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ANTIFUNGAL ASSESSMENT OF PLANT EXTRACTS, BIOCONTROL AGENTS AND FUNGICIDES AGAINST *Fusarium verticillioides* (Sacc.) CAUSING EAR ROT OF MAIZE

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ABSTRACT. Ear rot is a prominent biotic threat of maize causing significant yield loss and poor quality of grains. The antifungal activity of aqueous extract of *Acorus calamus* L., *Xanthoxylum armatum* DC., *Azadirachta indica* A. Juss., *Lantana camera* L. and *Artemisia indica* Willd at three different concentrations (1, 2 and 3% W/V), four chemical fungicides viz., Dithane M-45 (Mancozeb 75% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), ACME-COP (Copper oxychloride 50% WP) and Bavistin (Carbendazim 50% WP) at (500, 1000 and 1500 ppm) and three biocontrol agents (BCA) namely *Trichoderma viride, Trichoderma harzianum* and *Trichoderma koningii* were investigated against *Fusarium verticilliodes* (Sacc.) causing ear rot of maize. The experiment was carried out by poisoned food and dual culture techniques in a completely randomized design with five replications under laboratory conditions at National Maize Research Program, Rampur, Chitwan during the summer season of 2019. *F. verticillioides* showed significant growth inhibition in all the treatments compared to control. The *A. calamus* even at a lower dose (1% W/V on PDA) was able to check completely the growth of pathogen (4.00 mm). The mycelial growth inhibition per cent of *A. calamus*, *L. camera*, *X. armatum*, *A. indica*, and *Artemisia indica* at 3% W/V was 95.50, 51.13, 45.50, 42.12 and 35.36% respectively. In the case of fungicides, at 1500 ppm, the maximum antifungal potential was observed with SAAF (86.32%) followed by Dithane M-45 (80.27%), Bavistin (64.80%) and ACME-COP (59.42%). Antagonist *Trichoderma viride* completely overgrows *F. verticillioides* and covers the entire medium surface and exhibit more than 60% inhibition on the 7th day of incubation. The antifungal components from these plant extracts, fungicides and antagonists explored in this study need to be tested further in field experiments to control the ear rot of maize.

Introduction

Fusarium verticillioides Sacc. is the most commonly reported fungal species causing ear rot of maize (*Zea mays*). The species is known as *Gibberella fujikuroi* species complex and described as mating population A of the *Fusarium fujikuroi* species complex (Seifert *et al.*, 2003). The fungus on infected kernels emits the mutagenic chemical compound fusarin C (Gelderblom *et al.*, 1984), as well as a collection of disease-causing mycotoxins known as fumonisins (Ortiz *et al*., 2015). Among the various ear rotting organisms, *Fusarium verticillioides* Sacc. was found most common and destructive to the maize crop in Nepal (Subedi, 2015). Although Fusarium ear rot occurs throughout the country, it is particularly common in places with high rainfall, moist and foggy weather conditions, and long crop durations in the field, all of which give favourable conditions for disease incidence and severity (Subedi, 2015). Symptoms of the disease include ears with red tip discolouration and light weight, as well as tightly adhered husk leaves during harvest (NMRP, 2015). Multiple minute black fruiting bodies of the fungus, a

Gibberella phase, can sometimes be found on husk covering or other damaged plant parts (Agrios, 2005). The fungus is commonly found in wounded areas caused by ear/kernel/silk-cut insects or birds, as well as in germinating kernels of ears in stuck plants. The disease also appears later on harvested ears. The pathogen can also infect seeds, seedlings, and roots, causing discolourations, rots, and blights in the field during germination, especially at high altitudes with cold and foggy weather (NMRP, 2016). Because it may produce trichothecenes, deoxynivalenol, and nivalenol mycotoxin on mouldy kernels, the pathogen is also significant for maize quality. Trichothecenes mycotoxins were found in 16% of the 74 maize samples collected from various locations of Nepal (Desjardins *et al*., 2000). The toxic consequences of chemical pesticides on individuals and the environment, on the other hand, pushed the search for the development of environmentally sustainable fungus control alternatives. Many synthetic chemicals are known to cause carcinogenicity, teratogenicity on non-target species and pollute the environment, soil and groundwater due to their residual toxicity and non-biodegradable nature (Pimentel, Levitan, 1986). Furthermore, the use of many synthetic fungicides has been limited due to unfavourable characteristics such as high and acute toxicity, a long degradation time and accumulation in the food chain, and an unfavourable extension of their ability to kill useful microorganisms (Subedi *et al*., 2015). Alternative fungal control approaches are being explored owing to issues like chemical residues, biodegradation, phytotoxicity, and pollution connected with chemical control measures. The study primarily aimed to evaluate the efficacy of locally available botanicals and antagonists for their ability to check the growth of *F. verticillioides* as an alternative to the chemical fungicides. In this study, the effect of plant extracts, bio-control agents and chemical fungicides against the growth of *Fusarium verticillioides* Sacc. causing ear rot of maize was investigated under *in vitro* condition.

Material and methods

Isolation of the pathogen

The antifungal assessment of plant extracts, fungicides and biocontrol agents against *Fusarium verticillioides* Sacc. was done at the laboratory of the National Maize Research Program (NMRP), Rampur, Chitwan during the summer season of 2019. To isolate the pathogen, samples of ear rot disease specimens were collected from maize growing fields of NMRP, Rampur during the harvesting stage. Infected ears were brought to a plant pathology laboratory and *F. verticilliodes* was isolated under aseptic conditions and the pure culture colonies of the pathogen purified by single spore isolation method were subcultured aseptically for further study.

Plant extracts

The aqueous extract of *Acorus calamus* L., *Xanthoxylum armatum* DC., *Azadirachta indica* A. Juss., *Lantana camera* L. and *Artemisia indica* Willd at three different concentrations (1%, 2% and 3% W/V) were prepared as described by Subedi *et al*. (2019). The extracts were exposed to UV light for further sterilization.

Fungicides

The chemical fungicides used for the experiment were Dithane M-45 (Mancozeb 75% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), ACME-COP (Copper oxychloride 50% WP) and Bavistin (Carbendazim 50% WP). The concentration level maintained was 500, 1000 and 1500 ppm.

Bio-control agents

The bio-control agents used for the experiment were laboratory isolated culture of *Trichoderma viride, Trichoderma harzianum* and *Trichoderma koningii*.

***In vitro* test**

The experiment was carried out by poisoned food and dual culture techniques in a completely randomized design (CRD) with five replications. A cork borer cut a four-mm-diameter piece of test pathogen from a one-week-old culture which was picked up with an inoculating needle and put in the centre of the plate. To allow for better and faster pathogen-media contact, a cut piece of the test pathogen was placed upside down in the PDA plate supplemented with the various above-mentioned treatments. The plates were put in incubation at 25 °C for up to 7 days (Subedi *et al*., 2019).

Similarly, the antagonistic effect of bio-control agents against *Fusarium verticillioides* Sacc. was evaluated following the dual culture technique. A four-millimetre-diameter cut piece of test antagonists and pathogen from a one-week-old culture was placed on PDA medium in an equidistant with an inoculating needle under aseptic condition and incubated for seven days at 25 °C. The colony diameter of both the test fungus and bioagent on different days of incubation up to 7 days were measured and per cent inhibition was calculated.

Observation

The average radial growth (mm) of the test pathogen was measured using a measuring scale from the bottom of the Petri-plates on different incubation dates. The percentage of mycelial growth inhibition was determined using the formula given below.

$$MGI, \% = \frac{(dc - dt)}{dc} \times 100 \quad (1)$$

where MGI – mycelial growth inhibition; dc – average diameter of fungal colony in the control and dt – average diameter of fungal colony in the treatment group.

Statistical analysis

The recorded observations were analyzed statistically using Genstat 18th edition computer package program and treatment means were compared using Duncan's Multiple Range Test (DMRT) and Least Significance Difference (LSD) test at 1% levels of significance.

Results

The mycelial growth of *Fusarium verticillioides* Sacc. differed significantly over the control among the treatments at various concentrations (Table1). The sweet flag (*Acorus calamus* L.) even at a lower dose (1% W/V on PDA) was able to check completely the growth of the pathogen (4.00 mm). With the increase in concentration (3% W/V), the mean colony diameter of the *Fusarium verticillioides* Sacc. in the plate amended with lantana (*Lantana camera* L.) and prickly ash (*Xanthoxylum armatum* DC.) was significantly lower *i.e.* 43.40 mm and 48.40 mm (Table 1) respectively than the diameter recorded at control plate (88.80 mm).

Table 1. Effect of plant extracts incorporated PDA on the growth of *Fusarium verticillioides* Sacc. in different concentration level at 25 °C

Treatments	Mean colony diameter, mm		
	1% W/V	2% W/V	3% W/V
Acorus calamus	4.00^{e}	4.00^{e}	4.00^{f}
Xanthoxylum armatum	60.40^{d}	52.60^{d}	48.40^{d}
Azadirachta indica	74.60^{b}	55.40^{c}	51.40^{c}
Lantana camera	61.00^{d}	51.60^{d}	43.40^{e}
Artemisia indica	64.00^{c}	61.80^{b}	57.40^{b}
PDA only	89.40^{a}	88.80^{a}	88.80^{a}
Grand mean	58.90	52.37	48.90
P-value	<0.001	<0.001	<0.001
LSD (0.01)	2.16	2.08	1.97
CV,%	2.00	2.20	2.20

Means in column with the same superscript are not significantly differed by DMRT ($P \leq 0.01$). W/V – weight by volume, PDA – potato dextrose agar

The mycelial growth inhibition per cent of *A. calamus*, *L. camera*, *X. armatum*, *A. indica*, and *Artemisia indica* at the concentration of 3% W/V on PDA was 95.50, 51.13, 45.50, 42.12 and 35.36% respectively (Table 2).

Table 2. Effect of plant extracts incorporated PDA on mycelial growth inhibition of *Fusarium verticillioides* Sacc. in different concentration level at 25 °C

Treatments	Mycelial growth inhibition %		
	1% W/V	2% W/V	3% W/V
Acorus calamus	95.53	95.50	95.50
Xanthoxylum armatum	32.44	40.77	45.50
Azadirachta indica	16.55	37.61	42.12
Lantana camera	31.77	41.89	51.13
Artemisia indica	28.41	30.41	35.36
PDA only			

W/V – weight by volume, PDA – potato dextrose agar

All the tested fungicides incorporated PDA had a significant ($P < 0.01$) effect on the growth of the *Fusarium verticillioides* Sacc. at 25 °C as compared to control at different concentrations up to the 7th day of incubation (Table 3). The mean colony diameter of the pathogen in the plate amended with SAAF (Carbendazim 12% + Mancozeb 63% WP) and Dithane M-45 (Mancozeb 75% WP) was significantly lower ie 12.20 mm and 17.60 mm, respectively than the control plate (89.20 mm) on 7th day of incubation at 1500 ppm concentration level. Both fungicides Bavistin (Carbendazim 50% WP) and ACME-COP (Copper oxychloride 50% WP) also checked the growth of pathogen *i.e.* 31.40 mm and 36.20 mm respectively at 1500 ppm concentration compared to the control plate (Table 3).

Table 3. Effect of fungicides incorporated PDA on the growth of *Fusarium verticillioides* Sacc. in different concentration level at 25 °C

Treatments	Mean colony diameter, mm		
	500 ppm	1000 ppm	1500 ppm
SAAF (Carbendazim 12% + Mancozeb 63% WP)	28.00^{e}	20.80^{e}	12.20^{e}
Dithane M-45 (Mancozeb 75% WP)	34.40^{d}	28.60^{d}	17.60^{d}
Bavistin (Carbendazim 50% WP)	58.80^{c}	52.00^{c}	31.40^{c}
ACME-COP (Copper oxychloride 50% WP)	72.80^{b}	66.40^{b}	36.20^{b}
PDA only	89.20^{a}	89.20^{a}	89.20^{a}
Grand mean	56.72	51.40	37.32
P-value	<0.001	<0.001	<0.001
LSD (0.01)	2.11	2.33	1.97
CV,%	2.00	2.50	2.90

Means in column with the same superscript are not significantly differed by LSD ($P \leq 0.01$). WP – wettable powder, PDA – potato dextrose agar

Table 4. Effect of fungicides incorporated PDA on mycelial growth inhibition of *Fusarium verticillioides* Sacc. in different concentration level at 25 °C

Treatments	Mycelial growth inhibition %		
	500 ppm	1000 ppm	1500 ppm
SAAF (Carbendazim 12% + Mancozeb 63% WP)	68.61	76.68	86.32
Dithane M-45 (Mancozeb 75% WP)	61.43	67.94	80.27
Bavistin (Carbendazim 50% WP)	34.08	41.70	64.80
ACME-COP (Copper oxychloride 50% WP)	18.39	25.56	59.42
PDA only			

ppm – parts per million, WP – wettable powder, PDA – potato dextrose agar

The maximum antifungal potential was observed with SAAF which recorded excellent inhibitory activity (95.34%) against *Fusarium verticillioides* Sacc. (86.32%) followed by Dithane M-45 (80.27%), Bavistin (64.80%) and ACME-COP (59.42%) at the concentration of 1500 ppm (Table 4).

Table 5. Effect of *Trichoderma* species on radial colony growth of *Fusarium verticillioides* Sacc. in different incubation periods at 25 °C

Treatments	Mean colony diameter, cm		
	3rd day	5th day	7th day
Trichoderma viride	1.35^{d}	2.45^{d}	3.16^{d}
Trichoderma harzianum	2.15^{c}	3.03^{c}	3.75^{c}
Trichoderma koningii	2.73^{b}	3.75^{b}	4.35^{b}
Control (only *Fusarium monilliforme*)	3.85^{a}	4.45^{a}	8.37^{a}
Grand mean	2.52	3.42	4.91
P-value	<0.001	<0.001	<0.001
LSD (0.01)	0.32	0.29	0.30
CV,%	6.60	4.40	3.10

Means in column with the same superscript are not significantly differed by LSD ($P \leq 0.01$)

All of the *Trichoderma* species exhibited significant inhibition ($P < 0.01$) of the *Fusarium verticillioides* Sacc. as compared to control in different dates of incubation period at 25 °C. Less (3.16 cm) mean colony diameter of the pathogen was measured when the dual culture was done with *Trichoderma viride*. The diameter of the pathogen alone was 8.37 cm on the 7th day of incubation at 25 °C temperature. However, the tested

Trichoderma species differed in their abilities to suppress *Fusarium verticillioides* Sacc. Antagonist *Trichoderma harzianum* (3.75 cm) *and Trichoderma koningii* (4.35 cm) was also checked the growth of *Fusarium verticillioides* Sacc. on the 7th day of incubation at 25 °C temperature (Table 5). Antagonist *Trichoderma viride* completely overgrows *F. verticillioides* and covers the entire medium surface and exhibit more than 60% inhibition on the 7th day of incubation (Table 6).

Table 6. Effect of *Trichoderma* species on mycelial growth inhibition of *Fusarium verticillioides* Sacc. in different incubation periods at 25 °C

Treatments	Mycelial growth inhibition, %		
	3rd day	5th day	7th day
Trichoderma viride	64.94	44.94	62.25
Trichoderma harzianum	44.16	31.91	55.20
Trichoderma koningii	29.09	15.73	48.03
Control (only *Fusarium verticillioides*)			

Discussion

Botanical extracts are recognized as eco-friendly and safe for the management of plant diseases in the quest for better remedies. The results achieved in this study are in agreement with Satish *et al*., (2009) who screened 46 plants belonging to 32 different families against eight species of *Fusarium* and explored aqueous extracts of 12 different Asiatic plant species which showed significant antifungal potentialities against the test pathogen. The result of the experiment is also in line with the findings of many studies conducted with the extracts of *Azadirachta indica, Zingiber officinalis, Curcuma longa, Ocimum sanctum, Acorus calamus, Terminalia chebula, Lantana camera and Catharanthus roseus* which can be exploited in the control of fungicide resistant pathogens (Baligh *et al*., 1999; Bowers and Locke, 2000; Biswas *et al.*, 2002; Pothitirat and Gritsanapan, 2006). The alcoholic extract of *A. calamus* could inhibit many fungi including *Alternaria brassicae* Sacc, *Fusarium oxysporum f.sp. lycopersici, Rhizoctonia solani* Kuhn, *Sclerotinia sclerotiorum* de Bary at the concentration of 0.10% upward (Kungha, 1999). The major antifungal compound found in *A. calamus* is β-asarone which is considered to be the most biologically active compound in the rhizome of *A. calamus* and quantities of β-asarone vary 10–20% in the rhizome of European origin while in Asia, it was found in the range of 70–90% (Karwowska *et al*., 1997). The antifungal bioactive components especially monoterpenes like geranyl, terpinyl and bornyl acetate, bicyclic sesquiterpene ß-caryophyllene and a cyclic monoterpene like limonene extracted from the essential oil of *Lantana camera* restricted the growth of many tested fungi including *Fusarium* spp (Deena and Thoppil, 2000). The aqueous extract of *Acorus calamus* L. was found most effective followed by *Xanthoxylum armatum* and *Lantana camera* when tested against fungi *Exserohilum turcicum* and *Stemphylium botryosum* Walr. both *in vitro* and *in vivo* condition (Subedi *et al*., 2015; Subedi *et al*., 2019).

Bashir *et al*. (2018) studied the effects of fungicides on the mycelial growth of *Fusarium* spp and found a significant mycelial reduction by carbendazim. Studies have shown that carbendazim prevents microtubule formation and inhibits mitosis in fungal cells of the targeted fungi, which prevent mycelial growth (Kling and Jakobsen, 1997; Yang *et al*., 2011). Carbendazim specifically interacts with b-tubulin and stop the mycelial growth of fungi (Zhou *et al*., 2016; Vela-Corcia *et al*., 2018). The growth of *Exserohilum turcicum* causing northern leaf blight of maize was also effectively checked with the application of SAAF (Carbendazim 12% + Mancozeb 63% WP) and Dithane M-45 (Mancozeb 75% WP) under *in vitro* condition (Subedi *et al*., 2019). The bio-control agents *Trichoderma viride* Pers. showed the abundant potentials to suppress the radial colony growth of *F. verticillioides* under laboratory condition. *T. viride* developed more rapidly than *F. verticillioides* in single as well as in dual cultures. The intensive development of *Trichoderma* gives it a significant advantage in competition with pathogens for nutrients and space, besides the production of mycotoxins. Most of the studies showed these bioagents inhibitory activities were most likely due to competition and/or antibiosis (Subedi *et al*., 2019). The antagonism of *T. viride* found in the current study is consistent with the results of other researchers too (Biles and Hill, 1988; Mahamood *et al*., 1995; Ramachandra, 2000). The pathogen control mechanism of *Trichoderma* can involve attacking and binding pathogenic organisms via sugar linkage with the secretion of extracellular protease and lipase (Cal *et al*., 2004). The key reason for the ecological success of *Trichoderma* spp could be a combination of highly active mycoparasitism mechanisms and an efficient defensive strategy induced in the plants (Rosado *et al*., 2007). Trichoderma sp. grows over pathogenic fungal hyphae, coils around them, and degrades the cell walls, limiting the growth and activity of pathogenic fungi, a mechanism known as mycoparasitism, with the release of antibiotics (Harman, 2006).

Conclusions

The aqueous extracts of all the test plants, biocontrol agents and chemical fungicides were subjected to antifungal activity against *F. verticillioides* causing ear rot of maize. The extract of *Acorus calamus* L. at a lower dose also completely check the pathogen growth *in vitro* and found more effective compared to chemical fungicides. Antagonist *Trichoderma viride* suppresses the radial colony growth of *F. verticillioides* and develops more rapidly than the test pathogen. So these botanicals, antagonists and fungicides like SAAF (Carbendazim 12% + Mancozeb 63% WP) could be exploited for ecofriendly management of the diseases caused by the *F. verticillioides* and need to be tested further in field experiments as well as isolation of antifungal bioactive agents.

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Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

Author contributions
SS – study conception and design, analysis and interpretation of data, critical revision and approval of the final manuscript; SN – drafting of the manuscript, acquisition of data.

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