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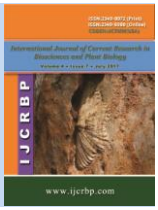

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Antifungal Activities of Plant Extracts against Coffee Berry Disease Caused by *Colletotrichum kahawae* L.

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Abstract

Coffee berry disease (CBD) caused by *Colletotrichum kahawae* L. is one of the major threads to the production of Arabica coffee in Africa. The antifungal potential of aqueous and ethanol extracts of three plant species (*Carica papaya, Cymbopogon citratus* and *Eucalyptus saligna*) against *Colletotrichum kahawae* was evaluated *in vitro* and on detached green berries. Plant extracts were applied at different concentration on *in vitro C. kahawae* and on detached green coffee berries simultaneously with pathogen inoculation. Results showed that aqueous extracts of *C. papaya, E. saligna* and *C. citratus* completely inhibited the *in vitro* growth of *C. kahawae* at the concentrations of 20 and 25 mg/ml while the ethanol extracts pathogen inhibition was at a concentration of 8 mg/ml. On detached coffee green berries, extracts from *E. saligna* and of *C. papaya* significantly reduced disease development. This study indicated the possibility of using *E. saligna* and of *C. papaya* extracts as an alternative for coffee berry disease management. However, a base for future tests under the natural conditions in the field is required.

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Keywords

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Coffee berry disease
Colletotrichum kahawae
Plants extracts

Introduction

Coffee (*Coffea* sp.) is one of the most important commodities in the international agricultural trade and represents a significant source of cash crop to several coffee producing countries. In Cameroon, coffee stands as the fourth largest agricultural export commodity after cocoa, bananas and cotton (ACP, 2010; Nganou, 2012). Chlorogenic acids and caffeine content in coffee seeds play an important role in the prevention of diabetes, Alzheimer and Parkinson's disease (Johnston et al., 2003; Farah and Donangelo, 2006; Bruneton, 2009; Rui, 2010). Despite its great economic importance, coffee production for the 2014-2015 season dropped from 32 808 to 23 866 tons (NCCB, 2015) due to several constraints among which, is coffee berry disease caused by *Colletotrichum kahawae* (Hindorf and Omondi, 2011). The disease attacks all parts of the crop (Gichimu et al., 2014). The symptoms first appear as small dark sunken patches on the pericarp of green berries that can later coalesce rapidly to cover the whole berry surface and subsequently destroy the bean (Masaba and Waller, 1992; Mouen Bedimo et al., 2007). Coffee berry disease is an anthracnose of the green and ripe coffee berries (Arega, 2006). Under cold and wet conditions, the fungus sporulates forming a mass of pink conidia that penetrate the interior of the berries destroying the coffee seeds (Mouen Bedimo et al., 2008). Maximum

production losses occur when infection takes place in expanding green berries, leading to premature, dropping and mummification of berries (Andreia et al., 2013).

The use of chemical pesticides (fungicides) is the most common practice for managing anthracnose disease, but this also causes the development of fungal resistance (Swami and Alane, 2013). In addition, continuous and inappropriate use of chemical fungicides is not considered to be a long-term solution because it increases not only the investment expenses, but also the risk of having high levels of toxic residues, the concerns for human health and environmental settings (Bieysse et al., 2002; Carvalho, 2004; Latha et al., 2009). Thus, there are several attempts for alternative measures for efficient control of the disease.

The utilization of natural products, especially plant extracts, has been shown to be effective against many plant pathogens and considered to be safe for consumers and the environment (Okemo et al., 2003; Hernandez-Albiter et al., 2007). Researchers have reported antifungal activities of several plant extracts against *C. kahawae* (Amsalu et al., 2011; Serawit and Tesfaye, 2014). Similarly, plants extracts have proven to be effective for the control of taro blight (*Phytophthora colocasiae)* on taro *in vitro* and on leaf fragment with *Laggera pterodonta*, *Cupressus lusitanica*, *Callistemon viminalis* and *Eucalyptus saligna* extract expressing suppression of mycelium growth and delaying mildew development on leaf fragment (Tsopmbeng et al., 2014). In Cameroon, information on antimicrobial activity of plants products against *Colletotrichum kahawae* is limited. However, the use of *Carica papaya, Cupressus lusitanica, Erigeron floribundus* and *Euphorbia hirta* extracts has a great potential in suppressing various plant pathogenic fungi including *Colletotrichum gloeosporioides* (Keuete et al., 2015). This may serve as biological alternative in substituting the application of chemical fungicides. The objective of this study was to evaluate the antifungal activities of *Carica papaya*, *Cymbopogon citratus* and *Eucalyptus saligna* extracts on the growth of *C. kahawae* and disease development on green coffee berries.

Materials and methods

Study area

The research was conducted at the University of Dschang, Cameroon, in Laboratory of Phytopathology and Agricultural Zoology (LAPHYZA). Dschang is located at 5°26′ N latitude and 10°26′ E longitude and at altitude 1400 m. The temperature ranges from 11.8°C to 26.8°C and with relative humidity of 90% and the mean rainfall of 1500 mm per annum.

Pathogen isolation

Colletotrichum kahawae was isolated from green infected coffee berries. Pericarp fragments from infected green berries were washed thoroughly in tap water and cut into small pieces of about 2 mm², surface disinfected with 5% sodium hypochlorite, rinsed in sterilized distilled water and plated on Petri dishes containing 20 ml potato dextrose agar (PDA) medium amended with Chloramphenicol (1 g/l) and incubated at 25°C for 3-5 days. Then hyphal tips of the growing fungus were transferred to freshly prepared PDA medium and this process was repeated several times to obtain pure culture. The fungus was identified under light microscope based on cultural and morphological characteristics, followed by the identification guides and species descriptions by Barnett and Hunter (1972) and Waller et al. (1993). To confirm the identity of the pathogen, Koch's postulate was conducted; detached green berries were inoculated with conidial suspension from pure culture of the pathogen and incubated at 25°C for 7 days. All incubated green berries developed black sunken patches on the pericarp similar in appearance to those observed on infected coffee berries in the field. Noninoculated control detached green berries were disease free.

Preparation of plant extracts

Fresh leaves of *Carica papaya*, *Cymbopogon citratus* and *Eucalyptus saligna* were collected from the University farm. The collected samples of each plant species were washed under tap water and surface sterilized with 2% sodium hypochlorite solution followed by thorough rinsing with sterile water. The plant samples were air-dried at room temperature and ground in a mortar with the use of a pestle. Thereafter, 100 g of the resulted powder were macerated in 500 ml of distilled water or ethanol and mixed thoroughly. The mixture was filtered using cheese cloth followed by Whatmann filter paper N°. 1 after 48 hrs incubation at room temperature. The ethanol extract was evaporated using a Rota vapor at 40°C. The stock extracts were transferred into labeled sterile bottles and store at 4°C (Amsalu et al., 2011).

In vitro antifungal assays

The effect of plant extracts on the growth of *C. kahawae* was assessed using the agar dilution method (Serawit and Tesfaye, 2014) on PDA with some modifications. Plant extracts were dissolved in dimethyl sulphoxide (DMSO), and then diluted at concentrations of 1, 2, 4 and 8 mg/ml for ethanol extracts and 10, 15, 20 and 25 mg/ml for aqueous extracts. One ml of these extracts at different concentrations were incorporated into 19 ml of warm PDA and poured into 9 cm sterile Petri dishes. After solidification, the plates were inoculated with mycelia discs (5 mm) of 10 day old culture of *C. kahawae* (Keuete et al. 2015). PDA plates mixed with fungicide (Beauchamp 72% WP) at 3.33 mg/ml and sterile distilled water served as positive and negative controls, respectively. The plates were incubated at 20 ± 2°C. The experiment had three repetitions. The radial growth was measured in two perpendicular directions on the reverse side of the Petri dishes every two day and the fungi toxicity was expressed as percentage inhibition of radial mycelia growth using the formula follows:

$$\mathbf{PI} = \frac{(DT-D)}{DT} \times 100$$

Where: DT and D, are the radial mycelia growth measurements in the control and treatment plates respectively.

Antifungal assays on detached coffee berries

Based on *in vitro* test results, a concentration of 25 mg/ml for aqueous extracts and 8 mg/ml for ethanol extracts were used for antifungal assays on detached coffee berries. Conidial suspension prepared from 10 days old cultures of *C. kahawae* was adjusted to a concentration of 5×10^4 conidia/ml using haemocytometer (Keuete et al., 2016). Apparently healthy coffee berries harvested from the University farm in Dschang were washed with tap water and surface sterilized with 5% hypochlorite solution followed by thorough rinsing with sterile distilled water. With the aid of a hand sprayer, the berries were simultaneously sprayed with 20 ml of 5×10^4 conidia/ml suspension and 20 ml of each plant extract at the concentrations mentioned above. This experiment was repeated three times. Seven days after inoculation at 20±2°C, disease severity was assessed on a scale of 1 to 5 (Akhtar and Alam, 2002). Where scale 1, 2, 3, and 4 represent 0, 1 to 3, 4 to 6 and 7 to 15 lesions, respectively. Scale 5 represents 30% of fruit surface covered with lesions. Disease severity was then obtained by the following formula.

$$\mathbf{S}\ (\%) = \frac{\sum(a+b)}{N.Z} \times 100$$

Where: ∑(a+b) = Sum of infected fruits and their corresponding score scale, N = Total number of sampled fruits and Z = Highest score scale.

Results

In vitro effect of plant extract on *C. kahawae* mycelia growth

Results revealed a gradual variation in *C. kahawae* mycelia growth inhibition with increasing concentration of the ethanol and aqueous extracts. There was a generally trend with extracts, from all the test plants significantly inhibiting mycelia growth of *C. kahawae* at a probability level of $p\leq0.05$ compared to the negative control. The inhibitory effect of aqueous extracts of the three plants recorded 34 to 100% for *Cymbopogon citratus*, 87 to 100% for *Eucalyptus saligna*, and 73 to 100% for *Carica papaya*. Complete inhibition of *C. kahawae* mycelia growth was noticed at minimal concentrations of 15, 20 and 25 mg/ml respectively for the aqueous extracts of *E. saligna*, *C. papaya* and *C. citratus* (Table 1).

Table 1. Effect of aqueous plant extracts and synthetic fungicide on the inhibition of radial mycelia growth of *C. kahawae* recorded in percentage.

Concentration (mg/ml)	Percentage inhibition (%)		
	E. saligna	***C. papaya***	***C. citratus***
0 (T-)	$0.00 \pm 0.00^{d*}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
10	87.26 ± 2.65^{b}	73.14 ± 3.09^{c}	34.31 ± 4.49^{d}
15	100.00 ± 0.00^{a}	83.14 ± 2.96^{b}	53.53 ± 4.81^{c}
20	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	74.31 ± 3.83^{b}
25	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
3.33 (T+)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}

*Values in the same column followed by different letters are significantly different ($p\leq0.05$) according to Duncan Multiple Range Test. T- = negative control (Distilled water); T+ = positive control (Beauchamp 72% WP (Metalaxyl 8% + Mancozeb 64%).

Ethanol extract, inhibition property on the mycelia growth scored 29 to 100% for *C. citratus*, 39 to 100% for *E. saligna*, and 77 to 100% for *C. papaya* with complete inhibition at a minimal dose 8 mg/ml (Table 2). Total inhibition of *C. kahawae* mycelia growth observed in this experiment was similar to that obtained with the synthetic fungicides Beauchamp 72% WP.

Table 2. Effect of ethanol plant extracts and synthetic fungicide (Beauchamp 72% WP) on the inhibition of radial mycelia growth of *C. kahawae* scored in percentage.

Concentration (mg/ml)	Percentage inhibition (%)		
	E. saligna	***C. papaya***	***C. citratus***
0 (T-)	$0.00 \pm 0.00^{f*}$	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
1	38.82 ± 12.90^{d}	76.86 ± 2.38^{b}	28.82 ± 3.28^{d}
2	52.55 ± 4.90^{c}	79.61 ± 6.18^{b}	56.08 ± 8.37^{c}
4	71.37 ± 5.59^{b}	100.00 ± 0.00^{a}	69.80 ± 11.84^{b}
8	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
3.33 (T+)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}

*Values in the same column followed by different letters are significantly different ($p \leq 0.05$) according to Duncan Multiple Range Test. T- = negative control (Distilled water); T+ = positive control (synthetic fungicide Beauchamp).

Effect of plant extracts on disease severity on detached coffee berries

The severity of the disease was lowest in berries sprayed with conidial suspension of *C. kahawae* and plant extract, which were statistically at par with the positive control. Within the extracts, coffee berries sprayed with the pathogen and ethanol extract had relatively lower severity of coffee anthracnose than those, which received conidial suspension and aqueous extracts (Fig. 1). The results showing percentage severity of anthracnose disease on coffee berries seven days after inoculation and application of the plant extract and synthetic fungicide are presented on Table 3. Coffee berries treated with distilled water that served as negative control express a higher disease severity (25.33%) than those, which received plant extracts treatments. Comparatively, the aqueous extracts of *Eucalyptus saligna* significantly reduced the severity of the disease (12.89%) on berries with no significant difference to the effect of synthetic fungicide (11.56%). *C papaya* ethanol extracts significantly reduced lesions development (2.67%) and a disease severity was recorded. Aqueous and ethanol extracts of *Cymbopogon citratus* consistently showed little or no effect on lesion development and thus, recorded no significant at $p \leq 0.05$ with that of the negative control.

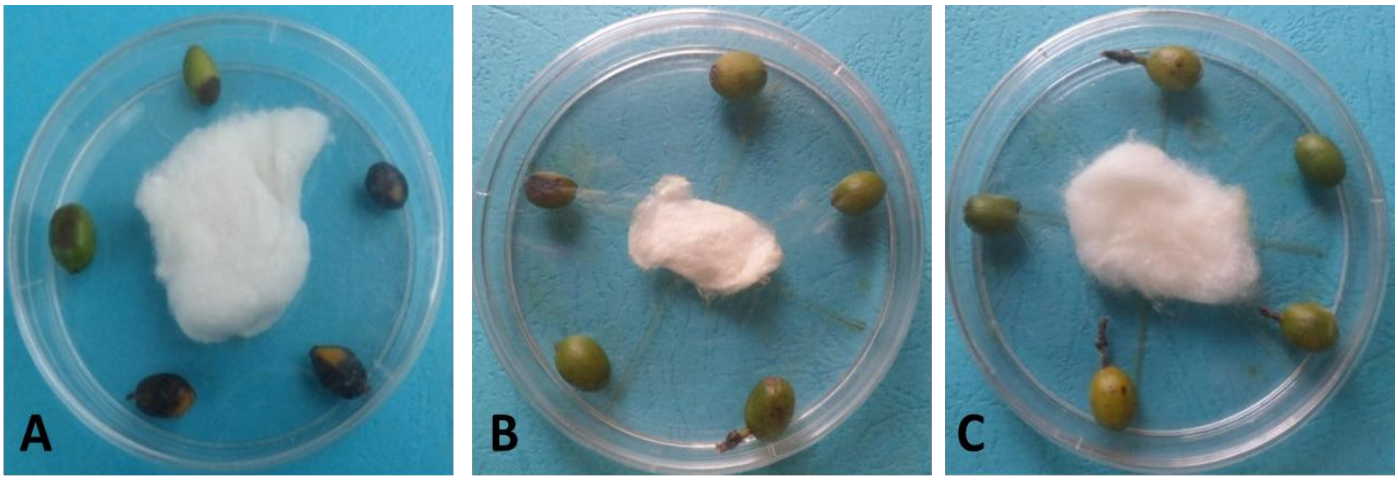

Fig. 1: Coffee berries treatments. Effect of plant extracts on anthracnose development on detached green coffee berries, 7 days view after simultaneous application of *C. kahawae* conidial suspension and plant extracts. A: untreated plates; B: Plate treated with *Eucalyptus saligna*, extract and *C*: Plate treated with *Carica papaya* extract.

Table 3. Effect of the plant extract and synthetic fungicide on severity of anthracnose on detached coffee berries.

Type of extract	Concentration (mg/ml)	Treatments	Disease severity (%)
Aqueous	0	T-	25.33 ± 9.24[a*]
	25	*C. papaya*	18.67 ± 4.81[ab]
	25	*E. saligna*	12.89 ± 6.30[b]
	25	*C. citratus*	19.78 ± 4.68[ab]
	3.33	Beauchamp 72% WP	11.56 ± 3.85[b]
Ethanol	0	T-	25.33 ± 9.24[a]
	8	*C. papaya*	2.67 ± 2.67[b]
	8	*E. saligna*	13.33 ± 4.62[ab]
	8	*C. citratus*	12.89 ± 1.43[ab]
	3.33	Beauchamp72% WP	11.56 ± 3.85[ab]

*Values in the same column followed by different letters are significantly different ($p \leq 0.05$) according to Duncan Multiple Range Test. T- = negative control (Distilled water); Conc = concentration.

Discussion

In this study the antifungal effect of the aqueous and ethanol extracts of three plants were evaluated against *Colletotrichum kahawae* under *in vitro* condition and on detached green coffee berry. The findings showed that the effect of plant extracts on *C. kahawae* radial growth and disease development vary depending on the type of plant species, method of extract preparation and applied dose of the extract.

These extracts were found to have considerable effect on inhibition of fungal radial growth *in vitro*. They equally possess characteristic of reducing disease development on detached green coffee berries. However, *Carica papaya* and *Eucalyptus saligna* extracts were the most effective in reducing the radial growth of *Colletotrichum kahawae in vitro* and the severity of the disease on detached green coffee berries for both aqueous and ethanol extracts. Overall, coffee berries that received conidial suspension of the pathogen and plant extracts had lower severity than the untreated control. Using aqueous extracts of *C. papaya* leaf proportion in PDA, Bautista-Baños et al. (2002) reported no inhibition of *C. gloeosporioides* after 7 days incubation compared to a control.

In an approach towards the development of eco-friendly antifungal compounds for controlling major fungal disease of fruit, Keuete et al. (2015) tested ethanol and aqueous extracts of *Carica papaya* against the pathogen *Colletotrichum gloeosporioides* for evaluation of antifungal properties. Results showed that aqueous extracts of *Carica papaya* was 100% inhibitory to spore mycelium growth (Keuete et al., 2015). This indicates that *Carica papaya and Eucalyptus saligna* possess antifungal activity against *C. kahawae*. Plants are rich sources of antimicrobial substances which are reported to inhibit *in vitro* fungal radial growth (Lapornik et al., 2005; Malkhan et al., 2012; Galani et al., 2013),

The results of the antifungal activity of plant extracts on detached coffee berries showed that, lesions developed seven days after simultaneously spraying of coffee berries with pathogen suspension and plant extract contrary to the control where these lesions were observed after five days. This suggests that plants used may contain some active compounds, which delay the development of disease. Extracts from *Eucalyptus saligna* and *Carica papaya* significantly reduced the disease severity compared to distilled water treatment. These plants extract to some extend showed higher and better antifungal activity compared to commercial fungicide Beauchamp 72% WP (Metalaxyl 8% + Mancozeb 64%). Nwachukwu and Umechuruba (2001) reported similar results on the antibiotic and antifungal properties of *Carica papaya* extracts on a number of plant disease causing pathogens, including tuber rot on yam and fruit rot on avocado pear. Similar report came up in 2015 by Keuete et al. (2015).

Extracts of *E. saligna* have been reported to contain eucalyptol, an antifungal substance that prevents or hinders the growth of many fungi (Enyiukwu et al., 2014). In addition, leaf extracts of *Carica papaya* contain proteolytic enzymes which are known to have antifungal activity against *Colletotrichum gloeosporioides* (Bautista-Banos et al., 2002) and alkaloids which have fungicidal activity against *Colletotrichum* and *Fusarium* species (Oliva et al., 2003).

Conclusion

The study suggest the possible use of extracts of *E. saligna* and *C. papaya* as an alternative means of coffee berry disease management and provides information that could constitute a base for future tests under natural field conditions.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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