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RESEARCH PAPERS

Extract from *Curcuma longa* L. triggers the sunflower immune system and induces defence-related genes against Fusarium root rot

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Summary. Sunflower (*Helianthus annuus* L.) has economic value worldwide Fusarium root rot, caused by *Fusarium solani* (Mart.) Sacc., is the most important disease in sunflower crops, causing considerable economic losses. Seed treatment with a turmeric aqueous extract was tested for control of Fusarium root rot. Gas chromatography-mass spectrometry analysis of the extract identified three major constituents; *ar*-curcumin, camphor and α-turmerone. The greenhouse experiment showed that incidence and severity of sunflower root rot were significantly reduced after treatment with turmeric extract. Plant growth parameters also increased 2 and 4 weeks after inoculation. In addition, treatment with turmeric extract triggered the sunflower immune system, as indicated by the induction of host phenolic content and activity of antioxidant enzymes (peroxidase and phenylalanine ammonia lyase). Differential display-PCR of the treated plants showed distinct profiles of gene expression in response to the treatments. Of the four bands randomly selected for sequencing and identification, three up-regulated genes that encode defence-related proteins (glutathione S-transferase 6, ascorbate peroxidase, and defensin) were detected. A time-course real-time quantitative PCR was carried out on mRNA of the defence-related genes defensin and chitinase of the treated sunflower seedlings. After 14 d, treatment with turmeric extract enhanced the expression levels of chitinase by > nine-fold and defensin genes by > four-fold. Based on these results, we recommend treatment of sunflower seeds with turmeric extract as a disease management method against Fusarium root rot.

Key words: chitinase, defensin, *Fusarium solani*, *Helianthus annuus*, qRT-PCR.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most economically important crops worldwide. It is used for edible oil production, as a livestock fodder, bird feed, and for various industrial purposes. Plant diseases, caused by different soil- and seed-borne fungi, are among the most important factors affecting sunflower production worldwide, and can result in high yield losses (Rashad *et al*., 2012; Al-Askar *et al*., 2014b). Fusarium root rot, caused by *Fusarium solani* (Mart.) Sacc., is the most important disease of sunflower (Ghoneem *et al*., 2014). The symptoms of this disease include root, collar, and stem rots, seedling damping-off and wilting, and stunting of the affected plants (Harveson and Woodward, 2016).

Control of fungal plant diseases using synthetic fungicides is possible but undesirable due to potential deleterious effects on human and animal health, microorganisms, and the environment (Maitlo *et al*., 2015). In addition, new resistant strains of fungal pathogens may develop resulting from repeated use of fungicides, especially those with specific sites of activity (Kitchen *et al*., 2016). Therefore, there is a requirement for new effective and eco-sustainable alternatives for control of fungal plant pathogens.

"Natural" control, using antifungal extracts of medicinal plants, may provides valuable solutions to address this issue (Al-Askar and Rashad, 2010;

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Al-Askar *et al*., 2014a; Ghoneem *et al*., 2016). These plant extracts can have good efficacy against the phytopathogenic fungi, due to the synergistic antifungal activity of their various phytochemical constituents (Gahukar, 2012; Baka and Rashad, 2016).

Curcuma longa L., common name turmeric, is an herbaceous plant in the Zingiberaceae. It is widely grown in south Asia and used as a spice, food flavouring, and as a human cosmetic colouring agent. Turmeric has been traditionally used for many pharmaceutical purposes, including as antioxidant, anti-inflammatory, anti-diabetic, hepatoprotective and anti-carcinogenic treatments (Nasri *et al*., 2014). In addition, antifungal, antibacterial, antiviral and antiparasitic activities have also been reported (Moghadamtousi *et al*., 2014). The present study was carried out to investigate the potential of an aqueous extract of turmeric for activity against Fusarium root rot of sunflower, and the genetic and biochemical effects of the extract on sunflower plants.

Materials and methods

Plant material and extraction

Fresh rhizomes of turmeric were obtained from a local market (El-Hawag company, Cairo, Egypt). These were cut into small pieces and air dried for 1 week. The air-dried samples were then ground to fine powder, of which 100 g was added to 1 L of sterilized distilled water and left for 24 h. The extract was filtered twice using a filter paper (Whatman no. 1). The resulting filtrate at this concentration (10%) was used as a crude extract and kept at 4°C for subsequent investigations.

Fungal inoculum

An isolate of *F. solani* (GenBank: KJ831188.1) was provided by the Department of Plant Protection and Biomolecular Diagnosis, Arid Lands Cultivation Research Institute, Egypt. The fungus was maintained on potato carrot agar held at 4°C until used. For fungal inoculum preparation, Petri plates containing potato dextrose agar (PDA, Difco) were inoculated with discs (5 mm diam.) of 7-d-old culture of *F. solani.* The plates were incubated for 10 d at 25±2ºC under cool white light. Fungal conidia were harvested using sterile water and the conidial suspension was adjusted at 3×10^5conidia mL^{-1}.

Analysis of the turmeric extract

Constituents of the turmeric aqueous extract were identified using a GC-MS-QP 2010 gas chromatography-mass spectrometry (GC-MS) system (Shimadzu), equipped with a flame-ionization detector and a Rtx-5MS column (30 m × 0.25 mm, 0.25 µm thickness), using helium gas as the carrier. The constituent compounds were identified by matching the mass spectrum of individual compounds with those in the NIST11 library (Gaithersburg, Maryland, USA).

Pot experiment

Plastic pots (15 cm diam.), previously sterilized using 5% formadehyde solution, were filled with sterile sandy-clay soil at 1 kg per pot. Sunflower seeds were obtained from a local market. They were surface sterilized using 1% NaOCl solution for 4 min, then washed twice using sterile water and dried between two sheets of sterile filter paper. In each pot, five apparently healthy sunflower seeds were sown and irrigated as necessary. For seed treatment with turmeric extract, sunflower seeds were soaked in the aqueous extract for 12 h before planting. For the experimental control treatment, five seeds were soaked in sterile water for 12 h before planting. Fungal infection was achieved by adding 5 mL of the *F. solani* conidial suspension around the hypocotyls of 3-week-old seedlings. Ten pots were treated with water only and served as an experimental control treatment. The applied treatments were as follow; untreated control (C), treated only with turmeric extract (E), inoculated with the pathogen (P), or treated with turmeric extract + inoculated with the pathogen (E+P). For each treatment ten replicate pots were used. The pots were arranged in a completely randomized design in a greenhouse maintained at 24/16°C (day/night), under 12 h photoperiod and at 60% relative humidity.

Analysis of plant growth parameters

Two and 4 weeks after inoculation, 15 plants for each treatment were carefully uprooted, washed using tap water and data of germination percentage, plant height, shoot and root weights and numbers of leaves were recorded. Shoot dry weights were recorded after oven drying at 80°C for 48 h.

Disease assessments

Disease severity (DS) and incidence (DI) of Fusarium root rot were assessed 2 and 4 weeks after inoculation. Disease severity was evaluated using the 0–5 scale described by Filion *et al*. (2003).

$$\text{Disease severity}(\%) = \frac{\Sigma ab}{AK} \times 100$$

where, a= number of diseased plants with the same infection degree, b = infection degree, A = total number of the evaluated plants, and K = the greatest infection degree. Disease incidence was calculated for each treatment according to the following equation:

$$\text{Disease incidence}(\%) = \frac{a}{A} \times 100$$

where, a = number of diseased plants, and A = total number of evaluated plants.

Biochemical analyses

For each treatment, 15 plants were collected 2 and 4 weeks after inoculation, and were analyzed for total phenol content using Folin Ciocalteau reagent (Sigma-Aldrich) according to the method of Maliak and Singh (1980). Extraction and assay for peroxidase (POX) was accomplished using the methods described by Maxwell and Bateman (1967), and for phenylalanine ammonia-lyase (PAL) enzyme as described by Beaudoin-Eagan and Thorpe (1985).

Molecular analyses

Plant samples from the four treatments at threetimes (7, 14, or 28 d after inoculation) were harvested and immediately frozen for subsequent differential display PCR (DD-PCR) molecular analyses.

RNA extraction and cDNA synthesis

Extraction of total RNA was carried out from the sunflower roots (0.5 g) using an RNA extraction kit (Qiagen) following the manufacturer's instructions. The obtained RNA was incubated with DNase for 1 h at 37°C and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). An RT-PCR kit (Omniscript RT; Qiagen) was used for the synthesis of cDNA. Each 20 µL reaction mixture contained: 2.5 µL of 5× buffer, 2.5 µL of $MgCl_2$, 2.5 µL of 2.5 mM dNTPs, 4 µL oligo (dT) 10 as primer (20 pmol μL^{-1}), 2 µg RNA and 0.2 µL (5 unit μL^{-1}) reverse transcriptase enzyme (Omniscript RT, Qiagen)., the RT-PCR amplification was performed using a thermal cycler (Promega), at 42°C for 1 h and 65°C for 20 min.

PCR amplification and sequence of potential resistance genes

PCR amplification of cDNA was carried out on RNA from sunflower samples collected 14 d after inoculation, using three arbitrary primers (A2, A4 and R2) (Table 1). These sequence 5′ primers were selected and used based on their reproducibility in previous investigations (Pinto *et al*., 2004; El-Bakatoushi, 2011). Each 25 µL reaction mixture contained 10 ng cDNA, 2.5 µL of 5× buffer, 2.5 µL of $MgCl_2$, 2.5 µL of 2.5 mM dNTPs, 1 µLprimer (10 pmolμL^{-1}), and 0.2 µL (5 unit μL^{-1}) Taq DNA polymerase (Promega). The PCR was carried out using a SureCycler 8800 thermocycler (Agilent Technologies) and the following conditions: one cycle at 94°C for 5 min, 35 cycles (each at 95°C for 30 sec, 30°C for 1 min, and 72°C for 2 min), and a final cycle at 72°C for 10 min. To overcome the non-reproducibility of the DD-PCR technique, biological (different plants of the same treatment) and technical (different amplifications of the same template DNA) replications were performed in triplicates. The PCR-amplified products were gel eluted and purified using a purification kit (Maxim Biotech Inc.). Sequencing of the PCR products was subsequently accomplished using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer) on an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer; Perkin Elmer). The resulting sequences were aligned and subjected to BLAST search to assign putative identities. The nucleotide sequences obtained were submitted to the GenBank database.

Quantitative Real-Time PCR (qRT-PCR)

Specific primers of the defence-related genes defensin and chitinase (Table 1) and the reference gene *β*-actine (forward 5′-GTGGGCCG CTCTAGGCACCAA-3′ and reverse 5′-CTCTTTGAT GTCACGCACGATTTC-3′), were used (Saleha, 2010). For sunflower plant samples collected at 7, 14, and 28 d after inoculation, qRT-PCR was carried out using TOPreal™ qPCR 2X PreMIX SYBR Green (Enzynomics) according to the manufacturer's instructions. The Rotor-Gene Real Time thermo-cycler (Qiagen) was programmed at 95°C for 1 min, followed by 40 cycles

Table 1. Nucleotide sequence of the primers used for DD-PCR.

Primer		Sequence	Reference
A2		5′-GAAACGGGTGGTGATCGC-3′	
A4		5′-GGACTGGAGTGTGATCGC-3′	
R2		5′-CAGGCCCTTC-3′	
Chit	F	5′-CTGCAGTGTCAGCAGCTGAT-3′	Mazeyrat *et al*., 1998
	R	5′-CTGCACCAGATGGGCGATTT-3′	
Def	F	5′-GTGAGAAGGCAAGCCAGACA-3′	Hu *et al*., 2003
	R	5′-TCAAGGTTTGGCTGTCGCCT-3′	

each at 95°C for 15 sec, 55°C for 15 sec and 72°C for 20 sec. The comparative method (Ct) was used to analyse the data (Schmittgen and Livak, 2008).

Statistical analyses

The data obtained were statistically analyzed using the CoStat system (CoHort Software), version 6.4 (CoStat, 2005). The data were firstly subjected to analysis of variance (ANOVA), and the means comparisons were carried out using the Duncan's multiple range test (Duncan, 1995) at $P \leq 0.05$.

Results and discussion

GC-MS analysis of turmeric extract

Seven compounds were identified in the aqueous extract from turmeric rhizomes (Table 2). The major constituents were *ar*-curcumin (26.9%), camphor (18.6%), and α-turmerone (14.9%). Four constituents, octadiene, methyl hexyne, β- pinene, and cyclooctene, were also present in lesser proportions. These results are similar to those of Singh *et al*. (2010).

At least 235 compounds have been identified from different parts of turmeric plants (Li *et al*., 2011). In rhizomes, curcuminoids are the main components with varying content depending on source of plants, agricultural conditions, geographical location, and extraction method. Curcuminoids are yellow diarylheptanoid pigments, which represent the principal active compounds (Lv and She, 2010).

Effects of turmeric extract treatment on root rot of sunflower

The results presented in Table 3 show that infection with *F. solani* led to high levels of disease incidence and severity that gradually increased with time after inoculation. Pre-treatment of sunflower seeds with turmeric aqueous extract before planting significantly reduced root rot incidence and severity compared to the untreated inoculated treatment.

These results are similar to those of Islam and Faruq (2012) for turmeric extracts used against seedling damping-off of tomato, eggplant and chilli pepper. Antifungal activity of turmeric extract has also been reported by Wongkaew and Sinsiri (2014) against the fungal pathogens *Alternaria alternata, Pythium* sp., and *F. oxysporum* f. sp. *lycopersici*. This fungitoxicity can be attributed to the chemical constituents of turmeric extract. Among curcuminoids that constitute the major components, the polyphenolic compound curcumin has been reported as an antifungal, antibac-

Table 2. Chemical constituents of aqueous extract from turmeric rhizomes.

Peak #	Retention time (min)	Peak area (%)	Height	Compound Name
1	6.40	5.80	23657	β- pinene
2	14.18	26.94	88121	*ar*-curcumin
3	21.30	18.55	63180	Camphor
4	25.76	1.62	35408	Cyclooctene
5	31.64	10.81	41003	Octadiene
6	48.35	14.85	31658	α-tumerone
7	51.70	9.60	13549	Methyl hexyne

Table 3. Mean Fusarium root rot incidence and severity in 2 or 4 weeks after different treatments were applied to sunflower seeds.

Treatment*	Disease incidence (%)		Disease severity (%)	
	2 weeks	4 weeks	2 weeks	4 weeks
Control (C)	0^{c**}	0^{c}	0^{c}	0^{c}
Extract (E)	0^{c}	0^{c}	0^{c}	0^{c}
Pathogen (P)	40.3 ± 1.13^{a}	55.4 ± 1.20^{a}	37.3 ± 0.93^{a}	44.3 ± 1.01^{a}
Pathogen + Extract (P+E)	22.9 ± 0.97^{b}	37.3 ± 1.06^{b}	16.8 ± 0.87^{b}	23.1 ± 0.94^{b}

* Values are the means of fifteen replicates ± standard errors
** Values of each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

terial, antiviral, and antimalarial agent (Moghadamtousi *et al*., 2014).

In addition to numerous biological properties, recent studies have demonstrated the antifungal activity of curcuminoids against several fungi including phytopathogens (Alnashi and Abdel Fattah, 2016; Amalraj *et al*., 2016). This activity may be attributed to the polycationic nature and/or to inhibition of the synthesis of particular enzymes (Tongnuanchan and Benjakul, 2014). Camphor is also one of the major components of the turmeric extract. This terpenoid has high antifungal and antibacterial activities, in synergism with other extract components (Chen *et al*., 2013). α-turmerone is another major component of the turmeric extract, which possesses antifungal activity. Dias Ferreira *et al*. (2013) reported reductions in mycelium growth, conidial viability, sporulation, and morphological changes in *Aspergillus flavus* treated with the essential oil from *C. longa*. In addition, β-pinene has also been reported as a potent fungitoxic agent. da Silva *et al*. (2012) reported the complete killing of *Candida albicans* inoculum within 1 h from treatment with (+)-β-pinene. Therefore, the control activity of the turmeric extract against root rot of sunflower is likely to be due to synergistic antifungal effects of theses chemical constituents.

Effects of turmeric extract treatment on sunflower growth parameters

Mean values of the measured sunflower plant growth parameters 2 and 4 weeks after inoculation with *F. solani* and/or treatment with turmeric extract are presented in Table 4. These included reductions in the growth parameters of the inoculated plants compared to uninoculated plants. The negative effects of the resulting disease increased with time from inoculation. In contrast, increases in the percentage of the means of percent seed germination, plant height, shoot and root weights, and numbers of leaves were recorded in the plants treated with turmeric extract compared to the non-treated control plants. These parameters increased with time from inoculation. Furthermore, the turmeric extract treatment reduced the negative effects of disease on the plants compared to the untreated, inoculated plants. In addition to disease control, turmeric increased seed germination and seedling growth for the non-inoculated treatment.

These results are similar to those reported by Pal *et al*. (2013), who recorded increased germination proportions of soybean and maize seeds when treated with 0.5% turmeric extract compared to untreated seeds.

Biochemical changes

In addition to the direct antifungal effects of turmeric extract, another possible indirect mechanism may be responsible for disease control. Turmeric extract may trigger the defence responses of sunflower plants against the root rot pathogen. To test this hypothesis, effects of turmeric extract treatment on the total phenol content and POX and PAL activities were evaluated.

The data presented in Table 5 show the effects of turmeric extract on the total phenol content and

Table 4. Mean seed germination and parameters for sunflower plants, the seedlings of which received different treatments of *Fusarium solani* inoculation and/or turmeric extract application, at 2 and 4 weeks after treatment.

Treatment*	Germination (%)		Plant height (cm)		Shoot fresh weight (g)		Shoot dry weight (g)	
	2 w	4 w	2 w	4 w	2 w	4 w	2 w	4 w
Control (C)	79.7 ±1.2^{b**}	82.6 ± 0.5^{b}	7.6±0.10^{b}	16.9±0.9^{b}	3.2±0.21^{b}	6.6±0.15^{b}	0.48±0.04^{b}	0.99±0.03^{b}
Extract (E)	90.0 ±1.1^{a}	91.6±1.1^{a}	8.9±0.11^{a}	19.0±0.7^{a}	4.1±0.23^{a}	7.5±0.22^{a}	0.64±0.03^{a}	1.1±0.04^{a}
Pathogen (P)	65.0 ±1.0^{d}	69.7± 0.7^{d}	5.5±0.09^{c}	10.9±0.6^{c}	2.8±0.11^{c}	5.4±0.13^{c}	0.39±0.02^{c}	0.76±0.01^{c}
Pathogen + Extract (P+E)	76.0 ±1.0^{c}	79.3± 0.6^{c}	6.5±0.10^{bc}	15.7±0.6^{b}	3.1±0.10^{b}	6.5±0.21^{b}	0.48±0.03^{b}	0.99±0.03^{b}

Treatment*	Root fresh weight (g)		Root dry weight (g)		No. of leaves	
	2 w	4 w	2 w	4 w	2 w	4 w
Control (C)	1.6±0.13^{ab}	2.2±0.14^{ab}	0.10±0.03^{ab}	0.15±0.03^{ab}	2.5±0.40^{b}	3.6±0.50^{b}
Extract (E)	1.7±0.21^{a}	2.4±0.09^{a}	0.11±0.03^{a}	0.16±0.05^{a}	3.0±0.61^{a}	5.6±0.71^{a}
Pathogen (P)	1.1±0.17^{d}	1.5±0.15^{d}	0.06±0.01^{d}	0.09±0.02^{d}	2.0±0.33^{c}	2.6±0.64^{c}
Pathogen + Extract (P+E)	1.4±0.12^{c}	1.9±0.12^{c}	0.08±0.02^{c}	0.12±0.03^{c}	2.3±0.54^{bc}	3.0±0.36^{bc}

* Values are the means of fifteen replicates ±SE.
** Values of each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 5. Mean amounts of total phenol, and activities of peroxidase (POX) and phenylalanine ammonia-lyase (PAL) of sunflower plants, the seedlings of which received different treatments of *Fusarium solani* inoculation and/or turmeric extract application, at 2 and 4 weeks after treatment.

Treatment*	Total phenol (mg g^{-1} fresh wt)		POX (U min^{-1} g^{-1} fresh wt)		PAL (U min^{-1} g^{-1} fresh wt)	
	2 w	4 w	2 w	4 w	2 w	4 w
Control (C)	7.22 ±0.11^{d**}	6.27 ±0.08^{d}	17.55±0.09^{d}	7.16 ±0.06^{d}	16.83±0.15^{d}	11.16±0.07^{d}
Extract (E)	10.46 ±0.15^{c}	8.29 ±0.10^{c}	37.7±0.07^{b}	18.57±0.15^{b}	21.43±0.12^{b}	17.55±0.13^{b}
Pathogen (P)	13.70 ±0.21^{b}	10.39 ±0.16^{b}	26.74±0.10^{c}	9.24±0.13^{c}	18.61±0.11^{c}	14.37±0.11^{c}
Pathogen + Extract (P+E)	15.37±0.15^{a}	11.47 ±0.14^{a}	53.27±0.11^{a}	22.77±0.09^{a}	25.32±0.21^{a}	20.13±0.10^{a}

* Values are the means of fifteen replicates ±SE.
** Values of each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

POX and PAL activities in sunflower plants. Two weeks after inoculation with *F. solani*, significantly greater total phenol content and activities of POX and PAL were recorded for the plants inoculated with *F. solani* than for the uninoculated plants. Treatment of seeds with turmeric extract also increased amounts of these compounds compared with the untreated controls. The greatest amounts of total phenol, and greatest POX and PAL activities were recorded for the inoculated plants treated

with turmeric extract. However, these parameters decreased after 4 weeks.

Devi *et al*. (2013) reported induction of these defence enzymes in sunflower plants infected with *Alternaria helianthi.* Plants have several defence mechanisms against invading fungal pathogens. Among these, production of antimicrobial compounds, such as phenolics, and the release of defence-related enzymes have been reported (Ghoneem *et al*., 2016). Antimicrobial activity of phenolic compounds has protective roles in plants by limiting the spread of invading pathogens from infected to healthy cells (Mazid *et al*., 2011). There is also positive correlation between hydroxylation of phenolic compounds and their toxicity to microorganisms (Mansfield, 2000). Induction of the enzyme POX is another biochemical mechanism of plant defence (Ghoneem *et al*., 2016). In the presence of hydrogen peroxide, POX oxidizes the phenols to toxic quinines. In addition, POX has role in lignin and suberin polymerization in plant cell walls, providing physical barriers to prevent the spread of pathogens (Fagerstedt *et al*., 2010). PAL has also been reported to contribute to plant resistance. This enzyme catalyses the conversion of L-phenylalanine to trans-cinnamic acid, the first step in the biosynthesis of polyphenol compounds including lignin, flavonoids, phenylpropanoids and phytoalexins (Ngadze*et al*., 2012).

Molecular investigations

Differential display-PCR

Differential display-PCR of the sunflower plants showed distinct profiles of gene expression in response to the applied treatments. Nine up-regulated genes and eight down-regulated genes ranging from 100 to 1500 bp were detected in the differential display band patterns (Figure 1a, 1b, and 1c). For the band profile of the A2 primer, four up- and three down-regulated genes were detected in the of turmeric extract plus *F. solani* (E+P) treatment compared to the experimental control (Figure 1a). The band profile for the A4 primer showed four up-regulated genes and two down-regulated genes (Figure 1b). One up- and three down-regulated genes were detected for the E+P treatment in the R2 band profile R2 compared to the control treatment (Figure 1c).

Four up- and down-regulated genes from the three band profiles were selected and sequenced. The sequences were analyzed using BLAST and FASTA,

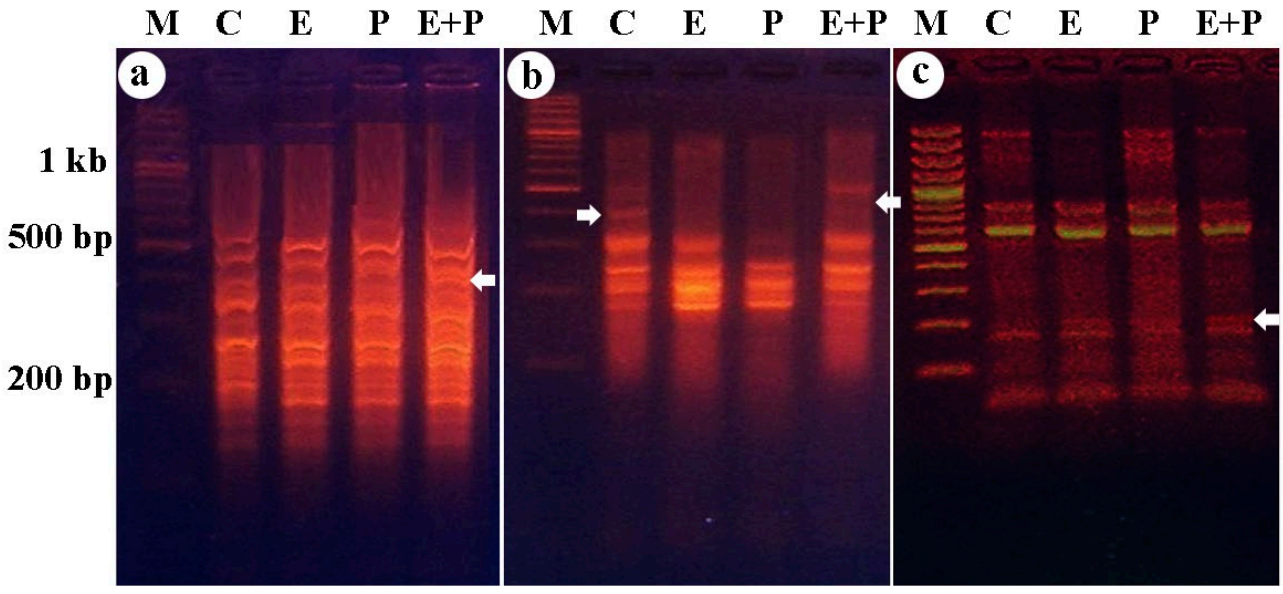

Figure 1. DNA fingerprinting of the DD-PCR using different arbitrary primers; A2 (a), A4 (b), and R2 (c) with reference to the selected bands for sequencing (arrows). M, 1.5 Kbp DNA marker; 1; untreated plant (C); 2, plant treated with turmeric extract (E); 3, plant inoculated with *Fusarium solani* (P), and 4, plant inoculated with *F solani* and treated with turmeric extract (E+P).

as well as through direct comparisons with other gene sequences available in the GenBank database. The four sequences were successfully identified according to the alignment analysis showing high similarity (97–100%), and were deposited in the Genbank database under the accession numbers listed in Table 6. The sequences were identified as the glutathione S-transferase 6 gene (up regulated), the Kunitz-type trypsin inhibitor gene (down regulated), the ascorbate peroxidase gene (up regulated), and the defensin-like protein encoding gene (up regulated).

The three up-regulated genes encode defence related proteins. The glutathione S-transferase gene family encodes a group of enzymes that are involved in key plant bioprocesses, such as hydroperoxide detoxification in response to various biotic and abiotic stresses (Islam *et al*., 2015). They have vital roles in the inhibition of necrosis caused by pathogens through detoxification of the organic hydroperoxides of fatty acids, thereby preventing cell death (Dixon *et al*., 2010). Induction of the glutathione S-transferase 6 gene is an important defence response to pathogen attack (Liao*et al*., 2014; Ahn *et al*., 2016). On the other hand, ascorbate peroxidase, an antioxidant enzyme, catalyses decomposition of hydrogen peroxide into water using ascorbate as an electron donor, protecting plant cells against adverse conditions caused by pathogen attack (Caverzan *et al*., 2012). Pechanova and Pechan, (2015) reported that triggering this enzyme is among the defence mechanisms of maize plants in response to infection by *A. flavus*, *Fusarium* spp., and *Curvularia lunata*. The defensin gene family encodes antimicrobial and cytotoxic proteins that are produced in plants as resistance mechanisms against pathogen attack (Abdallah *et al*., 2010). This is consistent with what has been observed in this study. Up-regulation of these genes confirms the triggering effect of turmeric extract on the immune system of sunflower plants against infection with *F. solani*.

Table 6. Nucleotide sequences of selected bands from the differential display-PCR.

No.	Gene	Length (bp)	Accession No.
1	Glutathione S-transferase 6 (GSTF6)	463	MF072371
2	Kunitz-type trypsin inhibitor mRNA	498	MF072366
3	L-ascorbate peroxidase 2 (APX) mRNA	538	MF072372
4	Defensin-like protein	220	MF072369

qRT-PCR

A time-course qRT-PCR was carried out with mRNA of the treated sunflower seedlings to assess the expression of defence-related genes of chitinase and defensin (Figure 2a and 2b). This showed an increase in the expression of both genes after 7 d, in response to inoculation with *F. solani* or treatment with the turmeric extract. Relative expression of both genes was greater in case of the inoculated seedlings treated with turmeric extract (treatment E+P) than in the other treatments. For all treatments, the relative expression

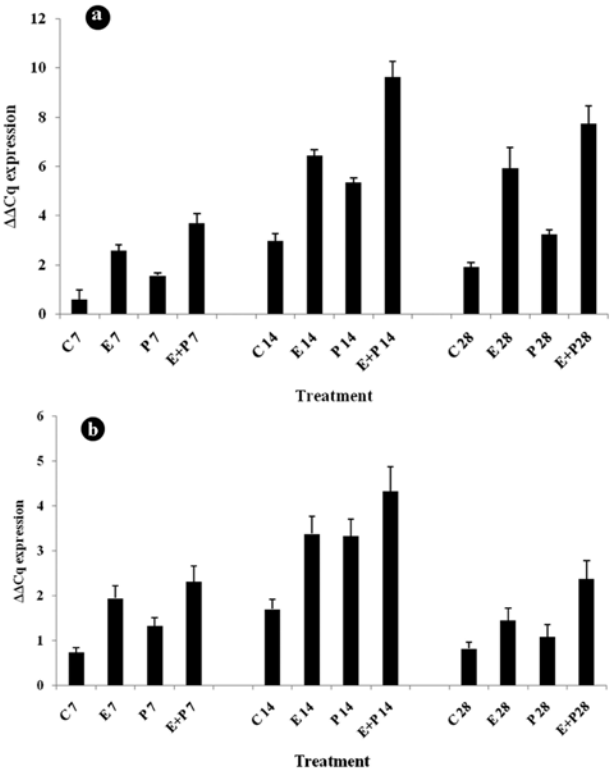

Figure 2. The relative gene expression of chitinase (a) and defensin(b) in sunflower plants inoculated with *Fusarium solani* and/or treated with turmeric extract at 7, 14 and 28 d after inoculation, using qRT-PCR. C = control, E = extract-treated, P = *F. solani* inoculated, and E+P = extract-treated + inoculated.

levels of both genes reached maximum at 14 d after inoculation. Compared to the control treatment, treatment (E+P) gave the greatest expression levels for chitinase and defensin genes, compared with the other treatments, resulting in a mean increase of 9.6-fold for chitinase and 4.3-fold for defensin. After 28 d, the expression levels of both genes decreased compared to those at 14 d, but they were still greater than at 7 d. The expression levels of both genes were greatest for the treatment (E+P), followed by the treatment (E).

Chitinase catalyses the degradation of chitin, the main constituent of fungal cell walls, and induction of chitinase is an important plant defence-related response against invading fungal pathogens. Chitinase therefore acts as an indicator of the plant defence response (Abdel-Fattah *et al*., 2011). Plant defensins are cysteine-rich peptides that have antimicrobial, enzyme inhibiting, and heavy metal resistance properties (Van der Weerden and Anderson, 2013). Despite of their multifunctional activities, the main effects of plant defensins are against phytopathogenic fungi (Lacerda *et al*., 2014). The role of defensins in plant innate immunity against pathogenic fungi has been extensively reported (Van der Weerden and Anderson, 2013; Lacerda *et al*., 2014). Two mechanisms of action of antifungal defensins are described; interaction with the negatively charged molecules in fungal cell walls, causing permeability disturbance and cell leakage, or interaction with phospholipids leading to induction of reactive oxygen species and activation of programmed cell death (Hegedus and Marx, 2013). Induction of chitinase and defensin genes in response to treatment with turmeric extract can explain the increased resistance of sunflower plants against infection with *F. solani.*

Conclusions

The present study has demonstrated that treatment of sunflower seeds with the turmeric extract induces resistance against *F. solani*. The triggered defence responses include increased phenol content and activation of the oxidative enzymes POX and PAL. In addition, some defence-related genes were over expressed. Based on their eco-safety and efficacy, we conclude that turmeric extracts have potential for management of the economically important disease Fusarium root rot of sunflower plants. Further evaluation of turmeric extracts should be carried out in field situations to evaluate efficacy in practical crop production.

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Compliance with ethical standards

This research did not involve any human and/or animal experimental subjects. The authors declare that they have no conflicts of interests.

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