy samples. This may be attributed to the fact that the disease may stimulate colonisation of fungi which can cause secondary infections such as grey mold (*Botrytis cinerea*), blue mould (*Penicillium aurantiogriseum*) and leaf spot (*Alternaria alternata* and *Cladosporium* spp.). In contrast, the healthy leaves were a source of antagonistic endophytic fungi such as *Trichoderma harzianum* and *T. koningii*.

Antagonistic effect of detected species against pathogenic fungi

Numerous studies have been consecrated to an investigation of biocontrol agents of plant pathogens as an alternative eco-friendly strategy to fungicide applications (Alwathnani & Perveen 2012, Siameto et al. 2010, Soria et al. 2012).

The antagonistic effect of 91 fungal isolates was tested in vitro against *Alternaria porri* AUMC 9301. *Trichoderma* showed the highest degree of competition as well as mycoparasitism against the mycelial growth of the pathogen, while *Epicoccum nigrum*, *Penicillium oxalicum* and *Stachybotrys chartarum* exhibited antibiosis, producing high inhibition zone values against *Alternaria porri* AUMC 9301.

In this respect, several reseachers have reported that *Trichoderma* has a good antagonistic effect on mycelial growth and conidial germination of *Alternaria porri* (Imtiaj & Lee 2008). *Trichoderma harzianum* exhibited a strong suppressive effect on the development of many foliar plant pathogens such as *Alternaria alternata* and *Stemphylium vesicarium* causing high inhibition of mycelial growth and conidial germination (Gveroska & Ziberoski 2012, Hussein et al. 2007, Rossi & Pattori 2009).

The use of *Epicoccum nigrum* as a biocontrol agents has been successfully applied against several plant pathogenic fungi as *Monilinia* spp. causing brown rot of peaches (Larena et al. 2005, Mari et al. 2007) and *Phytophthora infestans*, the pathogen of potato late blight (Li et al. 2013). *Penicillium oxalicum* has been reported to be a biocontrol agent for wilt disease (*Fusarium oxysporum* f. sp. *Lycopersici*) of tomato (De Cal et al. 1997, Sabuquillo et al. 2006, Sabuquillo et al. 2010), leaf spot (*Cercospora canescens*) of black gram (Rao & Mallaiah 1988), anthracnose disease (*Colletotrichum gloeosporioides*) of guava (Pandey et al.

1993) and wilt disease caused by *Fusarium oxysporum* f. sp. *niveum* on melon and watermelon (De Cal et al. 2009).

Our investigation showed that the mechanism of antagonistic potentiality of *Trichoderma* spp. against *Alternaria porri*, in vitro, may be conditioned by competition for nutrient and space and mycoparasitism. The competition mechanism of *Trichoderma* is based on a high growth rate causing a limitation of nutrients and space for the pathogen, and this may produce an inhibition of pathogen growth of up to 73.12%. The mycoparasitic activity of *Trichoderma* was detected morphologically by the formation of overgrowth on the mycelial growth of *Alternaria porri*. The mechanism of the antagonistic effect of *Epicoccum nigrum*, *Penicillium oxalicum* and *Stachybotrys chartarum* against the pathogen is antibiosis caused by production of effective antimicrobial secondary metabolites.

In this respect, numerous investigations have demonstrated that antagonistic *Trichoderma* spp. act directly on the plant pathogen by means of different mechanisms such as competition, lysis, antibiosis and hyperparasitism (Benítez et al. 2004, Siameto et al. 2010). *Trichoderma* spp. suppress the growth of pathogenic fungi through their capability of growing much faster than the pathogens, so that competition for limiting nutrients and space results in biological control of the target fungi (Harman 2006). *Trichoderma* may exert direct biocontrol by growing towards and parasitising the pathogen, coiling around its hyphae and penetrating the pathogen hyphae (Metcalf & Wilson 2001). Many researchers have reported that the antifungal effects of *Epicoccum nigrum* against plant pathogens may be ascribed to the production of bioactive compounds such as flavipin (Madrigal et al. 1991), epirodins (Burge et al. 1976) and epicorazines (Baute et al. 1978). The potentiality of *Penicillium oxalicum* to suppress and control many phytopathogens may be based on the production of lytic extracellular enzymes such as β-1,3-glucanases, chitinases and cellulases (Haggag & El Soud 2013), and the secretion of antibiotic and antifungal secondary metabolites (De Cal et al. 1997, Pandey et al. 1993, Shen at al. 2014). *Stachybotrys chartarum* competes and suppresses other microorganisms through biosynthesis and production of trichothecenes, e.g. satratoxines, verrucarins, trichoverrins and atranones, but some of them have proven to have toxicological impact on the environment, and human and animal health (Jarvis et al. 1998, Wilkins et al. 2003).

CONCLUSION

The quantitative and qualitative compositions of leaf surface and endophytic fungi associated with onion leaves were distinctly influenced by infection of *Alternaria porri*. Infection of onion leaves by *Alternaria porri* caused an increase in fungal counts and colonisation of opportunistic fungi producing secondary diseases. Phyllosphere, phylloplane and endophytic mycobiota associated with healthy and asymptomatic onion leaves are a natural source of eco-friendly bioagents which may control plant pathogens such as *Alternaria porri*. This investigation confirms that endophytic and leaf surface mycobiota such as *Epicoccum nigrum*, *Penicillium oxalicum* or *Trichoderma* species may be effective antagonists against *Alternaria porri* depending on production of non-toxic antifungal secondary metabolites or mycoparasitic ability, and thus be applied as an alternative to fungicides.

ACKNOWLEDGEMENT

We thank all the members of Assuit University Mycological Centre (AUMC) for assistance in identification of some fungal isolates.

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