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ANTIFUNGAL EVALUATION AND PHYTOCHEMICAL PROFILE OF *TRICHODERMA HARZIANUM* AND *GLOMUS VERSIFORME* SECONDARY METABOLITES ON COWPEA PATHOGENS

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Abstract – In this study, ethyl acetate crude extract of secondary metabolites obtained from strains of *Glomus versiforme* and *Trichoderma harzianum* was profiled for phytochemical constituents and antifungal potential against cowpea phytopathogens that causes powdery mildew and leaf spot diseases viz. *Erysiphe flexuosa*and*Cercospora canescens*. The phytochemical profile showed that Alkaloids, Flavonoids, Phenols, Tannins, Cardiac Glycosides, Steroids, and Saponins were all present. The antifungal inhibitory results of secondary metabolites from Glo-ver60 had the overall highest inhibition effects 55±0.05 mm against *C. canescens* and 59.7±0.03 mm against *E. flexuosa*. The metabolites obtained from *Trichoderma harzianum* gave the second overall highest antifungal inhibitory activity against cowpea pathogens with 53.3±0.07 for *C. canescens* and 50.5±0.05 mm for *E. flexuosa.* Generally, these results reveal that there was variation in the antifungal activity of the biofungicidal metabolites at different concentrations. The minimum inhibitory concentration and minimum fungicidal activity from these biological controlling strains of *Glomus versiforme* and *Trichoderma harzianum* on *C. canescens* and *E. flexuosa* indicated that Glo-ver60 strain gave better results compared to the other strains. Glo-ver60 gave the best result of 73.4±0.05 µg/mL and 98.4±0.05 µg/mL for *C. canescens* as well as 72.1±0.08 µg/mL and 97.1±0.07 µg/mL for *E. flexuosa* respectively. These results revealed that there was variation in the inhibitory activity of the metabolites from *Glomus versiforme* and *Trichoderma harzianum* against the cowpea pathogens and this was concentration-dependent. The results from this study have shown that secondary metabolites from these bioagents could be formulated as potential biofungicides against cowpea disease-causing pathogens.

INTRODUCTION

(*Vigna unguiculata* L. Walp), well known as cowpea or black-eyed pea is a key component of healthy human nutrition and is of vital importance in maintaining environmental sustainability (Boukar *et al.,* 2015; Gonçalves *et al.,* 2016). Cowpea is an important leguminous grain in most tropical and subtropical countries, especially in Africa (sub-Saharan Africa), part of America, Asia and Europe (Walker *et al.,* 2016; De Ron, 2015).

Cowpea contributes immensely towards ensuring food availability and nutritional security. It provides needed fodder for livestock farming, it enhances soil fertility, and also provide wealth and income for farmers and other players in the agri-food business chain (De Luca *et al.,* 2018; Naiker *et al.,* 2019).

The enormous contribution of cowpea to food and nutritional security is well known, especially in developing and low-income nations where it is a staple food and also serves as a cheaper alternative to animal protein, as well as source of vital dietary nutrients (Bhat and Karim 2009; Vaz Pato *et al.,* 2015).

In Nigeria, this crop is a staple food source and an alternative source of income, especially for low-income populations.

Nigeria produced about 42% of cowpea in the whole world, making her the foremost producer (Gabdo and Amaza, 2010). Despite being the number one producer of cowpea globally, Nigeria's

large population and its low domestic productivity/ supply are not adequate to meet the nation's demand (Gómez, 2004).

More so, cowpea production is mainly undertaken by small scale farmers with limited resources/capability, and this leads to low productivity in most developing nations like Nigeria. Under these situations, productivity is below the crop optimum potential (Singh, 2014).

In spite of its benefits and potential, the area under cowpea cultivation and its production output are declining worldwide at a significant rate. Among the main constraints, are the issues of changes in both local and global climate, pressure due to microbial pathogens, as well as pests' infestations both on the field and during postharvest storage (Afutu *et al*., 2017). This challenging scenario are a threat to the productivity and sustainability of this important leguminous crop (Abate *et al*., 2011).

Attack of cowpea by destructive pests, viruses, nematodes, bacteria, protozoans, and fungi, leads to low production output and economic losses. This negative trend is devastating for rural farmers in low-income nations, and the main microbial culprits are fungi pathogens (Strange and Scott, 2005).

Cercospora leaf spot (CLS), is associated with different economically vital food crops (Bakhshi *et al.,* 2015). *Cercospora canescens* and *Pseudocercospora cruenta* causes leaf spot disease and infest cowpea plant leading to enormous loss in yield and productivity (Omoigui *et al.,* 2019). It is widely distributed in Africa and can lead to upward of 40% loss in cowpea production. This disease is of vital importance in cowpea producing nations (Duangsong *et al.,* 2016).

Powdery mildew disease is of significant importance in leguminous plants. The causative agent of this important legume disease is different species of *Erysiphe* (Braun, 1987; Soylu *et al.,* 2004). There are quite a lot of species that are associated with diverse plants (Braun and cook, 2012). The disease-causing pathogen is widely distributed in warmer and drier climatic conditions (Sillero *et al.,* 2006), and leads to lower yield and reduced cowpea quality.

In recent times, the widely accepted means of tackling this threat to crop yield and productivity is by promoting sustainable alternative to synthetic fungicide use.

Bioformulations that are derived from beneficial plant-microbe interrelationship/organically produced bioformulants are being promoted.

(AM) fungi help protect plants from phytopathogens and buffer against harmful environmental conditions (Ren *et al.,* 2013). This positive function of mycorrhizal can be synergetically used together with beneficial rhizospheric microorganisms in improving the growth and productivity of plants (Hause and Fester, 2005).

It is well known that different *Trichoderma* species antagonize an array of disease-causing plant pathogens, with successes reported for different crops. *Trichoderma* spp areamong the most effective bioagents used in combating phytopathogens. They play key roles in agro-biotechnological applications being of positive influence in plant-microbe interactions (Reino *et al.,* 2008; Vieira *et al.,* 2013).

There will always be a need for new agents and new ways of controlling fungal pathogens in the light of the numerous biotic and abiotic challenges that restrict the sustainable development of food crops in feeding the growing global populace.

This study is therefore aimed at assessing the potential of secondary metabolites produce from *Glomus versiforme* and *Trichoderma harzianum* strains in inhibiting fungal pathogens responsible for powdery mildew and leaf spot disease of cowpea plant.

MATERIALS AND METHODS

Microbial pathogens and Biocontrol agent's collection

The biocontrol agents and the microbial pathogens causing both powdery mildew and leaf spot disease of cowpea used in this investigative study were earlier characterized in our previous research (Omomowo *et al*., 2018). The cowpea pathogenic organisms that were used are *E. flexuosa* and *C. canescens*, while the bioagents used were strains of *T. harzianum* and *G. versiforme* designated as Tric-harz, Glo-verW, Glo-ver30, Glo-ver60, and Glo-ver90 respectively.

Submerged fermentation and extraction of secondary metabolites from the bioagents

The production of secondary metabolites by submerged fermentation culture of the biofungicidal fungi isolates was carried out. Fresh mycelia of fungi bioagents were grown on PDA plates at 28 ± 2 ^{o}C for 3 – 7 days. The pure colonies

of the fungi were then transferred to the Potato Dextrose Broth and using an orbital shaker for agitation at 150 rpm for 7 days. The submerged fermentation product was harvested after 7 days of growth; the broth was separated from the mycelia biomass using cheese clothes to filter. The broth was centrifuged to remove anyremnants of fungal cells and mycelia (clarification). The supernatants were then collected in sterile flasks. 1:1 mixture of the broth with ethyl acetate was ensured i.e. 300 mL of ethyl acetate and 300 mL of broth was allowed to stand for one day. The above procedure was repeated until most of the metabolites were released into the solvent phase and was later obtained by the aid of separating funnel. Finally, ethyl acetate extracts were evaporated to dryness using rotary evaporator to obtain the crude extract. The crude extract was treated with anhydroussodium sulfate to remove the remnant moisture content (Ramesha and Srinivas, 2014).

Phytochemical profile

The ethylacetate metabolites from *G. versiforme* and *Trichoderma hazianum* were qualitatively assessed for phytoconstituents composition (Kokate, 1995; Ramesha and Srinivas, 2014).

Antifungal effect of the metabolites from *G. versiforme* and *T. harzianum* on *Cercospora canescens* and *Erysiphe flexuosa*

Plate and Broth bioassay of crude extracts of secondary metabolites of biofungicidal agent in ethyl acetate as a solvent and the pathogenisolates were carried out as done by Wani (2011) to determine the following:

(a) The minimum or lowest inhibitory concentration (MIC)
(b) The minimum or lowest fungicidal Concentration (MFC)

Plate Assay

The biofungicidal metabolites were incorporated into Potato Dextrose Agar at the following increasing concentration: 25 µg/mL up to 200 µg/mL. The agar plates were inoculated and incubated using (5 mm mycelial disc) of pathogenic fungi from the cowpea plant. The biocontrol inhibitory evaluation was done by estimating the pathogenmycelia radial growth.

Broth Assay

Also, the biofungicidal metabolite from the fungi bioagents was incorporated into Potato Dextrose Broth: using an increasing concentration of 25 µg/mL to 200 µg/mL. (5 mmmycelial discs) of pathogenic fungi from cowpea was used for inoculation, and incubation was for 7 days. The biocontrol inhibitory potential was assessed by estimating the mycelia dry weight of the pathogen.

Minimum Inhibitory Concentration (MIC) of biofungicidal treatments

Varying concentrations of biofungicidal metabolites ranging from 10.0 µg/mL to 100 µg/mL were used in determining the MIC. 0.5 mL of each metabolite concentration was incorporated into potato dextrose broth that contains the pathogenic fungi. The incubation of these experimental test tubes was at 28 °C, under aeration conditions for 72. Controls were equally set up by using solvents and test organisms without the treatments. The minimum inhibitory concentration was the lowest concentration of treatment that does not support growth after incubation.

Determination of (MFC)

Theminimum inhibitory concentration test-tubes that do not support growth were used to determine the MFC. From the (MIC) test-tubes, 0.5 mL inoculum was spread on PDA petri-plates containing pathogenic fungi and incubated at room temperature 28 °C for 5 days. Minimum fungicidal concentration (MFC), was the lowest concentration that inhibited pathogenic fungi growth.

Statistical data

Statistical inference on the data was done using variance analysis (ANOVA). $P<0.05$ were considered significant, and SPSS software was used.

RESULTS

Antifungal activity of crude extract of *Glomus versiforme* and *Trichoderma harzianum* strains on pathogens from cowpea.

Antifungal actions of metabolites from *Glomus versiforme* and *Trichoderma harzianum* was carried out on the isolated *C. canescens* and *E. flexuosa* disease-causing pathogens from cowpea using well diffusion method. Invitro inhibition of the cowpea pathogens showed that metabolites from AMFwild at 200µg/mL concentration showed the highest inhibition, while it also gave the lowest inhibition of

4.3±0.07 mm. The inhibitory results of metabolites from Glo-ver60 had the overall highest inhibition effects 55±0.05 mm against *C. canescens* and 59.7±0.03 mm against *E. flexuosa*. Metabolites obtained from *Trichoderma harzianum* (Tric-harz) gave the second overall highest inhibition against the pathogens with 53.3±0.07 for *C. canescens* and 50.5±0.05 mm for *E. flexuosa.* Generally, these results reveal that there is variation in the antifungal activity of the biofungicidal crude extract metabolites at different concentrations. These results are represented in (Tables 1, 2, 3, 4 and 5)

MIC/MFC of crude metabolite of *Glomus versiforme* and *Trichoderma harzianum.*

The MIC/MFC from strains of *Glomus versiforme* and *Trichoderma harzianum* on *C. canescens* and *E. flexuosa* were carried out. Glo-ver60 gave the best MIC result of 73.4±0.05 µg/mL and 98.4±0.05 µg/mL for *C. canescens* and 72.1±0.08 µg/mL and 97.1±0.07 µg/mL for *E. flexuosa* respectively. Generally, these results revealed that therewas variation in the antifungal action of the metabolites from *Glomus versiforme* and *Trichoderma harzianum* against the selected cowpea pathogens and this is concentration-dependent. The result is represented in (Table 6).

Phytochemical screening of the ethyl acetate crude extract of secondary metabolites from *Glomus versiforme* and *Trichoderma harzianum* strains

The ethyl acetate crude extract of secondary metabolites obtained from *Glomus versiforme* and *Trichoderma harzianum* strains was screened for their phytochemical constituents. The phytochemical profile showed that Alkaloids, Flavonoids, Steroids, Phenols, Tannins, Cardiac Glycosides, and Saponins were all present. This is shown in (Table 7).

DISCUSSION

Mankind is shifting towards the use of biobased, natural products and away from utilizing materials from synthetic sources. This is as a result of the awareness that the ecological environment and the ecosystem, in general, should be sustainably utilized and preserved (Omomowo and Babalola, 2019). Fungi is a key biological agent that is applied in combating pests and pathogens (Alguacil *et al.*

Table 1. Antifungal effect of the crude extract from *Glomus versiforme* strain (Glo-verW) on tested cowpea pathogens.

Organism	Zones of Inhibition (mm)				
	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	Control
Cercospora canescens	19.1±0.09	8.7±0.06	4.47±0.09	—	—
Erysiphe flexuosa	16.3±0.06	6.8±0.06	—	—	—
P level	***	***	***	—	—

Values expressed as mean±SEM*** = Means at P<0.05 is significant

Table 2. Antifungal effect of the crude extract from *Glomus versiforme* strain (Glo-ver30) on tested cowpea pathogens.

Organism	Zones of Inhibition (mm)				
	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	Control
Cercospora canescens	30.1±0.09	18.3±0.09	12.3±0.12	—	—
Erysiphe flexuosa	24.3±0.09	15.6±0.12	8.4±0.03	—	—
P level	***	***	***	—	—

Values expressed as mean±SEM*** = Means at P<0.05 is significant

Table 3. Antifungal effect of the crude extract from *Glomus versiforme* strain (Glo-ver60) on tested cowpea pathogens.

Organism	Zones of Inhibition (mm)				
	200 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL	Control
Cercospora canescens	53.5 ±0.09	46.2±0.09	32.3±0.15	30.0±0.06	—
Erysiphe flexuosa	50.3±0.12	37.5±0.12	28.4±0.09	20.1±0.09	—
P level	***	***	***	****	—

Values expressed as mean ± SEM*** = Means at P<0.05 is significant

2011; Dwivedi and Enespa, 2013).

Findings from this investigation showed that the application of crude extract of secondary metabolites obtained from submerged culture of Glomus *versiforme and Trichoderma harzianum* were effective as an antifungal agent against cowpea disease-causing pathogens. It was observed that *Glomus versiforme* strain (Glo-ver60) produced the metabolites with the highest inhibitory activity. Similar results that is in agreement with findings in this study were obtained by (Melo *et al*., 1997; Montealegre *et al*., 2009).

Also, the phytochemical qualitative analysis of secondary metabolites of *Glomus versiforme* and *Trichoderma harzianum* affirmed that alkaloids, flavonoids, phenols, tannins, cardiac glycosides, steroids, and saponinswere present. However, terpenoids and amino acids were absent. The presence of these phytoconstituents is an indication for its potential as a precursor for developing potential biofungicides.

The results from our investigations concur with the findings of Devi *et al*., (2012) that *Penicillium* species showed the presence of different phytochemicals, phenolic compounds, steroids, cardiac glycosides, tannins, alkaloids, and flavonoids. This result also agrees with anearlier reported study by Ramesha and Srinivas (2014) in which phytochemical analysis of ethyl acetate extracts of *C. gloeosporioides* extract that showed the presence of alkaloids and steroids. Our findings in this work were following previously reported phytoconstituents of fungi that showed the presence of different phytochemicals viz alkaloids, steroids, phenolic compounds and flavonoids (Leyliae and Zafari, 2018; Bhardwaz *et al*., 2015) and are known to possess strong antimicrobial inhibitory activities.

From our study, the significant antifungal inhibitory activity of metabolites obtained from *Glomus versiforme* and *Trichoderma harzianum* strains antagonistic to the cowpea pathogenic fungi may be due to their diverse defense mechanisms which

Table 4. Antifungal effect of the crude extract from *Glomus versiforme* strain (Glo-ver90) on tested cowpea pathogens.

Organism	Zones of Inhibition (mm)				
	200 µg/mL	100µg/mL	50µg/mL	25µg/mL	Control
Cercospora canescens	45.3±0.05	37.4±0.05	28.1±0.05	—	—
Erysiphe flexuosa	40.1±0.06	31.6±0.06	25.3±0.043	—	—
P level	***	****	***	—	—

Values expressed as mean±SEM.*** = Means at P<0.05 is significant

Table 5. Antifungal effect of the crude extract from *T. harzianum* strain (Tric-harz) on tested cowpea pathogens.

Organism	Zones of Inhibition (mm)				
	200µg/mL	100µg/mL	50 µg/mL	25 µg/mL	Control
Cercospora canescens	51.3±0.18	48.3±0.09	28.2±0.12	22.1±0.09	—
Erysiphe flexuosa	58.5±0.48	38.2±0.09	29.2±0.12	21.5±0.06	—
P level	***	***	***	***	—

Values expressed as mean±SEM*** = Means at P<0.05 is significant

Table 6. MIC/MFC from the crude metabolite of *Glomus versiforme* and *Trichoderma harzianum* strains on *Cercospora canescens* and *Erysiphe flexuosa*

Treatment	*Cercospora canescens*		*Erysiphe flexuosa*	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Glo-verW	24.6±0.04	40.3±0.06	20.3±0.04	39.8±0.09
Glo-ver30	30.1±0.03	50.6±0.07	26.7±0.03	48.9±0.08
Glo-ver60	73.4±0.05	98.4±0.05	72.1±0.08	97.1±0.07
Glo-ver90	57±0.012	72.7±0.04	49.4±0.05	62.9±0.04
Tric-harz	26.0±0.02	85.1±0.04	61.9±0.05	83.4±0.04
P level	***	***	***	-

Values are means±SEM ***=Means at P<0.05 is significant

Table 7. Phytochemical profile of the ethyl acetate secondary metabolite of *Glomus versiforme* and *Trichoderma harzianum* strains.

Secondary metabolite	Glo-verW	Glo-ver30	Glo-ver60	Glo-ver90	Tric-harz
Alkaloids	+	+++	+++	+++	+++
Flavonoids	+	+++	+++	+++	+++
Phenols	+	+++	+++	+++	+++
Tannins	-	—	+	++	++
Cardiac Glycosides	+	++	++	++	++
Steroids	+	—	-	+++	+++
Saponins	+	+	+	+++	+++
Terpenoids	-	—	-	-	-
Amino acids	-	—	-	-	-

Keys: +++ Strongly present, ++ averagely present, + Present, - Absent
Glomus versiforme strains: Glo-verW; Glo-ver30; Glo-ver60; Glo-ver90 and *Trichoderma harzianum* strain: Tric-harz

include completion and secretion of lytic compounds (Pawle and Singh 2014).

The cowpea fungi pathogen differences in response to secondary metabolites crude extract concentration could be a result of masking of antifungal bioactive metabolites of the extract or due to synergetic effects of the extract bioactive metabolites. This could also be attributed to differences in the metabolite concentration used.

Svetlana *et al*., (2010) reported the efficacy of metabolites obtained from *Trichoderma* species as antifungal agents against plant pathogens. For example, it inhibited *Fusarium solani* by (74.4%), *Alternaria solani* (70.0%), *Phythium aphanidermatum* (67.7%) and *Macrophomina phaseolina* (50.0%). Their investigations reported that *Trichoderma viride* metabolites inhibited phytopathogens growth by an average of 88 %.

The antifungal inhibitory effect of ethylacetate secondary metabolites obtained in this study against cowpea pathogen might be as a result of the production of antifungal antibiotics. A similar result, that is in agreement with our findings was reported by Upadhyay and Rai, (1987) using culture filtrate of *Trichoderma* spp to inhibit some selected pathogens and ascribed it to the production of antibiotics.

CONCLUSION

The results obtained in this study using the secondary metabolites of strains of *G. versiforme* and *T. harzianum* to combat important cowpea disease-causing pathogens *Cercosporacanescens* and *Erysiphe flexuosa* indicated a strong antifungal inhibitory activity potential. Therefore, this study is relevant because it has shown that secondary metabolites from these bioagents could be formulated as potential biofungicides that smallholder cowpea farmers can use against cowpea disease-causing pathogens.

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