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ORIGINAL ARTICLE

Plant extracts as potential control agents of Black Sigatoka in banana

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Abstract

Aqueous extracts of *Cinnamomum zeylanicum*, *Capsicum annuum* and *Azadirachta indica* were tested for efficacy for management of *Pseudocercospora fijiensis* in banana (*Musa* spp.) when applied as foliar sprays. Extracts of *C. annuum* and *A. indica* demonstrated fungicidal effects *in vitro*, without showing phytotoxic effects. The two extracts protected tissue culture banana plantlets of cultivar Musakala to a similar degree as the traditional fungicide difenoconazole. Extracts of *A. indica* and *C. annuum* (0.3 g/ml) reduced Black Sigatoka severity by 69.3% and 65.6%, respectively, and were thus comparable to the fungicide difenoconazole (72.7%). Soil drenching of the extracts did not significantly reduce Black Sigatoka severity. *In planta* effects of the extracts mainly consisted of delayed symptom appearance and reduced lesion number. Symptom development was dependent on extract concentration and days between extract application and inoculation. Effect of the tested extracts on height of plants and new leaves was not significant. Collectively, our data suggest that *A. indica* and *C. annuum* have interesting and unique properties as plant protection agents against *Pseudocercospora fijiensis*, but further research is needed to investigate their efficacy.

Keywords Banana · *Mycosphaerella fijiensis* · Plant extract · Protection · *Pseudocercospora fijiensis*

Introduction

Black Sigatoka, caused by the ascomycete *Pseudocercospora fijiensis* (M. Morelet) Deighton (syn. *Mycosphaerella fijiensis* M. Morelet), (Marin et al. 2003), is a major threat to plantain and banana production in Africa (Batte et al. 2019). In Uganda, Black Sigatoka is one of the most important constraints to production of the East African highland banana (AAA genome), particularly in the central and eastern parts of the country (Nowakunda et al. 2015). East African highland bananas (EAHB) are the most popular genotype grown by Ugandan farmers (Tushemereirwe et al. 2004). The first symptom of Black Sigatoka is the appearance of pale yellow or dark-brown streaks, 1–2 mm long, on the abaxial leaf surface. These streaks enlarge into dark-brown to black advanced streaks, which later merge into spots with a dark-brown to black centre often surrounded by yellow halos (Stover and Simmonds 1987). Six stages of lesion development following infection by *P. fijiensis* have been described (Churchill 2011). The first stage is characterized by faint reddish-brown specks on the abaxial side of the leaves. In the second stage, specks elongate and turn into reddish brown streaks. The streaks coalesce and change to dark brown colour in the third stage. In the fourth stage, lesions become elliptical with water soaked borders and the fifth stage is reached when the dark brown or black centre of the spots becomes depressed and the spots are surrounded by a yellow halo. The centre of the spot turns light grey, dries and becomes necrotic in the sixth stage. Additional symptoms include premature drying of leaves and incomplete filling of banana fingers (Nelson 2008).

In Uganda, Black Sigatoka is endemic and found in nearly all banana-growing regions (Kimunye et al. 2019; Tushemereirwe 1996). Areas free of the disease have been reported, and the highland areas are less impacted by the

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disease (Kimunye et al. 2019). Tushemereirwe (1996) reported banana yield reduction in EAHB of 30–50% in Uganda. While the disease does not immediately kill infected plants, it negatively affects bunch weight and yield by interfering with photosynthesis (Vézina and Van den Bergh 2018). Elsewhere Black Sigatoka has been reported to cause a bunch weight loss of up to 37% and a decline in fruit quality (Churchill 2011). Banana, particularly, the cooking banana, is a staple food crop in Uganda. According to FAO (2018), 75% of households in Uganda cultivated banana in 2017 on approximately 1.5 million hectares. Annual banana production in 2010 was estimated at 9.512 million tonnes (Uganda Bureau of Statistics 2010). Black Sigatoka is therefore, a threat to food security in Uganda (Barekye 2009).

Fungicide use and breeding for resistance are the main control strategies for the disease.

However, the use of fungicides is not a practical option in Uganda, since fungicides are expensive and beyond the reach of resource-constrained subsistence farmers. Currently, the main control strategy for Black Sigatoka in Uganda is breeding for resistance and the use of cultural practices (Mortensen et al. 2013). Resistance to Black Sigatoka among pre-existing banana genotypes is poor, resulting in poor control. Some resistant cultivars of plantains and bananas such as FHIA-17 (AAAA genome), FHIA-25 (AAB genome), M9 hybrid (AAAA genome) and M2 hybrid (AAAA genome) are available and could be used in subsistence agriculture. However, they are usually less desirable in terms of longevity, marketability (Gold et al. 2002) and taste (Edmeades 2006) than those that are susceptible (Nowakunda and Tushemereirwe 2004; Akankwasa et al. 2013). Cultural practices including pruning, improvement of drainage and correct plant spacing have been used to manage the disease. None of these methods is considered effective and chemical control is often harmful to the environment, animal and human health. Given the threat posed by the disease to food security as well as environmental concerns of using chemicals for control, there is a need to develop alternative control strategies where eradication is not possible. The search for new, naturally derived, locally available and environmentally friendly products can be an option for the control of Black Sigatoka since their use is considered an important component of sustainable agriculture (Nwokeji et al. 2011).

Although extracts from plants offer alternative control options to synthetic chemicals (Khan and Nasreen 2010; Gurjar et al. 2012), they have not been exploited for control of *P. fijiensis* in banana. Indigenous plant extracts with antifungal activity against *P. fijiensis* may be exploited further for developing an integrated disease management strategy. Limited information exists in the literature regarding control of banana diseases using plant extracts. Many studies have only tested plant extracts in vitro, not providing information about the control potential of plant extracts in the crop itself. *In planta* tests are necessary to understand the potentency of a control agent as the interaction between pathogen, plant and environment may greatly influence efficacy. Preliminary studies have shown that plant extracts can partially control Black Sigatoka in banana (Nwokeji et al. 2011; Gumisiriya 2014). In both studies, only one extract application method was tested with one preventative application timing. In addition, comparison of plant extract and fungicide efficacy and effect of extracts on growth parameters were not investigated. This paper, describes the effect of aqueous extracts of *Cinnamomum zeylanicum*, *Azadirachta indica* and *Capsicum annuum* on Black Sigatoka in banana plantlets under controlled conditions in Uganda when applied as foliar spray and when incorporated in the soil.

Materials and Methods

In vitro tests of effects of extracts of plants on *P. fijiensis*

Extracts were prepared from three-year-old *C. zeylanicum*, two-year-old *A. indica* and four-month-old *C. annuum* plants. Fresh leaves from *A. indica*, bark from *C. zeylanicum* and fruits from *C. annuum* were harvested during late afternoon (5:00 pm) to ensure physiologically comparable tissues from northern Uganda for *A. indica* and *C. annuum* and central Uganda for *C. zeylanicum*. Extracts were prepared according to Kamalakannan and Shanmugam (2009), with slight modifications. Briefly, soil contamination was removed by washing with domestic tap water, followed by rinsing with sterile distilled water. Clean samples were air-dried for 7 days in a screenhouse at temperature 22–28 °C. A blender (Waring 1-Liter Laboratory Blender (3390D43), USA) was used to thoroughly pulverise the dried samples of each plant species into powder. The powder was stored in waterproof plastic bags at 4 °C until use. 250 g of the pulverised plant parts was dissolved in 500 ml of distilled water to extract potential fungicidal compounds. Water was used because has been previously used as an extractant of biologically active compounds (Lidiya et al. 2015; Mihaylova and Lante 2019). The mixture was incubated at room temperature (25–28 °C) for 24 h, yielding 0.5 g/ml plant extract. Extract mixtures were filtered through four layers of cheese cloth to remove plant debris and subsequently adjusted to a concentration of a 0.5 g/ml solution by adding 500 ml of sterile distilled water. The prepared extract was either used immediately or stored at 4 °C for later use.

Pseudocercospora fijiensis (syn. *Mycosphaerella fijiensis*, local isolate Mak 01) was cultured on potato dextrose agar (PDA, Sigma-Aldrich, USA) at 25 °C. Agar disks (3 mm in

diameter) of 14-day-old cultures of *P. fijiensis* from actively growing PDA cultures at 25 °C were aseptically transferred to Malt Extract Agar plates (Sigma-Aldrich, USA). Twenty-five plates for each experiment were incubated at 25 °C for 21 days in darkness. Mycelium was harvested by scraping with a sterile scalpel followed by bulking and weighing on pre-weighed sterile filter paper. Harvested mycelium was fragmented at full speed for 3 min in a blender (commercial blender). A mycelium suspension of 15 mg/ml was prepared from a master mycelium suspension.

Four concentrations of plant extracts were examined for their direct effects on growth of *P. fijiensis* in vitro on PDA plates. Extracts from *C. zeylanicum*, *A. indica* and *C. annuum* were filter sterilized using a 0.2 µm Millipore Nalgene Syringe Filter (Thermo Scientific, USA). Extracts were tested against *P. fijiensis* in the concentrations of 0.05, 0.1, 0.2 and 0.3 g/ml. Water served as a control and the fungicide difenoconazole (Score®240 EC, Syngenta, USA, 0.1%, v/v) was included for comparison. A sterile mycelial plug (3 mm) picked from the middle of the colony was placed centrally in the agar plates. Growth of mycelia (orthogonal colony diameters) was assessed every seven days up to 21 days. Each treatment was replicated four times.

Effect of foliar spraying of plant extracts on Black Sigatoka in banana

The effect of foliar application of extracts was tested under screenhouse conditions. Tissue culture banana plantlets of cultivar Musakala (EAHB-AAA) were used for the assay. Musakala is susceptible to Black Sigatoka and would provide a verifiable proof of disease control. Two-month-old plantlets were raised in individual pots containing pre-sterilized loam soil. The plantlets were raised in the screenhouse (temperature 22–28 °C) for one month under natural light conditions. Plantlets with 4–6 fully expanded leaves were used for experimentation. Plant extracts were prepared as described in "In vitro tests of effects of extracts of plants on *P. fijiensis*". A randomised complete block design with split-split plot arrangement (3 replications) was adopted for the experiment. Three extracts (*A. indica*, *C. zeylanicum* and *C. annuum)* were randomly assigned to whole plots within each block. Each whole plot was divided into 4 sub-plots, in which the different concentrations (0, 0.1, 0.2 and 0.3 g/ml) were randomly assigned. Each sub-plot was further divided into 4 sub-sub plots for which 4 time points (0, 1, 3 and 6 days before inoculation with *P. fijiensis*) were randomly assigned. Each treatment was applied to 3 plantlets. Banana plantlets were sprayed with extracts (50 ml) using a plastic hand-sprayer until run-off with the individual extracts. Controls were sprayed with distilled water.

Extract-treated and control treated plantlets were inoculated with 1 ml of *P. fijiensis* mycelial suspension (15 mg/ml) by spraying the abaxial surfaces of the first and second fully unfolded leaves using an airbrush sprayer (Fen Yuan, China). After inoculation, plants were incubated for 48 h in a humid chamber made of a wood frame covered with a polythene sheet (temperature of 28–31 °C and RH of approximately 90%). To maintain a continuously high relative humidity, two open 5-l plastic beakers containing 10 l of water were placed in the humid chamber. A wall thermometer was placed inside the chamber to monitor temperature. Plantlets were then moved to a bench in the screenhouse and observed for weekly symptom development on both leaf surfaces for two weeks and thereafter daily. To assess disease, incubation period and severity (number of lesions) were used. In addition, data on increase in plant height (cm) and number of new leaves formed were recorded. Height of young plants was measured using a tape measure by measuring from the soil surface to the node of the developing leaf. Efficacy of the extracts was calculated as follows: Efficacy (%) = [(B-A) × 100]/B, where A and B are disease incidence/lesion number for the treatment and the untreated control, respectively. The experiment was performed in duplicate.

Effect of soil drenching plant extracts on Black Sigatoka in banana

The effect of soil drenching with the extracts was tested in the screenhouse using the tissue culture banana plantlets of cultivar, Musakala. Banana plantlets and *P. fijiensis* inoculum were as described in "In vitro tests of effects of extracts of plants on *P. fijiensis*" and "Effect of foliar spraying of plant extracts on Black Sigatoka in banana", respectively. The experiment was established following a randomised complete block design with 4 replications. Three extracts (*A. indica*, *C. zeylanicum* and *C. annuum)* were tested. The three extracts were prepared following the same procedure as outlined in "In vitro tests of effects of extracts of plants on *P. fijiensis*". Extract volumes (100 or 50 ml) at the concentrations 0.1, 0.2 and 0.3 g/ml were evaluated for each extract. Controls were drenched with water (100 or 50 ml). Each treatment was administered to 3 banana plantlets. Extract-treated and control plantlets were inoculated with 1 ml of *P. fijiensis* mycelial suspension (15 mg/ml) at 24 h after treatment. Inoculated plants were incubated following the same procedure as outlined in "In vitro tests of effects of extracts of plants on *P. fijiensis*". To assess disease, incubation period and severity (number of lesions) were used. In addition, data on increase in plant height (cm) and number of new leaves formed were recorded. Height of plants was measured following the same procedure as described in "Effect of foliar spraying of plant extracts on Black Sigatoka in banana". The experiment was performed twice.

Effect of foliar spraying of extract combinations on Black Sigatoka in banana

The effect of foliar application of extract combinations was tested in the screenhouse on the tissue culture banana plantlets of Musakala. Banana plantlets and *P. fijiensis* innoculum were prepared as described in "In vitro tests of effects of extracts of plants on *P. fijiensis*" and "Effect of foliar spraying of plant extracts on Black Sigatoka in banana, respectively". Three extracts (*A. indica*, *C. zeylanicum* and *C. annuum)* were tested. The three extracts were prepared following the same procedure as outlined in "In vitro tests of effects of extracts of plants on *P. fijiensis*". A randomised complete block design with 3 replications was adopted for the experiment. Four extract combinations were tested and compared with single extracts at 0.3 g/ml (Table 1).

Extract combinations were prepared from 0.3 g/ml stocks by mixing extracts using a ratio of 1:1, except for the combination of three extracts (*A. indica* + *C. annuum* + *C. zeylanicum*) where a ratio of 1:1:1 was used. Banana plantlets were sprayed using a hand-sprayer until run-off with individual extracts or combinations (50 ml). Controls were sprayed with 50 ml distilled water. Each treatment was administered to 3 banana plantlets. Treated banana plantlets were kept on the bench in the screenhouse (temperature 22–28 °C) until inoculation. Extract-treated and control plantlets were inoculated with 1 ml *P. fijiensis* mycelial suspension (15 mg/ml) at 24 h after extract treatment. Incubation of inoculated plants was done following the same procedure as outlined in "Effect of foliar spraying of plant extracts on Black Sigatoka in banana" To assess disease, incubation period and severity (number of lesions) were used. In addition, data on increase in plant height (cm) and number of new leaves formed were recorded. Height of plants was measured following the same procedure as outlined in "Effect of foliar spraying of plant extracts on Black Sigatoka in banana" The experiment was performed in duplicate. Extract mixture efficacy was determined using the Colby analysis (Flint et al. 1988) using the equation $E = (X \times Y)/100$, where X and Y are the effects of the extracts applied alone (expressed as percent-of-control).

Comparing efficacy of plant extracts with synthetic fungicide

The efficacy of plant extracts was compared with a synthetic fungicide in the screenhouse using tissue culture banana plantlets of Musakala (EAHB-AAA). Banana plantlets and *P. fijiensis* were prepared following the procedures outlined in "Effect of foliar spraying of plant extracts on Black Sigatoka in banana" and 2.1, respectively. *A. indica*, *C. zeylanicum* and *C. annuum* extracts were prepared as described in "In vitro tests of effects of extracts of plants on *P. fijiensis*". The experiment was established following a randomised complete block design with 4 replications. *A. indica*, *C. zeylanicum* and *C. annuum* at a concentration of 0.3 g/ml were tested for efficacy compared to the fungicide difenoconazole (Score® 240 EC, Syngenta, USA, 0.1% [v/v]) at 0.3 µg/ml. Each treatment was administered to 4 banana plantlets. Banana plantlets were sprayed with extracts (50 ml) using a hand-sprayer until run-off. Controls were sprayed with fungicide. Extract and fungicide-treated plantlets were inoculated with 1 ml of *P. fijiensis* mycelial suspension (15 mg/ml) at

Table 1 Mean colony growth of *Pseudocercospora fijiensis* on PDA amended with different concentrations of plant extract in vitro

Treatment (PDA amendment)	Concentration (g/ml)	Week 1	Week 2	Week 3	ED50 (g/ml)
		Mean fungal colony growth (mm)			
C. zeylanicum	0.05	1.0	8.0	17.3	0.446
C. zeylanicum	0.1	0.7	8.5	17.0	
C. zeylanicum	0.2	0.6	6.2	13.8	
C. zeylanicum	0.3	0.5	5.6	11.7	
A. indica	0.05	0.8	7.6	14.1	0.098
A. indica	0.1	0.5	4.8	8.6	
A. indica	0.2	0.0	2.0	4.3	
A. indica	0.3	0.0	0.0	0.0	
C. annuum	0.05	0.5	3.8	12.0	0.077
C. annuum	0.1	0.1	2.1	6.1	
C. annuum	0.2	0.0	1.0	1.9	
C. annuum	0.3	0.0	0.0	0.0	
Difenoconazole[a] (µg/ml)	0.3	0.0	0.0	0.0	0.154
Control (PDA)[b]	0.0	0.9	7.9	17.7	
$LSD_{0.05}$		0.2	0.8	1.3	

a = medium amended with fungicide (difenoconazole), b = untreated control (PDA), ED50 = effective dose 50%

24 h after treatment. Disease was assessed as incubation period and severity (number of lesions). In addition, data on increase in plant height (cm) and number of new leaves formed were recorded. Efficacy of the extract and fungicide was calculated as follows: efficacy (%) = 100 × (1—a/b), with a = disease severity (lesion number) for the treatment and b = disease severity (lesion number) for the untreated control (Barbara et al. 2006). The experiment was performed twice.

Phytochemical screening of plant extracts

Phytochemical analysis of plant extracts was done to determine the diversity of antimicrobial compounds. Quantitative estimation of the percentage crude chemical constituents of the extracts was carried out as described by Edeoga et al. (2005). Two grams of the samples were defatted with 100 ml diethyl ether (Sigma-Aldrich) for 2 h. Total phenols were determined using the method described by Obadoni and Ochuko (2001). Fat free samples were boiled with 50 ml ether (Sigma-Aldrich) for 15 min. Five millilitres of the extract was pipetted into a 50 ml flask and then 10 ml distilled water was added. Two millilitres of ammonium hydroxide solution (Sigma-Aldrich) and 5 ml concentrated amyl alcohol (Thermo Fischer Scientific) were also added.

To measure total phenolics, the absorbance of the samples were measured in a spectrophotometer at 505 nm (Thermo Fischer Scientific). Total alkaloids were determined using the method described by Adeniyi et al. (2012). Five grams of the sample was weighed into a 250 ml beaker and 200 ml 10% (v/v) acetic acid (Ricca) in ethanol (Sigma-Aldrich) was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a boiling water bath to one-quarter of the original volume. Concentrated ammonium hydroxide (Sigma-Aldrich) was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with 0.1 M ammonium hydroxide (Sigma-Aldrich) and then filtered. The residue represents the alkaloids and was dried and weighed.

Tannin content was determined using the method described by Van-Buren and Robinson (1969). Five hundred milligrams of the sample was weighed into a 100 ml plastic bottle. Fifty millilitres of distilled water was added and shaken for 1 h in a mechanical shaker (200 rpm). This was filtered into a 50 ml volumetric flask and made up to 50 ml with distilled water. Then 5 ml of the filtered solution was pipetted into a test tube and mixed with 2 ml 0.1 M iron (III) chloride ($FeCl_3$) (Sigma-Aldrich) in 0.1 N HCl (Sigma-Aldrich) and 0.008 M potassium ferrocyanide (Sigma-Aldrich). The absorbance was measured in a spectrophotometer at 120 nm (Thermo Fischer Scientific) within 10 min. Flavonoid content was determined using the method described by Bohm and Koupai-Abyazani (1994). Ten grams of the plant sample was extracted using 100 ml 80% (v/v) aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a boiling water bath and weighed.

Saponin content was determined as described by Obadoni and Ochuko (2001). Twenty grams of the ground sample was added to 200 ml 20% (v/v) ethanol and the suspension was heated in a water bath for 4 h with continuous stirring at 55 °C. The mixture was filtered and the residue re-extracted with another 200 ml 20% (v/v) ethanol. The combined extracts were reduced to 40 ml in a water bath at 90 °C. The concentrate was transferred to a 250 ml separator funnel and 20 ml diethyl ether (Sigma-Aldrich) was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. Sixty millilitres n-butanol (Sigma-Aldrich) was added. The mixture of n-butanol and extracts was washed twice with 10 ml 5% (v/v) aqueous sodium chloride. The remaining solution was heated in a water bath at about 90 °C. The samples were dried in an oven at 100 °C until a constant weight is obtained. The saponin content was calculated in percent (Obadoni and Ochuko 2001).

The effect of crude extracts of total flavonoids, tannins, phenolics and alkaloids on growth of *P. fijiensis* was assayed in vitro on PDA plates following the procedure described by Maddox et al. (2010). The experiment was established following a randomised complete block design, with three replications. Extracts of the chemical constituents were filter sterilized using a 0.2 µm Millipore Nalgene Syringe Filter (Thermo Scientific, USA). A concentration of 0.5 mg/ml of crude extracts of total flavonoids, tannins, phenolics and alkaloids was tested together with water as a control. Ten millilitres of crude extracts from a stock solution (1.0 mg/ml) were mixed with 80 ml PDA for each chemical constituent in separate 100 ml conical flasks to prepare concentrations of 0.2 mg/ml extract in PDA (Mukherjee et al. 2011). Three Petri dishes (9 cm) were prepared for each treatment. A sterile mycelial plug (3 mm) was placed in the centre of agar plates. Growth of mycelium (colony diameter) was assessed every seven days up to 21 days. Each treatment was replicated three times and was performed in duplicate.

Statistical analyses

Data on incubation period, plant height, percentage leaf area infected and percentage disease reduction were analysed by analysis of variance (ANOVA) assuming normal distribution. Number of lesions and number of new leaves formed were analysed by logistic regression. Data analysis was performed using GENSTAT statistical package 16th edition. The data were subjected to ANOVA, and residual plots were used to check ANOVA assumptions. Hypotheses were

rejected at $P \leq 0.05$ and means compared by Tukey's test. All experiments were performed in duplicate and mean values of results presented. Duplicate experiments were analysed together as there were no interactions between treatment and experiment.

Results

Fungicidal activity of plant extracts in vitro

The results of the in vitro effect of plant extracts are presented in Table 1. The crude extracts of *Cinnamomum zeylanicum, Azadirachta indica* and *Capsicum annuum* significantly inhibited the growth of *Pseudocercospora fijiensis* in vitro. The inhibitory effect of the extracts varied with concentrations and type of extract. For example, *C. zeylanicum* significantly inhibited the growth of *P. fijiensis* at 0.2 g/ml and 0.3 g/ml but had no significant effect at lower concentrations (0.1 g/ml and 0.05 g/ml). On the other hand, *A. indica* and *C. annuum* significantly inhibited growth of *P. fijiensis* at 0.05, 0.1, 0.2 and 0.3 g/ml over the three week period of incubation. In general, growth inhibition increased with the concentration of each extract although the plant extracts exhibited differing degrees of antifungal activity against *P. fijiensis*. The most vigorous growth was recorded for the control (17.7 mm) and the lowest for *A. indica* (0.0 mm), *C. annum* (0.0 mm) at 0.3 g/ml and difenaconazole (0.0 mm) at 0.1% (v/v). Overall, inhibition ranged from 1.3% to 33.0% for *C.zeylanicum*, 14.8% to 100% for *A. indica* and 38.6% to 100% for *C. annuum*.

Disease reducing efficacy of the plant extracts *in planta* based on single applications

The efficacy of single extract effects *in planta* is presented in Table 2. Black Sigatoka severity on plants treated with water (control) was significantly higher than in plants where extracts were applied as foliar spray, except for *C. zeylanicum* when applied 3 and 6 days before inoculation (DBI). Extract efficacy increased with concentration. The highest percentage leaf area infected was recorded on the water control (80.0%) and the lowest on *A. indica* treated plants (16.7%) when 0.3 g/ml of extracts was applied 0 DBI. Percentage leaf infected area for *C. zeylanicum* and *C. annuum* were 56.6% and 21.7% respectively. Overall, percentage leaf infected area of all the extracts increased with increasing interval between extract application and inoculation. Furthermore, the incubation period differed significantly among the plant extracts. Symptom development was delayed by *A. indica*, *C. annuum* and *C. zeylanicum* at a concentration of 0.3 g/ml when the extracts were applied as foliar sprays 0 DBI.

Table 2 Percentage leaf area infected and incubation period of banana plantlets sprayed with different plant extracts

Extract	Concentration (g/ml)	Mean percentage leaf area infected					Mean incubation period (days)					Mean plant growth parameters	
		Extract application DBI					Extract application DBI						
		0	1	3	6	$LSD_{0.05}$	0	1	3	6	$LSD_{0.05}$	Height (cm)	No. of new leaves
Control	0.0	80.0 ± 0.8a	85.0 ± 0.8a	82.7 ± 0.4a	85.0 ± 0.5a	4.7	22.0 ± 0.0d	21.3 ± 0.5d	21.3 ± 0.9c	21.7 ± 0.5a	0.8	13.1 ± 0.2a	3.5 ± 0.6a
A. indica	0.1	52.3 ± 0.5d	64.3 ± 0.5c	71.0 ± 1.2b	79.9 ± 0.9b	4.3	26.3 ± 0.5c	23.3 ± 0.5 cd	23.0 ± 0.0bc	23.0 ± 0.0a	1.0	14.6 ± 0.3a	3.4 ± 0.5a
A. indica	0.2	39.7 ± 0.5e	45.7 ± 0.5e	60.0 ± 0.8c	70.0 ± 0.4 cd	4.2	31.3 ± 0.5b	28.3 ± 0.5b	23.7 ± 0.5bc	23.0 ± 0.0a	2.3	13.6 ± 0.4a	3.6 ± 0.5a
A. indica	0.3	16.7 ± 0.5 g	27.9 ± 0.2 g	47.1 ± 0.9e	66.0 ± 0.9d	3.2	35.7 ± 0.9a	32.7 ± 0.5a	26.0 ± 0.9ab	23.0 ± 0.0a	1.9	14.2 ± 0.2a	3.5 ± 0.4a
C. annuum	0.1	57.4 ± 0.4d	60.0 ± 1.1c	70.5 ± 0.6b	81.6 ± 0.5ab	3.4	30.7 ± 0.8	26.3 ± 0.5b	25.0 ± 0.0b	23.0 ± 0.0a	2.2	13.2 ± 0.2a	3.5 ± 0.6a
C. annuum	0.2	41.8 ± 0.7e	51.6 ± 0.5d	62.9 ± 0.7c	73.1 ± 0.6c	3.5	33.3 ± 0.5b	28.3 ± 0.5b	25.7 ± 0.5ab	23.3 ± 0.5a	2.6	14.2 ± 0.2a	3.4 ± 0.5a
C. annuum	0.3	21.7 ± 0.4f	38.0 ± 0.8f	52.1 ± 0.4d	63.5 ± 0.6d	3.1	36.7 ± 0.5a	30.7 ± 0.5a	27.3 ± 0.5a	23.3 ± 0.5a	2.3	13.0 ± 0.0a	3.6 ± 0.5a
C. zeylanicum	0.1	66.8 ± 0.5b	75.0 ± 0.8b	81.2 ± 0.9a	83.6 ± 0.5ab	3.2	26.0 ± 0.0c	23.7 ± 0.5c	23.0 ± 0.8bc	22.0 ± 0.0a	1.1	13.8 ± 0.2a	3.5 ± 0.6a
C. zeylanicum	0.2	62.0 ± 0.8c	71.7 ± 0.4b	78.6 ± 0.7a	81.3 ± 0.6ab	3.7	26.7 ± 0.4c	23.3 ± 0.5 cd	22.3 ± 0.5c	22.0 ± 0.0a	1.2	13.6 ± 0.3a	3.6 ± 0.5a
C. zeylanicum	0.3	56.6 ± 0.5d	61.0 ± 0.3c	69.2 ± 0.4b	80.8 ± 0.2ab	3.8	26.3 ± 0.3c	23.0 ± 0.0 cd	23.0 ± 0.0bc	22.3 ± 0.5a	2.0	12.9 ± 0.3a	3.4 ± 0.5a

DBI Days Before Inoculation

Means within the same column followed by the same letter are not significantly different at 5% significance level

Table 3 Effect of plant extract drenching on Black Sigatoka severity and plant growth

Extract	Concentration (g/ml)	Mean percentage infected leaf area	Mean incubation period (days)	Mean plant height (cm)	Mean no. of new leaves
A. indica	0.0	79.0 ± 1.0a	23.0 ± 0.0a	14.3 ± 0.2bc	3.9 ± 0.3b
C. annuum	0.0	78.0 ± 0.6a	23.0 ± 1.0a	14.3 ± 0.2bc	3.9 ± 0.2b
C. zeylanicum	0.0	79.5 ± 0.9a	22.0 ± 1.0a	14.3 ± 0.2bc	3.9 ± 0.1b
A. indica	0.1	78.8 ± 0.8a	23.0 ± 1.0a	17.1 ± 0.2ab	4.0 ± 0.2b
C. annuum	0.1	80.2 ± 0.3a	23.3 ± 0.6a	13.1 ± 0.3c	3.9 ± 0.1b
C. zeylanicum	0.1	80.0 ± 1.0a	23.0 ± 1.0a	14.3 ± 0.1bc	4.5 ± 0.5a
A. indica	0.2	78.3 ± 0.3a	23.3 ± 0.6a	17.5 ± 0.5a	3.7 ± 0.6b
C. annuum	0.2	78.0 ± 1.0a	22.3 ± 0.6a	14.1 ± 0.3bc	3.8 ± 0.3b
C. zeylanicum	0.2	78.0 ± 0.9a	22.0 ± 0.0a	14.8 ± 0.3bc	4.6 ± 0.2a
A. indica	0.3	80.5 ± 0.5a	22.3 ± 0.6a	13.7 ± 0.3bc	4.5 ± 0.5a
C. annuum	0.3	80.2 ± 0.8a	23.0 ± 1.0a	15.5 ± 0.5b	4.9 ± 0.2a
C. zeylanicum	0.3	79.5 ± 0.9a	23.0 ± 0.0a	15.1 ± 0.2b	4.8 ± 0.2a

Means within the same column followed by the same letter are not significantly different at 5% significance level

The longest incubation period at 0.3 g/ml was seen for *C. annuum* (36.7 days) and the shortest on *C. zeylanicum* (26.3 days). The incubation period for the water control and *A. indica* were 21.0 and 35.7 days respectively. A similar trend was seen for 0.1, 0.2 and 0.3 g/ml. Overall, the incubation period varied among and within extracts for the concentrations of 0.1, 0.2 and 0.3 g/ml. Disease appearance and development was delayed by *A. indica*, *C. annuum* and *C. zeylanicum* at a concentration of 0.3 g/ml when the extracts were applied as foliar sprays at 0 DBI. Similar observations were made for *A. indica* and C. *annuum* at 0.2 g/ml. *Azadirachta indica*, *C. annuum* and *C. zeylanicum* concentrations of 0.1 g/ml did not significantly delay disease appearance and development. The extract effect on plant growth (plant height and number of new leaves) was, however, not significant. The plant height ranged between 12.9 and 14.6 cm. Number of new leaves ranged from 3.4 to 3.6.

Effect of soil drenching single plant extracts on Black Sigatoka severity and plant growth

The result of extracts soil drenching is presented in Table 3. When the extracts were incorporated in the soil, there was no significant reductions in disease severity. Soil incorporation of plant extracts (*A. indica, C. annuum and C. zeylanicum*) at concentrations of 0.1, 0.2, 0.3 g/ml did not significantly affect Black Sigatoka development (percentage leaf area infected and incubation period) when compared to water-treated control plants. However, soil incorporation of the extracts significantly affected plant height. *Azadirachta indica* significantly increased plant height at concentrations of 0.1 and 0.2 g/ml. The largest and smallest increase in height was recorded for a concentration of 0.2 g/ml (3.2 cm) and 0.1 g/ml (2.8 cm), respectively. The concentration of 0.3 mg/ml did not significantly affect plant height. On the other hand, *C. annuum and C. zeylanicum* did not significantly affect plant height. Furthermore, there was a significant effect of extract concentration on number of new leaves. Thus, *C. zeylanicum* significantly increased the number of new leaves at 0.1, 0.2 and 0.3 g/ml. The largest and smallest increase in the number of new leaves was seen for 0.3 g/ml (0.9 leaves) and 0.1 g/ml (0.6 leaves), respectively. The other extracts, *C. annuum* and *A. indica* only significantly increased the number new leaves at 0.3 g/ml.

Table 4 Effect of **c**ombined plant extracts on Black Sigatoka severity and plant growth

Extract	Mean percentage infected leaf area	Mean plant height (cm)	Mean no. of new leaves
A. indica	27.5 ± 0.6e	13.7 ± 0.2a	3.1 ± 0.3a
C. annuum	28.0 ± 0.8e	13.7 ± 0.1a	3.3 ± 0.5a
C. zeylanicum	73.0 ± 0.4b	14.4 ± 0.2a	3.3 ± 0.5a
A. indica + *C. annuum*	21.8 ± 0.5f	13.1 ± 0.2a	3.3 ± 0.5a
C. annuum + *C. zeylanicum*	68.6 ± 0.5c	13.7 ± 0.1a	3.8 ± 0.2a
A. indica + *C. zeylanicum*	80.0 ± 0.2a	13.7 ± 0.2a	3.3 ± 0.4a
A. indica + *C. annuum* + *C. zeylanicum*	47.8 ± 0.5d	14.1 ± 0.2a	3.3 ± 0.5a
Control	82.4 ± 0.4a	13.7 ± 0.1a	3.3 ± 0.5a

Means within the same column followed by the same letter are not significantly different at 5% significance level

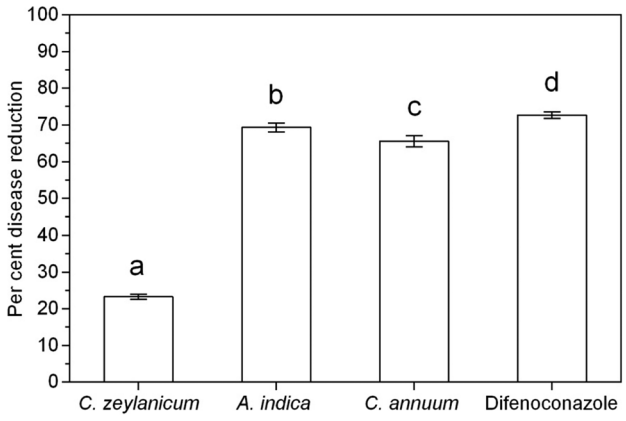

Fig. 1 Effect of extracts from *Cinnamomum zeylanicum*, *Azadirachta indica* and *Capsicum annuum* on *Pseudocercospora fijiensis* infection in banana in the screenhouse. Banana plantlets were treated with extracts of *A. indica*, *C. annuum* or *C. zeylanicum* (0.3 g/ml), the fungicide difenoconazole (0.1%, v/v) or water. Pathogen inoculation was done 24 h after extract treatment and disease assessment was done 45 days after pathogen application. Bars show percent disease reduction relative to the untreated control. Error bars represent standard error of the mean. Means marked with different letters are significantly different

Efficacy of the plant extracts *in planta* based on combinations of extracts

The result of the effect of the efficacy of extract combinations *in planta* is presented in Table 4. The extract combination *A. indica* + *C. annuum* significantly reduced Black Sigatoka severity compared to single extracts. The most and least effective extract combination was *A. indica* + *C. annuum* and *C. zeylanicum* + *A. indica,* respectively. The combination of *A. indica* + *C. annuum* gave a significantly lower disease severity (21.8%) at the concentration 0.3 g/ml. On the contrary, *C. zeylanicum* + *A. indica* was not significantly different from the control. The impact of extract combinations on plant height and number of new leaves was not significant.

Efficacy of plant extracts compared with a synthetic fungicide

In three-month-old banana plantlets, treatment with the fungicide difenoconazole gave 72.7% disease reduction compared to the water control (Fig. 1). Treatment with plant extracts at 30% (w/v) all gave significantly lower disease reductions compared to the fungicide, although reductions for *A. indica* and *C. annuum* extracts were only marginally lower (69.3 and 65.6% reductions, respectively). *C. zeylanicum* reduced Black Sigatoka by 23.3%. only.

Percentage crude chemical constituents of the extracts

The results of quantitative phytochemical analysis of *A. indica* leaf extract, *C. annuum* fruit extract and *C. zeylanicum* bark extract is presented in Table 5. The percentage of total flavonoids, alkaloids, tannins and phenolics in the three extracts were significantly different. For example, high levels of total flavonoids (*A. indica*—8.2% and *C. annuum*—7.5%) and phenolics (*A. indica*—9.3% and *C. annuum*—7.2%) and low levels of tannins (*A. indica*—4.1% and *C. annuum*—0.7%) and alkaloids (*A. indica*—4.4%) were found in *A. indica* and *C. annuum*. On the other hand, *C. zeylanicum* extracts showed high level of alkaloids (20.3%) and low level of flavonoids (4.5%), tannins (4.2%) and phenolic compounds (0.8%). Total saponins percentage was however, not significant. Additionally, total flavonoids, total tannins, total alkaloids, total saponins and total phenolics directly inhibited the vegetative growth of *P. fijiensis* (Fig. 2). The highest percentage inhibition was observed with phenolics (11.3%) and the lowest with tannins (4.6%).

Discussion

Extracts from three plants (*Cinnamomum zeylanicum*, *Capsicum annuum* and *Azadirachta indica)* were studied for their ability to control Black Sigatoka in banana. These plants were selected for this study because they are widely grown in Uganda and therefore, readily available in banana production agroecologies. Aqueous extracts, rather than extracts obtained using an organic solvent, were used since this would allow direct applicability by farmers. Extracts of *A. indica* and *C. annuum* could protect East African highland bananas against *P. fijiensis* under screenhouse conditions in Uganda. Two extract application methods (foliar spraying

Table 5 Chemical constituents in the extracts of *Azadirachta indica, Cinnamomum zeylanicum* and *Capsicum annuum*

Plant extract	Chemical constituents (%)				
	Total flavonoids	Total alkaloids	Total tannins	Total phenolics	Total saponins
C. annuum	7.5 ± 0.1a	8.7 ± 0.1b	0.7 ± 0.0b	7.2 ± 0.1b	1.6 ± 0.1a
A. indica	8.2 ± 0.1a	4.4 ± 0.2c	4.1 ± 0.1a	9.3 ± 0.2a	2.3 ± 0.1a
C. zeylanicum	4.5 ± 0.2b	20.3 ± 0.1a	4.2 ± 0.1a	0.8 ± 0.0c	1.1 ± 0.1a

Means within the same column followed by the same letter are not significantly different at 5% significance level

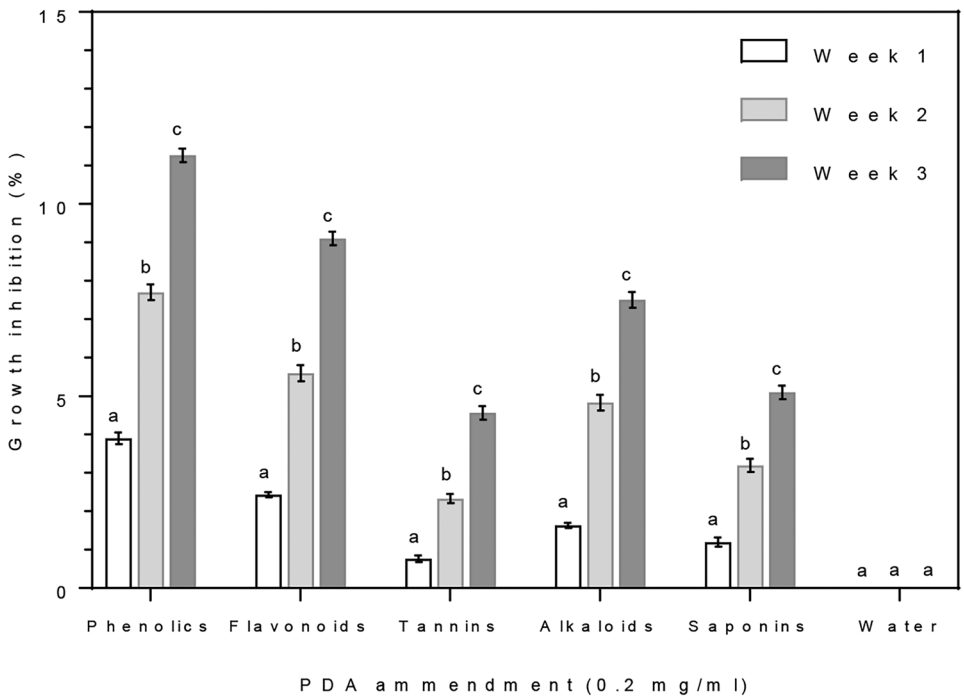

Fig. 2 Mean colony growth inhibition of *Pseudocercospora fijiensis* on PDA amended with plant extract constituents in vitro. Bars show percent disease reduction relative to the untreated control. Error bars represent standard error of the mean. Means marked with different letters are significantly different within each group of compounds

and soil drenching) were tested, but only foliar sprays were effective. Furthermore, it was found that the duration between extract application and inoculation with *P. fijiensis* influenced the protection efficacy, the level of efficacy decreasing with increasing interval between extract application and inoculation. *A. indica* and *C. annuum* caused a significant reduction in symptomatic leaf area when applied to leaves at 0, 1 or 3 DBI. Disease reduction increased with increasing concentration of extract. The increase in disease severity with increasing interval between treatments could be due to the degradation of the active ingredients of the extracts. Soil incorporation of the extracts did not significantly reduce Black Sigatoka severity when applied in the soil at 0, 1 or 3 DBI. This observation suggests that the inhibitory effect of *A. indica* and *C. annuum* extracts was as a result of direct toxic action on the causal organism. Direct toxic effects of *A. indica* (Mahmoud et al. 2011; Fieira et al. 2013), *C. zeylanicum* (Wisal 2018) and *C. annum* (Abayhne and Chauhan 2016) have been reported before against various fungal pathogens. *A. indica* and *C. annuum* exhibited direct inhibitory effects on *P. fijiensis* growth in vitro, providing further indication of direct toxic effects in *planta*. According to Kumakech et al. (2017), the inhibitory effect of *C. annuum* and *A. indica* against *P. fijiensis* was not plant-mediated through induced resistance. It cannot be excluded that *A. indica* and *C. annuum* may induce host resistance mechanisms to some degree as demonstrated by Guleria and Kumar (2006). Thus, Guleria and Kumar (2006) demonstrated that protection of *Sesamum indicum* against *Alternaria sesami* by an aqueous leaf extract of *A. indica* correlated with the activation of the enzymes phenylalanine ammonia lyase (PAL), peroxidase (POX) as well as with phenolic compounds.

The effect of the extracts depended on the mode of application. For example foliar spraying reduced disease and had no effect on growth parameters (height and number of new leaves). In contrast, when applied to the soil, *A. indica* significantly increased plant height and formation of new leaves whereas *Capsicum annuum* and *C. zeylanicum* significantly increased only number of new leaves. Therefore, in addition to disease reduction, the extracts also promoted plant growth. The results suggest that *A. indica, C. annuum* and *C. zeylanicum* contain substances with hormone-like properties that can stimulate growth in plants. The ability of plant extracts to promote plant growth has been reported. For example Lashin et al. (2013) reported increase in plant height, number of leaves and lateral branches of *Vigna unguiculata* when treated with *Malva sylvestris* and *Artemisia absinthium* extracts. No phytotoxic effects were observed in relation to the use of *A. indica* and *C. annuum* extracts on leaves and in soil. In fact, banana plantlets did not develop any symptoms of phytotoxicity in all the screen-house experiments. Thus, a commercial application of the two extracts for banana production might be possible.

The efficacy of the two extracts (*A. indica* and *C. annuum* extracts) was comparable to the effect of the standard fungicide difenoconazole. Difenoconazole is a broad-spectrum, systemic fungicide for preventative and curative control of several diseases, including leaf spots, powdery mildews, rusts and scab of annual and perennial crops (Huan et al.

2013). Use of fungicides is the most effective and widely used method for the control of Black Sigatoka of banana (Pérez-Vicente 2013; Ploetz 2001 The use of fungicides is quite problematic because of persistence in the environment and their effects on non-target organisms. The replacement of fungicides by potentially environmentally friendly natural products is a major focus of researchers worldwide (Tal 2018). The results of the present investigation clearly indicate the potential of plant extracts for control Black Sigatoka. Alternative control options for organic agriculture are limited (Thuerig et al. 2006). According to Thuerig et al. (2006), plant protection products must fulfil a number of criteria to be recommended for use in organic agriculture. Although only natural products may be used, such natural products should not be obtained from genetically modified organisms. *A. indica* and *C. annuum* extracts comply with these guidelines, in contrast to other plant protection agents such as the plant activator Actigard 50WG, which contains the synthetic active compound Acibenzolar-S-methyl (Bektas and Eulgem 2015). In particular, *A. indica* is of interest locally, since it is widely planted in Uganda and is readily available in the immediate environment of the farmer.

When *A. indica* and *C. annuum* extracts were used in combination, they had a significantly better effect than when used alone. This suggests that there are synergistic effects of extracts when used in combination. Gali et al. (2010) reported similar effects by mixtures of *C. annuum* and *Terminalia chebula* extracts against *Apergillus flavus* and *A. parasiticus* in vitro. According to Mukherjee et al. (2011), synergism is assumed to occur if the activity of extract components in combination is significantly increased when compared to the effect of individual components. Other combinations (*A. indica* and *C. zeylanicum, C. annuum* and *C. zeylanicum*) did not have significantly better effect on Black Sigatoka than the extracts used alone. This is probably because the activities of their principal components when acting in combination were not significantly enhanced in comparison to individual component activities. Selection of an appropriate combination is therefore crucial and this requires understanding the potential interaction between the plant extracts.

A direct inhibitory effect of *A. indica* extract on *M. fijiensis* was observed in this study. Previously, it has been well documented that antimicrobial compounds present in plants play an active role in the defence of plants against pathogens (Fiori et al. 2000; Sangeetha et al. 2013). Indeed, several plant extracts have been reported to be capable of reducing diseases in plants through direct action on the causal organisms (Guleria and Kumar 2006). Thousands of diverse natural products are produced by plants and many of these are involved in plant defence. In this study, the phytochemical analysis of the aqueous extracts of *C. zeylanicum*, *A. indica* and *C. annuum* showed the presence of different groups of secondary metabolites such as flavonoids, tannins, alkaloids, saponins and phenolics of which can be antifungal. The results of the present study are in agreement with the results published by the other research groups (Susmitha et al. 2013; Prashanth and Krishnaiah 2014). Total flavonoids, tannins, alkaloids, saponins and phenolics from *C. zeylanicum*, *A. indica* and *C. annuum* inhibited growth of *P. fijiensis* in vitro. Indeed, the inhibitory effect of *C. annuum* on *P. fijiensis* could be due to the presence of capsaicinoids such as capsaicin, a phenolic compound, which could have reduced the rate of disease development (Veloso et al. 2014). In this study, phytochemical analysis revealed high level of phenolic compounds in *C. annum*. The results are consistent with those reported for *Penicillium expansum* of apple, the growth of which was reduced by capsaicin (Fieira et al. 2013). Furthermore, *A. indica* extracts have been reported to inhibit growth of a wider spectrum of microorganisms (Mahmoud et al. 2011; Salam et al. 2014). In the current study, the fungitoxic nature of *A. indica* was evidenced by the high percentage fungal growth inhibition of *P. fijiensis,* an indication that the effect could be due to several antimicrobial active ingredients in the leaves of *A. indica*. In fact, the analysis of *A. indica* leaf extract revealed high levels of phenolic compounds and flavonoids. Antifungal activity of phenolic compounds against *Alternaria solani, Botrytis cinerea* and *Fusarium culmorum* was reported by Winkelhause et al. (2005). A similar study by Filho et al. (2015) reported a fungistatic activity of flavonoids against *Candida krusei* strains. Accordingly, the bioefficacy of the *A. indica* and *C. annum* extract on *P. fijiensis* can be attributed to the fact that *A. indica* and *C. annum* have phenolic compounds and flavonoids as their components, which are antifungal. The low bioefficacy of *C. zeylanicum* could be attributed to the very low level of phenolics (0.8%) in the extract.

Examination of the ability of plant extracts to inhibit *P. fijiensis* in this study showed promising prospects for their utilization in Black Sigatoka control in banana. *A. indica* and *C. annuum* significantly reduced disease development, while maintaining overall plant growth. The result of the present study can therefore be exploited further for formulating an integrated disease management strategy of Black Sigatoka in banana in a manner that can be adopted easily by resource-poor farmers. Integration of natural compouds in plant disease management is the focus of researchers worldwide. However, further investigations are still required to validate the findings of this study, identify highly efficacious extract formulation for use against *P. fijiensis* and recommendations for field applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-022-01211-1.

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