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Potential of herb crude extracts against Thai isolates of *Fusarium* wilt pathogens

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Abstract

Fusarium has damaged a wide host range and economically impacted on many important vegetables and plants in Thailand such as tomato. The goals of this study were to isolate and identify *Fusarium* spp. from local soil and plant diseased samples, and to study the efficacy of plant crude extracts against *Fusarium* for sustainable and environmentally friendly control. Forty-four *Fusarium* isolates from soil and diseased plants from selected agricultural fields in Thailand were characterized by using morphological and phylogenetical analyses. The isolates were assigned to clades with *Fusarium* reference isolates, and representative eight species were selected including *F. chlamydosporum* (3 isolates), *F. decemcellulare* (8), *F. falciforme* (2), *F. incarnatum* (7), *F. longipes* (4), *F. proliferatum* (3), *F. solani* (13) and *F. verticillioides* (4). In 7-day greenhouse experiments, all the *Fusarium* isolates were pathogenic to cucumber, chili pepper and tomato plants. Plant crude extracts from roselle, clove, turmeric and lemongrass were evaluated for their abilities to inhibit the mycelial growth of 8 representative isolates of *Fusarium* plant pathogens. Inhibition of all *Fusarium* isolates was observed at a concentration of 10 g L^{-1} for both clove and roselle crudes, absolutely. Whilst turmeric crude extract could completely inhibit growth of *F. decemcellulare*, *F. falciforme*, *F. incarnatum* and *F. verticillioide*. However, lemongrass crude extract was a less potent, showing about 60-80% inhibition. The results indicated that these herb crude extracts deserve further study singly or in combination towards development of natural biofungicides for management of agricultural plant diseases caused by *Fusarium*.

Keywords – biocontrol – *Fusarium* taxa – plant crude extract – vascular wilt disease

Introduction

Fusarium is a large cosmopolitan genus of asexual morph fungi that comprises many species widely distributed on plants and in the soil. These species have diverse ecological roles ranging from saprophytes and endophytes to animal and plant pathogens (Samson et al. 2004, Summerell et al. 2010, Fisher et al. 2012). Many *Fusarium* species, for example *F. oxysporum* f. sp. *cubense*, *F.*

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oxysporum f. sp. *elaeidis* and *F. solani* have been reported to cause high losses in global agriculture crops by causing diseases such as vascular wilt, root and stem rot, fruit blotch, fruit rot, damping-off, head blight and post-harvest rot (Burgess & Summerell 1992, Aoki et al. 2014, Gordon 2017). Additionally, some species of *Fusarium* such as *F. sporotrichioides*, *F. graminearum* and *F. verticillioides* produce food-borne mycotoxins, including trichothecenes, zearalenones, fumonisins and other mycotoxins (i.e., enniatins, moniliformin, beauvericin and fusaproliferin) that threaten human and animal health (O'Donnell et al. 2000, Bhat et al. 2009, Selvaraj et al. 2015, Ferrigo et al. 2016, Moretti et al. 2017).

Currently, chemical biocides are commonly used for the prevention and control of *Fusarium* agents. However, resistant fungal pathogens have arisen that are increasingly difficult to control (Servin et al. 2015). In the long term, pathogen resistance is anticipated to have major consequences for human health and food security (Li et al. 2011, Mudili et al. 2014). To overcome the problem of resistance, biocontrol is a promising alternative to chemical biocides. Various plant extracts exhibit antifungal activity, indicating their potential as biocontrol agents for fungal diseases. For example, crude extracts of clove are active against *F. oxysporum* and *F. equiseti* at 1 g L^{-1} (Kritzinger et al. 2002), essential oil extracted from citronella grass is active against *F. oxysporum* (Istianto & Emilda 2011) and crude leaf extract of lemongrass inhibits mycelial growth of *F. verticillioides*, *F. proliferatum*, and *F. graminearum* (Nwachukwu & Umechuruba 2001, Velluti et al. 2004). The efficacy and safety of these plant extracts as biocontrol agents are not proven as these studies were limited to laboratory and/or greenhouse experiments. In the present paper, an alternative biocontrol of plant crude extracts is suggested. Growth and mycelial inhibition of *Fusarium* pathogens may be affected by herb crude extracts.

Phytochemical extracts from plants cultivated in Thailand were tested for activity against *Fusarium* pathogens towards the ultimate aim of developing safer and more effective biocontrol agents. Crude extracts of roselle (*Hibiscus sabdariffa* L.), clove (*Syzygium aromaticum* (L.) Merrill & Perry), turmeric (*Curcuma longa* L.), and lemongrass (*Cymbopogon citratus* (DC.) Stapf) were tested at four different concentrations (0.01, 0.1, 1 and 10 g L^{-1}) for inhibition of mycelial growth of local *Fusarium* isolates. These isolates were obtained from soil and diseased plant samples randomly collected from selected Thai agricultural fields. The isolates were identified based on gross microscopic morphology and multi–gene DNA sequence phylogeny.

Materials & Methods

Fungal collection and isolation

Fusarium species were isolated from soil and plant samples associated with symptoms of *Fusarium* wilt in selected agriculture fields located in four provinces of Thailand (Kanchanaburi, Chanthaburi, Nonthaburi and Sakon Nakhon) between September 19, 2016 and January 21, 2017. Soil samples were taken at a depth of 10 cm at each site, placed into sterile polyethylene bags and returned to the fungal laboratory at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University (KU), Thailand. Soil samples (10 g) were suspended in 100 mL of 0.1% agar (Difco) distilled water medium and homogenized by shaking for 15 min (Innova 2100 Shaker, New Brunwick Scientific, USA). The homogenate was serially diluted in quadruplicate, and 0.1 mL of each dilution was pipetted onto the center of a Glucose Ammonium Nitrate Agar plate (GAN; himedia, India) medium supplemented with streptomycin (0.25 g L^{-1}) and spread with a sterile spreader on the agar surface. The plates were incubated at 25±2ºC for 2 days (Elad et al. 1981). For tissue transplantation, cross-sections (1×1 cm^2) of plant tissue samples were obtained from the margins of browning stem bases that were surface sterilized with 70% ethanol (W/V) for 2 min. The plant samples were sterilized in 2% sodium hypochlorite solution for 5 min, followed by two final rinses with sterile distilled water. Stem tissues were transferred to Potato Dextrose Agar (PDA, difco, USA) plates and incubated at 25±2ºC for 2–3 days. Fungal isolates were selected, transferred onto fresh PDA and purified by the single-spore isolation technique as described in Choi et al. (1999).

Morphological identification

Fusarium species were identified using morphological characters displayed on PDA and Carnation Leaf Agar (CLA; sterile 3-5 mm carnation leaf-pieces onto agar) as described by Fisher et al. (1982). Monoconidial isolates were sub-cultured on PDA plates and incubated at 25±2ºC for 7 days. A 0.5-mm-diameter plug from the colony margin was placed in the center of a 90-mm-diameter PDA plate and incubated at 25±2ºC under light to examine colony characteristics, growth rates, pigmentation and microconidial arrangement. Colony diameters were measured after 7 days of incubation period and the mean growth rates were calculated from three replicates. In addition, fungal cultures were incubated at 25±2ºC with a 12-h photoperiod for 14 days on CLA to investigate the shape and size of macroconidia and chlamydospores using a compound microscope (Carl Zeiss: Scope.A1, Jena, Germany), according to standard mycological manuals, guidebooks, and publications of Burgess (1981) and Leslie & Summerell (2006). All fungal axenic cultures were deposited at the Kasetsart University Fungal Culture Collection (KUFC), the Department of Plant Pathology, Faculty of Agriculture, KU (deposition codes F1 to F44).

DNA extraction, amplification, and nucleotide sequencing

Genomic DNA was extracted using the CTAB method described in Boonyuen et al. (2011). Translation elongation factor 1-alpha (*TEF1α*), DNA-directed RNA polymerase II largest gene (*RPB1*) and the partial RNA polymerase II second largest subunit (*RPB2*) were amplified using primers EF1 and EF2 (Park et al. 2011), FA and R8 (O'Donnell et al. 2010) and 5F2 and 7CR (Geiser et al. 2013), respectively. Polymerase chain reaction (PCR) was conducted in 50 µL reaction volumes containing 25 µL of DreamTaq DNA polymerase (1.25 U; Thermo Fischer Scientific), 21 µL of ddH2O, 1 µL of each primer (0.2 µM), 1 µL of dNTPs (0.2 mM), and 1 µL genomic DNA extract. PCR amplifications were performed in a DNA Engine DYAD ALD 1244 Thermocycler (MJ Research, USA) using the following program for amplification of *TEF1α*: an initial denaturation step at 95ºC for 2 min followed by 35 cycles of 95ºC for 30 s, 57ºC for 30 s and 72ºC for 1 min, and a final extension of 72ºC for 10 min. Thermocycling parameters for amplification of *RPB1* were 95ºC for 2 min followed by 35 cycles of 95ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, and a final extension of 72ºC for 10 min. The *RPB2* gene was amplified by an initial denaturation of 94ºC for 5 min followed by 35 cycles of 94ºC for 1 min, 54ºC for 1 min and 72ºC for 2 min, and a final extension of 72ºC for 5 min. 5 µL samples of the PCR reaction products were separated by electrophoresis in 1% agarose gel. The PCR products were sequenced by Macrogen, Inc., Korea, using the same primers as used for the PCR.

DNA sequence data and phylogenetic analyses

DNA sequence similarity to known sequences was examined by National Center for Biotechnology Information (NCBI) nucleotide BLAST search (Altschul et al. 1997; https://blast.ncbi.nlm.nih.gov/). Each sequence was checked for ambiguous bases and assembled using BioEdit 7.0.5.3 (Hall 1999). The consensus sequences for each DNA region were initially aligned with ClustalW v. 1.6 (Thompson et al. 1994) and optimized using MUSCLE (Edgar 2004). Manual gap adjustments were made to improve the alignment with ambiguously aligned regions excluded. Missing data at the 5′- and 3′-ends of the partial sequences were coded by BioEdit 7.0.5.3. The final dataset for phylogenetic analysis covered an aligned length of 2,373 characters, of which 1,024 characters were constant, 462 were variable parsimony-uninformative, and 887 were parsimony-informative. Phylogenetic analysis of the combined *TEF1α, RPB1* and *RPB2* sequence dataset was conducted using maximum parsimony (MP) methods based on a protocol in O'Donnell et al. (2010) and Torbati et al. (2019). Tree construction was performed in PAUP∗ version 4.0b10 (Swofford 2002), with gaps treated as missing data. Trees were generated using 100 replicates of random stepwise additions of sequence and the tree-bisection reconnection branch-swapping algorithm, with all the characteristics given equal weight. Branch support for all the parsimony analyses was estimated by performing 1,000 bootstrap replicates (Felsenstein 1985) with a heuristic search of 10 random-addition replicates for each bootstrap replicate. The consistency indices (CI),

retention indices (RI) and rescaled consistency indices (RC) were calculated for each tree (Kluge & Farris 1969, Farris 1989). All newly generated sequences of *TEF1α, RPB1* and *RPB2* were submitted to GenBank (NCBI; https://www.ncbi.nlm.nih.gov/) under accession numbers MW377509 – MW377525 for *RPB2*; MW377526 – MW377543 for *RPB1* and MW377544 – MW37756 for *TEF1α.*

Pathogenicity tests

All *Fusarium* isolates were evaluated for their pathogenicity on healthy and uniform 30-day-old seedling plants of cucumber (*Cucumis sativus* L.), chili (*Capsicum frutescens* L.) and tomato (*Lycopersicon esculentum* L.). *Fusarium* isolates were cultured on PDA agar for 14 days. The spore suspensions were quantified by haemocytometer and diluted to a final concentration of 1×10^6 spore mL^{-1}. The spore suspension was then mixed with sterile soil and inoculated onto tested plants using non-wounded inoculation. Five replicates of each isolate were tested, and the control plants were planted in the sterile soil without any added spore suspension. Plants were maintained in the greenhouse at 30±5ºC. The occurrence of *Fusarium* wilt symptoms was assessed at 3, 5 and 7 days after inoculation. Fungi were re-isolated from wilted plants to satisfy Koch's postulates as described by Agrios (2005) and Jacomo et al. (2002).

Preparation of Thai herb crude extracts

Fresh plants of roselle (*Hibiscus sabdariffa* L.), clove (*Syzygium aromaticum* (L.) Merrill & Perry), turmeric (*Curcuma longa* L.), and lemongrass (*Cymbopogon citratus* (DC.) Stapf) were purchased from the Talad Thai market, Khlong Luang district (N14º04'55.7", E100º37'11.3"), Pathum Thani province, Thailand and transported to the laboratory in polythene bags. Plant parts such as sample of blossoms, roots, leaves and stems were washed thoroughly under running tap water, chopped into small pieces, and dried at room temperature. Dried plant samples were pulverized using a blender (LB-2068, China). Two hundred grams of each powdered sample were weighed using an electronic weighing balance (ES-1200HA, China) and soaked in 200 mL of 95% ethanol at a 1:1 ratio. The sample was kept overnight at 25±2ºC and filtered using Whatman filter paper No. 1. The organic phase was evaporated in a rotary vacuum evaporator (BUCHI, Operation Manual Rotavapor® R-210/215, Vacuum Pump V-700/710, Switzerland) under a pressure of 45 mbar at 40ºC, followed by storage at 4ºC until use.

***Fusarium* growth inhibition tests using plant crude extract**

PDA amended with plant crude extracts at four different concentrations (0.01, 0.1, 1 and 10 g L^{-1}) were used to assess inhibitory activities against eight isolates selected from different *Fusarium* species sorted them in morphotypes and identified the taxa based on DNA sequence analyses, including *F. chlamydosporum* (isolate F23), *F. decemcellulare* (F4), *F. falciforme* (F9), *F. incarnatum* (F19), *F. longipes* (F2), *F. proliferatum* (F22), *F. solani* (F1) and *F. verticillioides* (F11). The center of Petri plates was inoculated with a mycelial plug obtained from a colony edge of a 7-day-old culture of the fungi. Plates without plant extract served as negative controls. Positive control plates for these tests comprised PDA supplemented with the fungicides captan and prochloraz at the concentration of 1 g L^{-1}. Tests were conducted in triplicate. The diameter of the mycelial colonies (mm) was measured and recorded after 7 day of incubation period. Antifungal activity was recorded in terms of inhibition of mycelial growth (%) and calculated as the following formula. Inhibition of mycelial growth (%) = C-T/C x 100; where 'C' is average diameter of fungal colony in the control plates and 'T' is average diameter of fungal colony in the treatment plates (Elizabeth et al. 2005, Kawakami et al. 2015). Data were analysed with SPSS (version 22) statistical software. The effect of different concentrations of crude extracts on the growth of fungi were tested using one-way ANOVA. Duncan's multiple range test was used to compare the differences among treatments. P-values less than 0.05 were considered statistically significant.

Results

Isolation, morphology and genetic identity of the *Fusarium* isolates

Forty-four isolates of eight species of the genus *Fusarium* were identified from soil samples, including *F. chlamydosporum* (3 isolates), *F. decemcellulare* (8), *F. falciforme* (2), *F. incarnatum* (7), *F. longipes* (4), *F. proliferatum* (3), *F. solani* (13) and *F. verticillioides* (4). Morphological characteristics of isolated *Fusarium* spp. were illustrated in Table 1 and Fig. 1. *Fusarium chlamydosporum* (isolate F23) was characterized by the formation of white or pink colonies on PDA and by single-celled, oval-shaped microconidia and fusiform macroconidia measuring 15−20 × 2−4.7 μm and consisting of 6 to 7 cells (i.e., 5–6-septate macroconidia). The macroconidial dimensions for *F. decemcellulare* (F4), *F. incarnatum* (F19), *F. longipes* (F2) were 24.5−50 × 3.5−4 μm, 23.5−58 × 3.5−6 μm and 40−60 × 4-6 μm, respectively. The macroconidial size for *F. falciforme* (F009) was 17−32 × 3−6 μm) and for *F. solani* (F1) was 25−36 × 4−6 μm. For *F. proliferatum* (F22) macroconidia measured 20−40 × 3−5 μm and were 3 to 4 celled (i.e., 2–3-septate). They also had curved apical cells and basal cells that were typically foot-shaped or occasionally slightly curved. Chlamydospores were also formed. For *F. verticillioides* (F11), spores were 20.2−60 × 35 μm, and mostly intercalary or terminal, and rough-walled.

Table 1 Morphological data of *Fusarium* isolates derived from soil and plant samples

***Fusarium* isolate**	**Species**	**Micro-conidium**	**Chlamydo-spore**	**Apical cell**	**Basal cell**	**Macroconidium**	
						Size (μm)	**Septae**
F23, F24, F29	*F. chlamydosporum*	ellipsoidal	clump, rough	curved, pointed	foot shaped	15–20×2–4.7	3–4
F4, F5, F6, F7, F8, F10, F14, F17	*F. decemcellulare*	oval	ND	blunt	foot shaped	24.5–50×3.5–4	5–6
F9, F13	*F. falciforme*	oval, obovoid	smooth	curved, pointed	barely notched	17–32×3–6	3–4
F3, F18, F19, F20, F21, F25, F30	*F. incarnatum*	ND	clump, smooth	curved	foot cell	23.5–58×3.5–6	5–6
F2, F12, F26, F28	*F. longipes*	ND	clump, smooth	tapered, curved	elongated	40–60×4–6	5–6
F22, F27, F32	*F. proliferatum*	clavate	chain	curved	foot shape	20–40×3–5	5–6
F1, F15, F31, F33, F34, F35, F36, F37, F38, F39, F40, F41, F42	*F. solani*	cylindrical, oval	clump, rough	tapered, curved	foot cell	25–36×4–6	3–4
F11, F16, F43, F44	*F. verticillioides*	club shaped	smooth	pointed	foot cell	20.2–60×3–5	3–4

Remark: ND = not detected

To complement the morphological data for taxonomic identification of the fungal isolates, DNA sequence data from 18 isolates representative of the eight species identified from morphology were analyzed. The sequence data were analyzed by BLAST search against sequences in the NCBI database. All 18 isolates produced top hits from one or more gene sequences to sequences from the corresponding species described by the morphological characteristics of the isolate described above (Table 2). For example, the *TEF1α* gene data for *F. chlamydosporum* (isolate F23) showed 96% identity with *F. chlamydosporum* (GQ505427). The *RPB1* and *RPB2* gene sequences also gave top

hits for *F. chlamydosporum* (JX171454) at 100% identity and for *F. chlamydosporum* (GQ505427) at 96% identity. Additionally, DNA sequence comparison of the *TEF1α, RPB1* and *RPB2* regions of *F. solani* (isolate F1) revealed that the three loci had 100% similarity with the GenBank accession numbers MG195132, LN828050 and LN828050 from *F. solani*.

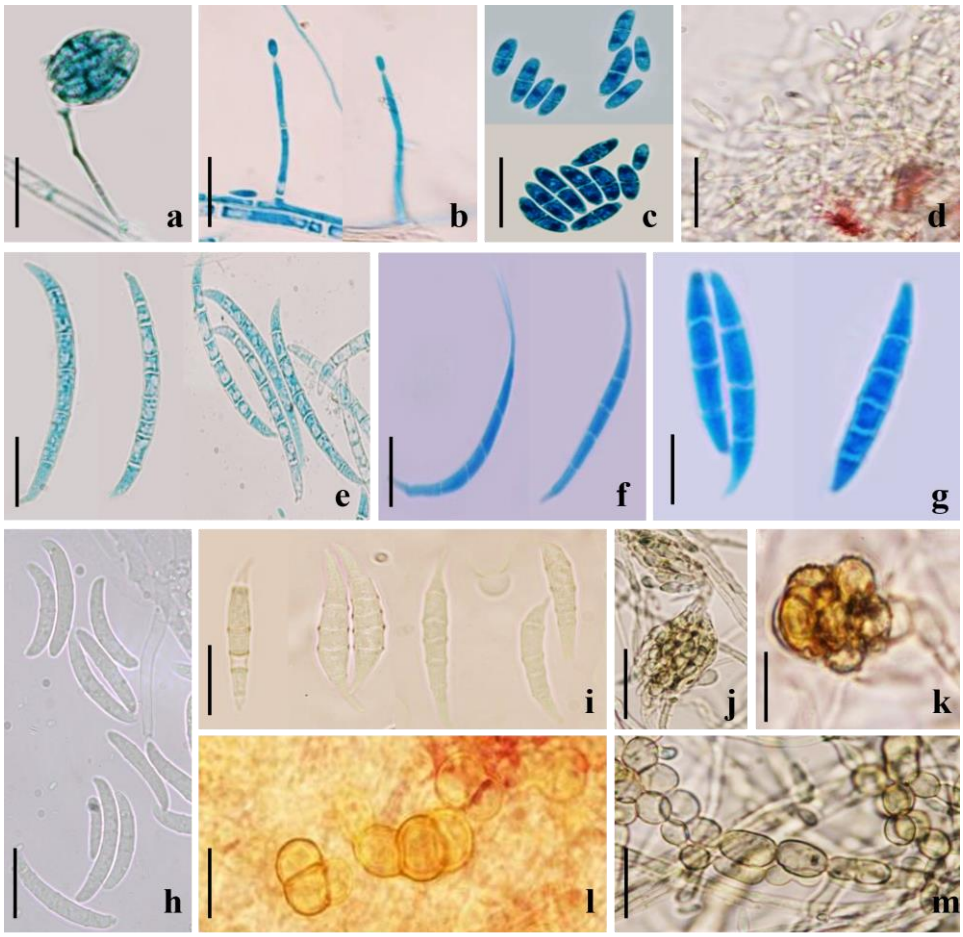

Fig. 1 – Morphological characteristics of selected *Fusarium* spp. in this study identified as *F. solani* (a, c, h); *F. proliferatum* (b, g); *F. verticillioides* (d, k, l); *F. decemcellulare* (e); *F. longipes* (f); *F. incarnatum* (i). Arrows indicate the following features: erect conidiophores bearing microconidia arranged in false heads (a); conidiophores (b); macroconidia with foot-shaped basal cell (f); 5-6 septated macroconidia (g); Chlamydospores (k, l, m). Scale bars = 10 μm.

Phylogenetic relationship of eight selected *Fusarium* taxa

The concatenated *TEF1α*, *RPB1* and *RPB2* sequence data were used for phylogenetic analysis to investigate the taxonomies of the 18 representative isolates in more detail (Fig. 2). The MP tree revealed by the parsimony analysis of 51 aligned sequences was selected to represent the relationships among these taxa and is shown in Fig. 2. The results showed that all isolates were assigned to six different species complexes (SC) of the genus *Fusarium* with 90% or greater branch support. Isolates F23 and F29 were placed in the FCSC clade that includes *F. clamydosporum, F. atrovinosum, F. sporodochiale* and *F. spinosum*. Whilst isolates F19, F30 and F3 were placed in the clade of FIESC that includes the *F. incarnatum* type species CBS132194. Isolates F2 and F12 were identified as *F. longipes* in FSAMSC, a sister clade to FIESC. Isolates F22 and F27 clustered with *F. proliferatum* type species NRRL22944 in the GFSC clade. Isolates F011 and F016 were also assigned to the GFSC clade, although they shared the same branch with *F. verticillioides*. Isolates F4, F14 and F17 were assigned to clade FDSC, consists of a single species, *F. decemcellulare.* Isolates F1, F9, F15 and F30 were assigned to the FSSC clade which includes the closely related species *F. solani* and *F. falciforme*.

Table 2 BLAST search results (The closest BLAST hit) against the NCBI GenBank nucleotide database for *TEF1α*, *RPB1* and *RPB2* sequence data from 18 isolates identified as *Fusarium* spp. using morphological characters.

Morphological identification	Code of isolate	Nearest BLAST match	GenBank accession no.	Identity (%) of *TEF1α*	Nearest BLAST match	GenBank accession no.	Identity (%) of *RPB1*	Nearest BLAST match	GenBank accession no.	Identity (%) of *RPB2*
F. chlamydosporum	F23	*F. chlamydosporum*	GQ505427	96	*F. chlamydosporum*	JX171454	100	*F. chlamydosporum*	GQ505427	96
	F29	*Fusarium* sp.	GQ505427	100	*F. chlamydosporum*	HM347187	100	*F. chlamydosporum*	EF470217	99
F. decemcellulare	F4	*F. decemcellulare*	KM231938	99	*F. decemcellulare*	LC212975	99	*F. decemcellulare*	KM232378	99
	F14	*F. decemcellulare*	KM231938	99	*F. decemcellulare*	JX171453	99	*F. decemcellulare*	LC214751	99
	F17	*F. decemcellulare*	MG857299	100	*F. decemcellulare*	LC212975	99	*F. decemcellulare*	JX171567	98
F. falciforme	F9	*Fusarium* sp.	KC820977	99	*F. falciforme*	JX1715416	99	*Fusarium* sp.	EU3296441	99
F. incarnatum	F3	*F. incarnatum*	JX2689961	99	*F. incarnatum*	HM347160	100	*F. incarnatum*	EF470121	100
	F19	*F. incarnatum*	GQ505628	100	*Fusarium* sp.	HM347160	99	*Fusarium* sp.	EF470121	99
	F30	*F. incarnatum*	JX268996	99	*Fusarium* sp.	JF741014	100	*F. equiseti*	LS479865	99
F. longipes	F2	*F. longipes*	GQ915509	99	*F. longipes*	MG282381	100	*F. longipes*	GQ915493	100
	F12	*F. longipes*	GQ915509	99	*F. longipes*	GQ915509	97	*F. longipes*	GQ915493	100
F. proliferatum	F22	*F. proliferatum*	JX8690165	100	*F. proliferatum*	KU171687	100	*F. proliferatum*	MG2824052	100
	F27	*F. proliferatum*	FN552086	98	*F. proliferatum*	KF466402	100	*F. proliferatum*	EF470122	98
F. solani	F1	*F. solani*	MG195132	100	*F. solani*	LN828050	100	*F. solani*	LN828050	100
	F15	*F. solani*	KM065871	99	*Fusarium* sp.	KC808296	98	*F. solani*	MF467484	99
	F31	*F. solani*	MG195132	100	*Fusarium* sp.	JX1715416	100	*Fusarium* sp.	JX1716531	100
F. verticillioides	F11	*F. verticillioides*	MH856066	100	*F. verticillioides*	KF466402	100	*F. verticillioides*	EF470192	99
	F16	*F. verticillioides*	MH582327	99	*F. verticillioides*	KF466402	100	*F. verticillioides*	KU604217	98

Pathogenicity test

The 44 *Fusarium* isolates revealed pathogenicity in the greenhouse trials when added to the soil in potted plants. The pathogens entered via the roots and interfered with the water conducting vessels of the plant. This caused wilting and yellowing symptoms of leaves, crown rot, and even root rot as shown in Fig. 3.

Effect of plant crude extracts on mycelial inhibition

Clove, turmeric, roselle, and lemongrass crude extracts were tested for mycelial inhibition against eight *Fusarium* isolates each belonging to different species. Inhibition was observed at 0.1 g L^{-1} or greater for each plant extract (Fig. 4). However, Fig. 5 showed the plates treated with the maximum dose of each extract (10 g L^{-1}). The plates with negative and positive controls were shown in Fig. 6 for comparison. Solvents used

to extract and dilute plant crude extracts including 95% ethanol and 2% DMSO did not affect the growth of *Fusarium* since PDA with a solvent showed a normal growth similar to the growth in PDA with water. All the four plant crude extracts showed significant inhibitory effects at the concentrations of 1 and 10 g L^{-1} compared to the control after 7−14 days of incubation, which is comparable in potency to the chemicals captan and prochloraz at the concentration of 1 g L^{-1} which were used as positive controls (Figs 4, 6).

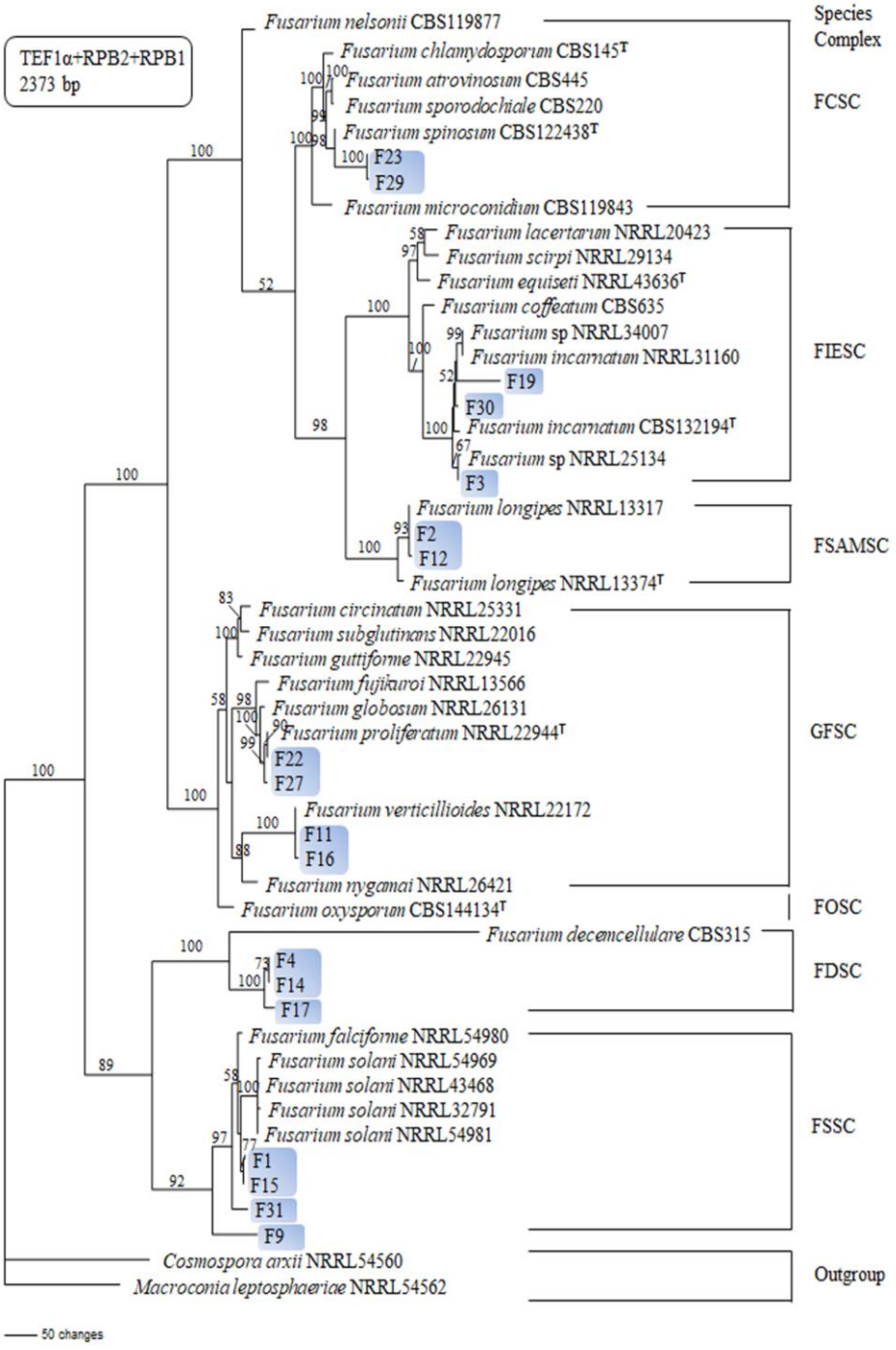

Fig. 2 – A maximum parsimony (MP) phylogeny obtained from concatenated *TEF1α*, *RPB1* and *RPB2* sequences of 51 taxa belonging to *Fusarium chlamydosporum* (FCSC), *F. incarnatum-equiseti* (FIESC), *F. sambucinum* (FSAMSC), *F. oxysporum* (FOSC), *F. dimerum* (FDSC) species complexes and the *Gibberella fujikuroi* (GFSC) species complexes. The final alignment for

phylogeny consisted of 2373 bp (*TEF1α* = 567 bp, *RPB1* = 913 bp, and *RPB2* = 881 bp). Numbers above branches indicate bootstrap values (>50%) from 1,000 replicates. Ex-type strains are indicated with T. The tree is rooted with *Cosmospora arxii* NRRL54560 and *Macroconia sphaeriae* NRRL54562. Descriptive tree statistics for the MP tree are shown as follows: TL = 41, CI = 0.605, HI = 0.395, RI = 0.865, and RC = 0.523.

Fig. 3 – Example of wilt disease symptoms in seedlings caused by *Fusarium* species at 7 days after inoculation in seedlings of *Cucumis sativus* (a); *Capsicum frutescens* (b); *Lycopersicon esculentum* (c); and control plants (d, e, f).

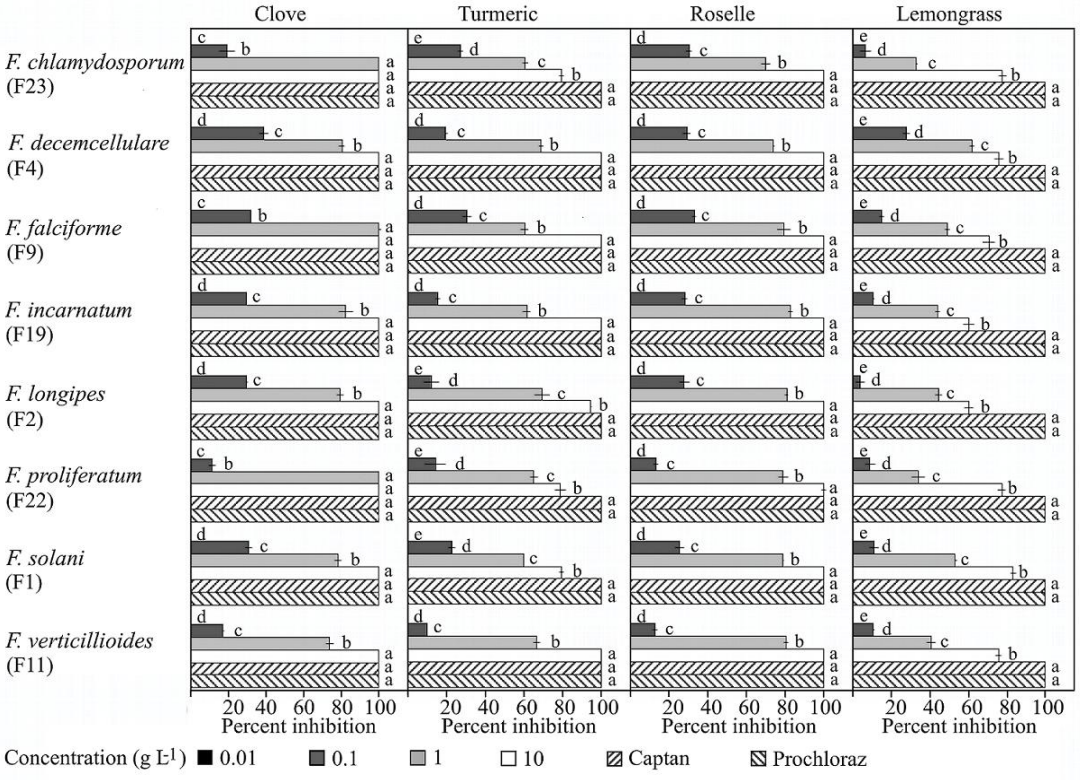

Fig. 4 – Inhibition of mycelial growth of *Fusarium* spp. on PDA amended with plant crude extracts at 0.01, 0.1, 1, and 10 g L^{-1} as well as captan and prochloraz at 1 g L^{-1}. The different lowercase letters denote significant difference (p-value < 0.05).

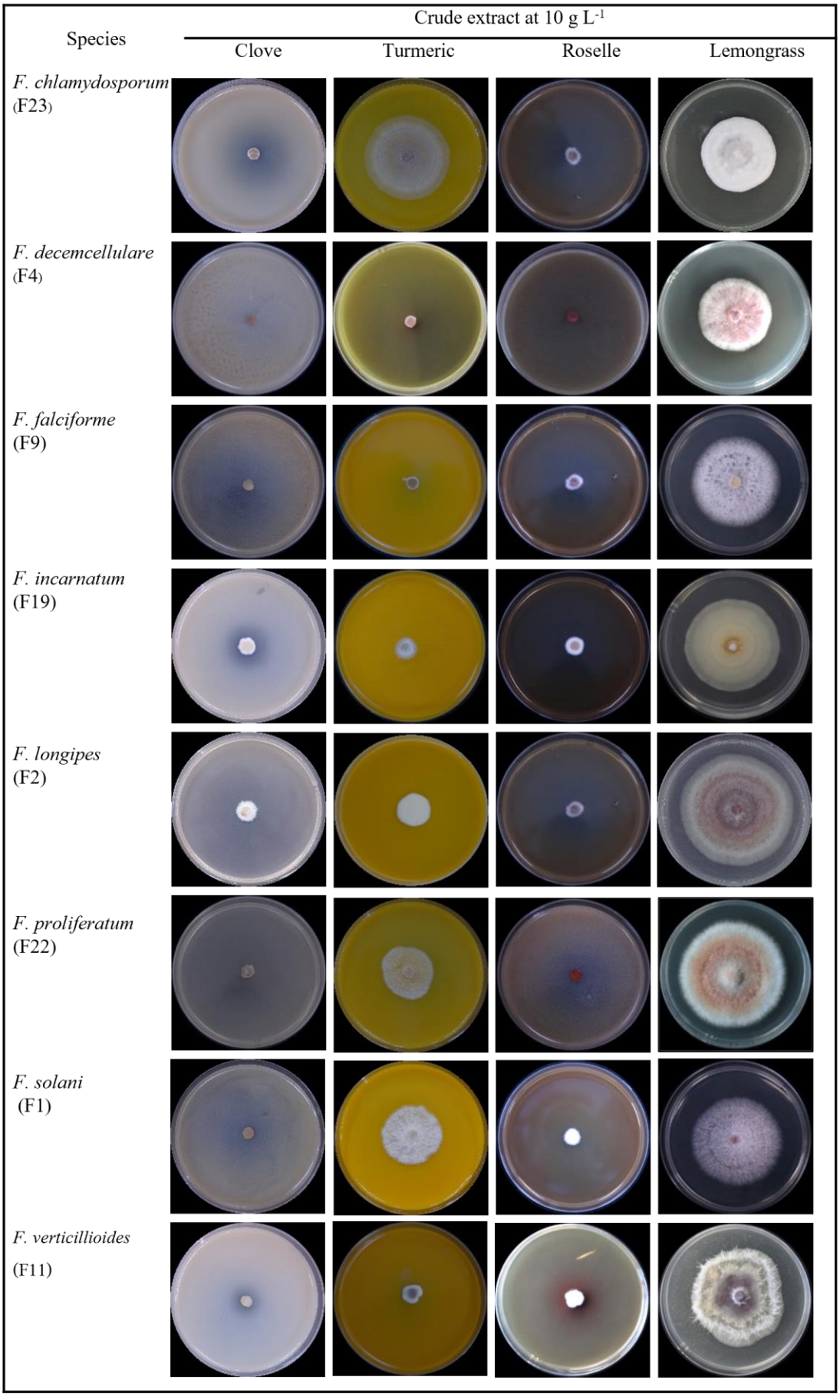

Fig. 5 – Inhibition of mycelial growth of *Fusarium* spp. on PDA with different plant crude extracts applied at 10 g L^{-1}.

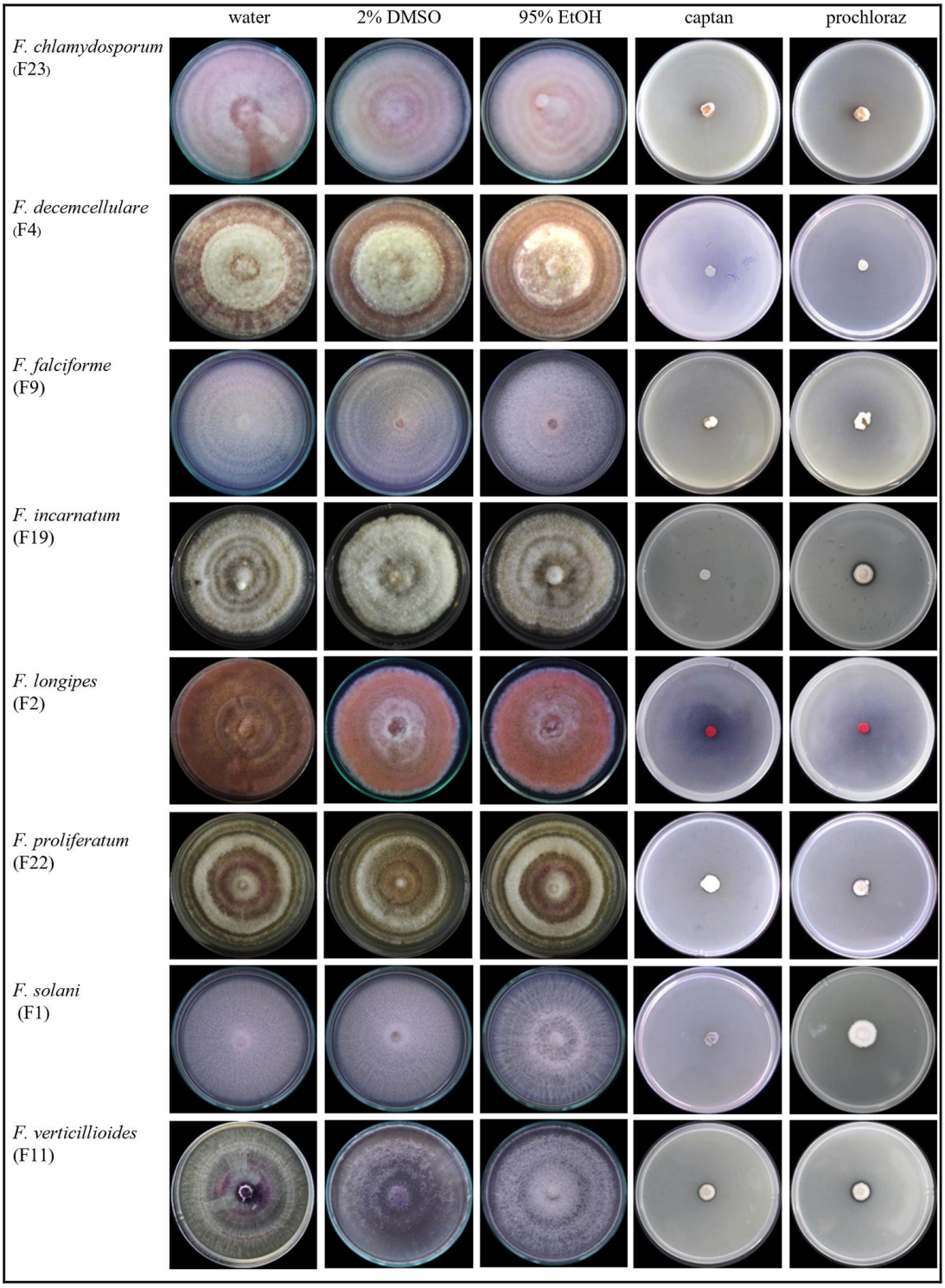

Fig. 6 – Inhibition of mycelial growth of *Fusarium* spp. on PDA with control treatments. Negative controls include water, DMSO, and ethanol. Captan and prochloraz (1 g L^{-1}) were tested as two positive controls.

Discussion

Clove and roselle extracts were the most potent extracts and could completely inhibit all tested eight *Fusarium* isolates. These results concur with those of Abhishek et al. (2017) who showed that clove crude extract completely inhibited mycelial growth and spore germination at 125 ppm with the IC50 value of 18.2 ppm. Similarly, Velluti et al. (2004) and Bhuiyan et al. (2010) observed that clove oils and cinnamon leaves could reduce the colony growth of *F. verticillioides*, *F. proliferatum* and *F. graminearum* under all tested conditions including water activity and

temperature. Clove and cinnamon oils reduced fungal colony growth by 62 and 80%, respectively. However, some researchs reported that the antifungal activities of clove and roselle crude extracts are attributed to the presence of the antifungal phenolic compounds such as eugenol, eugenyl acetate, and caryophyllene. These compounds can damage fungal cell walls and cell membranes, resulting in cytoplasm leakage and inhibition of DNA, RNA, protein and peptidoglycan biosynthesis (Fang et al. 2006, Lopez et al. 2007, Bakkali et al. 2008).

In addition, turmeric extract also displayed complete inhibition of four *Fusarium* isolates (*Fusarium decemcellulare* (F4), *F. falciforme* (F9), *F. incarnatum* (F19), and *F. verticillioides* (F11); Figs 4, 5). According to Chen et al. (2018), alcoholic extract of turmeric at the concentration of 0.5 g L^{-1} inhibited five *Fusarium* species, including *F. chlamydosporum*, *F. culmorum*, *F. graminearum*, *F. oxysporum* and *F. tricinctum* at 67.97, 63.50, 63.80, 41.20, and 65.40%, respectively. Turmeric crude extract contains the bioactive compound curcumin, which has previously been reported to inhibit mycelial growth of *F. verticillioides* at 1 g L^{-1} (Singh et al. 2002). Similarly, at 2 g L^{-1} the turmeric crude extract also inhibited growth of *Aspergillus niger*, *F. oxysporum*, *F. culmorum* and *F. graminearum* (Ferreira et al. 2013, Perczak et al. 2019).

For lemongrass crude extracts, growth of *F. solani* (F1) and *F. falciforme* (F9) was inhibited by 83.27 and 73.67% (Figs 4, 5), respectively, after 7 days of incubation period compared to the control as shown in Fig. 6. Studies by Krzyśko-Łupicka et al. (2020) and Ameziane et al. (2007) showed that lemongrass extracts had antifungal properties against *F. solani*, *F. graminearum*, and *F. oxysporum* f. sp. *tulipae*, which is probably due to the activity of phenolic and flavonoid constituents. Notwithstanding, polyphenolic compounds found in plant essential oils have been previously reported to exhibit microbial and antifungal activity (Mehani et al. 2014, Seseni et al. 2015).

Medicinal plant crude extracts are recognized to have relatively low or little toxicity, increasing the chance of success for the development of botanical fungicides by using plant-derived metabolites (Yoon et al. 2013). However, the development of botanicals as replacements for chemical fungicides such as captan in the future will be carefully required selection of solvents, including methods for extraction, phytochemical screening, fractionation, and identification of bioactive compounds. In addition, botanical fungicides should be assessed for cost, applicability, safety and phytotoxicity against crops.

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