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RESEARCH PAPERS

Antifungal potential and defense gene induction in maize against Rhizoctonia root rot by seed extract of *Ammi visnaga* (L.) Lam.

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Summary. Methanol extracts from five medicinal plants (*Eucalyptus tereticornis* Sm., *Ammi visnaga* (L.) Lam., *Azadirachta indica* A. Juss., *Rheum Palmatum* L., and *Adansonia digitata* L.) were assessed *in vitro* for antifungal activity against *Rhizoctonia solani* Kühn, the causal agent of Rhizoctonia root rot of maize. All tested extracts showed antifungal activity with varied extents. *Ammi visnaga* (khella) extract showed the greatest activity compared with the untreated experimental controls. Observations using transmission electron microscopy showed ultrastructural changes in hyphal cells as a response to exposure to khella extract. Gas chromatography-mass spectrometric analysis of khella extract showed the presence of 69 compounds. The antifungal properties of the extracts were mainly attributed to their content of coumarins and fatty acids. In the greenhouse experiment, treatment of maize plants with khella extract at 15% gave the least incidence of Rhizoctonia root rot. Results of DD-PCR showed up- and down- regulations of some genes in maize as response to the treatment with khela extract. Identification of the randomly selected genes from DD-PCR revealed that they were defense-related, as S-domain class receptor-like kinase 3 and glutathione-S-transferase1. Real-time PCR showed induction in the gene expression of the pathogenesis-related protein chitinases (2.36 fold) and thaumatin-like proteins (8.99 fold) by treatment with khella extract at 15%, which was greater expression than detected at 10 and 20%. This indicates triggering effects from the extract on the maize immune system against the *R. solani* infection in a concentration-dependent manner. The efficient, low-cost and eco-friendly characteristics of khella extract indicate that it could be used for the control of *Rhizoctonia* root rot of maize.

Key words: chitinase, electron microscopy, real-time PCR, *Rhizoctonia solani*, thaumatin-like proteins.

Introduction

Maize (*Zea mays* L.) is one of the most important cereals in the world, and is the second most important cultivated crop in Egypt. The area under maize cultivation in 2015 was 1,039,241 ha with a total production of 8,100,000 tons (FAOSTAT, 2016).

Rhizoctonia root rot, caused by *Rhizoctonia solani* Kühn, is an economically important disease worldwide, which causes serious yield losses in many crops (Rashad *et al*., 2012; Al-Askar *et al*., 2014a). The common disease control strategy mainly relies on the use of chemical fungicides such as benomyl, carbendazim and tolclofos-methyl. The use of chemical fungicides poses concerns about the risks for human and animal health, degradation and persistence in the environment and possible carcinogenicity.

Medicinal plant extracts may provide an eco-friendly, effective, and economic approach for the control of Rhizoctonia root rot of maize. Their rich content of bioactive compounds, including quinines, phenols, tannins and flavonoids, may have multifunctional activities against the phytopathogenic fungi (Al-Askar and Rashad, 2010; Al-Askar *et al*., 2014b; Baka and Rashad, 2016).

In addition to direct antifungal activity, medicinal plant extracts may induce plant resistance against invading fungal pathogens, acting as "biotic elicitors"

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(Stangarlin *et al*., 2011). One of the most important defense responses is the induction of pathogenesis-related (PR) proteins in plants. Kagale *et al.* (2011) demonstrated that application of aqueous extracts from *Zizyphus jujuba* and *Ipomoea carnea* induced PR proteins in rice plants against *R. solani*. Akladious *et al.* (2015) reported that seed-soaking in *Ocimum basilicum* extract induced various PR proteins in tomato plants against *Fusarium* wilt. Using SDS-PAGE analysis, a new protein band was expressed from treated tomato plants compared to untreated plants.

The aims of the present study were to determine the *in vitro* antifungal activities of the methanol extracts from the medicinal plants forest red gum, khella, neem, rhubarb, and tabaldi against *R. solani*, to evaluate the efficacy of application of the most active extracts against Rhizoctonia root rot of maize under greenhouse conditions, and to investigate inductive effects of extracts on the PR protein synthesis in maize plants.

Materials and methods

Fungal isolate and maize cultivar

An isolate of the fungal pathogen *R. solani* AG-2-2 IIIB (No. PPR1723), isolated from a diseased maize plant, was obtained from the Plant Pathology Reseach Institute, Agricultural Research Center, Egypt. The isolate was maintained on potato dextrose agar (PDA) slants and stored at 4°C until needed. Maize seeds (var. Giza 2) were provided by the Central Administration for Seed Certification, Egypt.

Medicinal plant extracts

Healthy tissue samples of the medicinal plants listed in Table 1, namely; forest red gum (leaves and seeds), khella (seeds), neem (fruits and leaves), rhubarb (roots), and tabaldi (fruits), were collected from the market or from some local farms in Egypt. For each sample, 50 g of the air-dried material were ground and added to 200 mL of 80% (v/v) methanol in a dark bottle, and extracted by shaking for 72 h. The crude extracts were then each filtered using Whatman filter paper No. 1, then centrifuged at 5000 rpm for 30 min. The supernatant was collected in a clean dark bottle. Using a rotary evaporator unit, the solvent was evaporated from the sample and the residue was dissolved in 50 mL of distilled water and stored at 4°C until used.

Antifungal activity assay

Medicinal plant extracts were tested *in vitro* for the inhibition of *R. solani* mycelial growth using an agar plate technique. A suitable quantity of the plant extract was added to sterilized PDA medium before solidification to obtain a final concentration of 10%

Table 1. Medicinal plants tested in the present study.

Latin name	Common name	Description	Family	Therapeutic uses	References
Eucalyptus tereticornis Sm.	Forest red gum	Fast-growing tree	Myrtaceae	Antibacterial, antifungal, insecticidal, acaricidal, herbicidal effects.	Barbosa *et al*., 2016
Ammi visnaga (L.) Lam.	Khella	Annual herb	Apiaceae	Respiratory, cardiovascular, liver and kidney disorders, antifungal, antibacterial, and antiviral.	Hashim*et al*., 2014; Sabry*et al*., 2014
*Azadirachta indica*A. Juss.	Neem	Medium-sized tree	Meliaceae	Antifungal, antibacterial, anthelmintic, antiperiodic, antiseptic, diuretic action.	Alzohairy, 2016
Rheum Palmatum L.	Chinese rhubarb	Rhizomatous perennial herb	Polygonaceae	Antifungal, antibacterial, antiviral, antipyretic, antineoplastic, purgative, and anti-inflammatory.	Miraj, 2016
Adansonia digitata L.	Baobab or tabaldi	Massive tree	Malvaceae	Antifungal, antibacterial, antiviral, anti-oxidant, anti-malarial, and anti-inflammatory.	Rahul *et al*., 2015

(v/v) of each extract. PDA plates supplemented with 1 mL of the the fungicide, Rhizolex-T (0.2% : a.i. tolclofos-methyl) were used as positive controls, and another set of PDA plates treated with sterile water was used as negative controls.The PDA plates were then each inoculated with a 8 mm-diameter disk of 5-d-old culture of *R. solani*. Three replicate plates were used for each treatment. The plates were then incubated at 25±2°C for 72 h. The diameter of each fungal colony was measured daily and the average growth reduction relative to the negative controls was calculated.The most active plant extract was tested again at the concentrations 11, 13, and 15% to determine the concentration at which complete inhibition of fungal growth occurred.

Transmission electron microscopy (TEM)

The inhibitory effects of the khella extract on mycelium of *R. solani* was examined using TEM. Sample fixation was carried out using the method described by Hayat(2000), with glutaraldehyde in 3% phosphate buffer at pH 6.8, followed by 1% osmium tetroxide in the same buffer. The samples were then dehydrated in a gradual ethanol series (10–100%, for 10 min each). The specimens were embedded in epoxy resin. Sections were obtained using an ultramicrotome, and were then stained with uranyl acetate followed by lead citrate. The sections were examined using JEOL model JEM-1230 transmission electron microscope.

Gas chromatography-mass spectrometry (GC-MS)

The khella extract was analyzed using the GC-MS-QP 2010 system (Shimadzu, Japan) to identify its constituents. The sample was injected at the rate of 0.9 $mLmin^{-1}$ via an Rtx-5MS column (30 m × 0.25 mm, 0.25 μm thick) using helium as a carrier at 260°C. The oven temperature was 61°C using the split mode of injection at 50:1. The ion source temperature was 230°C, while the interface temperature was 250°C, at an ionization volatage of 70 eV. The retention times and mass spectra of the constituents were used to identify the extract constituents using the NIST11library (Gaithersburg, USA).

Greenhouse experiment

For inoculum preparation, *R. solani* was grown in flasks containing sterilized maize grains:sand (2:1 v/v), and incubated at 25°C for 12 d. Plastic pots (20 cm diam.) filled with sterile soil (clay:sand, 2:1 v/v) were used. Fourteen days before planting, the soil was infested by the inoculum at the rate of 2% (v/v). Maize grains were individually soaked in the khella extract at concentrations of 0, 10, 15 or 20% for 12 h. The chemical fungicide Rhizolex-T was used as a positive control at the recommended dose of 3 gkg^{-1} seeds (seed dressing). For each treatment, five maize grains were sown in each pot. Five replicates were used for each treatment. All pots were watered as required and kept under greenhouse condition at 26/20°C day/night temperature. A completely randomized experimental design was used in this experiment. Forty five days after planting, all pots were evaluated for the incidence of *Rhizoctonia* root-rot. Percentages of seed rot, pre- and post-emergence damping off and plant survival were also recorded.

Differential Display Polymerase Chain Reaction (dd-PCR)

Total RNA extraction

Total RNA was extracted from maize leaves using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The obtained RNA was dissolved in diethyl dicarbonate-treated water then incubated with DNase for 1 h at 37°C to remove any DNA residues, and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Reverse Transcription (cDNA synthesis)

With a total volume of 25 μL, the reaction mixture contained; RNA (3 μL), 10mM dNTPs (2.5 μL), 10× buffer with $MgCl_2$ (2.5 μL), oligo (dT) primer (4 μL) and reverse transcriptase enzyme (Biolabs) (0.2 μL). The PCR was carried out using a SureCycler 8800 thermocycler (Agilent Technologies), at 37°C for 2 h and 65°C for 20 min.

dd-PCR

The dd-PCR was carried out using seven different primers (Table 2). With a total volume of 25 μL, the reaction mixture contained; 10× buffer (5 μL), 10 pmol primer (5 μL), 25mM $MgCl_2$ (2.5 μL), 10mM dNTPs (2 μL), cDNA (1 μL) and Taq DNA polymerase (0.2 μL) (Promega). The PCR was carried out using a SureCycler 8800 thermocycler (Agilent Technologies), with one cycle at 95°C for 3 min, 40 cycles (95°C for 45 sec, 30°C for 60 sec, 72°C for 1 min), final cycle 72°C for

Table 2. Primers used in the molecular studies

Primer	Type	Sequence (5'-3')
Endogluconase(PR1)	Sense	CTTATCAGATCTCAATGGAGAAATC
Polyphenol oxidase (PPO)	Sense	CATGCTCTTGATGAGGCGTA
	Antisense	CCATCTATGGAACGGGAAGA
Chitinase A(PR3)	Sense	GCCCATGGAAGGAATCAGTTATGCGCAAAT
	Antisense	GCGGATCCCAACGCACTGCAACCGATTAT
Thaumatin-like protein(PR5)	Sense	ACCTCTTCCGCTGTCCTC
	Antisense	GAAGACGACTTGGTAGTTGC

5 min. The PCR products were separated using 2% agarose gel electrophoresis with a DNA marker, photographed using a gel documentation system, and the unique bands were cut and kept at -20°C.

Sequencing and phylogenetic analysis

To purify the selected bands, a gel extraction purification kit (Maxim Biotech INC) was used. The nucleotide sequences were aligned and identified compared to the other genes available in the GenBank database. The obtained sequence was subjected to NCBI-BLAST searches, aligned and compared with other similar sequences from GenBank using MEGA 4 software (Tamura *et al.*, 2007). The phylogenetic analysis was carried out using the bootstrap method, and the phylogenetic tree was generated based on the UPGMA statistic method.

Quantitative Real-Time PCR (qRT-PCR)

The qRT-PCR was carried out using a TOPreal™ qPCR 2X PreMIX SYBR Green (Enzynomics) according to the manufacturer's instructions. Chitinase A and thaumatin-like proteins primers were used (Table 2). The qRT-PCR was carried out using a real-time PCR system (Rotor-Gene Q, Qiagen), with one cycle at 95°C for 15 min, and 45 cycles (95°C for 10 sec, 60°C for 15 sec, 72°C for 30 sec). A β-actine gene was used as a reference gene (forward 5'-GTGGGCCGCTCTAGGCACCAA-3' and reverse 5'-CTCTTTGATGTCACGCACGATTTC-3') (Saleha, 2010). The comparative method (Ct) was used to analyze the data (Schmittgen and Livak 2008).

Statistical analyses

All results were analyzed using the statistical analysis software CoStat (version 6.4). Comparisons between means was made using Duncan's multiple range test at $P \leq 0.05$ (Duncan, 1955).

Results

Screening of antifungal activity of plant extracts

The methanol extracts of the tested medicinal plants were screened for their antifungal activities. The average reductions of *R. solani* growth in response to treatment with the methanol extracts at 10% concentration are shown in Table 3. All tested extracts showed inhibitory activity to varied extents, compared to the untreated experimental control. Inhibition of mycelium growth increased with time. At day 3, the greatest growth inhibition (79.5%) was obtained with the khella extract, while the least growth inhibition was recorded for the forest red gum extract (leaves). In order to achieve complete inhibition of mycelium growth, khella extract was tested at concentrations of 11, 13, and 15% (Table 4). Complete inhibition was obtained at the concentration of 15%.

Electron microscopy

TEM observations of the fungal cells from untreated controls showed normal morphology. Thin electron-lucent cell walls and plasma membranes enclosing the cytoplasm were observed, with each cell containing a nucleus, electron-lucent glycogen par-

Table 3. Mean growth reduction (%) of *Rhizoctonia solani* mycelium when exposed to methanol extracts of various medicinal plants at 10% concentrations.

Plant extract	Incubation time (d)		
	1	2	3
Control	0.0 f	0.0 f	0.0 f
Rhizolex-T (0.2 %)	60.5 a	73.7 a	80.6 a
Baobab (fruit)	12.7 d	18.4 d	25.3 d
Eucalyptus (leaves)	7.8 e	11.4 e	13.2 e
Eucalyptus (seeds)	32.5 c	37.7 c	44.3 c
Khella (seeds)	58.4 a	71.1 a	79.5 a
Neem (fruits)	37.5 b	44.6 b	54.5 b
Neem (leaves)	35.4 bc	40.3 c	46.3 c
Rhubarb (roots)	35.2 bc	40.2 c	45.9 c

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 4. Mean growth reduction (%) of *Rhizoctonia solani* mycelium when exposed to different concentrations of Khella methanol extracts.

Plant extract	Extract concentration (%)		
	11	13	15
Control	0.0[1] b[2]	0.0 c	0.0 c
Rhizolex-T (0.2%)	80.3 a	80.3 b	80.3 b
Khella (seeds)	86.5 a	94.6 a	100 a

[1] Each value represents the mean of three replicates.
[2] Values within each column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

ticles and electron-dense inclusions inside the vacuoles (Figure 1A). After the treatment of the fungal mycelia with khella extract considerable ultrastructural changes were obsrved. The cellular responses included thickening of the cell walls (electron-dense), increases in the numbers of mitochondria, presence of electron-dense bodies and absence of the glycogen particles (Figure 1B).

GC-MS

The methanol extract of khella was analyzed using GC-MS to identify chemical components. Sixty-nine chemical compounds in different pproportions were detected (Table 5; Figure 2). The major constituents (with proportions) included; 8-methoxypsoralen (17.5%), glycerin (8.5%), 3-piperidin-1-yl-1-(4-piperidin-1-yl-phenyl)-pyrrolidine-2,5-dione (7.2%), 2-monopalmitin (6.6%), palmitic acid (6.3%), khellin(5.5%), xanthyletin (4.5%), cyclopenta[c] pyran-7-carboxaldehy (4.5%), oleic Acid (3.5%), and 2-(1-Hydroxy-1-methylethyl)-2,3-dihydrofuro3,2-chromen-7-one (3.3%). Other constituents present in small proportions were; acetic acid (2.4%), pyranone (2.1%), stearic acid (1.8%), methyl acetate (1.7%), oxycarboxin(1.5%), 7-Hydroxycoumarin (1.4%), and benzoic acid (1.2%). Other components were present in minor proportions.

Greenhouse experiment

Effects of the seed treatments with khella extract at three concentrations (10, 15, and 20%) on the Rhizoctonia root rot were investigated. Means of disease incidence are presented in Table 6. Compared with the untreated control, treatment of the uninfected maize grains with the khella extract at concentration of 15% led to significant increase in the mean percentage of germinated seeds. All of the tested treatments of khella extract reduced disease symptoms (percentages of pre- and post-emergence seed rot) in the infected plants, and increased proportions of surviving plants compared with the untreated-infected controls. Treatment with the khella extract at 15% was the most effective treatment in comparison with the chemical fungicide, resulting in 80% plant survival, followed by khella extract at concentrations of 20 or 10%.

dd-PCR

The dd-PCR profiles of the maize samples revealed considerable variation of gene expression in response to the infection with *R. solani* and/or to treatment with the khella methanol extract at the three tested concentrations. A total of ten up-regulated genes, in the size range of 250–700 bp, and five down-regulated genes ≅ 220–450 bp, were observed

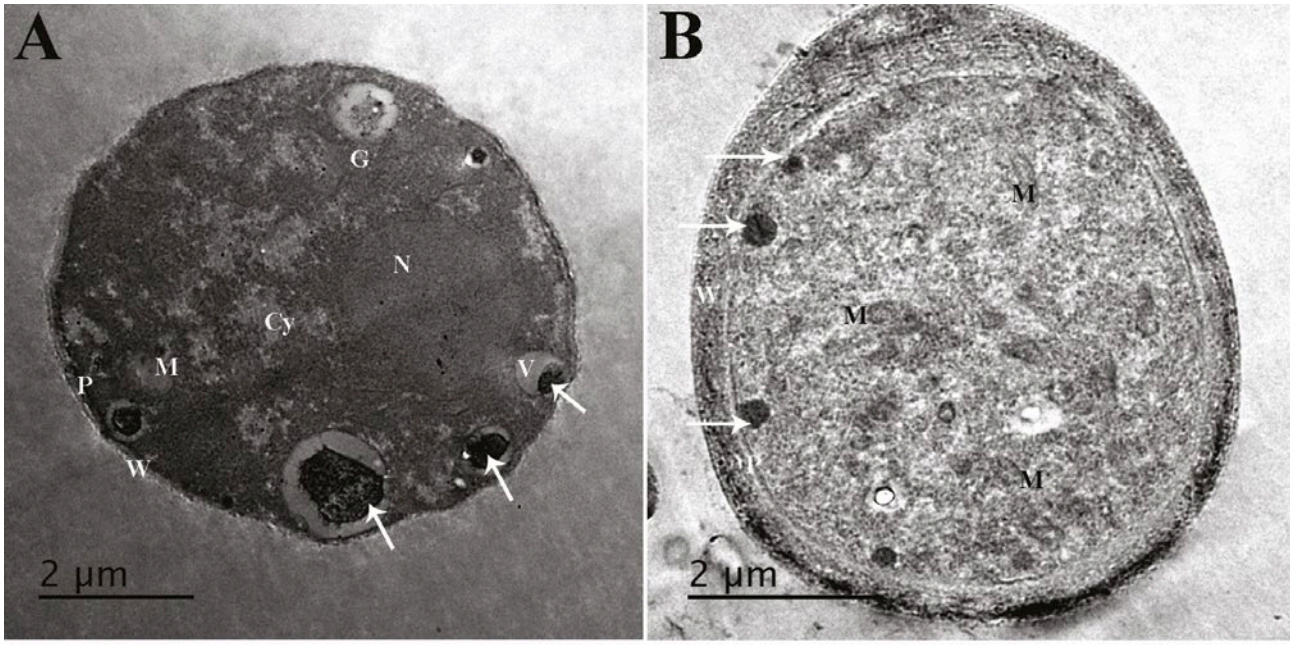

Figure 1. TE micrograph of a transverse sections of *Rhizoctonia solani* hyphae. A. Untreated (control) showing a thin electron-lucent cell wall (W) with a plasma membrane (P). The cytoplasm (Cy) contains a nucleus (N) and electron-lucent glycogen particles (G). Note electron-dense inclusions (arrows) inside the vacuoles (V). B. Hypha treated with the khella extract (10%), showing a thick electron-dense cell wall (W), plasma membrane (P), numerous mitochondria (M) and electron-dense bodies (arrows). Note the absence of glycogen particles.

in the band profile of the PR5 forward primer (Figure 3A). Compared with the controls, three up- and two down-regulated genes from the treatment with P+K10, six up- and three down-regulated genes from the P+K15 treatment, and one up- and three down-regulated genes from the P+K20 treatment were recorded. In the band profile of the PR5 reverse primer, a total of 11 up-regulated genes in the range of 300–1500 bp, and 23 down-regulated genes ≅ 200–600 bp were observed (Figure 3B). Three up- and five down-regulated genes from the treatment P+K10 treatment, three up- and ten down-regulated genes from the P+K15 treatment, and five up- and eight down-regulated genes from the P+K20 treatment were observed, compared to the control treatment. In the band profile of the PR3 forward primer, a total of six up-regulated genes of ≅ 220–550 bp, 14 down-regulated genes ≅ 280–400 bp and one monomorphic gene with molecular size of 220 bp were observed (Figure 3C). Four up- and two down-regulated genes from in the P+K10 treatment, two up- and three down-regulated genes from the P+K15 treatment, nine down-regulated genes from the P+K20 treatment, and one monomorphic gene from all of the treatments were observed, compared to the controls. In the band profile of the PR3 reverse primer, a total of five up-regulated genes ≅ 300–700 bp, and 12 down-regulated genes ≅100–300 bp were observed (Figure 3D). Nine down-regulated genes from the P+K10 treatment, three up- regulated genes from the P+K15 treatment, and two up- and ten down-regulated genes from the P+K20 treatment were observed in comparison with the controls. In the band profile of the PPO forward primer, the results showed a total of six up-regulated genes ≅ 300–600 bp and three down-regulated genes ≅ 250–400 bp were observed (Figure 3E). Three down-regulated genes were observed from both the treatments P+K10 and P+K15, and five up-and three down-regulated genes were observed from the P+K20 treatment. In the band profile of the PPO reverse primer, a total of six up-regulated genes ≅ 250–800 bp and eight down-regulated genes ≅ 100–500 bp were observed (Figure 3E). Two up- and three down-regulated genes were observed from the P+K10 and P+K15) treatments, and three up- and two down-regulated genes were observed

Table 5. Chemical constituents of the methanol extract of khella, determined using GC-MS.

Peak #	Retention time (min)	Peak area (%)	Compound name
1	1.539	1.66	Methyl acetate
2	1.746	0.32	Propanal,2-methyl-3-phenyl
3	1.859	2.42	Acetic acid
4	1.934	0.73	Chloroform
5	2.194	0.10	1-Butanol-3-, methyl-, acetate
6	2.267	0.71	Methyl neopentyl ketone
7	4.202	0.17	Pyrazine, methyl-
8	4.802	0.27	2-Furanmethanol
9	5.678	0.41	2-butoxyethanol
10	5.835	0.32	Dihydro- 2(3H)-furanone
11	6.110	0.72	Pentanoic acid, 5-oxo-, methyl ester
12	8.314	8.48	Glycerin
13	9.497	0.38	Isopentanal
14	9.691	0.42	1,2-Cyclobutanedicarboxylic acid
15	10.267	0.12	α-Amino-γ-butyrolacton
16	10.476	2.07	Pyranone
17	11.149	1.23	Benzoic acid
18	11.864	0.31	Benzofuran, 2,3-dihydro-
19	12.355	0.29	(R)-carvone
20	13.130	1.24	1-Di(tert-butyl) silyloxypentadecane
21	13.270	0.08	1,1-suberoylbis(1H-benzotriazol)
22	13.591	0.34	4-Hydroxy-2-methylacetophenone
23	14.075	0.19	Triacetin
24	14.226	0.24	2,6-Dimethoxy phenol
25	15.731	0.17	2-Propenoic acid, 3-(2-hydroxyphen)
26	16.393	0.09	Dihydro-beta-ionone
27	16.784	1.09	Levoglucosan
28	17.625	0.18	4-Methyl-2,5-dimethoxybenzaldehyd
29	18.162	0.24	1,2,3,4-Butanetetrol, tetraacetate
30	18.523	0.44	Apiol
31	18.581	0.35	Megastigmatrienone
32	20.284	0.03	Myristic acid
33	20.378	0.33	4-Acetylisocoumarin
34	20.551	0.10	Syringic acid
35	20.854	0.08	Pregn-4-ene-3,20-dione
36	21.276	0.06	4-[n-Propylamino]-2,5-dimethoxyan
37	21.448	0.13	Isopsoralen
38	21.752	1.37	7-Hydroxycoumarin
39	22.136	0.08	Isocytosine
40	22.555	0.28	trans-2,3-Epoxyoctane
41	22.903	6.34	Palmitic acid
42	23.053	0.33	2,3-Dihydro-1-Benzofuran
43	23.105	4.45	Cyclopenta[c]pyran-7-carboxaldehy
44	23.622	0.44	2H,8H-Benzo[1,2-b:5,4-b′] dipyran-2
45	24.093	17.5	8-methoxypsoralen
46	24.518	4.45	Xanthyletin
47	24.983	3.55	Oleic Acid
48	25.040	1.49	Oxycarboxin
49	25.224	1.77	Stearic acid
50	25.290	0.61	Tricyclo[7.2.1.0(3,8)]dodeca-3(8),4,6-triene-2,12-dione,6-methoxy-9-methyl
51	25.465	0.49	Dimethylmalonic acid, 4-acetylphen
52	25.650	0.17	2-isopropenyl-2 3-dihydrofuro 3 2-g chromen-7-one
53	25.706	0.66	1,3-Isobenzofurandione, 5,5′-[(1-methylethylidene)
54	25.962	5.53	khellin
55	26.686	0.45	1,3-Diolein

(Continued)

Table 5. (Continued).

Peak #	Retention time (min)	Peak area (%)	Compound name
56	26.969	0.37	2-Acetylphenyl diethylborinate
57	27.135	3.34	2-(1-Hydroxy-1-methylethyl)-2,3-dihydrofuro3,2-chromen-7-one.
58	27.391	0.17	1-Methylverbenol
59	27.455	0.40	7H-Furo[3,2-g][1]benzopyran-7-one
60	27.874	0.18	Ledol
61	28.203	0.61	2-Tetradecyloxirane
62	28.352	0.07	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester
63	28.420	6.64	2-Monopalmitin
64	28.538	0.29	Pyrrolo[3,2-g]quinoline, 9-methoxy-2,3.5,7-tetramethyl
65	28.711	1.15	Psoralen
66	29.344	7.21	3-piperidin-1-yl-1-(4-piperidin-1-yl-phenyl)-pyrrolidine-2,5-dione
67	29.440	0.81	α-Monostearin
68	29.704	0.63	4-(2 3-dihydroxy-3-methylbutoxy)furo(3 2-g) chromen-7-one
69	31.434	0.21	trimethylsilyl ether

from the P + K20 treatment, compared to the control treatment. In addition, one gene (350 bp) was highly expressed in all treatments except P+K10, where this gene completely disappeared. In the band profile of the PR1 forward primer, a total of seven up-regulated genes ≅ 200–1200 bp, and nine down-regulated genes ≅100–600 bp were observed (Figure 3G). Three down-regulated genes from the P+K10 treatment, one down-regulated gene from the P+K15 treatment, and three up- and four down-regulated genes from the P+K20 treatment were observed compared to the control treatment. In the band profiles obtained for all tested primers, different up- and/or down-regulated genes were observed in response to treatments with K10, K15, or K20 in the absence of the fungal pathogen, compared with the untreated control.

Two up- and two down-regulated genes were randomly selected for sequencing and identification (Table 7). The partial nucleotide sequences of the selected genes were aligned, compared with other genes available in the GenBank database, and the phylogentic trees were constructed (Figure 4A and 4B). The phylogentic analysis showed that the two up-regulated genes were S-domain class receptor-like kinase 3, which is closely related to the S-domain class receptor-like kinase 3 gene of *Z. mays* (NM001112185), with nucleotide sequence similarity of 70%, and glutathione-S-transferase 2, which is closely related to the GSTF2 genes EU960197, EU953165 and NM001111942 of *Z. mays* with nucleotide sequence similarity of 88% (Figure 4A). The two down-regulated genes were kinesin-related protein 2, which is related to kinesin motor protein gene (U92845) of *Ustilago maydis* with nucleotide sequence similarity of 69%, and MADS-domain transcription factor 4, which is closely related to the MADS4 gene (XM014087306) of *Trichoderma atroviride* with nucleotide sequence similarity of 90% (Figure 4 B).

qRT-PCR

To provide accurate and reproducible detection at the gene level, mRNA of chitinase and thaumatin-like protein genes were amplified and quantified using qRT-PCR (Figure 5A and 5B). Both genes displayed susceptibility-associated expression patterns in the case after the P+K15 treatment. Data obtained revealed a significant increase (2.36-fold) in chitinase gene expression after the P+K15 treatment compared with the control treatment (Figure 5A). On the other hand, data illustrated in Figure 5B showed that the P+K15 treatment led to a significant increase (8.99-fold) in the expression of the thaumatin-like protein gene compared with the control treatment. In contrast, this gene exhibited much less activation after the K10 or P+K10 treatments.

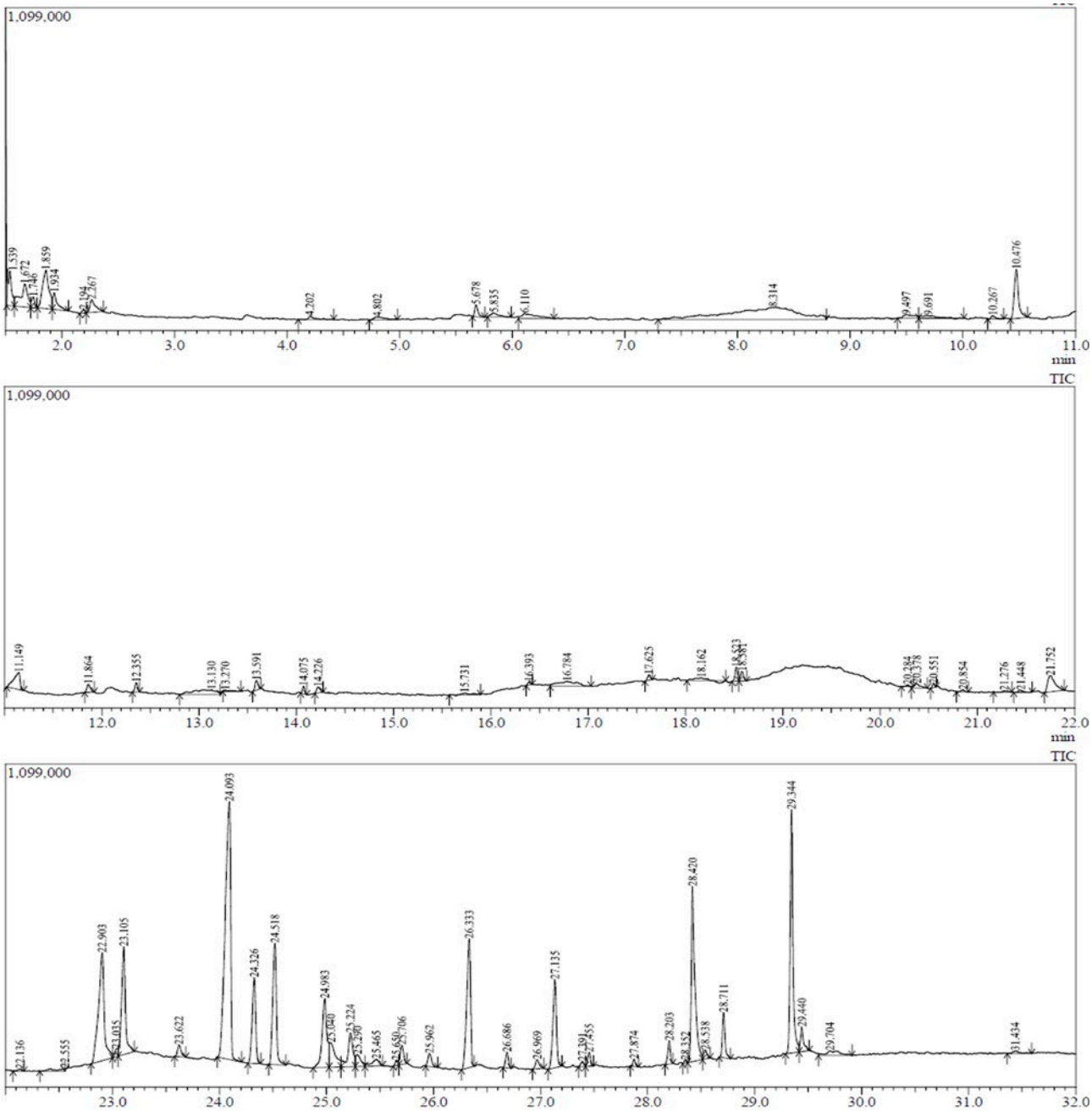

Figure 2. GC-MS chromatogram of the chemical constituents of the khella methanol extract.

Discussion

This study has examined the use of some medicinal plant extracts as a potential biocontrol agents against the Rhizoctonia root rot of maize. The *in vitro* results confirmed the antifungal activity of all tested plant methanol extracts against *R. solani* with varied extents. These results are in accordance with those reported in other studies (Al-Askar and Rashad, 2010; Atiq *et al*., 2014). Various medicinal plant extracts exhibit antifungal activities against phytopathogenic

Table 6. Mean incidence of different expressions of Rhizoctonia root rot for maize plants treated with different concentrations of the khella methanol extract.**

Treatment[a]	Seed rot (%)	Pre-emergence (%)	Post-emergence (%)	Survival of plants (%)
C	4[b] c[c]	0 d	0 e	96 b
K 10%	4 c	0 d	0 e	96 b
K 15%	0 d	0 d	0 e	100 a
K 20%	0 d	0 d	0 e	100 a
P	16 a	48 a	32 a	4f
P + F	4 c	8 c	8 d	80 c
P + K 10%	8 b	12 b	16 b	64 e
P + K 15%	4 c	8 c	8 d	80 c
P + K 20%	4 c	8 c	12 c	76 d

[a] C = Control, K = Khella, P = Pathogen, and F = Fungicide (Rhizolex-T).
[b] Each value represents the mean of five replicates.
[c] Values in each column followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

fungi. This fungitoxicity may be attributed to one or more of the respective bioactive compounds, such as quinines, phenols, tannins and flavonoids. Different mechanisms for their antifungal activities have been reported. These includes; disruption of cell wall/membrane integrity (Cho *et al*., 2013), inhibition of enzyme activities (Muhsin *et al*., 2001), induction of oxidative stress (Lemar *et al*., 2005), DNA damage (Cardoso-Lopes *et al*., 2008), inhibition of protein synthesis (Upadhyay *et al*., 2015), and/or down-regulation of the expression of virulence- or toxin-related genes (Yin *et al*., 2015). Among the medicinal plant extracts tested in the present study, the methanol extract from khella was the most inhibitory of mycelial growth of *R. solani*. These results are in agreement with those of El-Mougy and Abdel-Kader (2007), who reported the antifungal activity of the methanol extract from khella at a concentration of 8% against the plant pathogenic fungi *Alternaria solani, Fusarium solani, Macrophomina phaseolina* and *R. solani*.

Our TEM observations showed the antifungal impacts of the khella extract on the ultrastructure of *R. solani* hyphae. Different cellular changes were observed, indicating more than one mechanism by which the bioactive constituents in the khella extract can exert their antifungal effects. The cell wall thickening in the treated fungal mycelium supports the disruption of the cell wall integrity mechanism. Li *et al.* (2009) reported the induction of the cell wall thickening in *Aspergillus nidulans* as the mode of action of the antifungal compound dihydromaltophilin. Cell wall thickening affects fungal cells by limiting the exchange between the intra- and extra-cellular contents and inhibiting fungal growth (Beauvais and Latgé, 1991).The increase in the numbers of mitochondria indicates the induction of oxidative stress mechanisms. Alteration in the mitochondrial dynamics (fission/fusion) is the first marker of oxidative stress in eukaryotic cells. In fungi, mitochondrial fission, which is a cellular stress response, is probably a pro-survival action against harmful oxidative stress (Knorre *et al*., 2013). In addition, our TEM observations revealed the presence of electron-dense particles and the absence of glycogen granules in the fungal cells treated with the khella extract. Under normal conditions, fungal cells accumulate carbohydrates as glycogen granules, while under stress, they tend to stimulate recycling of glycogen molecules instead of inducing their accumulation (Parrou *et al*., 1997). All of these observations provide evidence for the antifungal activity of the khella extract against the cells of *R. solani*.

Our results from the GC-MS analysis of the khella extract showed the presence of 69 compounds. Of these, there were some that possess well-known and widely investigated antifungal activities against various phytopathogenic fungi, including *R. solani*. The major antifungal constituents included; the coumarins 8-methoxypsoralen, khellin, xanthyletin, um-

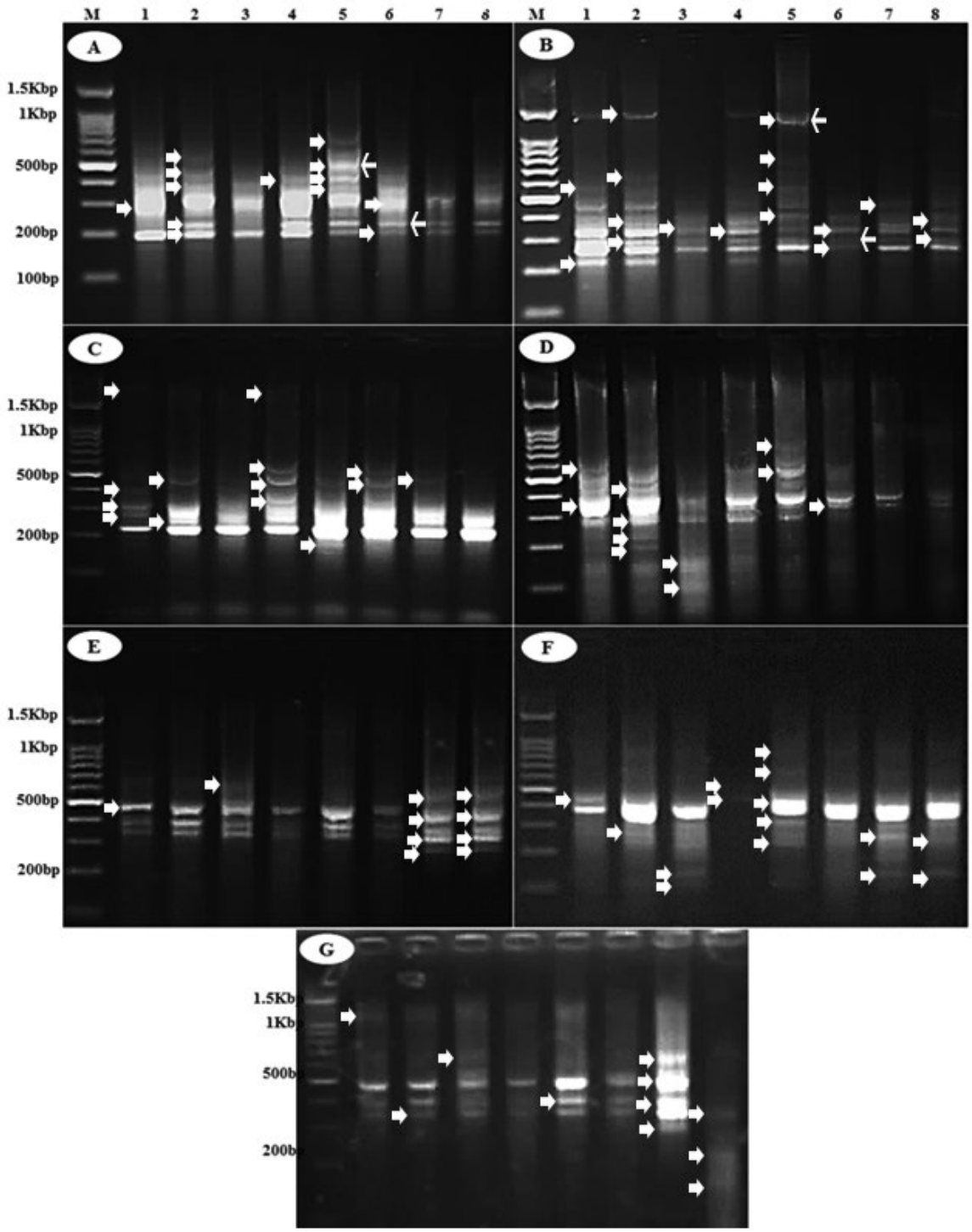

Figure 3. DNA fingerprinting from dd-PCR using different arbitrary primers; PR5F (A), PR5R (B), PR3F (C), PR3R (D), PPOF (E), PPOR (F), and PR1F (G) with reference to the affected bands (thick arrows) and the bands selected for sequencing (thin arrows). M, 1.5 Kbp DNA marker; 1,untreated maize (control); 2,infected with *R. solani*; 3,treated with khella extract 10%; 4,infected and treated with khella extract 10%; 5, treated with khella extract 15%; 6, infected and treated with khella extract 15%; 7, treated with khella extract 20%; and 8,infected and treated with khella extract 20%.

Table 7. DNA nucleotide sequences of the selected genes from dd-PCR.

No.	Gene	Function	Similarity (%)	Length (bp)	Regulation
1	S-domain class receptor-like kinase 3 (pk3)	Receptors-mediated signaling (recognation and binding with the pathogen-associated molecular patterns).	91	207	Up
2	Kinesin-related protein 2 (kin2)	Motor protein (organizing the mitotic spindle assembly during the cell division).	90	295	Down
3	MADS-domain transcription factor 4	MADS-box transcription factors are involved in various vital processes in fungi including cell development and pathogenicity.	100	100	Down
4	Glutathione-S-transferase 1 (GSTs)	GSTs gene family regulates a group of multifunctional enzymes in plant that have significant roles in the detoxification of xenobiotics in response to different stresses.	90	1200	Up

belliferone (Montagner *et al.*, 2008; Arif *et al.*, 2009) and the fatty acids palmitic acid, oleic acid, stearic acid and 2-monopalmitin (Walters *et al.*, 2004; Jung *et al.*, 2013). In addition, some minor antifungal components viz. benzoic acid, oxycarboxin, and levoglucosan (Shabana *et al.*, 2008; Kumar and Bhaskar, 2012; Jadhav and Gawai, 2015) were also detected. The antifungal activity of the khella extract can probably be attributed to the synergistic effects of these fungitoxic compounds. The antifungal mechanisms that may be utilized by coumarins and phenolic acids include; disruption of cell membranes, suppression of cell wall formation and the mitochondrial dysfunction (Freiesleben and Jäger, 2014). Antifungal mechanisms that may be attributed to fatty acids include; disruption of cell membrane permeability, inhibition of enzymes and fatty acid metabolism (Pohl *et al.*, 2011).

The greenhouse results revealed the efficiency of khella extract at a concentration of 10% for control of Rhizoctonia root rot in maize. In spite of the large number of studies dealing with the therapeutic effects of khella against some human diseases, there are few reports that document their effects for reducing plant diseases. Low incidence of *Tomato yellow leaf curl virus* was recorded by El-Dougdoug *et al.* (2007) within a period of spraying of khella extract. In addition to the direct antifungal activity of medicinal plant extracts against the fungal pathogens, they can have indirect effects on host plants. They may trigger host defense against pathogens via induction of plant immune systems (Akladious *et al.*, 2015; Abkhoo and Jahani, 2017). Plant responses include up-regulation of several defense-related genes, induction of various pathogenesis-related (PR) proteins, accumulation of phytoalexins, deposition of lignin and programmed cell death (Goel and Paul, 2014, 2015).

At the molecular level, results of dd-PCR showed varied up- and down- regulation of some genes in response to the treatment of maize with khela extract, indicating the induction/suppression of some genes. Identification of the randomly selected genes from dd-PCR revealed that the genes were mostly defense-related. Of these, two genes were up-regulated in maize plants after treatment with khella extract. Receptor-like kinases, which are cell surface localized and transmembrane proteins, play important roles in plant resistance and growth. As the first steps of triggering the immune responses, they rapidly recognize and bind with pathogen-associated molecular patterns (Greeff *et al.*, 2012). Moreover, Zhou *et al.* (2017) reported that successful microbial infections depend on avoiding or blocking of these plant recognition receptors by production of microbial effector proteins to hide the pathogenic patterns or suppress these receptors-mediated signals. These reports are in agreement with results from the present study, where

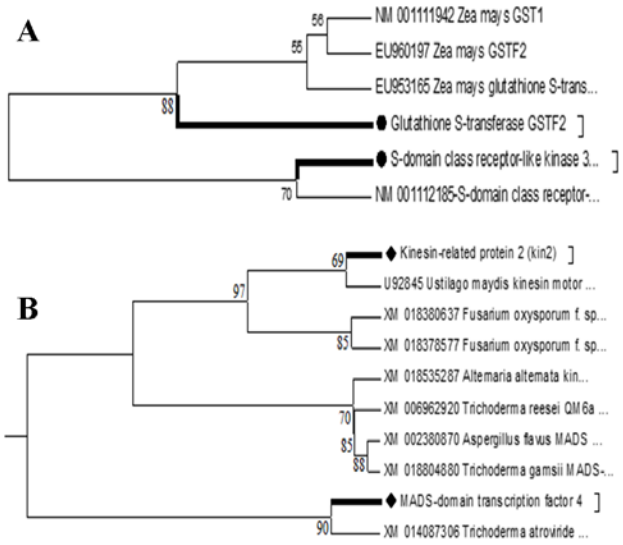

Figure 4. Dendrograms illustrating the phylogenetic relationships of the selected up- (A) and down-regulated (B) genes in comparison with the DNA nucleotide sequences of other genes listed in the GenBank database.

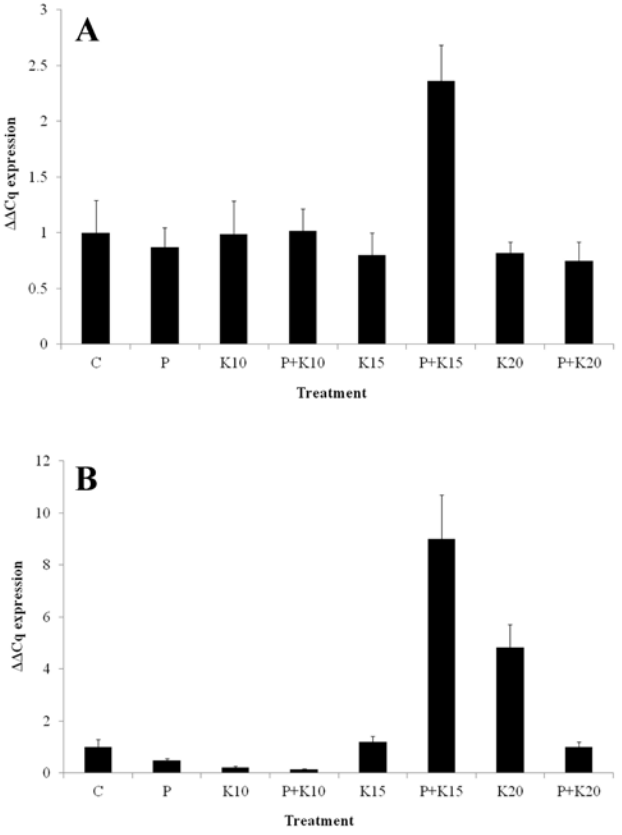

Figure 5. Gene expression using qRT-PCR of chitinases(A) and thaumatin-like proteins(B) in maize plants infected with *R. solani* and/or treated with khella extract at 10, 15 or 20%. C = control, P = pathogen, and K = khella extract.

treatment of maize with khela extract up-regulated the expression of S-domain of kinase receptor gene in maize plants to prevent the *R. solani* infection.The glutathione-S-transferase 1 gene is involved in the detoxification of xenobiotics in plants in response to the oxidative stress (Islam *et al*., 2015). Ahn *et al*. (2016) reported the expression of the glutathione S-transferase gene in the leaves of *Vitis flexuosa* in response to the infection with *B. cinerea, Elsinoe ampelina,* or *Rhizobium vitis* as important defense responses. This report is in accordance with the results from the present this study, and supports the triggering effect for plant resistance induced by the khela extract. On the other hand, two identified genes were down-regulated in the pathogen cells by the treatment with the khela extract. Kinesin-related protein 2 (kin2), which belongs to a group of motor proteins, plays an important role in organizing the mitotic spindle assembly during the cell division (Blackwell *et al*., 2017). Lehmler *et al.* (1997) reported that Δkin2 mutants of the phytopathogenic fungus *U. maydis* were severely affected in their hyphal extension and pathogenicity. Blocking or down-regulation of this gene negatively affects fungal growth and virulence. These results are in accordance with our findings. Treatment with the khela extract down regulated the expression of this gene in the pathogenic fungus, which explains the antifungal property. The second down-regulated gene was MADS-domain transcription factor 4 of *R. solani*. Members of the MADS-box family are significant transcription factors and are involved in various vital processes in fungal cells. Zhang *et al*. (2016) reported that the MADS-box gene, Bcmads1, is necessary for growth and virulence potential of *Botrytis cinerea*, and is indispensable for sclerotium production in this pathogen. Rocha *et al*. (2016) found that the MADS-box gene, rlmA, regulates cell wall integrity and virulence in *A. fumigates*. Down regulation of this gene in *R. solani* cells by the treatment with the khela extractalso support their antifungal properties.

One of the plant defense responses to pathogen attack is the production of antifungal compounds to suppress infections. Chitinases and thaumatin-like proteins are pathogenesis-related proteins produced

by attacked plants, giving protection by inhibiting hyphal growth of invading pathogens (Jalil *et al*., 2015; Souza *et al*., 2017). These data are in accordance with our findings from qRT-PCR, which showed induction in expression of both genes (especially the thaumatin-like protein gene) in maize plants infected-treated with khella extract at 15% concentration, indicating their triggering of the plant immune system against the *R. solani* infection. In contrast, treatments with khella extract at 10 or 20% led to gene depression for the two genes, indicating that the induction of both gene expressions by khella extract is concentration-dependent. Our results are in agreement with other studies that have reported concentration-dependent stimulation of defense-related gene expression by inducers. β-aminobutyric acid induced chitinase gene expression when applied at 20 mM to grape fruit against *Penicillium digitatum*, the green mold of grape fruit, more than at greater or lesser concentrations (Porat *et al*., 2003). Yamakawa *et al.* (1998) also reported induction of the acidic PR1 gene expression and accumulation of acidic PR2, PR3, and PR5 proteins against tobacco mosaic virus after the exogenous application of spermine on tobacco leaves, in a dose-dependent manner.

In conclusion, the present study has demonstrated the effects of methanol extracts of khella in triggering the maize immune system against *R. solani*. Considering their efficient, economical and eco-frindly characteristics, we recommend khella extract has considerable potential for controlling Rhizoctonia root rot of maize.

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