Changes in pathogenesis-related gene expression in response to bioformulations in the apoplast of maize leaves against *Fusarium oxysporum*

Usmara Riaz Butt, Rabia Naza, Asia Nosheen, Humaira Yasmin, Rumana Keyani, Ishtiaq Hussain and Muhammad Nadeem Hassan

Abstract

In present study, we initially tested four different plant species *Aristolochia indica* (leaf), *Cuscuta pedicellata* (stem), *Melilotus indicus* (leaf) and *Tribulus terrestris* (leaf and fruit) for their antifungal potential against *F. oxysporum*. Among the studied plant species, *T. terrestris* (fruit) and *C. pedicellata* showed the maximum antifungal potential by 92% and 89%, respectively. We then investigated the potential of CHI, MeJA, and plant extracts (*C. pedicellata* and *T. terrestris*) alone as well as in combinations in pot experiment. Treatments with the extracts of *C. pedicellata* and *T. terrestris* in combination with MeJA resulted in decrease in disease severity by 93% and 89%, respectively, and enhanced the plant growth. We observed significant elevated levels of expression of pathogenesis-related (PR-1) proteins (*β*-1-3-glucanase, chitinase, phenyl-alanine ammonia lyase (PAL), lipoxygenase, lipid transfer protein and PR protein) and defense enzymes (*β*-1-3-glucanase, chitinase, peroxidase, polyphenoloxidase and PAL) in the CHI and MeJA in combination with plant extracts-treated plants for defense signaling pathways in the apoplast of maize leaves compared to the inoculated control. We found CHI in combination with *C. pedicellata* most effectively induced the plant defense mechanisms against *F. oxysporum* through increased induction in the level of expression of PR-proteins.

Introduction

In Pakistan, maize (corn) is the fourth largest grown crop after wheat, cotton and rice. Maize is easy to process, high yielding, cheaper as compared to other cereals. Moreover, every part of it has economical value for making edible and non-edible products. With this impact of maize, it has always been overwhelmed by a wide range of diseases particularly caused by pathogenic fungi from the Fusarium genus that is a serious threat to maize cultivation and productivity worldwide (Vurro et al. 2010). Fusarium vascular wilt and root rot of corn is caused by numerous Fusarium species i.e. *Fusarium proliferatum*, *Fusarium culmorum* and *Fusarium avenaceum*, most commonly *Fusarium oxysporum* that is adapted to grow in the vascular system of their host (Lichtenzveig et al. 2006; Orole and Adejumo 2009). This disease produces small necrotic or chlorotic spots and lesions causing significant damage to maize crop up to 60%. In Pakistan, the symptoms similar to wilt disease are reported in maize, sugarcane, sesame, castor, soybean, pigeon pea, mango and papaya (Hayat et al. 2007). *Fusarium solani* and *F. oxysporum*, which are very common in agriculture fields of Pakistan, are known to cause root rot, stem rot and wilt disease on a wide range of plants (Nelson et al. 1983; Ghaflar 1992). Among many other Fusarium species, *F. oxysporum* Schlecht has been reported from Pakistan on maize seed that is known to cause root rot and wilt disease in maize plants (Niaz and Dawar 2009).

Commonly plant diseases are being controlled by the applications of chemical fungicides (El-Mougy et al. 2004), but fungicides do not kill the fungus, they form the barrier between plant and fungus to evade disease (Damon 2013).
However, fungicide applications may cause harmful effects to human health and increase environmental pollution. Therefore, it is important to find some alternative crop protection strategies, to not only prevent resistance development by pathogens, but also to promote the green farming.

Many researchers have reported the natural resistance mechanisms of plants against pathogen invasions via induced responses (Kunkel and Brooks 2002; Gennenn 2006). These elicitors, or signal molecules, generally enhance non-specific plant resistance due to their effect of imitating a pathogenic attack, thereby priming plant defense before infection (Dangl and Jones 2001). Chitosan, a high molecular polymer, nontoxic, bioactive agent has become a useful choice due to its fungicidal effects and elicitation of defense mechanisms in plants. It can induce host defense responses, including the accumulation of antifungal hydrolysates and phytoalexins (Li and Yu 2000). Several studies have demonstrated the protective effects of chitosan against pathogen attack on various crops. Jayaraj et al. (2009) reported the decreased incidence of fungal pathogens in carrots when sprayed with chitosan. El-Tantawy (2009) has also reported the reduced disease incidence and increased tomato plant growth and yield after pathogen attack.

Elicitor’s effects are generally mediated by signaling pathways which include the ethylene (ET) jasmonic acid (JA) and salicylic acid (SA), pathways. These pathways play key roles in the induction of local and systemic defense responses, either alone or in combination. Methyl jasmonate (MeJA), a volatile form of JA, that has been extensively used to study the jasmonate signaling pathways and plant defense mechanisms. MeJA are known to activate the signaling cascade of genes associated with plant cell membrane. After activation, the expression level of defensive genes, such as pathogenesis-related (PR) proteins and proteinase inhibitors, change to control the plant immune and defense responses (Vijayan et al. 1998). Similar to chitosan, the application of pre and postharvest plant growth regulators have also been associated with plant protection and growth improvement (Figueroa et al. 2012; Karaman et al. 2012).

Recent research is focused on to discover natural plant products that are environmentally safe and easily biodegradable alternatives to synthetic fungicides (Rai and Carpinella 2006; Al-Samarrai et al. 2012). The in vitro antifungal potential of various plant extracts against a number of plant pathogens has been reported (Yang and Clausen 2007). Plant extracts containing the antimicrobial and resistance-inducing activities providing protection against infections, are considered as the sustainable method for controlling plant diseases (Muthukrishnan et al. 2001).

Our main aim was to determine the molecular responses of maize inoculated with \( F. \text{oxysporum} \) to the selected plant extracts (\textit{Cuscuta pedicellata} and \textit{Tribulus terrestris}) and chemical elicitors (chitosan and methyl jasmonate) applications. The specific experimental objectives were to (1) determine the effects of leaf extracts and chemical elicitors on the mycelial growth of \( F. \text{oxysporum} \) in vitro; (2) describe and compare symptom development on inoculated plants following the various treatments; (3) quantify the effects of the plant extract formulations on the expression of defense-related genes in the inoculated maize leaves and (4) quantify the enzyme activities of selected PR proteins (β-1,3-glucanases, chitinases, peroxidases, PAL and polyphenol oxidase) in the apoplast of treated and inoculated maize leaves.

### Materials and methods

#### Collection of host plant seeds and pathogen

Wilting and root rot susceptible maize cultivar (CM 165), was obtained from National Agricultural Research Centre (NARC) Islamabad, Pakistan.

Wilted maize plants were collected from South Punjab, Pakistan. The infected plants were uprooted and carefully washed in running tap water to remove any soil remains. Affected parts of the infected roots were cut into small pieces (2 mm in length) surface sterilized with 5% of Chlorox, washed 2–3 times with distilled water and then dried between sterilized filter papers. The sterilized pieces were transferred onto potato dextrose agar (PDA) medium supplemented with penicillin (20 μg/mL) and incubated at 25 ± 1°C, then examined daily for fungal growth.

#### Isolation, purification and identification of the causal organism

The fungal colonies were purified using single spore or hyphal tip isolation technique and then identified according to their morphological and microscopical characters as described by Booth (1985) and Barnett and Hunter (1986). Identification was confirmed by Crop Disease Research Institute, NARC Islamabad, Pakistan. The obtained isolates were maintained on PDA slants and kept in a refrigerator at 4°C for further study.

#### Collection of plant materials and extract preparation

Four different plant species \textit{Aristolochia indica} (leaf), \textit{C. pedicellata} (stem), \textit{Meliolites indicus} (leaf) and \textit{Tribulus terrestris} (fruit) were collected from the villages around Chowk Azam, District Layyah (South Punjab) Pakistan. Plants were identified by Dr. Mir Ajab Khan, Department of Plant Sciences, Quaid-i-Azam University Islamabad.

Collected plant parts were washed thoroughly with distilled water and were shade-dried for 3 d at room temperature. The dried plant material was uniformly ground using an electric grinder. The powdered plant material (250 g) was extracted for 4 d in 100% methanol and for 24 h in sterile distilled water (Harbone 1998). The separated extracts were then filtered using Whatman No. I filter paper and the methanol filtrate evaporated to dryness using a rotary evaporator at room temperature (30°C). Dried extract was stored in an air-tight container at 4°C until further use. Concentrations of 5% and 15% (v/v) in (separate) methanol and water extracts were used for the in vitro antifungal assay.

#### In vitro fungicidal potential of plant extracts

The agar tube dilution method was used for the determination of antifungal activity of selected plant extracts (Washington and Sutter 1980). Concentrations of 5% and 15% (v/v) in (separate) methanol and water extracts were prepared in dimethyl sulfoxide (DMSO) and sterile water respectively, for the in vitro antifungal assay. A solution (10 mL) of Sabouraud dextrose agar (6.5%) (Merck) was dispensed in screw-capped tubes or cotton-plugged test tubes and was autoclaved at 121°C for 21 min. Tubes were allowed to cool at 50°C and the Sabouraud dextrose agar solution was loaded with 67 μL of sample pipetted from each of the plant extract at 5% and
15% (v/v) methanol and water extracts and, chemical elicitors. The tubes containing the media were then allowed to solidify in slanting position at room temperature.

Three slants of the sample from each concentration of methanol and water extracts were prepared. The tubes containing solidified media and plant extract were inoculated with a 4 mm-diameter piece of inoculum, taken from a 7-day-old culture of the fungus. Slants without extract/sample were used as positive controls (Terbinaine) and negative controls (DMSO for methanol extracts and sterile water for water extracts). The test tubes were incubated at 28°C for 7 d. Cultures were examined twice weekly to observe fungal growth during incubation. Mean growth values were obtained after 7 d and converted to percentage inhibition of mycelial growth in relation to the control treatment by using the following formula: \( \text{MGI} = \left( \frac{(mc - mt)}{mc} \right) \times 100 \), where MGI is minimum growth inhibition, and \( mc \) and \( mt \) represent mycelial growth in control and treated samples, respectively.

**Inoculum preparation for glasshouse experiment**

An inoculum suspension in potato dextrose broth (PDB, DIFCO) from *F. oxysporum* culture was prepared following the method of Hao et al. (2005) with some modifications. The suspension was incubated at room temperature on a shaker at 25°C ± 2°C for 5–7 d until the microconidia were produced. Four layers of cheesecloth were used to strain and separate the mycelial mass from the microconidia. The concentration was adjusted to the required dose (1 × 10^6 conidia mL⁻¹) by diluting the suspension with phosphate-buffered saline (PBS) solution.

**Preparation of elicitor and plant extract formulations**

Formulations were prepared by using the extracts of selected plant species (*C. pedicellata* (stem) and *T. terresteris* (fruit)) after in vitro screening, fungicide (Mancozeb), chitosan, methyl jasmonate and Tween-80 (0.6% v/v) was used as an emulsifier. All plant extract formulations were prepared alone as well in combination with chitosan and methyl jasmonate, and stored in dark at room temperature prior to application.

Seeds of maize cv. CM 165 were surface sterilized prior to sowing for pot experiment with 95% ethanol (v/v) followed by shaking in 10% (v/v) chlorox for 2–3 min, thereafter; the seeds were thoroughly rinsed 3X with sterile distilled water.

**Pot experiment**

The above-mentioned formulations of the selected plant extracts, fungicide and chitosan were tested against the *F. oxysporum* in pot experiment under glasshouse conditions. A mixture of soil and sand at 3:1 (w/w), pH 7.0, with available nutrients Na, K, P, Mg and Ca at 19, 12, 9, 0.7 and 35 µg/g, respectively, was added to sterilized plastic pots (20 × 25 cm^2). Seven surface-sterilized seeds were sown in each pot and thinned to five plants per pot after germination. For each pot, 50 mL of the inoculum was added to the root zone of four-week-old maize seedlings. All the prepared formulations (Table 1; 50 mL) were applied as drench application after 24 h of *F. oxysporum* inoculation. Control plants were treated with sterile distilled water instead of the plant extract formulations. Deionized water was used daily to adjust the pots to field capacity. A completely randomized design (CRD) was followed with ten treatments and three replications per treatment, with an average temperature of 25–30°C and day-length ranging from 10–13 h.

**Phenotyping**

Final observations on disease development were made 14 d after pathogen inoculation as described by Majdah and Al-Tuwaijri (2015). First symptoms (leaf yellowing and wilting) start appearing at about 14 d post pathogen inoculation. The severity of developed symptoms was evaluated on a 0–5 rating scale for the wilting/yellowing phenotypes. A score of 0 indicated a healthy plant with no signs of disease, 1 = approximately 10% of the plant showing symptoms of disease, 2 = approximately 25% of the plant showing symptoms of disease, 3 = approximately 50% of the plant showing symptoms, 4 = approximately 75% of the plant showing symptoms, and 5 = 100% of the plant showing disease symptoms. Three replicates per treatment were evaluated individually then averaged to determine the disease severity for each treatment.

\[
\text{Disease severity} = \left[ \frac{\sum \text{(No. infected plants)} \times \text{their infected degree or disease grade}}{\sum \text{(No. of total examined plants)} \times \text{maximum infected degree}} \right] \times 100.
\]

**Growth parameters**

After 14 d of Fusarium inoculations, plants were harvested to measure the root length, root fresh weights (RFW), root dry weight (RDW) and shoot length, shoot fresh weight (SFW), shoot dry weight (SDW). After measuring the lengths and fresh weights of root and shoot, the samples were dried till the constant weight was obtained using microwave method on medium heat for 1.5 min ± 30 s (Hudson and Drost 2009).

**Sample collection**

Plant leaf samples were collected at 3 d (72 h) of treatment application. Whole plants were randomly harvested from different pots at the respective sampling times (during the period 10:00–12:00 AM). Plants were collected from each replication and treatment and were used to study the

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**Table 1.** The following formulations were prepared and applied as drench application after 24 h of pathogen inoculation.

| S/N treatments | Symbols used |
|---------------|-------------|
| 1. Control | C |
| 2. Infection control with *F. oxysporum* | Fo |
| 3. Mancozeb (0.3%) + *F. oxysporum* | Mz + Fo |
| 4. Chitosan (0.2%) + *F. oxysporum* | Chi + Fo |
| 5. Methyl Jasmonate (20 µM) + *F. oxysporum* | MeJ + Fo |
| 6. *T. terresteris* (15%) + *F. oxysporum* | TtJ + Fo |
| 7. *C. pedicellata* (15%) + *F. oxysporum* | Cp + Fo |
| 8. Methyl Jasmonate (10 µM) + Chitosan (0.1%) + *F. oxysporum* | MeJ + Chi + Fo |
| 9. *T. terresteris* (7.5%) + Chitosan (0.1%) + *F. oxysporum* | TtJ + Chi + Fo |
| 10. *C. pedicellata* (7.5%) + Chitosan (0.1%) + *F. oxysporum* | Cp + Chi + Fo |
| 11. *T. terresteris* (7.5%) + Methyl Jasmonate (10 µM) + *F. oxysporum* | TtJ + MeJ + Fo |
| 12. *C. pedicellata* (7.5%) + Methyl Jasmonate (10 µM) + *F. oxysporum* | Cp + MeJ + Fo |

Note: Tween-80 (0.6%) was added to each formulation as an emulsifier.
expression of PR and defense-related genes by RT-qPCR, and for the extraction of IWF to determine changes in protein concentration and activities of defense-related enzymes.

**Infiltration of maize leaves and extraction of intercellular washing fluid**

Intercellular wash fluids (IWF) was collected from all the leaves of infested and treated plants according to the method of Witzel et al. (2011) with some modifications. Collected leaves were washed with distilled water and cut into the segments of 5.5 cm. The IWF was collected twice from the same leaves by centrifugation after vacuum infiltration with chilled infiltration sodium phosphate buffer (pH 6.5). A vacuum was applied manually for 8 min using a plastic syringe (60 mL) filled with 40 mL of sodium phosphate buffer. Pressure was generated inside the syringe by pulling the plunger that would create low pressure approximately 20 kPa to remove generated inside the syringe by pulling the plunger that would create low pressure approximately 20 kPa to remove the air from the apoplastic spaces during the evacuation. A gradual release of the vacuum over a period of 20 s allowed the apoplastic spaces to be filled with infiltration buffer. Leaf segments were blotted dry and arranged in bundles in a nylon filter mesh. The leaf bundles were placed in a 20 mL syringe that was placed in a 50 mL Falcon tube and centrifuged at 3000 g for 30 min at 4°C. The extracted IWF was re-centrifuged at 13,000 rpm for 5 min in a microcentrifuge and stored at −70°C for further analysis.

**RNA extraction and RT-qPCR**

RNA was extracted from leaves using TRIZOL reagent (Invitrogen, USA) according to the manufacturer’s instructions and quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) at a 1:10 (v/v) dilution. Purified RNA was used as a template to synthesize cDNA using the cDNA synthesis kit (Thermo Scientific) as per the manufacturer’s instructions. Six defense-related genes (and housekeeping gene, Actin; the internal control) were selected for RT-qPCR analysis (primer sequences are given in Table S1). Real-time reactions were performed using 12.5 μL of the 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientist), 0.4 μL of forward primer (0.3 μM), 0.4 μL of reverse primer (0.3 μM), 2.0 μL of template (30 ng) and nuclease-free water to 25 μL. Reactions were performed in triplicate for each sample, including negative controls in which cDNA was substituted by the same volume of water. The StepOnePlus™ Real-Time PCR System (Applied BioSystems) was used with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s and 72°C for 20 s and extension at 72°C for 1 min. The results were interpreted using delta ct values.

**Total soluble protein content**

Protein estimation was done by using the Bradford reagent. IWF 50 μL and 2.5 mL of Bradford reagent were added in the test tubes, for each sample and absorbance was measured at 595 nm within 60 min. following the method of Wang et al. (2003) with some modifications. Apoplast soluble proteins (200 μL) were precipitated in 2 vol. of 10% (w/v) TCA, in acetone overnight at −20°C and pelleted by centrifugation at 5000 g for 10 min. The supernatant was discarded and the apoplastic soluble protein pellet was re-suspended in 0.07% 2-mercaptoethanol (v/v) in cold acetone. The samples were stored at −20°C for 1 h, and then centrifugation was done for 15 min at 10,000 g at 4°C, this step was repeated twice. The supernatant was discarded, and pellet was dried under vacuum for ~1 h and stored at −80°C.

**Electrophoretic analyses**

For one-dimensional separation of proteins, the pellets were dissolved in 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue, 63 mM Tris-HCl (pH 6.8) and heated at 70°C for 10 min. A sample containing 10 μg of protein was separated by SDS-PAGE according to Laemmli (1970).

4–12% Acryl and Bis-acryl amide gels (pH 7) were used and proteins were resolved under 200 V (constant) in a Mini-Cell electrophoresis system until the sample buffer containing bromophenol blue reached the bottom of the gel (40–50 min). Protein bands were visualized by staining with a solution containing 0.1% (w/v) coomassie R250, 40% ethanol and 10% acetic acid and a de-staining solution containing 10% (v/v) ethanol and 7.5% (v/v) acetic acid.

**Estimation of β-1,3 glucanase activity**

Gluconase activity was assayed according to the method of Abeles and Forrence (1979) with some modifications. An aliquot (400 μL) of IWF plus 100 μL of 2 mg/mL laminarin (a linear polysaccharide with a β(1→3)-β(1→6) ratio of 3:1; Sigma) was incubated at 37°C for 15 min. An aliquot (400 μL) of dinitrosalicylate (DNS) was added to reaction mixture and boiled for 5 min. After cooling, absorbance of the solution was measured at 500 nm in a plate reader. Glucanase activity was expressed as units per mg protein, where one unit is defined as the reducing sugar equivalent to 10 nmole of glucose produced per second under the assay conditions.

**Estimation of chitinase activity**

Chitinase activity was determined by colorimetric assays using the purple dye-labeled biopolymeric substrate, CM-chitin-RBV (Loewe Biochemical, Germany) (Stangarlin et al. 2000). An aliquot (200 μL) of CM-chitin-RBV (2 mg/mL) was mixed with 300 mL of IWF and 300 mL of Tris-HCl (10 mM, pH 7.5), Triton 1%. The mixture was incubated at 37°C for 3 h. The reaction was stopped by adding 200 mL of 2 M HCl. Samples were cooled on ice for 15 min, centrifuged at 20,000 g for 10 min to remove the non-degraded substrate. The supernatant was collected, and the absorbance was recorded at 550 nm. One unit of chitinase activity is defined as giving an increase of absorbance of 0.1 at 550 nm (Lopes et al. 2008).

**Estimation of peroxidase (POD) activity**

POD activity was measured by the method of Gorin and Heidema (1976). The assay mixture contained 0.1 mL of IWF, 1.35 mL 0.1 M mM MES buffer (pH 5.5), 0.05% H₂O₂ and...
0.1% phenylenediamine. Changes in the absorbance in each sample from three replicates per treatment were recorded at 485 nm for 3 min.

**Phenyl-alanine ammonia lyase (PAL) activity**

PAL activity was done by the method of Peixoto et al. (1999) with some modification. IWF 200 µL was mixed with 200 µL of 0.2 M borate buffer and 200 µL of phenylalanine to prepare the reaction mixture. This mixture was incubated at 39°C for 60 min. After incubation, 20 µL of 6N HCl was added to stop the enzymatic reactions. Absorbance (three replicates/treatments) was determined by the spectrophotometer at 290 nm against the borate buffer as blank.

**Polyphenol oxidase (PPO) activity**

PPO activity was determined following the method of Kara and Mishra (1976) with some modifications. The 3-mL reaction mixture contained 25 mM phosphate buffer (pH 6.8), 0.1 mM pyrogallol, 0.1 mL IWF and blank without pyrogallol. The absorbance of each sample (three replicates/treatments) was recorded at 420 nm.

**Statistical analysis**

Results were expressed as ±standard deviation of the mean (SD). The data were then subjected to ANOVA and statistical analysis using Statistix version 8.1. Comparisons among mean values of treatments were made by Least Significant Difference (LSD) to test significant differences at $p < .05$ (Gomez and Gomez, 1984).

**Results**

**In vitro antifungal potential of selected plant extracts and synthetic fungicides against F. oxysporum**

The methanolic and aqueous extracts of all the selected plant extracts at 15% exhibited maximum reduction in the mycelial growth of *F. oxysporum* (Figure 1).

The aqueous fruit extracts of *T. terrestris* was found to be highly effective in inhibiting the mycelial growth of *F. oxysporum* by 92% followed by *C. pedicellata* and *M. indicus* (89% and 87%, respectively). Positive control (Terbinafen) significantly inhibited the fungal growth by 97%.

The methanolic *T. terrestris* fruit extract exhibited the maximum reduction (98%) in the mycelial growth of *F. oxysporum* that was followed by *C. pedicellata* and *M. indicus* (94% and 90%, respectively). Positive control (Terbinafen) inhibited the mycelial growth of *F. oxysporum* by 99%.

**Effect of plant extracts and chemical elicitor formulations on the disease severity of maize plants inoculated with F. oxysporum**

Different levels of disease severity in plants were observed in the treated as well as inoculated plants compared to the untreated inoculated control plants (Figure 2). Single as well as combined formulations showed decrease in disease severity over the inoculated control. Full dose formulations of fungicide (mancozeb), chitosan, *C. pedicellata*, *T. terrestris* extracts and methyl jasmonate, exhibited 91, 87, 61, 72 and 85%, respectively, decrease in disease severity compared to infected control. While, *C. pedicellata*, *T. terrestris* and methyl jasmonate in combination with chitosan showed decrease in disease severity by 84, 91 and 90%, respectively, over the inoculated control. Treatments with the extracts of *C. pedicellata* and *T. terrestris* in combination with methyl jasmonate resulted in decrease in disease severity by 93 and 89%, respectively.

**Effect of chemical elicitor and plant extract formulations on the growth parameters of maize plants inoculated with F. oxysporum**

**Root and shoot lengths**

Different heights of plants were observed in the treated as well as inoculated plants (Figure 3). The inoculated control plant showed 48% decrease in root length compared to the healthy control plants. All treatments exhibited an increase in root length over the inoculated control. Single formulations of
mancozeb, chitosan, *C. pedicellata*, *T. terresteris* extracts and methyl jasmonate gave an increase in root length by 67%, 91%, 108%, 154% and 162%, respectively. While the combined formulation of *C. pedicellata*, *T. terresteris* extracts and methyl jasmonate with chitosan resulted in increase in root length by 169%, 184% and 154%, respectively, over the infected control. Whereas, the two plant extract formulations (*C. pedicellata* and *T. terresteris*) with methyl jasmonate gave even more increase in root length compared to inoculated control plants (174% and 206%, respectively).

*F. oxysporum* inoculation exhibited 29% decrease in shoot length compared to the healthy control plants (Figure 3). All treatments gave increase in shoot length over the inoculated control. Single formulations of mancozeb, chitosan, *C. pedicellata*, *T. terresteris* extracts and methyl jasmonate gave an increase in shoot length by 22%, 24%, 49%, 52% and 58%, respectively. *C. pedicellata*, *T. terresteris* extracts and methyl jasmonate combined with chitosan resulted in increase in shoot length by 51%, 66% and 66%, respectively, over the infected control, while the two half-dose plant extract formulations with methyl jasmonate gave even more increase in shoot length (72% and 100%, respectively) over the inoculated control plants.

**Root, shoot fresh and dry weight**

*F. oxysporum* inoculation reduced root fresh and dry weights by 61 and 37%, respectively, compared to the healthy control. All treatments increased root fresh and dry weight compared to the inoculated plants (Table 2). Treatments with *C. pedicellata*, *T. terresteris* extracts combined with methyl jasmonate were the most effective, increasing root fresh weight by 219% and 230%, respectively, and root dry weight by 126% and 184%, respectively, as compared to the inoculated control.

Shoot fresh and dry weights were reduced by 35% and 42%, respectively, in the *F. oxysporum* inoculated plants.
compared to the healthy control plants. All treatments increased shoot fresh and dry weight compared to the inoculated plants (Table 2). Treatments with *C. pedicellata*, *T. terresteris* extracts combined with methyl jasmonate were the most effective in increasing shoot fresh weight by 177% and 256%, respectively, and shoot dry weight by 223% and 283%, respectively, as compared to the inoculated control.

### Differential expression of defense-related genes at the transcript level

Expression patterns of defense-related genes following plant extract formulations (with chemical elicitors i.e. chitosan and methyl jasmonate) and inoculation with *F. oxysporum* were analyzed using RT-qPCR (Figure 4). The induction patterns in maize leaves were evaluated 72 h after inoculation. The housekeeping gene *Actin* was used as an RT-qPCR internal control. All the treatments resulted in substantial up-regulation of the selected genes in response to *F. oxysporum* inoculation.

**Expression of β-glucanases, chitinase and phenylalanine ammonia lyase**

In response to various plant extract formulations with chemical elicitors (chitosan and methyl jasmonate), β-1,3-glucanase, chitinase and phenylalanine ammonia lyase (PAL) genes were up-regulated 72 h after *F. oxysporum* inoculation. The formulations of *C. pedicellata, T. terresteris* extracts and methyl jasmonate in combination with chitosan up-regulated the glucanase expression by 8, 4 and 1-fold, respectively, chitinase by 27, 15 and 14-fold, respectively, and PAL by 33, 22 and 9-fold, respectively, compared to the inoculated control. While the combined treatments of *C. pedicellata, T. terresteris* with methyl jasmonate significantly up-regulated the glucanase expression in leaves by 4 and 3-fold, respectively,
chitinase by 8 and 10-fold, respectively, and PAL by 11 and 16-fold, respectively, compared to the untreated, inoculated control (Figure 4(A–C), respectively).

Expression of lipoxygenase, lipid-transfer protein and pathogenesis-related maize protein
Lipoxygenase, lipid-transfer protein (LTP) and pathogenesis-related maize protein (PR-1) were up-regulated 72 h after inoculation in maize plants treated with full- as well as half-dose formulations of the plant extracts and chemical elicitors. The formulations of *C. pedicellata*, *T. terresteris* extracts and methyl jasmonate in combination with chitosan up-regulated the expression of lipoxygenase by 22-, 10- and 9-fold, respectively, LTP by 8-, 17- and 12-fold, respectively, and PR protein by 23-, 21- and 10-fold, respectively, compared to the uninoculated control. While the combined treatments of *C. pedicellata* and *T. terresteris* with methyl jasmonate significantly up-regulated the lipoxygenase expression in leaves by 8- and 7-fold, respectively, LTP by 10- and 11-fold, respectively, and PR-1 protein by 16- and 14-fold, respectively, compared to the inoculated control (Figure 4(D–F), respectively).

Effect of plant extracts and chemical elicitor formulations on protein content and protein complement (SDS-PAGE) in the apoplast of maize leaves
*F. oxysporum* inoculation reduced the protein content by 36% compared to uninoculated control (Figure 5). The fungicide, chitosan, extracts of *C. pedicellata*, *T. terresteris* and methyl jasmonate treatment increased protein content by 118%, 189%, 233%, 370% and 280%, respectively, compared to the inoculated control. The half-dose formulations of the aqueous extracts of *C. pedicellata*, *T. terresteris* and methyl jasmonate combined with chitosan more significantly increased the protein content by 509%, 590% and 541%, respectively. While, the combined formulations of *C. pedicellatae* and *T. terresteris* extracts with methyl jasmonate gave even more increase in protein content by 647% and 633%, respectively, compared to the inoculated control.

Coomassie stained 1-DE gel revealed several protein bands ranging in molecular mass from 10 to 100 kDa (Figure 6). Fungicide, chitosan, methyl jasmonate and plant extracts (*C. pedicellata*, *T. terresteris*) induced some new protein after the pathogen inoculation. A newly induced protein of 160 kDa molecular weight (assigned as PR-a) was observed in all the treatments/lanes (from lane 2 to lane 12). Expression of newly induced protein PR-1a was higher in lane 6–12 (*T. terresteris* + *F. oxysporum*, *C. pedicellata* + *F. oxysporum*, Methyl Jasmonate + Chitosan + *F. oxysporum*, *T. terresteris* + Chitosan + *F. oxysporum*, *C. pedicellata* + Chitosan + *F. oxysporum*, *T. terresteris* + Methyl Jasmonate + *F. oxysporum*, *C. pedicellata* + Methyl Jasmonate + *F. oxysporum*). Another protein PR-b of 110 kDa was observed in response to methyl jasmonate, and plant extract formulations (*C. pedicellata*, and *T. terresteris* fruit extracts, applied alone as well as in combination with chitosan and methyl jasmonate), representing lane 5–12, respectively, while this protein was absent in rest of the treatments. PR-c of 55 kDa was observed in lane 6–12. While PR-d and PR-e were only observed in response to *C. pedicellata* and *T. terresteris* extracts in combination with chitosan and methyl jasmonate. The molecular weight of protein 35 (lane 8–12) and 20 kDa (lane 8–12) induced in response to different treatments. These induced proteins are corresponding to PR proteins indicating the highly induced defense enzymes in response to plant extract formulations after pathogen inoculation.

Effect of plant extracts and chemical elicitor formulations on the activity of defense-related enzymes and pathogenesis-related proteins in the apoplast of maize leaves
Inoculation of *F. oxysporum* stimulated activities of all the defense-related enzymes tested in all treatments compared to the uninoculated control. Pathogen inoculation induced higher β-1,3-glucanase and chitinase activities in treated plants compared to untreated plants (Figure 7(A,B)).
fungicide, chitosan, extracts of *C. pedicellata*, *T. terresteris* and methyl jasmonate treatments increased β-1,3-glucanase activity by 21%, 37%, 30%, 39% and 30%, respectively, and chitinase activity by 22%, 33%, 18%, 23% and 19%, respectively. The half-dose formulations of the aqueous extracts of *C. pedicellata*, *T. terresteris* and methyl jasmonate combined...
with chitosan significantly increased β-1,3-glucanase activity by 51%, 67% and 45%, respectively and chitinase activity by 31%, 44% and 31%, respectively, after pathogen inoculation. While, the combined formulations of C. pedicellata and T. terresteris extracts with methyl jasmonate gave even greater increase in β-1,3-glucanase activity by 81% and 69%, respectively, and chitinase activity by 59% and 48%, respectively, compared to the inoculated control.

Pathogen inoculations induced higher PAL, POD and PPO activity in treated plants compared to untreated plants (Figure 7(C-E), respectively). The fungicide, chitosan, extracts of C. pedicellata, T. terresteris and methyl jasmonate treatment increased PAL activity by 21%, 37%, 30%, 39% and 30%, respectively, POD activity by 22%, 33%, 18%, 23% and 19%, respectively, and PPO activity by 22%, 33%, 18%, 23% and 19%, respectively. The combined formulations of the aqueous extracts of C. pedicellata, T. terresteris and methyl jasmonate with chitosan significantly increased β-1,3-glucanase activity by 51%, 67% and 45%, respectively and chitinase activity by 31%, 44% and 31%, respectively, after pathogen inoculation. While, the combined formulations of C. pedicellata and T. terresteris extracts with methyl jasmonate gave even greater increase in β-1,3-glucanase activity by 81% and 69%, respectively, and chitinase activity by 59% and 48%, respectively, compared to the inoculated control.

**Discussion**

In recent years, chemical fungicides for controlling the pathogenic fungal diseases in plants are being replaced by natural plant products owing to environmental concerns. Therefore, considerable research to identify biocides that are environmentally safe and easily biodegradable has been carried out during the last two decades. The treatment of agricultural crops with naturally occurring inducers of pathogen resistance to supplement or substitute the effect of chemical fungicide is of special interest to control plant diseases. The main feature of induced resistance of plant tissues to disease is priming with the elicitors that allows the quick development of active defense mechanisms against pathogens.

In this study, we examined the plant extracts (Tribulus terrestris, C. pedicellata), growth regulator (methyl jasmonate (MeJA)) and chemical elicitor (chitosan) formulations, to protect maize against Fusarium root rot and wilt. *F. oxysporum* (rarely determined on maize plants at the maturity phase) with its considerable number of isolates (10) deserves attention as its high intensity of development according to Kwasna et al. (1991) and Fiedorow et al. (2001), coincides with maize emergence and may lead to seedling root rot in plants. Elicitors are eco-friendly chemicals that are known to induce effective immune responses in plants (Ramirez-Estrada et al. 2016). Chitosan has been shown to be an effective protective agent in agriculture because of its anti-fungal and elicitor activities (Ramjegathesh and Jayaraman 2015). In our study, elicitor and plant extract formulation as a drench application was found to be an efficient way to control Fusarium root rot and wilt, while reducing the required dose of chemical fungicide.

We first observed that among the selected plant extracts *T. terrestris* extract effectively inhibited the mycelial growth of *F. oxysporum* in vitro that was followed by *C. pedicellata* using Terbinafine as standard fungicide positive control (Figure 1). Terbinafine is active in vitro against a wide range of pathogenic fungi (Jo Siu et al. 2013).

We then observed the lower disease severities (less wilting and mild chlorosis) in the chitosan, MeJA and plant extract treated maize plants when compared to fungicide and untreated controls. The combined formulation of plant extracts with chitosan and MeJA gave significantly higher levels of inhibition of root rot infection. Apart from their protective ability, the mentioned formulations also exhibited the enhanced maize growth. The plant growth promoting capability of methyl jasmonate has been demonstrated by previous studies and has been attributed to increased cell elongation, cell division, and protein biosynthesis (Zapata et al. 2014). Similar finding was observed by Bautista-Baños et al. (2006) who reported the mixed formulations of plant extracts (custard apple leaves, papaya leaves and papaya seeds) with chitosan suppressed the growth of Colletotrichum gloeosporioides when compared with all the tested plant extracts applied alone. The reduced disease severity has also been reported in the bioformulation treated tomato plants against Fusarium wilt (Anitha and Rabeeth, 2010). Udo et al. (2014) reported the increased shoot length, fresh and dry weight in response to plant extracts treated plants as compared to un-treated plants. Neem leaf extracts have been found to be effective against Fusarium sp. and *Colletotrichum graminicola* (Masum et al. 2009), giving >90% reduction in seed-borne infections. Other researchers have also reported the substantial inhibition potential of *Datura stramonium* leaf extracts against *F. solani*, which causes Fusarium rot of *Luffa aegyptiaca* (Rawal and Thakore 2003; Abo-Elyoury et al. 2009; Mandal et al. 2009).

Gene expression analysis at the mRNA level and defense enzyme activities of maize plants inoculated with *F. oxysporum* and treated with elicitor and plant extract formulations revealed that all the treatments upregulated the expression of genes encoding defense-related proteins (glucanase, chitinase, PAL, lipoxygenase, LTP, PR-maize protein) and induced higher activities of defense enzymes β-1,3-glucanase, chitinase, PAL, POD and PPO as determined 72 h after pathogen inoculation. Plant extracts (*C. pedicellata* and T. terresteris) in combination with chitosan and MeJA gave even more upregulation in the expressions of genes encoding defense-related proteins and enhanced induction of defense enzyme activities. The combination of these two responses would have made the maize plants even more resistant to the pathogens.

We propose that disease incidence reduction was due to the chitosan and plant extract’s unique elicitation of physiological, biochemical and molecular changes in maize leading to induced resistance. These findings are in accordance with those of Chen et al. (2016), who reported an increased defense enzyme activity exhibiting resistance in *Begonia hemalis* treated with chitosan in response to *Botrytis cinerea*. Chitosan is reported to induce activities of various defense enzymes (chitinase, glucanase, POD, PAL and PPO) in plants (Mandal et al. 2013; Zhang et al. 2015; Ramkisson et al. 2016) and to elicit the expression of proteinase inhibitors and ethylene response (PIN II and ETR-1) pathogen defense genes (Ramkisson et al. 2016). Glucanase and chitinase enzymes are capable to hydrolyze the bacterial and fungal cell wall components thereby contributing to the defense mechanisms of plants (Xu et al., 2016).

Plant extract exogenous applications have been reported to induce systemic resistance in host plants, disease
reduction and increased plant growth (Ramamoorthy et al. 2002). Activation of defense responses, such as accumulation of PR-proteins (PR-1, PR-5 and PR-10) transcripts in Fusarium-infected maize, has been reported by other scientists (Poloni 2015; Maschietto et al. 2016). When activated by various factors, plants defense genes that are inactive in healthy uninoculated/untreated plants can induce systemic resistance against pathogens. Higher levels of POD, PPO, and PAL enzyme activities (Ngadze et al. 2012), as well as expression of β-1,3-galactanase and chitinase genes (Fan et al., 2002; O’Kennedy et al. 2011) have been reported to be effective against various fungal diseases. The onset of induced resistance in plants correlates with the increased defense enzyme activity and expression of PR proteins such as chitinases, β-1,3-galcanases, PAL and peroxidases; consequently, PR proteins are generally used as ISR (induced systemic resistance) markers (Chassot et al. 2007; Naz et al. 2014).

The plant extract’s ability to augment the efficacy of chitosan and MeJA to reduce disease was attributed to the elicitation of JA/ET mediated ISR which in turn increased the activity of a broad range of defense enzymes. We extended the available knowledge regarding the role of elicitors in combination with plant extracts in inducing defense. Based on this study we concluded that chitosan in combination with C. pedicellata extract can provide a broad-range protection against F. oxysporum through ISR mechanisms. Therefore, these findings provide good support for the integration of chitosan, MeJA and plant extract formulations for the sustainable disease management in maize. This is especially relevant in the tropics where there is a high use of pesticides and inorganic fertilizers. We believe that such biofungicide formulations in pre-pack mixtures could be commercially sold with other contact fungicides. Moreover, the exogenous applications of such biofungicide formulations can indirectly diminish the harmful effects of chemical fungicides as well as their residual effects on the human health and environment. Further investigations are needed to assess the potential benefits of these formulations in other crops grown under different tropical conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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