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FUNGITOXIC PROPERTIES OF FOUR CRUDE PLANT EXTRACTS ON *FUSARIUM OXYSPORUM* SCHL. F. *SP PHASEOLI*

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ABSTRACT

Fusarium yellows is a disease of common beans (*Phaseolus vulgaris*, L.) caused by *Fusarium oxysporum* Schl. F. *sp. phaseoli,* it has been found to be important in Busia district of Western province, Kenya. The study on fungitoxic properties of four locally available crude plant extracts was aimed at evaluating their efficacy in controlling Fusarium yellows infestation under the field conditions. Crude plant extracts from *Azadirachta indica, Tagetes minuta, Nicotiana tobacum and Vinca rosea* were tested against *Fusarium oxysporum* Schl. F. *sp. phaseoli*. Participatory On-Farm Trials (POFT) in six (6) divisions were carried out in August-September 2005 and March-June 2006; a total of thirty (30) farms were randomly surveyed. Minimum Inhibitory Concentration (MIC) of crude plant extracts against Fusarium was determined by broth microdilution method. Analysis of variance (ANOVA) was performed on the data, using Genstat 8th edition statistical program (Release 8.11, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK). Means were separated using LSD. Crude plant extracts exhibited fungitoxic activity against *Fusarium oxysporum* Schl. F. *sp. phaseoli*, with varying degree of efficacy. *Nicotiana tobacum* and *Vinca rosea* were not effective, *Azadirachta indica* and *Tagetes minuta* exhibited significant control over Fusarium. *Azadirachta indica* performed better amongst all the plant extracts. Common bean treatment with Benomyl 1 significantly reduced ($P \leq 0.05$) wilt incidence and increased growth in comparison to negative (–ve) control. *Azadirachta indica* formulation gave a significant reduction in wilt incidence compared to the other three crude plant extracts formulations. It reduced the wilt incidence by 17.24% in comparison to *Tagetes minuta, Nicotiana tobacum and Vinca rosea* whose wilt incidence reduction ranged from 5.84-9.8%. *Azadirachta indica* inhibited Fusarium growth at lower dosage than *Tagetes minuta, Nicotiana tobacum* and *Vinca rosea.* Extracts from *Azadirachta indica* and *Tagetes minuta* are effective, cheap and ecofriendly promising methods for protecting common bean against *Fusarium oxysporum* Schl. F. *sp. phaseoli.*

Key words: Ecofriendly, Efficacy, Plant extracts, *Fusarium oxysporum*

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important food and cash crop particularly in Eastern, Southern and Great lake region [1]. It is considered to be the second most important source of human dietary protein (after maize) and third most important source of calories after maize and cassava [2]. The common bean is grown by more than 90% of the population worldwide, mostly by small scale farmers for home consumption, with some selling the surplus to meet domestic financial requirements [3]. It is the main source of affordable protein for rural and urban households and for institutions such as hospitals, schools and the army [4]. Compared to other pulses, the common bean is the most widely grown pulse in Kenya, found in most of the districts with production decreasing towards Coast and Northern parts of the country.

A survey conducted in central province of Kenya in 1980 revealed a high percentage incidence of Fusarium yellows in all districts where beans are grown but most seriously in Muranga and Kiambu where significant reduction in yields was also reported [5]. In western Kenya the incidence was noted to be high in Busia, Vihiga and Kakamega [6]. Diseases such as anthracnose, angular leaf spot, bean rust, bean scab, sclerotinia rot and common bean mosaic virus are some of important bean diseases that have received attention, but little has been done to control Fusarium yellows caused by *Fusarium oxysporum* Schl. F. *sp. phaseoli.* Economic losses in yield due to *Fusarium oxysporum Schl. F.sp. phaseoli* was first reported by Kendrick & Snyder in 1942 in Colorado [7] and subsequently in Egypt [8], Colombia [9], Brazil [10], Central Africa [11] and Kenya [5]. Synthetic chemical fungicides are being used successfully for the control of *Fusarium oxysporum* Schl. F. *sp. phaseoli* but indiscriminate use of these chemicals has led to the development of fungicide resistance and more importantly, environmental pollution, posing potential risk to animal and human health [12].

The four selected plants are locally available in sufficient quantities. Scientific studies have revealed that the four plants can control a number of fungal pathogens in vivo [13]. Their active ingredients content ranges from moderate to high, making them potential candidates for On Farm Trials (OFT). The major setback in the control of *Fusarium oxysporum* Schl. F. *sp. phaseoli* in the study area has been low per capita income; with household poverty incidence among subsistence farmers in the district being 68.2%, even though it is classified as high potential zone in terms of Agricultural productivity [14]. This situation has hindered small-scale subsistence farmers' ability to acquire synthetic chemicals to control this fungal infection of beans. This whole scenario, therefore, calls for alternative approaches for the control of soil borne pathogens. The approach need to be economically feasible and ecofriendly to eliminate or reduce the incidence of this economic pathogen, so as to increase yield of common bean. The leaves, flowers, seeds and twigs were used to yield 'concentrated' extract due to the fact that they contain high level of active ingredients needed for pathogen control

This paper reports the fungitoxic activity of four locally available plant species against *Fusarium oxysporum* Schl. F. *sp. phaseoli.*

MATERIALS AND METHODS

The study was conducted in Busia district, western part of Kenya in the field during two rainy seasons of 2005 and 2006. The district receives 1150 mm of rainfall annually on average and experiences an average of a maximum temperature of 26^{o}C and a minimum of 16^{o}C [15]. Bean-growing sites in Busia municipality, Budalangi, Matayos, Funyula, Nambale and Butula divisions were used during this study.

Pathogen identification

Pathogen identification was done in Commonwealth Agriculture Bureau International (CABI) Laboratory, Nairobi and Botany Laboratory at Maseno University, Kenya. In Botany Laboratory at Maseno University, Kenya; five plants were uprooted from each of the surveyed farm and dried in plant press for isolation experiments. Diseased plant specimens were isolated to confirm the identification of the pathogens present.

Potato Dextrose Agar (PDA) was used for the isolation, PDA slants in Mcartney bottles loosely screwed with caps fitted with an inner rubber lining and sterilized by autoclaving at 121°C at one bar gauge pressure (10N/m²) for 15 minutes and left to cool a little then placed in a slanting position, forming a sloping medium surface were used to subculture pure *Fusarium oxysporum* fungus.

Pure *Fusarium oxysporum* Schl. F *sp. phaseoli* fungus prepared in Botany Laboratory at Maseno University was allowed to grow for 10 days at CABI Laboratory. To induce conidiation Carnation Leaf Agar (CLA) media was used [16]. For microscopic observation, Fusarium was grown on CLA at 25°C for 5 days, and agar blocks (~2 x 2 mm) containing fungal propagules were cut out from the media. They were attached onto specimen stubs, frozen in liquid nitrogen, and observed with a scanning microscope (S-4000, Hitachi, Ibaraki, Japan) after gold coating.

Fusarium oxysporum Schl. F *sp. phaseoli* penetrates into the host through the root or the hypocotyls but the presence of wounds may be necessary in order to permit penetration into exposed xylem vessels. The fungus grows principally inside the vascular elements causing discoloration and browning of the affected areas. Subsequent deposits of gum-like materials can cause plugging of the vascular system. To cause wilt, the fungus must also establish in the xylem and induce tracheomylosis a process completed only by the strain specialized for that host plant [17]. The severity of the disease depends on inoculum's density and soil type with some soils being more suppressive than others [18].

Sandy soils are more conducive in spreading wilt than loams or clays; this is due to better drainage in former. Fusarium wilt has also been reported to be more severe in

wet rather than in dry soils. The fungus penetrates the base and invades the xylem vessels. The plugging of the vessels by the fungal mycelium and in some cases production of toxins results in wilting symptoms in some plants and yellowing of leaves and shoots in others.

With the occurrence of unfavourable conditions and in the absence of a host, the fungus forms chlamydospores in the soil [19].

Chlamydospores are hyphal or spore cells with thickened cell walls and which are resistant to adverse physical and chemical conditions. Germination of chlamydospores is stimulated by nutrients from the host root exudates or by extraneous food base introduced into the soil.

Plant material

Azadirachta indica (Neem tree), *Tagetes minuta* (Mexican marigold), *Nicotiana tobacum* (Tobacco) and *Vinca rosea* (Peri winkle) materials were collected from the wild in Budalangi and Butula areas of Busia district. Taxonomic authentification of plant specimens was performed by taxonomist at Department of Botany-Herbarium and Botanic garden, Maseno University, Kenya.

Crude plant extracts evaluated comprised of *Azadirachta indica, Tagetes minuta, Nicotiana tobacum* and *Vinca rosea* and Benomyl 1 was used as control. Plant parts were harvested from mature plants at random by either uprooting entire plant (*Tagetes minuta, Nicotiana tobacum* and *Vinca rosea)* or *plucking* by hand *(Azadirachta indica)*. They were stored under room temperature in sisal sacks. Crude extracts of single plant (*A. indica, T. minuta* and *N. tobacum*) were prepared by thoroughly washing leaves and /or flowers or twigs then air-drying them on sterile blotter under shade (Table 3). Within 36 hours after harvesting, a sample of 1kg of each of the three plants was blended in waring blender (Waring International, New Hartford, CT, USA) for 10min without distilled water. Individual plant extracts were filtered using Buchner funnel to yield 'concentrated' extract.

The extracts were stored in brown bottles at room temperature until use. The 'concentrated' extracts from 1kg plant parts were diluted in 4 litres of water individually and applied to bean crops within 7 days after preparation [20]. *Vinca rosea* was prepared by adding a proportion of 1 g (approx. 1 teaspoonful) of oven dried (leaves, flowers and twigs) to 1 ml of sterile distilled water [21]. Control constituted bean plants subjected to spray with Benomyl 1 (2.5 g/l) and distilled water.

Participatory On-Farm Trials (POFT)

Participatory On-Farm Trials (POFT) was conducted on 30 farms. The lay out for POFT were based on modified paired-comparison trial approach (Figure 1). Each treatment, strips ran across with dimensions of 25 x 2.25 m and replicated 3 times.

Each strip comprised of 2 bean rows and 2 maize rows; the strips were separated from each other with a buffer strip (1 row of beans and 1 row of maize). Within the strip; 1 bean row was subjected to normal dosage and the other double dosage. Intrastrip buffer zone constituted maize row between the two bean strips.

This approach was chosen based on fact that it eliminates field variability effect as a result of nutrient and moisture differences also given that strips lay close to each other; field variability between treatment strips is minimized.

Replications were randomized so as to avoid biasing effects that are not anticipated. The treatments evaluated included *Azadirachta indica, Nicotiana tobacum, Vinca rosea,* Benomyl 1 and water.

Crude plant extracts application

First spraying of crude plant extracts commenced upon appearance of >30% wilt symptoms, based on disease severity scale 0–4 (0 = no symptoms; 1 = 1–33% foliage affected; 2 = 34–66% foliage affected; 3 = 67–100% foliage affected; 4 = dead plant) [18], on the control and continued on once a week basis up to second month (Pod forming stage), with application rate of 1000g/l and 2000g/l for four plant extracts; 2.5g/l and 5g/l for Benomyl 1 and spraying was done using hand sprayer [22].

Sampling procedure

To determine efficacy of plant crude extracts; sample plot of 2 bean rows each measuring 25 m in length, within row spacing of 25 cm; was used to determine the number of plants showing Fusarium yellows over time with spraying. Data were collected from 3 sample rows in each farm and average value computed. The data were collected at 4^{th}, 6^{th} and 8^{th} week of weeks after planting (WAP) so as to show trend of plants recovery. Percentage disease incidence was determined using the model: I /Tx100% where I = Number of plants showing wilting, T = total sample plant population.

Minimum Inhibition Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the formulations and Benomyl 1, against Fusarium yellows were determined by the broth microdilution method [23]. The formulations were serially diluted with distilled water to prepare the solutions, with a series of concentrations ranging from 0.5 to10 mg ml^{-1} per testing well. After

shaking, 4 ml of the antifungal agent solutions were added to the wells of 360-well plates. The suspension of *Fusarium oxysporum* was adjusted to 2.5×10^6 spores/ml and then 5 ml added to the individual wells and incubated at 24–28°C. MIC is the lowest concentration that completely inhibited visible fungal growth in the wells after 72 hrs of incubation on observing under compound microscope. *Fusarium oxysporum* was also cultured with a control solution containing distilled water (-ve control) to certify that it did not affect fungal growth and Benomyl 1 (+ve control); the treatments were performed in triplicate.

Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using Genstat 8th edition statistical program (Release 8.11, Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) to identify differences between treatments.

LSD (Least Significant Difference) procedure for comparison of means was applied to separate means ($P<0.05$). Treatments differing significantly are indicated in tables by designating different sets of letters.

RESULTS

Participatory On-Farm Trials (POFT)

Fusarium wilt incidence in the 30 farms sampled one month (4th week) after planting was recorded to range between 28-31% (Figure 1), at this stage of growth bean plants are susceptible to pathogen attack due to tender tissues. Spraying with extracts commenced at 4th week of growth and the incidence significantly reduced at 8th week with continued spraying (8th week) to lowest incidence of 10% as seen in *Azadirachta indica.*

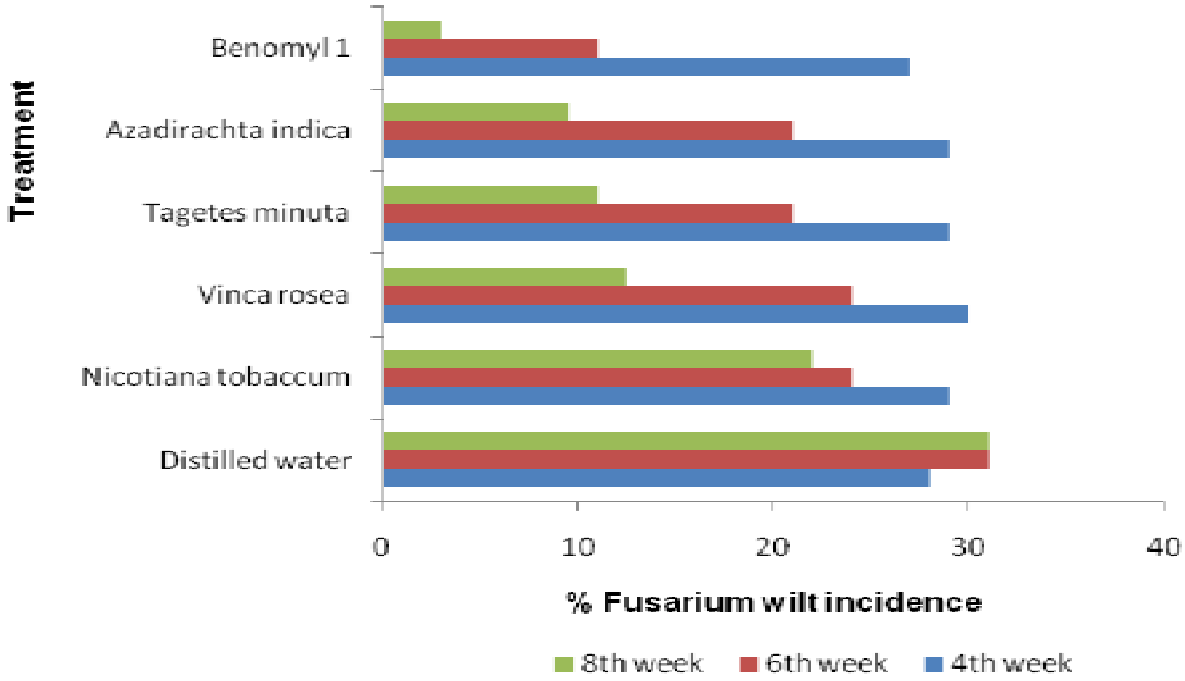

Figure 1: Fusarium yellows incidence overtime with normal spraying regime in farmers fields during March-June 2006 season

Table 1 shows results of Participatory On-Farm Trials in terms of % Fusarium yellows incidence with spraying over 5 weeks period. Benomyl 1 significantly ($P \leq 0.05$) reduced disease incidence in comparison to all other tested treatments. *Azadirachta indica* performed better amongst all the plant extracts. It reduced the incidence by 17.24% in comparison to *Tagetes minuta, Nicotiana tobacum and Vinca rosea* whose % reduction ranged from 5.84-9.8 considering distilled water as bench mark. Variation in dosage regime (normal/double) did not significantly ($P \leq 0.05$) affect disease incidence in the field.

Minimum Inhibitory Concentration (MIC)

As shown in Table 2 Benomyl 1 exhibited the strongest inhibition against Fusarium yellows with MIC of 1.0 mg ml^{-1}. The fungicidal activity against Fusarium yellows by *Tagetes minuta* and *Vinca rosea* was rated as 5·0 mg ml^{-1} compared to 10 mg ml^{-1} for *Nicotiana tobacum*.

The MIC of *Azadirachta indica* 2.5 mg ml^{-1} was higher than that of Benomyl 1, however, much lower in comparison to all other plant extracts. This finding suggests that *Azadirachta indica* is inhibitory to Fusarium growth at lower dosage than *Nicotiana tobacum*, *Tagetes minuta* and *Vinca rosea* with MIC ranging between 5.0 and 10.0 mg ml^{-1}. Distilled water did not affect the growth of Fusarium yellows.

DISCUSSION

Common bean treatment with fungicide significantly decreased ($P \leq 0.05$) the Fusarium yellows incidence and increased the plant growth in comparison to the –ve control. Crude plant extracts from *Tagetes minuta, Nicotiana tobacum, Vinca rosea* and *Azadirachta indica* were effective in controlling *Fusarium oxysporum* with varying degree of efficacy over different growth stages of the bean plants.

Azadirachta indica formulation gave a significant reduction in wilt incidence compared to the other three crude plant extracts formulations. Reports by Achook, Godrej Agrovet Ltd., Mumbai, India, 1999 indicate that a water-soluble Neem formulation containing Azadirachtin, Azadiradione, and a mixture of Nimbocinol and epinimbocinol affects the growth and development of some noctuid larvae or inhibits the growth of phytopathogenic fungi. Neem, as a natural botanical pesticide with a low risk of toxicity to humans and animals, could be one of the important plant protection agents in Integrated Pest Management (IPM) programmes. Studies show that on application as spray; neem is systemically translocated into the plant hence inhibiting mycelial growth in the vascular system [24, 25].

Tagetes minuta performed better in controlling *F. oxysporum* but with lower efficacy in comparison to *A. indica*. Bioactive extracts from different *Tagetes* species have been shown to have insecticidal, nematicidal, and fungicidal properties. The main bioactive extract from *T. minuta* is oil consisting mainly of (*Z*)-ocimene (60%), followed by dihydrotagetone (15%), (*E*) - and (*Z*)-tagetenones (12%), (*E*) - and (*Z*)-tagetones (10%) and sesquiterpenes (2%) [26]. No previous reports on fungitoxic activity of *T. minuta* could be traced in literature; to date documented information on medicinal properties of *T. minuta* is on its use in pest control initiatives. This study has dealt with the fungitoxic activity of *Tagetes minuta* and results indicated that it is promising botanical that can be utilized in control of Fusarium yellows.

Although no significant ($P \leq 0.05$) difference among *T. minuta* and *V. rosea* against *F. oxysporum* were observed in wilt incidence, putting in consideration other evaluated parameter such as MIC; *T. minuta* can be rated superior to *V. rosea*.

Vinca rosea has been documented to posse's fungitoxic characteristics and has been able to control (*in-vivo*) a number of fungi. Studies on fungitoxic characteristics of *V. rosea* on *Helminthosporium modulosum, Sclerotium rolfsii, Pestalotia sp., Fusarium oxysporum, Colletotrichum sp*. and *Aspergillus niger* showed that it inhibited spore germination, sporulation and mycelial growth in some of test fungi (*in-vivo*) [14]. Growth of *H. modulosum, S. rolfsii, Pestalotia sp. and Colletotrichum sp*. were completely inhibited at 8ml extract, but *F. oxysporum and A. niger* exhibited growth [20]. Findings from this study concur with results of Narain and Satapathy [1977]; *V. rosea* controlled *F. oxysporum* though at a much higher concentration (MIC of 5.0 mg ml^{-1}) in comparison to *A. indica* and Benomyl 1.

Nicotiana tobacum is known to be highly poisonous due to alkaloid nicotine, a powerful neurotoxin that is particularly harmful to insects. Other active alkaloids in tobacco include Harmala alkaloids. It is also documented to contain nitrosamines and other carcinogenic compounds.

Research on insecticidal activity of tobacco showed that if tobacco powder is used as admixture, it reduces egg laying and hatchability of *Callosobruchus maculatus* on cowpeas [20]. Research indicates tobacco has insecticidal activity that control *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). Little however is documented about its fungitoxic properties against plant pathogens [27]. Results from this study showed that *N. tobacum* formulation did not significantly affect *F. oxysporum* in comparison to –ve control.

CONCLUSION AND RECOMMENDATIONS

Participatory On-Farm Trials (POFT) showed that crude plant extracts are effective in controlling *Fusarium oxysporum* Schl. F. *sp. phaseoli*, with varying degree of efficacy. Spraying with crude plant extracts reduced the incidence of Fusarium in the field and also resulting in complete recovery of initially infected bean plants. *Azadirachta indica* and *Tagetes minuta* were found to be the highly effective plant extracts amongst tested crude plant extracts. Besides inhibiting growth of Fusarium in vascular system of the plants they also resulted in low wilt incidence.

In order to institutionalise utilization of these findings in crop production systems and poverty alleviation initiatives; future work should focus on; awareness creation, dissemination of findings, capacity building of both extension staff and community on preparation and conservation of plant sources of these extracts; evaluation of efficacy of combined application of plant extracts and fractionating crude extracts from *Tagetes minuta* in order to identify the active ingredients responsible for mycelial growth inhibition with view of packaging it as a fungicide will increase its chance of being utilised.

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Table 1: Mean Fusarium yellows incidence (%) for five weeks (Participatory on- Farm Trials)

Treatment	Dosage (g/l)	Incidence (%) (Mean±SE)	Difference level 0.05
Water (- ve control)	500	30.47±2.3	a
Nicotiana tobacum	1000	24.63±2.2	bc
	2000	21.57±1.3	bc
Vinca rosea	1000	21.17±0.9	cb
	2000	20.67±0.7	cb
Tagetes minuta	1000	20.67±0.7	dc
	2000	21.63±0.9	dc
Azadirachta indica	1000	13.23±0.7	e
	2000	15.07±0.7	e
Benomyl 1(+ve control)	2.5	10.70±0.3	f
	5.0	11.70±0.3	f
LSD			4.38

Means followed by same letter in column are not significantly different(ns) at P≤ 0.05

Table 2: Minimum inhibitory concentration (MIC) of various treatments estimated by broth microdilution method

Treatment	MIC[1] (Mean±SE)
Nicotiana tobacum	10·0 ± 0.4
Tagetes minuta	5·0 ± 0.3
Vinca rosea	5·0 ± 0.2
Azadirachta indica	2.5 ± 0.2
Benomyl 1	1.0 ± 0.1

MIC[1] (mg ml^{-1}) mean of 3 replicates

Table 3: Plant parts used to prepare extracts

Plant species	Plant parts used
Nicotiana tobacum	Whole plant
Tagetes minuta	Whole plant
Vinca rosea	Whole plant
Azadirachta indica	Leaves/seeds

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