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In vitro evaluation of plant-extracts activity on two tomato pathogenic fungi in Togo

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ABSTRACT

The effects of plant-based formulation on two common fungi of tomato were carried out *in vitro*. A total of five concentrations of the formulation: 0.2, 0.4, 0.6, 0.8 and 1% were tested using agar dilution and agar diffusion methods. Significant antifungal effects were observed. The plant-based extract formulation was more effective with the agar dilution method in comparison to the diffusion one and also depend on the higher concentrations. The antifungal effects decreased with the incubation time and a high effect was recorded after three days of incubation with mycelial growth inhibition from 63.60 to 77.76% and 80.08 to 100% for *Alternaria solani*, and from 47.72 to 74.83 and 78.8 to 100% for *Fusarium oxysporum*, with agar dilution and diffusion methods, respectively.

Keywords: Tomato, collar fungi rot, plant extracts, *Alternaria, Fusarium*, control.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetables worldwide and contributes to a human healthy diet (Abuley and Nielsen, 2017). Tomatoes are used as an ingredient in several meals in Togo and their demand increased in recent years. Thus, tomato cultivation is performed on the national level to meet the population's needs. Tomato production increased from 7,620.4 tons in 2013 to 13,328.2 tons in 2017 in Togo (DSID, 2018), revealing the importance of this vegetable in the country. Unfortunately, tomato is attacked by various diseases caused by pathogens including fungi, bacteria and viruses. Two common and devastating diseases of tomato in Togo are early blight and *Fusarium* wilt caused by *Alternaria solani* and *Fusarium oxysporum,* respectively (Singh et al., 1980; Maiero and Barksdale, 1989). These two diseases cause a severe reduction in yield and high economic losses. Chemical fungicides are used to control these diseases. Tomato fruits ripen gradually with long harvesting times and are usually picked weekly. Thus, the intervals between pesticide spray and harvesting are very short. The presence of pesticide residues in the fruits constitutes a potential risk to humans, and the environment (Al-Wabel et al., 2011; Kolani et al., 2016a; Zhu et al., 2016).

In sustainable agriculture, natural plant extracts have latterly gained importance for crop protection against pests and pathogens including several fungi species; because of their safety, target specificity, and antimicrobial activity of their phenolic compounds (Hassanein et al., 2010; Lima et al., 2016). Neem (*Azadirachta indica*) and camel grass (*Cymbopogon schoenanthus)* are originally used for the treatment of human-affecting pathogens (El-Askary et al., 2003; Elhardallou, 2011; Sabry et al., 2014), and their uses were later extended to the protection of cultivated plants (Chattopadhyay, 1999; Sanda et al., 2006; Hassanein et al., 2010; Laba et al., 2012). The botanical-based emulsifiable concentrate formulation PiperaAzad 85EC from camel grass and neem oils used in the present study, is an experimental formulation developed to be as an alternative to synthetic chemical pesticides and has

shown good effectiveness against crop pests such as *Plutella xylostella* and *Podagrica* sp. (Kolani et al., 2016b), however, it has not yet been tested against plant pathogens. Thus, the present study aimed to *in vitro* evaluate the antifungal activity of this botanical formulation on two pathogenic fungi *A. solani and F. oxysporum* of the tomato.

MATERIALS AND METHODS

Plant extracts formulation and Fungi culturing

We prepared the botanical formulation following the method developed by Kolani et al. (2016a). It consisted of mixing camel grass essential oil (42.5% w/w) and neem oil (42.5% w/w) to which, the SUPROLCABS emulsifier (15% w/w) was added. The whole mixture was further vigorously homogenized with a Stuart brand SS10 stirrer at 1000 rpm for 10 minutes.

Both fungal strains *A. solani* and *F. oxysporum* used in this study were isolated from samples of tomato diseased plants collected during a survey in the Central Region of Togo, isolated and identified by Bamazi (2021). These strains were stored in the refrigerator at 4°C. For laboratory testing, stock isolates were subcultured onto the new medium before use.

Antifungal activity tests

The antifungal susceptibility testing of the botanical formulation was evaluated using two methods: (i) agar dilution method, and (ii) agar diffusion method. The pre-growth agar dilution method consisted of adding different amounts of botanical formulation and chemical control to Potato Dextrose Agar (PDA) (45°C) to give a final concentration of 0.2, 0.4, 0.6, 0.8 and 1% for botanical formulation and 7.5% for Mancozeb 80 WP. The resulting media were poured into Petri dishes and incubated for 7 days. A 6 mm diameter disc of inoculum of each strain of the tested fungus was cut with a cork borer from the periphery of an actively growing culture on PDA plates and was placed at the centre of each Petri plate. The negative control consists of culture medium without product. Each treatment has four replications. The plates were incubated at 25°C. When the fungus had grown on control plates close to the margin of the plate, the percentage of fungal growth inhibition was calculated in each treated plate according to Yahyazadeh al. (2008) formula as follows:

$$\mathbf{I} = \frac{\mathbf{d} - \mathbf{d}'}{\mathbf{d}} \times 100$$

where I = inhibition rate (%); d = mycelial growth diameter in the negative control; d' = mycelial growth diameter in the treatment.

The post-growth agar diffusion test was done according to the method described by Hashim et al. (2017) with some modifications. Sterile nutrient agar was inoculated with each strain of the tested fungus and then immediately covered and incubated (25°C, 24 h) and subsequently sprayed with 200 µl of different concentrations of fungicides as described in the agar dilution method and incubated again for 7 days. The concentration of the fungicide Mancozeb (positive chemical control) has been determined according to manufacturers' instructions, while the botanical formulation concentrations were fixed according to the previous work on the inhibition of mycelial growth of *Fusarium* sp (Afole, 2021).

Statistical analysis

GENSTAT software was used with the factorial experiment as the completely randomized design. ANOVA was performed to examine the significance of the inhibition data on the radial mycelial growth of the fungi. Duncans' Multiple Range Test was used to discriminate the mean values of the mycelial growth at a significance level of 1%.

RESULTS

Agar dilution (pre-growth) activity of botanical formulation on fungi

Botanical formulation showed significant activity against the two fungal strains tested (Table 1) in the agar dilution method. On the 3rd day, the percentage inhibition of mycelial growth significantly increased from 81.4 to 89.3% (*F. oxysporum*) and 88.08 to 94.45% (*A. solani*) for concentrations varying from 0.2 to 0.4% of the botanical formulation. The level of inhibition came down from 78.4 to 84.0 (*F. oxysporum*); 75.25 to 88.7% (*A. solani*) on the 5th day and 71.4 to 71.0% (*F. oxysporum*) and 74.98 to 82.47% (*A. solani*) on the 7th day for the same concentration. Generally, the difference between the percentage inhibition of mycelial growth induced by concentrations 0.2 and 0.4% was significant for *A. solani* and insignificant for *F. oxysporum* strains. Between the 3rd and the 7th day. The percentage of inhibition decreased by 12.29 and 20.49% (*F. oxysporum*) and by 14.87 and 12.68% (*A. solani*) respectively for 0.2 and 0.4% concentrations. The higher concentrations of the botanical formulation of 0.6 to 1% as well as the fungicide Mancozeb 7.5% induced highly significant and constant inhibition of mycelial growth of 100% during all the periods of incubation in comparison to the lower concentrations of 0.2 and 0.4%.

Table 1. Inhibition rate (%) of mycelial growth of *F. oxysporum* and *A. solani* with agar dilution method *in vitro.*

Fungicides	Concentrations (%)	*F. oxysporum*			*A. solani*		
		Incubation days			Incubation days		
		3	5	7	3	5	7
Botanical formulation	0.2	81.4^{c}*	78.4^{b}	71.4^{b}	88.08^{c}	75.25^{c}	74.98^{c}
	0.4	89.3^{b}	84.0^{b}	71.0^{b}	94.45^{b}	88.7^{b}	82.47^{b}
	0.6	100^{a}	100^{a}	98.0^{a}	100^{a}	100^{a}	100^{a}
	0.8	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	1	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Mancozeb	7.5	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

*Values with the same letter are not significantly different with Duncan's test at 1%.

Agar diffusion (post-growth) activity of botanical formulation on fungi

The botanical formulation showed a significant influence on the two strains studied through the agar diffusion method. The post-growth activity of the botanical formulation resulted in the inhibition of mycelial growth from 47.73 to 74.84% (*F. oxysporum*) and 63.60 to 77.76% (*A. solani*) respectively for concentrations 0.2 to 1% on the 3rd day after treatment. The percentage inhibition decreased with the incubation time despite the presence of botanical pesticides to give 40 to 69.41% and 37.25 to 58.82% (*F. oxysporum*); 63.22 to 74.02% and 43.86 to 73.39% (*A. solani*) respectively for concentrations of 0.2 to 1% of botanical formulation on the 5th and 7th day of incubation while it remained relatively constant with the chemical control Mancozeb (94.12 to 92.95 and 77.83 à 73.81 respectively for *F. oxysporum* and *A. solani* from 3rd to 7th days after incubation). For each strain, the increase in the concentration of botanical formulation is followed by a significant increase in the percentage inhibition of mycelial growth (Table 2). The chemical control Mancozeb (7.5%) gave significant inhibition of mycelial growth compared to the botanical formulation for *F. oxysporum* strain, but for *A. solani*, the inhibition induced par Mancozeb was comparable to the higher concentration of the botanical formulation (1%) (Table 2).

Table 2. Inhibition rate (%) of mycelial growth of *F. oxysporum* and *A. solani* with agar diffusion method *in vitro.*

Fungicides	Concentrations (%)	*F. oxysporum*			*A. solani*		
		Incubation days			Incubation days		
		3	5	7	3	5	7
Botanical formulation	0.2	47.73^{d}	40^{e}	37.25^{c}	63.60^{d}	63.22^{c}	43.86^{e}
	0.4	55.29^{c}	44.71^{de}	38.04^{c}	67.34^{c}	67.47^{b}	54.68^{d}
	0.6	57.54^{c}	47.45^{d}	40.78^{c}	68.45^{c}	70.22^{b}	60.59^{c}
	0.8	72.06^{b}	61.96^{c}	58.43^{b}	72.39^{b}	69.73^{b}	68.47^{b}
	1	74.84^{b}	69.41^{b}	58.82^{b}	77.76^{a}	74.02^{a}	73.39^{a}
Mancozeb	7.5	94.12^{a}	94.12^{a}	92.95^{a}	77.83^{a}	75.67^{a}	73.81^{a}

Values followed by the same letter in a column are not significantly different with Duncan's test at 1%.

Comparative activity (agar dilution and agar diffusion method) of botanical formulation on fungi

The ratio of agar dilution/agar diffusion of percentage inhibition of mycelial growth was calculated in order to establish a comparison of the efficacy between the two methods (Table 3). The calculated ratios were higher than 1, i.e. 1.34 to 2.40 and 1.29 to 1.71 for *F. oxysporum* and *A. solani* for botanical formulation and 1.06 to 1.08 and 1.28 to 1.35 respectively for *F. oxysporum* and *A. solani* for the chemical control Mancozeb) indicating that the percentages of inhibition of mycelial growth in agar dilution treatments are higher than those of agar diffusion treatment. *F. oxysporum* strain (1.34 to 2.40) showed a higher ratio compared to *A. solani* (1.29 to 1.71) for botanical formulation. For the chemical control

Table 3. Agar dilution/agar diffusion ratio of mycelial growth inhibition rate.

Fungicides	Concentrations (%)	*F. oxysporum*			*A. solani*		
		Incubation days			Incubation days		
		3	5	7	3	5	7
Botanical formulation	0.2	1.71	1.96	1.92	1.38	1.19	1.71
	0.4	1.62	1.88	1.87	1.40	1.31	1.51
	0.6	1.74	2.11	2.40	1.46	1.42	1.65
	0.8	1.39	1.61	1.71	1.38	1.43	1.46
	1	1.34	1.44	1.70	1.29	1.35	1.36
Mancozeb	7.5	1.06	1.06	1.08	1.28	1.32	1.35

Mancozeb, an inverse trend was observed (1.6 at 1.08 and 1.28 to 1.32 respectively for *F. oxysporum* and *A. solani*). The highest ratio for both strains *F. oxysporum* and *A. solani* was observed with a 0.6% concentration of botanical formulation.

DISCUSSION

The results of the present study indicate that the botanical formulation made by the mixture of *A. indica* and *C. schoenanthus* oils exhibited antifungal activity with a significant effect on the inhibition of mycelial growth of *F. oxysporum* and *A. Solani*. All concentrations of the botanical formulation substantially inhibited the mycelial growth of the two pathogens and at 0.6% concentration, the botanical formulations significantly suppressed mycelial growth of both of the fungi in the agar dilution method. Strong activity of the aqueous extract of *A. indica* leaves on *A. solani* and *F. oxysporium* was reported by Hassanein et al. (2010). Antifungal activity of *A. indica* has been attributed to some bioactive compounds such as nimbidin (Murthy and Sirsi, 1958), gedunin (Rao Nazma and Rao, 1977), cyclic trisulphide andcyclic tetrasulphide (Pant et al., 1986). El-Said (2011) studied a natural piperitone, a major component of essential oil of *C. scheonanthus* (Koba et al., 2004; Hashim et al., 2017) and reported its strong activity against four strains of fungi, such as *F. graminearium, Bipolaris sorokiniana, Rhizoctonia solani AG-4* and *F. oxysporum*. According to Yoon et al. (2013), the terpenes, phenols, alcohols, alkaloids, tannins and other secondary metabolites found in botanical pesticides induce toxicity to fungal cell walls, cell membranes and cell organelles. These metabolites also inhibit spore germination, mycelial development, germ tube elongation, delay sporulation and also inhibit the production of important enzymes, DNA and protein synthesis (Martinez, 2012). The botanical formulation at a lower concentration (at most 1%) in comparison to the chemical control Mancozeb (7.5%) induced the same performance in inhibiting mycelial growth. The botanical formulation is a mixture of two extracts (*A. indica* oil and essential oil of *C. schoenanthus*) each having a biological activity. The active compounds of these two oils may act synergistically to confer strong activity to the botanical formulation (Romagnoli et al., 2005).

The results showed a positive relationship between the concentration of botanical formulation and its antifungal activities. Ojaghian et al. (2014) reported similar findings with crude extracts derived from neem (*A. indica*) leaves and ginger (*Zingiber officinale*) rhizomes against three isolates of *Sclerotinia sclerotiorum*. The effectiveness of botanical formulation on the inhibition of mycelial growth of fungi may depend on the concentration of the product in the culture medium. The study revealed a significant decrease in inhibiting mycelial growth with the botanical formulation treatment in comparison to Mancozeb at delayed incubation time. The botanical pesticides are not persistent and degrade in a very short time. This makes them not accumulate in water and soils, therefore do not pose a risk of pollution (Sokoviš et. al., 2010). Their exposure to air, sunlight, moisture and high temperatures is enough to break down their constituents (Gunasekara, 2005). In general, this study showed that the botanical formulation can significantly reduce the mycelial growth of the pathogens through the pre-growth agar dilution medium than post-growth agar diffusion. This is possibly due to the lower initial percentage of the fungus in the agar plate at the moment of application of botanical formulation in the case of agar dilution. This demonstrates that the use of the botanical formulation could guarantee a better effect in a preventive control process or at the initiation of the fungal pathogen.

CONCLUSION

The botanical formulation showed potential effectiveness in controlling *F. oxysporum* and *A. Solani*, since their lower concentrations reduce significantly the growth of these fungi. Although, both methods - agar dilution and agar diffusion were found to have an antifungal activity agar dilution method revealed higher effects than the

diffusion one. The present findings are prerequisites for further investigations on the use of the targeted formulation based on *C. schoenanthus* and *A. indica* oils in controlling the pathogenic fungi of cultivated crops.

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