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Evaluation of Endophytes Isolated from Rice Leaves for Their Antifungal Activities Against *Pyricularia oryzae* Causative Blast Disease

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Rice crop is attacked during its growing season by many fungal diseases. Blast disease, caused by *Pyricularia oryzae* (*Magnaporthe grisea* Hebert), is the major serious one. The aim of this work was to replace fungicide applications by ecofriendly and natural substances against the disease. Fifty endophyte isolates of different microorganisms were isolated from rice leaves at Sakha Agricultural Research Farm on five different isolation media. *In vitro* test was performed to screen the antifungal activity of these isolates against rice blast fungus. Of these, only five isolates showed highly antagonistic activity *in vitro* and *in vivo* experiments. The secondary antifungal metabolites of these endophyte isolates were extracted by TLC and tested for their efficiency against the pathogen in *in vitro* test. Two isolates were the most potent and identified as *Streptomyces albidoflavus* strain Emeranaa MK203832.1 and *Bacillus subtillus*. GC-MS extract analysis for these two isolates identified 24 and 13 compounds, respectively.

Keywords: Antimicrobial compounds, Endophytes, GC-MS analysis, *Pyricularia oryzae,* Rice blast and TLC test.

Application of fungicides, which leads to many human and animal diseases besides disorder in the bioenvironmental balance, has forced many investigators to explore a solution from this environment (Sukanya *et al.*, 2011 and Zhang *et al.*, 2016). To produce new bioactive molecules with higher efficiency, natural products i.e. plants and microbes are used. One of those important sources of natural bioactive compounds is endophytes. Endophytes are microorganisms which can colonize inner tissues of plants for part or all of their life time producing various enzymes, siderophores or antibiotics, which provide a protection to plants from phytopathogens or insects (Kafur & Khan, 2011). Also, they may enhance their host growth by improving nutrient absorption. Rice plants are inhabited with various endophyte populations which enhance nitrogen fixation, promote plant growth and induce disease resistance (Tian *et al.*, 2004; Fernandez *et al.*, 2006 and Naik *et al.*, 2009). Endophytic bacteria and streptomyces with antibacterial or antifungal activity were detected in many crops i.e. banana; common bean and rice (Tian, *et al.*, 2004; Harish *et al.*, 2008; Suryadi *et al.*, 2011 and Costa *et al.*, 2012).

Beneficial *Bacillus* spp. strains can compete other microbes that could affect crops; they can inhibit phytopathogenic attacks, or induce host-plant defense system against potential pathogenic attacks, stimulate plant growth, improve nutrient uptake and reduce some negative environmental traits (Brader *et al.*, 2014 and Santoyo *et al.*,

2016). *Bacillus* species are known to have a wide spectrum of plant protection and growth promoting abilities. Species of *Bacillus* have the ability to produce endospores with a prolong viability and high resistance (Sella *et al.*, 2014 and Noell *et al.*, 2015) which makes them easy to be formulated in various types and stored in simple conditions, marketing them similar to chemical fungicides in a certain manner. *Bacillus* species could act directly against pathogens by producing extracellular lytic enzymes and secondary metabolites with inhibitory growth action (Kumar *et al.*, 2012). They can also improve nutrient uptake or compete plant pathogens for the available nutrients (Borriss, 2011 and Chowdhury *et al.*, 2013). In addition, reducing infection process by inducing defense responses in their host plants (Choudhary and Johri, 2009).

Streptomyces is the largest genus of Actinobacteria following Streptomycetaceae family (Hong *et al.*, 2009). Over 500 species of *Streptomyces* genus have been characterized and about two third of the naturally antibiotics are produced by Streptomycetes (Mohanraj and Sekar, 2013).

Rice plants are subjected to attack by many fungal diseases during the season. Blast disease incited by *Pyricularia oryzae* Cavara is the most major and destructive one occurring all over rice growing areas of the world causing yield losses up to 50% (Ou, 1985). Therefore, the present work has been performed to isolate these endophytes, identify, evaluate their antagonistic ability in vitro and in vivo against rice blast causal fungus and finally to purify and detect its antifungal metabolites.

Materials and Methods

Sample collection:

About three healthy rice leaves adjacent to blast infected ones were collected from each of 15 individual plants of Sakha 101 cv. during 2017 rice growing season at Sakha Agricultural Research Station Farm. Samples were packed in clean paper bags, then directly transferred to the laboratory for isolation.

Isolation of endophytes:

To isolate the available number of endophytes, five different media; Water-Yeast Extract Agar (WYE), Yeast Extract-Malt Extract (ISP2), Zhang′ Starch Soil Extract Agar (ZSSE agar), Bendict′s modified from that of Lindenbein medium and Casein Starch medium (Crawford *et el*.,1993; Shriling and Gottlieb, 1966; Zhang, 2011; Porter *et al.,* 1960 and Kuster & Williams, 1964) were used. All isolation media were prepared in distilled water and adjusted with 0.1N NaOH to pH 7.1 -7.2 prior to autoclaving. Ten- gram samples of the collected healthy rice leaves were washed with running tap water for 10 minutes. Leaves were left till dryness, then cut into small pieces (2 cm) and surface sterilized by immersing in 0.5% sodium hypochlorite solution for 2 minutes (Okazaki, 2003). Sterilized pieces were directly rinsed with sterilized water three times. The samples were mashed in 90 ml sterile distilled water with a mortar and pestle. A 0.1 ml of this suspension was inoculated using the spread plate method. Three replicates of each of the aforementioned media were inoculated. Plates were incubated at 28˚C for 15-30 days. Pure cultures were maintained on PDA slants as stock cultures and then were stored at 4˚C for further studies.

Antifungal activity against Pyricularia oryzae in vitro:

The obtained microbial endophyte isolates were screened for their antifungal activity against *Pyricularia oryzae* using the dual culture technique (Gupta *et al.*, 2001). All isolates were subjected to standardized test as follows: PDA medium was poured into 9 cm Petri dishes (15 ml/ dish). After solidification, each plate was inoculated in the center with a disc (6 mm in diameter) from 7- day old cultures of *P. oryzae* isolate. Two days later, plates were inoculated with a streak from each of the tested endophytic isolates (4 Isolate/plate) at the periphery. Three replicates were performed. Plates free from endophytic isolates were served as control. Plates were incubated at 28°C until full growth of control treatments. Inhibition zone of each antagonistic isolate was measured and the relative power of antibiosis (RPA) of each isolate was estimated through the ratio as described by Saleh, 2012 as follows:

$$\text{RPA} = \frac{z}{c}$$

Where:
Z= Diameter of inhibition zone.
C= Diameter of spotted antagonistic isolate.

Identification of the potent bioagents:

Pure cultures of the two most efficient antagonistic endophyte isolates were identified. On the basis of 16S r RNA amplification, the first isolate (no.12) was identified at GATC company, Germany. A pair of universal primers 27f (5'-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G- 3') and 1392r (5'-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3') were used. The sequence data were aligned and identified using the basic local alignment search tool (BLAST) in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA X software was used to estimate the phylogenetic relations of the endophytic isolates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 isolate sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1576 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018). Whereas, the other isolate (no.17) was identified at Cairo Mircen, Faculty of Agriculture, Ain Shams University by GEN III Biolog system (Biolog, Inc., USA), Plaza *et al.*, 2015.

Effect of the filtrates of the potent endophyte isolates on blast disease fungus:

Agar disk-diffusion method was followed for testing the antifungal activity of the two potent isolate filtrates (Bauer *et al.,* 1966). The effect of culture filtrates of the two potent endophyte isolates on the radial growth of *P. oryzae* was studied following the method of Burgess *et al.* (1997). Flasks containing 100 ml sterilized PD broth medium were inoculated with 0.5 cm diameter PDA mycelial disks of each isolate of isolates no.12 and 17. Each flask received one disk individually. The flasks were incubated at 30˚C for 10 days for actinomyces isolate, while the bacterial isolate was incubated at 28˚C for two days using shaking incubator (100 rpm/min).

The cultural mats were removed and the rest of the medium was subjected to sterilization using 0.22Mm Millipore filter. The sterilized filtrate of each isolate was added individually to semi cold-autoclaved PDA medium with a concentration of 15 ml/45 ml medium, and, then poured into Petri plates. Sterilized water was added to check treatment. A 6 mm in diameter of PDA medium *P. oryzae* mycelium disks were placed in the center of each treated plate. Plates were incubated at 28˚C. Three replicates were prepared for each treatment. Experiment was noticed till full growth of control plates.

Scanning Electron Microscope (SEM):

To observe the significant effect of the most two potent antagonistic endophyte isolates (no.12 and 17) on the internal morphological changes of *P. oryzae* mycelium, Scaning Electron Microscope (SEM) was used. Samples were prepared according to Piroeva *et al.*, (2013). The samples were then examined and photographed using a JEOL, JSM- 5200 LV scanning electron microscope, Japan at SEM Unit, Faculty of Science, Tanta University.

Antifungal activity against rice blast disease in vivo:

Susceptible rice cultivar Sakha 101 grains were sown in plastic trays. The trays were kept in the greenhouse at 25-30^{o}C. The seedlings were treated with the isolates, 3-4 weeks after sowing. The most five effective antifungal endophytes (no. 6, 12, 17,19 and 34) were tested for their efficiency in managing the blast disease infection on rice plants. The experiment was arranged in a randomized complete design with three replicates for each treatment. Two treatments were conducted comparing with beam fungicide. Firstly, we sprayed the filtrates of these endophyte isolates one day before inoculation with *P. oryzae* spores as a protective treatment. Secondly, the filtrates of the same five bioagent isolates were sprayed two days after inoculation with *P. oryzae* spores as a curative treatment. Beam fungicide was sprayed at 2g/l. Spore suspension of *P. oryzae* was adjusted to 5 x 10^4 spores/ml and sprayed using electrical spray gun. Trays with only the pathogen were left as a check treatment; likewise trays inoculated with only the bioagent were included to test for any probable side effects on plants. The inoculated trays were held in a dark moist chamber with 90-95% R.H. and 25-28^{0}C overnight and then moved to the greenhouse conditions with saving sprinkling to enhance infection development. Blast reaction, as the typical blast lesions, according to the standard evaluation system using 0-9 scale (Anonymous, 1996) was recorded seven days after inoculation. Disease incidence and disease severity were recorded after full appearance of infection symptoms.

Thin Layer Chromatography:

The most five effective antifungal endophyte isolates (no. 6, 12, 17, 19 and 34) were grown on PD broth medium for 10 days at 30°C, while, isolate no.17 was grown for 48 hours (150 rpm, 28-30°C). Then, filtrates were centrifuged at 10,000 rpm for 10 min. The culture media were extracted by mixing with ethyl acetate 1:1 (v/v) to separate the effective secondary metabolites of these five isolates using a thin layer chromatography (TLC) silica gel 60 F254 sheets (Merck, Darmstadt, Germany) (Wu *et al.*, 2013). The resultant bands were viewed under UV light (wavelength λ-254 and λ-365 nm) and bands boundaries were marked (Qin and Judith, 1999). Rf

value was recorded. Then, discs of silica gel (where the separated secondary metabolites were adhered) were cut and tested for their antagonistic effect against 6 mm discs of *P. oryzae* from 7 days old cultures in Petri dishes using the dual culture technique in triplicate. Inhibition zone was visually recorded. Plates inoculated only with *P. oryzae* were left for comparison.

Gas chromatography:

The whole filtrates of the isolates that proved antifungal effect in both in vitro and in vivo experiments were analysed after extraction process with solvent on Agilant GC-MS system at The National Research Center, Egypt. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data, (Kaaria *et al.,* 2012).

Statistical analysis:

The randomized complete design was applied to both laboratory and greenhouse experiments. Means were compared using multiple range tests according to Duncn (1955).

Results

Isolation of endophytes and antifungal activity against rice blast disease in vitro:

Isolation trials from healthy rice leaves grown on five different media resulted in the presence of fifty different endophytes isolates of both actinomyces and bacteria. These isolates were tested for their antifungal activity against *Pyricularia oryzae in vitro* (Table 1). Five isolates including four actinomycetes (no. 6, 12, 19 and 34) and one bacterial isolate (no.17) exhibited clear zones of fungal inhibition and highly RPA values. Of the five efficient antagonistic isolates, two isolates actinomyces no. 12 and bacteria no. 17 were the most potent as they recorded the highest RPA values, 10.88 and 12.83, respectively.

Identification of the potent bioagents:

The isolates of the highest RPA ratio (no. 12 and17) were identified. Based on 16S rRNA sequence analysis of potent bioagent isolates, actinomyces isolate no.12 was identified as *Streptomyces albidoflavus* 16S *strain Emeranaa* with accession no MK203832.1 in the Gene Bank. However, bacterial isolate no. 17 was identified by Biolog System as *Bacillus subtillus*. BLAST search analysis showed that the sequence was 94% similar to the 16S sequence of both *Streptomyces albidoflavus* with accession no LN626361.1 and LN626360.1, respectively. While, it was 95% similar to the 16S sequence of *Streptomyces albidoflavus* isolate M-33 with accession no HG965213.1), (Fig.1).

Table 1. Relative power of antibiosis (RPA) of fifty endophyte isolates against *P. oryzae*

Isolate no.	RPA	Isolate no.	RPA
1	2.05 jkl	26	0.01 q
2	2.5 jk	27	4.30 fg
3	1.3 1 mno	28	4.60 f
4	2.47 jk	29	1 nop
5	0.66 opq	30	0.01 q
6	9.64 c	31	0.01 q
7	1.33 mno	32	0.01 q
8	1.02 nop	33	0.01 q
9	0.44 pq	34	5.30 de
10	0.88 nop	35	0.01 q
11	0.01 q	36	0.01 q
12	10.88 b	37	0.01 q
13	0.01 q	38	1.83 klm
14	0.01 q	39	2.19 jk
15	0.01 q	40	4.75 ef
16	0.01 q	41	2.55 jk
17	12.83 a	42	2.2 jk
18	1.50 lmn	43	2.56 jk
19	5.47 d	44	0.01 q
20	1.26 mno	45	1.26 mno
21	1.21 mno	46	3.62 h
22	1.08 nop	47	2.25 jk
23	1.16 mno	48	3.87 gh
24	1.03 nop	49	2.64 ij
25	3.25 hi	50	2.73 ij

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Effect of filtrates of the potent endophyte isolates on blast disease fungus:

The effect of *Streptomyces albidoflavus* 16S strain *Emeranaa* MK203832 and *Bacillus subtillus* filtrates on mycelial growth of *P. oryzae* is illustrated in Fig. 2. There was no growth in treated dishes for both isolates in comparison with the control treatment. However, *Streptomyces albidoflavus* 16S strain *Emeranaa* apparently exceeded the bacterial isolate which allowed growth of mycelium only on the disk.

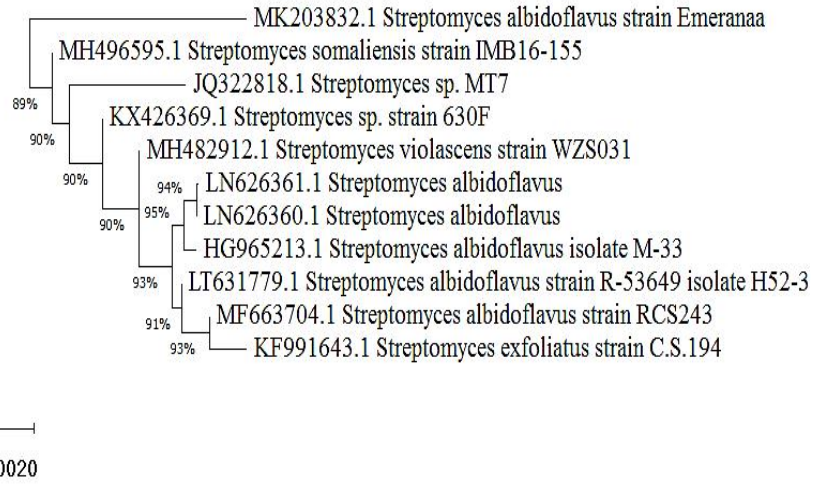

Fig.1. Phylogenetic neighbour-joining tree showing the relationship of strain K203832 with elven members of the genus *Streptomyces*.

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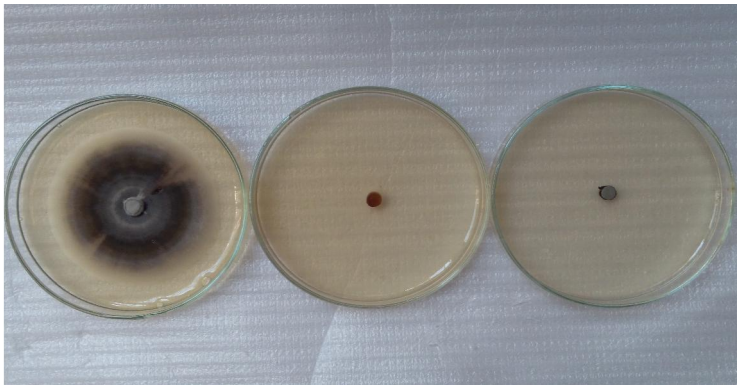

Fig. 2. Effect of culture filtrates of *Streptomyces albidoflavus* strain *Emeranaa* MK203832 (middle) and *Bacillus subtillus* (right) on growth of *P. oryzae* comparing with control treatment (left).

Scanning Electron Microscope:

Using scanning electron macrograph, samples free from bioagents showed normal hyphae growth (Fig. 3a). Contrariwise, *P. oryzae* hyphae, was distorted when inoculated with the bioagents. Scanning confirmed the aggregation of the fungal mycelium. Hyphae were coiled, thickened (Fig.3: 12b&17d) and cleaved (Fig.3: 12c &17e) in the presence of *Streptomyces albidoflavus* 16S strain *Emeranaa* MK203832 and *Bacillus subtillus*, respectively.

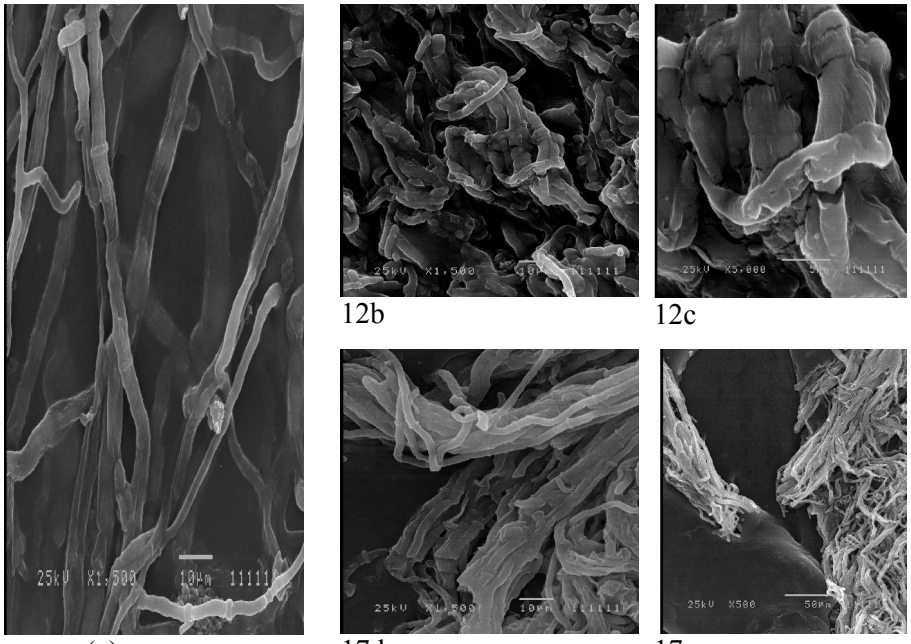

Fig. 3. Scanning electron microscope photographs indicating the effect of *Streptomyces albidoflavus* 16S strain *Emeranaa* MK203832 (no. 12) and *Bacillus subtillus* (no.17) on *P. oryzae* mycelium: (a) control; 12b & 17d, hyphae aggregation, coiling and thickening. 12c & 17e, hyphae cleavage.

Antifungal activity against rice blast disease in vivo:

Data presented in Table 2 show that, all the five tested endophytes were efficient against the rice blast disease in both treatments (spraying before or after inoculation with the pathogen), particularly, spraying after inoculation with the pathogen in comparison with the check treatment. However, *Streptomyces albidoflavus* 16S strain *Emeranaa* MK203832 isolate no.12 and *Bacillus subtillus* isolate no.17 proved to be the most efficient in managing the disease. They significantly recorded the least percentage of infection and severity after the fungicide treatment, alike they were applied before or after inoculation with the pathogen comparing with the other tested bioagents. Data also showed that treating plants with these isolates

after pathogen inoculation was more efficient in controlling the disease than before pathogen inoculation. No side effects appeared on plants treated only with bioagents.

Table 2. Effect of antagonistic endophytes on rice blast disease incidence under greenhouse conditions

Bioagent no.	One day Before inoculation		Two days After inoculation	
	%Infection	Severity	% Infection	Severity
1- Actinomyces no. 6	27.01d	1.34b	9.91b	0.79b
2-*Streptomyces albidoflavus* strain *Emeranaa* MK203832 (no.12)	24.85d	1.25b	0.001d	0.001e
3- *Bacillus subtillus* (no.17)	13.27e	1.32b	0.001d	0.001e
4- Actinomyces no. 19	48.26b	1.46b	6.73c	0.67c
5- Actinomyces no. 34	38.96c	1.15b	0.93d	0.22d
6- control	75.00a	3.50a	76.00a	1.96a
7- Beam (fungicide)	0.001f	0.001c	0.001d	0.001e

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Thin Layer Chromatography:

The ethyl acetate extraction method was the best in extracting and separating the crude antimicrobial compounds from filtrates of the five antifungal endophytes isolates (act. no. 6, 12, 17, 19 and 34) by TLC. One or two bands were observed. Bioassay of TLC extract proved that the compounds showing antifungal activity migrated through the plate with Rf value ranging from 0.27 to 1.00 for all isolates. The compounds activity was confirmed by agar diffusion method for *P. oryzae*, Table 3 and Fig. (4).

Table 3. The five endophytes Rf value of formative peaks by TLC and their inhibition zone against the rice blast fungus

Isolate peak, assigned substance	R.f. value	Inhibition zone
Actino. 6	0.27	+
Actino 6	0.93	+
Actino 12	0.29	++
Actino 12	0.96	++
Bacteria 17	1	++
Actino 19	0.89	+
Actino 19	0.78	+
Actino 34	0.90	+
Actino 34	0.92	+

Isol.no.:6- 12- 17- 19- Isol.no.:6- 12- 17- 19-34 Isol.no.:6- 12- 17- 19-34

Fig. 4. Chromatogram of separated antifungal compounds by TLC

Identification of bioactive metabolites GC:

The identification of the compounds excreted by the most active biocontrol endophytes was confirmed based on the molecular formula and peak area %. This area clarifies the quantity of the compound present in the active band. GC- MS analysis proved the presence of different compounds for both isolates which introduced 24 and 13 different compounds for *Streptomyces albidoflavus strain Emeranaa* MK203832.1 and *Bacillus subtillus* (Tables 4&5, respectively). These compounds enclosed phenols, antioxidants, fatty acids and antifungal compounds.

Table 4. Antimicrobial compounds identified from *Streptomyces albidoflavus* strain *Emeranaa* MK203832.1 through GC-MS analysis

Peak	Compound name	Molecular formula	% Peak area	Property
1	Benzaldehyde,3-methoxy-4-[(trimethylsilyl)oxy]-, O-methyloxime Or Vanillin, MO TMS	$C_{12}H_{19}NO_3Si$	2.03	Phenolic aldehyde, antifungal, Carpinella *et al.*, 2003
2	Undecane	$C_{11}H_{24}$	13.80	Antifungal, Manna and Kim, 2018
3	2-Pyridinepropanoic acid, .alpha.-methyl-.beta.-oxo-, ethyl ester or N-acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	0.72	Antimicrobial antioxidant
4	1,7-Di(2,5-dimethylphenyl)-2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisila	$C_{22}H_{36}O_4Si_3$	1.14	N.I.
5	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	0.88	Precursor in industry and medical field. CSR, 2010
6	1,1,3,3,5,5,7,7-Octamethyl-7-(2-methylpropoxy)tetrasiloxan-1-ol	$C_{12}H_{34}O_5Si_4$	1.33	N.I.
7	1,1,1,3,5,7,9,9,9-Nonamethylpentasiloxane	$C_9H_{30}O_4Si_5$	0.86	N.I.

Continued

8	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	4.14	Antifungal, antioxidant, biosurfactant, allelochemical, Varsha *et al.*, 2015
9	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	0.89	Antifungal Moustafa *et al.*, 2013
10	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	0.68	Antibacterial, Hassan, 2016
11	Hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	$C_{12}H_{38}O_5Si_6$	3.75	Antifungal, Sivanandhan *et al.*, 2018
12	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	$C_{16}H_{32}O_2$	1.16	Antifungal, antioxidant, Liu *et al.* (2008)
13	2-Pyridinepropanoic acid, .alpha.-methyl-.beta.-oxo-, ethyl ester or N-acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	1.25	Antimicrobial antioxidant
14	Hexadecanoic acid, methyl ester or palmitic acid	$C_{17}H_{34}O_2$	11.67	Antioxidant, Antimicrobial, pesticidal, nematicidal Banaras *et al.*, 2017
15	Silicone grease, siliconfett		0.59	N.I.
16	Cyclohexane, 1,1'-[1-(2,2-dimethylbutyl)-1,3- propanediyl] bis-	$C_{21}H_{40}$	0.76	N.I.
17	2-Pyridinepropanoic acid, .alpha.-methyl-.beta.-oxo-, ethyl ester or N-acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	0.61	Antimicrobial antioxidant
18	Hexadecanoic acid, 14-methyl-, methyl ester or methyl palmitate	$C_{18}H_{36}O_2$	0.81	Antimicrobial, Banaras *et al.*, 2017
19	9-Octadecenoic acid (Z)-, phenylmethyl ester or Oleic acid	$C_{25}H_{40}O_2$	0.76	Antifungal, Liu *et al.*, 2008
20	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]m or Linolinic acid	$C_{27}H_{52}O_4Si_2$	0.62	Antifungal-Antioxidant, Liu *et al.*, 2008 and Pohl *et al.*, 2011
21	9-Octadecenoic acid (Z)-, methyl ester	$C_{18}H_{34}O_2$	4.80	Antioxidant, Antimicrobial Abubakar & Majinda, (2016)
22	trans-9-Octadecenoic acid, pentyl ester	$C_{23}H_{44}O_2$	0.85	Amyl elaidate
23	Methyl stearate or Stearic acid	$C_{19}H_{38}O_2$	5.36	Antifungal- antioxidant
24	Decyl oleate or Oleic acid	$C_{28}H_{54}O_2$	40.54	Antifungal, Liu *et al.*,2008

N.I. = Non identified

Table 5. Antimicrobial compounds identified from *Bacillus subtillus* through GC-MS analysis.

Peak	Compound Name	Molecular Formula	Area %	Property
1	Butyl alcohol 1-D1	$C_4H_{10}O$	44.57	Biosurfactant
2	Glycolaldehyde dimer	$C_4H_8O_4$	5.25	Morpholin synthesis, Kim *et al.,* 2001
3	Butanone,3-hydroxy-		9.4	Biosurfactant
4	1,2,3-Propanetriol or glycerol	$C_3H_8O_3$	1.51	Glycerin
5	Acetic acid, hydroxy- or Glycolic acid	$C_2H_4O_3$	2.06	Industrial uses
6	2,3-Butanediol, [R-(R*,R*)]-	$C_4H_{10}O_2$	29.75	Biosurfactant
7	Undecane	$CH_3(CH_2)_9CH_3$	0.65	Antifungal, Mannaa and Kim, 2018
8	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	0.65	Antifungal, antioxidant Biosurfactant, Dharni *et al.*, 2014
9	Hexadecanoic acid, methyl ester or palmitic acid	$C_{17}H_{34}O_2$	0.98	Antifungal, Liu *et al.,*2008 Pohl, *et al.,* 2011
10	Octadecanoic acid, methyl ester or Oleic acid	$C_{27}H_{56}O_2$	0.48	Antifungal, Liu *et al.*, 2008
11	Butyl citrate	$C_{18}H_{32}O_7$	0.79	Biosurfactant,plasticizer, Takeshita *et al.*, 2011
12	Hexanedioic acid, bis(2-ethylhexyl) ester or DEHA	$C_{22}H_{42}O_4$	0.74	Plasticizer, *Bizzari et al.* 2009, cosmetic products, Gottschalck and McEwen 2004, and pesticides PMRA 2005
13	Decyl oleate	$C_{28}H_{54}O_2$	3.17	Antifungal and cosmetics Liu *et al.*, 2008

Discussion

The obtained results showed that fifty endophytes were isolated from rice leaves on different five media. Only five isolates were effective against *Pyricularia oryzae* by inhibiting its growth on the tested media. Of these five isolates, two isolates were the most potent against *P. oryzae* and identified by 16S rRNA sequence as *Streptomyces albidoflavus Emeranaa* with accession no. MK203832.1 (isolate no.12) and was confirmed to the species level. Similar findings were obtained by Cook and Meyers (2003). However, *Bacillus subtillus* (isolate no.17) was identified according to biolog system as adopted by Wang *et al.* (2012).

Concerning the effect of filtrates of these two isolates, the obtained results showed that they were effective in inhibiting the mycelial growth of *P. oryzae* on individually treated PDA plates with superiority of *S. albidoflavus Emeranaa* strain MK203832.1 in *P. oryzae* growth obstruction. This may be due to their ability to

produce various metabolites that degrade the fungal cell walls, consequently inhibited the fungal growth of the pathogen. However, Alina *et al.* (2015) reviewed the ability of *B. subtillus* to produce different antifungal compounds *i.e*., chitinases, cellulolytic enzymes and antibiotic compounds. This was assured by scaning electron microscope technique which illustrated the distortion of *P. oryzae* hyphae. Thus, the production of antifungal metabolites by these endophytes makes them very promising biocontrol agents (Rajer *et al.*, 2017).

The efficiency of spraying the filtrates of the five active endophytes in reducing blast disease incidence in both treatments was promising. Particularly, the filtrates of *S. albidoflavus Emeranaa* strain MK203832.1 and *B. subtillus* when applied two days after inoculation with the pathogen. This may be due to the ability of these endophytes to produce plethora of bioactive secondary metabolites. These metabolites hinder the pathogen growth, so no infection symptoms appear. Similar results were found on several crops by many investigators (Bressan, 2003; Taechowisan *et al.*, 2003; Taechowisan, *et al.*, 2005 and Gangwar *et al.*, 2011) and this was assured by scanning electron microscope study.

S. albidoflavus Emeranaa strain MK203832.1 and *B. subtillus* recorded the highest Rf values in Thin layer chromatography test, which proved their most efficiency. So, this was promoting to more studies using GC-MS analysis to detect the bioactive compounds from the whole filtrates of these two isolates.

The detected compounds were categorized into phenols, antioxidants, alcohols and fatty acids. *S. albidoflavus* Emeranaa strain MK203832.1 and *B. subtilis* filtrates are known to produce many volatile organic compounds efficient in plant protection against pathogens whether as antioxidants or as antimicrobials (Agoramoorthy *et al.*, 2007; chandrasekharan *et al.*, 2008 and Lima *et al.*, 2011). These toxic volatile compounds; hexadecanoic acid, 9- octadecenoic acid and 2,4-Di-tert-butylphenol; were detected also in some plants; *Chenopodium album* (root extract), grassy weeds or other microorganisms extracts, *i.e*. *Lactococcus* sp.; *Pseudomonas monteilli* and actinomycetes (Ali *et al.*, 2017; Varsha *et al.*, 2015). In the present study, the two bioagents contain some similar compounds (*i.e*., palmitic acid, oleic acid, 2,4-Di-tert-butylphenol, undecane and decyl oleate) which supposed them to be a promising source of antifungal compounds and industry for better human life (*i.e*., vanillin, glycolic acid and glycoaldehyde dimmer).

These findings are in accordance with many workers (Kadoma *et al.*, 2009; Sang *et al.*, 2011; Sang and Kim, 2012; Dharni *et al.*, 2014 and Varsha *et al.*, 2015) who reported that, 2,4-Di-tert-butylphenol (DTBP) is an antioxidant or antifungal biosurfactant compound. As it inhibits sporulation and hyphal growth of many pathogenic fungi. *Bacillus subtilis* was reported as a potential antibiofilm against Streptococcus group A biofilm formation on seaweed. DTBP is the active principle compound for this antibiofilm resulting in changes in cell surface architecture with reducing thickness after treatment as reported by Viszwapriya *et al.* (2016). *Bacillus subtillus* filtrate proved to contain fatty acids *i.e*., palmitic and oleic acids which have role in plant protection against pathogens whether as antioxidant or antimicrobial as

mentioned by many workers (Agoramoorthy *et al.*, 2007, Chandrasekharan *et al.*, 2008; Liu *et al.* 2008 and Lima *et al.*, 2011).

GC-MS analysis detected some other compounds in *B. subtillus* filtrate such as: butyl alcohol, glycerin, butyl citrate, acetic acid hydroxyl (glycolic acid). Glycerin is used as a natural antimicrobial in food and cosmetics technologies (Idan *et al.*, 2017) in addition to its medical benefits (Stout and McKessor, 2012 and Mortensen *et al.*, 2017). This was in accordance with Cazorla *et al.* (2007) and Elkahoui1 *et al.* (2014) who found that *Bacillus* sp secrete butanolic compounds in their extracts and lipopeptides belonging to the surfactin, iturin and fengycin families. For example, fengycin is an antifungal lipopeptide complex produced by *B. subtillus* F-29-3 and the compound iso- hexadecanoic acid (1-16) is one of its structures (Vanittanakon *et al.*, 1986).

Streptomyces albidoflavus Emeranaa strain MK203832.1 filtrate proved to contain another fatty acid, stearic acid, which have role in plant protection against pathogens whether as antioxidants or as antimicrobials as Agoramoorthy *et al.* (2007) and Ali *et al.* (2017) found. Data are in accordance with Idan *et al.* (2017) who found that the presence of fatty acids *i.e.* stearic acid, oleic acid, palmitic acid in PDA medium treated with *Aspergillus niger* filtrate reduced the mycelium growth. In actinomyces filtrates, some other compounds were detected, such as, undecane, acetyl phenylalanine (a product of phenylalanine N- acetyltransferase in the pathway of phenylalanine metabolism) and vanillin which can be used as a precursor in morphine synthesis, which have a role in systemic resistance induction and have herbicidal and fungicidal activities (Carpinella *et al.*, 2003; Deba *et al.*, 2007 and Lee *et al.*, 2012).

Detection of components of these efficient endophytes filtrates interprets their mode of action in the lab or greenhouse experiments. All compounds play direct or indirect role in plant protection. Some actions belong to induced systemic resistance (ISR) with phenolic compounds which have antioxidant abilities (Pan *et al.*, 2017).

Others depend on chemical reactions created by butanolic compounds or fatty acids which are efficient as antibiotics. Some of these detected compounds act as biosurfactants *i.e.*, DTBP, butyl alcohol and butanone which are used as biodegradables. They are environmentally safe, stable under different conditions and can be produced from renewable inexpensive substrates when they are compared with synthetic compounds (Benincasa *et al.*, 2010 and De Faria *et al.* 2011).

To date, no report is available on the isolation of endophytes from rice plants especially, *S. albidoflavus* and *B. subtillus* and their potentiality to control blast disease in Egypt. However, optimization of fermentation, media and extraction process is needed for maximum better results.

A c k n o w l e d g e m e n t

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تقييم فعل الكائنات الميكروبية الداخلية المعزولة من أوراق الأرز ضد الفطر *Pyricularia oryzae* المسبب لمرض اللفحة

قسم أمراض الأرز- معهد بحوث أمراض النباتات- مركز البحوث الزراعية- الجيزة - مصر

يُعد مرض لفحة الأرز المتسبب عن بيركيولاريا أوريزا (ماجنابورس جريزيا هيبيرت) أكثر الأمراض خطورة والذي يُهاجم نبات الأرز أثناء موسم زراعته. ويهدف هذا البحث لاستبدال تطبيق المبيدات بمواد طبيعية وصديقة للبيئة ضد المرض. تم عزل خمسين عزلة ميكروبية مختلفة داخلية من أوراق الأرز بمحطة بحوث سخا الزراعية على خمس بيئات مختلفة. تم اجراء اختبارات المعمل لدراسة النشاط التضادى لهذه العزلات ضد فطر اللفحة، حيث أظهرت خمس عزلات فقط نشاط تضادي بتجارب المعمل والصوبة. وقد فصلت المواد المضادة الثانوية لهذه العزلات بالتحليل الكروماتوجرافى واختبار كفاءتها ضد المسبب المرضى بالمعمل. عزلتان كانتا الأكثر فاعلية تم تعريفهما: ستربتومايسس البيدوفلافاس سلالة ايميرانا أم ك ١.٢٠٣٨٣٢ والأخرى باسيلس ساتلس. وبتحليل الجى سى ام اس لراشح هاتين العزلتين تم تعريف ٢٤ و١٣ مركب على التوالي لهذه العزلات.