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FUNGICIDAL ACTIVITY OF CRUDE EXTRACTS FROM SOME INDIGENOUS MEDICINAL PLANTS AGAINST *Aspergillus niger* and *Rhizopus stolonifer* CAUSING WET ROT DISEASE ON TOMATO (*Solanum lycopersicum*) FRUITS

***[1]Tijjani, A., [1,2]Usman, G.U,[3] Gurama, A.U.,[4]Babura, S.R., [1]Aliyu, M. and [1]Adamu, A.D.**

[1]Department of Crop Production, Faculty of Agriculture and Agricultural Technology, Abubakar Tafawa Balewa University PMB 0248, Bauchi, Bauchi State, Nigeria.
[2]Laboratory of Climate-Smart Food Crops Production, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
[3]Department of Agronomy, Federal University Kashere P M B 0182, Kashere, Gombe State, Nigeria.
[4]Department of Biological Sciences, Bayero University, P.M.B. 3011, Kano, Nigeria.
*Correspondence author: tijjaniahmadu@gmail.com

ABSTRACT
The objective of this study was to determine the antifungal effect of crude extracts from Chinese date (Ziziphus jujube) and African ebony (Diospyros mespiliformis) each with four varying concentrations(50g/L, 100g/L, 150g/L and 200 g/L), a standard fungicide (Benomyl 50WP at 2g/L) on tomato inoculated with Aspergillus niger and Rhizopus stolonifer and a control (untreated tomato) which constituted the treatments. The treatments were laid in a Completely Randomized Design (CRD) with three replications. The results obtained showed significant difference (P<0.01) on radial growth and weight loss of tomato when the aqueous plant extracts where used. Ziziphus jujube gave the best reduction for radial growth (1.55) and weight loss (3.00) between the two plant extracts tested and was as good as standard fungicide (Benomyl 50WP) (1.50) at P<0.01. Also from the result obtained, it is evident that the plant extracts possessed antifungal properties which if fully exploited could be used in integrated pest management and post-harvest treatment for tomato before storage and on transit as control measures for Aspergillus niger and Rhizopus stolonifer to reduce the overdependence of farmers on chemical fungicides.
Keywords: antifungal, Aspergillus niger, in vitro, in vivo, plant extracts, post-harvest treatment, Rhizopus stolonifer

INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important and also a popular vegetable crop grown worldwide which has a high consumers acceptance and demand due to its sufficient and well balanced nutrition containing minerals like potassium, zinc, magnesium e.t.c (Osemwegie *et al*., 2010) and rich in vitamins A,B,C and E (John *et al*., 2010; Uke and Chiejina, 2012) which help to minimize the possibility of prostate and breast cancer (Giovannucci,1999). Additionally, tomato fruit contains lycopene (red pigment) an excellent antioxidant that has attracted interest recently because of its activity against radicals that cause aging or muscular degradation, cancer and heart diseases (Uke and Chiejina, 2012). Tomato is the second most highly consumed vegetable crop globally after potato, and it is cultivated in almost every region of the world (FAOSTAT, 2013; Pirondi *et al*., 2017).Tomato is widely grown as an important commercial as well as vegetable crop in the world (Celis *et al*., 2014;Borghesi*et al*., 2016).The world leading producer is China with 50,000,000 million tons, followed by India with 17,500,000 million tons, then United States of America with 13,206,950 million tons, then Turkey with 11,350,000 million tons and Egypt with 8,625,219 million tons in that order (FAO, 2012).

Despite the nutritional values of tomato in human diet, the yield potentials of the crop is affected by the activities of plant pathogens (fungi, bacteria, viruses e.t.c) that often cause the crop failure and have been worldwide (Zebena *et al*., 2014; Sundin *et al*., 2016). Tomato is susceptible to many destructive plant pathogens, especially more prone to postharvest fungal pathogens (Taskeen-Un-Nisa *et al*., 2011).

Postharvest fruit rot diseases caused by fungal pathogens are the major share of crop losses in yield and quality in the field as well as in the storage and transport on a global scale (Saxena, 2016; Sundin *et al*., 2016). Agrios (2005) estimated an overall worldwide loss of 30-40% due to postharvest diseases of tomato.Wet rot disease of the fruit is one of the major postharvest rot diseases caused by *Aspergillus niger* and *Rhizopus stolonifer*. The pathogens may initiate the infection in the green fruit but remain latent or quiescent till ripening of the fruits. During ripening the lesion starts to develop very fast and may cause total ripe fruit decay in the field and during post-harvest stages of storage and transport. The high moisture content, nutrient composition and pH of the ripe fruits support the growth of the fungal pathogens thereby causing rots and fruit contaminations by mycotoxins production (Amadi*et al*., 2014; Choudhury*et al.,* 2017).

Drawbacks associated the with available control methods that affect their potency in disease management necessitate the increased interest in developing further alternative control methods, particularly those that are eco-friendly, biodegradable, feasible to the farmers, non-toxic to human and animals, specific in their action and have a broad spectrum of antimicrobial activity (Abhishek*et al*., 2013). The plants contain natural phytochemicals which could be exploited for use as biopesticides (Satish*et al*., 2007; Pereira *et al*., 2015). For example medicinal plants like *Ziziphus jujube* and *Diospyros mespiliformis* extracts containing a quantum of crucial phytochemicals rich in phenolic compounds, flavonoids, tannins, saponins and alkaloids are now emerging as secure, safer and more compatible way to manage plant pathogens. All parts of these plants including flowers, roots, bark, leaves, stem, seeds and essential oils have antimicrobial qualities and/or properties and are therefore used for medicinal and other purposes (Anwar *et al*., 2007; Dwivedi and Enepsa, 2012). The choice and use of these potential plants for the control of postharvest rots of tomato and other perishables is due to: (1) their safety for human consumption, (2) non environmental pollution. (3) More acceptable to the local farmers because they are indigenous (4) their biological activity and dispersion in harvested tissue (5) our ability to develop formulations that allow the delivery of non-toxic concentrations but at the same time interfere with fungal development (Bhattacharjee and Dey, 2014; Sogvar *et al*., 2016).Plant extracts are the most pressing sources of natural active compounds and can be screened from local traditional plants (Mari *et al*., 2016). These plant compounds have been extracted using different solvents and are shown to acquire antimicrobial, antifungal, antibacterial properties against various pathogens (Bhattacharjee and Dey, 2014).

The objective of the present research work is to evaluate the effectiveness of crude extracts from some indigenous and widely available plants (*Ziziphus jujube* and *Diospyros mespiliformis*) in the management of wet rot disease of tomato caused by *Aspergillus niger* and *Rhizopus stolonifer* under laboratory condition.

MATERIALS AND METHODS

Tomato Fruits

Tomato fruits (UC-82-B) at 80% maturity were purchased from Muda Lawal market In Bauchi town, Bauchi State, Nigeria. The fruits were then surface sterilized with sodium hypochlorite 1 % (v/v) rinsed three times in distilled water, dried on paper towel at 28±2°C and used in the study.

Isolation of the Pathogens from Infected Tomato Samples

Potato Dextrose Agar (PDA, Difco™, Becton, USA) was prepared 39 g/L, autoclaved at 121°C for 15 min and used for the isolation of the fungal pathogens from infected tomato fruits. Tissue fragments (approximately 5 mm ×5 mm) were taken from the margin of the diseased fruits, sterilized with 1% Sodium hypochlorite (NaOCl) for 1 min, washed three times with distilled water and then plated on PDA medium amended with 1mg.ml^{-1} streptomycin to suppress bacterial growth and incubated at 28±2°C under normal fluorescent light for 5 days. The identity of the fungal pathogens was confirmed based on the colony morphology and spore characters. Repeated subcultures were done until pure cultures of *Aspergillus niger* and *Rhizopus stolonifer* were obtained. The pure cultures of *Aspergillus niger* and *Rhizopus stolonifer* were maintained on PDA slants and stored for further studies. All steps were performed in a laminar flow to avoid contaminations.

Pathogenicity Assay

To confirm the ability of isolated pathogens to cause wet rot disease in healthy tomato fruits pathogenicity assay was carried out as a confirmatory test. The tomato fruits surface sterilized with 1% sodium hypochlorite solution were used for the assay. The method of Okigbo and Ikediugwu (2000) was employed, in which holes were made in the tomato fruits with the aid of a flamed 6mm cork borer, and aseptically inoculated with a disc of 7 day-old cultures of *Aspergillus niger* and *Rhizopus stolonifer* isolates separately.

The inoculated fruits were labeled accordingly and incubated at room temperature. They were observed for symptoms of wet rot like colour change, softening, characteristic foul odour, etc. A control experiment was also set up by removing the core in the fruit without introducing any organism in it except replacement with 6mm sterile PDA. Fungal isolates which caused clearly visible wet rot were considered pathogenic compared with the control. The pathogens were re-isolated, compared with original isolates and those with high pathogenicity were used as test organisms for treatment with the plant extracts.

Preparation of Plant Extracts

Extracts were obtained from leaves of Chinese date and African ebony collected during the dry season of 2016 at Chokel, Azare district from Katagum local government area of Bauchi State. This extraction was performed with water as the solvent by dissolving 50, 100, 150 and 200 g of dried leaves of *Ziziphus jujube* and *Diospyros mespiliformis* separately in 1 litre of sterilized distilled water in 2 litre Erienmeyer flasks, vigorously shaken and left for 24 h at 28±2°C. After 24 h, the mixture was filtered with two layered Muslin's cheese cloth. The obtained filtrate was then centrifuge at 6000 rpm for 15 min (Avanti J-26 XPI centrifuge, Beekman Coulter, USA). The supernatant collected was filtered again with a Whatman's No. 1 filter paper (ALBERT^{R}). The crude extracts were filtered with a sterilized 0.22 µm syringe filter (Sartouris® Syringe filters) to remove unwanted material.

***In vitro* Assessment of Plant Extracts**

Antifungal efficacy of the crude extracts from different plants against *Aspergillus niger* and *Rhizopus stolonifer* was done using the poisoned food technique (Choudhury *et al.,* 2017) at four different concentrations: 50g/L, 100g/L, 150g/L and 200 g/L mixed with plant extracts as well as Benomyl 50WP (2g/L) and sterile distilled water as check. The mixture was gently swirled to obtain efficient miscibility of the agar and the extracts. Five-day old fungal cultures of *Aspergillus niger* and *Rhizopus stolonifer* isolates were aseptically punched with a sterile cork borer of 6 mm (0.6 cm) diameter and the fungal discs were placed at the centre of PDA gelled plates. Perpendicular lines were drawn at the bottom of each plate and the point of intersection was taken as the Centre of the plate to ease precise measurement. The plates were incubated at 28±2°C until the control plates were filled. The diameter of the colony was recorded on daily basis by taking the measurement of the 2 paradoxical circumference of the colony growth along the lines drawn at the bottom of each plate from four replicates for each fungus. Radial growth was taken by subtracting the new radial increase from the initial, using a meter ruler and the difference was recorded for analysis.

***In vivo* Assessment of Plant Extracts**

For the *in vivo* bioassay of the plant extracts, the tomato fruits surface sterilized with sodium hypochlorite 1 % (v/v) were used. Cylindricalcores were made with flamed cork borer 60 mm (0.6 cm)in each healthy tomato fruit. One set of fruits were treated first with plant extracts(50g/L, 100g/L, 150g/L and 200 g/L) by dipping method before inoculation (preventive) and another sets of fruits were first inoculated with 0.6 cm mycelial plug of the test fungal pathogens and dipped in plant extracts (50g/L, 100g/L, 150g/L and 200 g/L) (curative). A standard fungicide Benomyl 50WP (Benlate) 2 g/L and a control was used to compare with the treatments. The daily difference in radial growth was obtained as in the in vitro tests. Data were also collected on the weight loss of the tomato fruits at interval of 24 h for 7days. The weight was obtained with the help of top pan electronic balance (Model BP2100, Sartorius, Germany)and the initial weight of tomato prior to treatment was subtracted from the daily weight of the fruits and recorded as weight loss for analysis.

Experimental Design and Statistical Analysis

All experiments (*in vitro* and *in vivo*) were arranged incompletely randomized design (CRD) and data were subjected to analysis of variance (ANOVA) using SPSS software (Version 23.0) and Means with significant difference were separated with Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Pathogenicity studies revealed that the fungal isolates of *Aspergillus niger and Rhizopus stolonifer* tested were pathogenic to inoculated tomato (UC-82-B) considered in this study. After 10 days of inoculation, un-inoculated (control) tomato fruits were symptomless for wet rot while inoculated tomatoes were with wet rot symptoms. Fungal isolates (*Aspergillus niger and Rhizopusstolonifer*) were re-isolated from the infected plants not from the control to confirm Koch's postulates (Koch, 1882; Roberts and Boothroyd, 1972).

The results obtained from Table1 indicated that with increase in concentration of plant extracts, radial growth of *Aspergillus niger and Rhizopusstolonifer* decreases compared with the controls. The effectiveness of *Ziziphus jujube* (0.33) is comparable to standard fungicide (Benomyl 50WP) (0.30).

Although there is no statistical difference (P<0.01) among the concentrations with lowest radial growth (0.33) under *Aspergillusniger*and (2.66) under *Rhizopusstolonifer* compared with the highest radial growth (8.16) observed in the control under *Rhizopusstolonifer*. The inhibitory activity of plant extracts may be due to direct toxic effect on the pathogens (Bhutia*et al.,* 2015; Chowdhury*et al.,* 2017). The antifungal activities of the plant extracts may also be due to the presence of secondary plant metabolites like terpinoides, phenols, flavonoides, alkaloids that was earlier reported by Mohamed and EI-Hadidy (2008). These corroborate with earlier reports indicating the fungicidal properties of natural plant products and their potential to control plant diseases (Tunwari and Nahunnaro, 2014).Also earlier reports that are in consistent with the results of the present study are that of Tijjani*et al.* (2014) and Chowdhury*et al.* (2017) which indicated that with increase in concentration of plant extracts implied an increase in the active ingredients of the crude extracts which act on the test pathogens thereby affecting its physiological processes, lowering the growth of the pathogens.

Table 2 presented the antifungal efficacy of the plant extracts with respect to different concentrations and methods of application as either preventive or curative.It was generally observed that *Ziziphus jujube* leaves extracts applied at 200g/L significantly (P<0.01) reduces radial growth of *Aspergillus niger* (1.71) better than other concentrations under preventive control method and is comparable to standard fungicide (Benomyl 50WP) (1.66)(Table 2).Application of plant extracts at varying concentrations reduced wet rot disease under preventive method of control better than under curative method of control. This is probably as a result of the microbes being killed on exposure to a higher concentrations of these plant extracts when the inoculum was introduced on the treated parts of the tomato (i.e under preventive) which inhibit their ability to establish a nutritional relationship that will subsequently enable the pathogen to get nourishment or nutrient required for its growth and development. This is in agreement with the reports of Tijjani*et al.* (2010) on the use of *Moringaoleifera* and Neem seed extracts to control wet rot disease on irish potato caused by *Rhizopusstolonifer* and the report ofAmienyo*et al.* (2007) on the use of *Z. officinale, Annonamuricata, Gacinia cola, Alchorneacordifolia*and *Allium sativum* to control wet rot on sweet potatoes caused rot fungal pathogens.

The results in table 3 showed the effects of different concentrations of plant extracts and method application on weight loss of tomato infected with *Aspergillusniger* and *Rhizopusstolonifer*. Treatment of tomato fruit with*Ziziphus jujube*leave extracts significantly (P<0.01) reduced weight loss (3.00) better than all concentrationsunder preventive method for both *Aspergillusniger* and *Rhizopusstolonifer*. Increase in the concentrations of the plant leaves extracts correspondingly decreased radial growth of the test pathogens as well asthe weight loss of tomato fruit and this correspond to the works of Ijato *et al.* (2010) on the use of *Azadrachtaindica* and *Chromolacnaadorata* against postharvest and transit rot of tomato and Ebele (2011) on the use of *Carica papaya, C. adorata* and *Acalyphaciliata* on the control of pawpaw fruit rot fungi.

Table 1: Effect of different concentration of plant extracts on radial growth of *Aspergillusniger* and *RhizopusstoloniferIn-vitro*

Treatment	Concentration (g/L)	Radial growth (cm)	
		Aspergillusniger	*Rhizopusstolonifer*
Ziziphus jujube	50	1.33^{cde}	5.00^{b}
	100	0.33^{e}	4.66^{bc}
	150	0.33^{e}	4.66^{bc}
	200	0.33^{e}	2.66^{ed}
Diospyrosmespiliformis	50	2.00^{cd}	5.66^{b}
	100	2.00^{cd}	5.00^{b}
	150	2.00^{cd}	4.66^{bc}
	200	1.00^{cde}	4.66^{bc}
Benomyl 50WP	2	0.30^{e}	0.32^{e}
Control(distilled water)	0	7.66^{a}	8.16^{a}
Level of significance		* *	*
SE±		0.95	1.5

Means with different superscripts in the same Colum are significantly different. * * = Significant at 1%, * = Significant at 0.05%, SE = Standard error

Table 2: *In-vivo* effect of different concentration of plant extracts and method of control on radial growth of *Aspergillusniger*and *Rizopusstolonifer*

Treatment	Concentration (g/L)	Radial growth (cm)			
		Aspergillusniger		*Rhizopusstolonifer*	
		Curative	Preventive	Curative	Preventive
Ziziphus jujube	50	4.33^{bcd}	2.66^{cd}	6.46^{bc}	5.66^{b}
	100	4.00^{bcd}	2.33^{bcd}	6.06^{bc}	4.33^{bcd}
	150	3.33^{cd}	2.33^{bcd}	6.06^{bc}	4.33^{bcd}
	200	2.66^{d}	1.71^{e}	5.13^{c}	$3.\ 73^{cd}$
Diospyrosmespiliformis	50	6.33^{b}	3.33^{bc}	7.00^{b}	5.70^{b}
	100	6.00^{bc}	2.66^{cd}	7.00^{b}	5.46^{bc}
	150	5.66^{bc}	2.66^{cd}	4.66^{bc}	5.10^{c}
	200	4.33^{b}	2.00^{d}	4.66^{bc}	5.03^{c}
Benomyl 50WP	2	2.66^{d}	1.66^{e}	3.00^{d}	2.06^{d}
Control(distilled water)	0	8.00^{a}	8.33^{a}	8.33^{a}	8.00^{a}
Level of Significance	* *	* *	* *	* *	* *
SE±		0.54	0.73	0.7	0.60

Means with different superscripts in the same Colum are significantly different. * * = Significant at 1%, SE = Standard error

Table 3: *In-vivo* effect of different concentration of plant extracts and method of control on weight loss of tomato inoculated with*Aspergillusniger*and *Rizopusstolonifer*

Treatment	Concentration (g/L)	Weight loss (g)			
		Aspergillusniger		*Rhizopusstolonifer*	
		Curative	Preventive	Curative	Preventive
Ziziphus jujube	50	8.90^{ab}	5.66^{b}	8.73^{ab}	7.66^{c}
	100	5.90^{bc}	4.00^{c}	6.46^{b}	5.33^{d}
	150	5.13^{c}	3.33^{cd}	6.06^{b}	4.33^{de}
	200	3.66^{d}	3.00^{d}	5.13^{c}	3.00^{e}
Diospyrosmespiliformis	50	8.30^{ab}	4.33^{c}	5.70^{bc}	8.57^{ab}
	100	6.30^{b}	3.66^{cd}	5.46^{bc}	4.66^{de}
	150	4.60^{cd}	3.33^{cd}	5.10^{c}	4.00^{de}
	200	4.36^{cd}	3.33^{cd}	5.03^{c}	3.00^{e}
Benomyl 50WP	2	2.66^{e}	1.00^{e}	2.06^{d}	2.66^{ef}
Control (distilled water)	0	14.50^{a}	10.66^{a}	9.83^{a}	9.33^{a}
Level of Significance	* *	* *	* *	* *	* *
SE±		0.43	1.50	1.72	0.50

Means with different superscripts in the same Colum are significantly different. * * = Significant at 1%, SE = Standard error

CONCLUSION

Based on the present study, it was discovered that there was significant difference in the potency of the extracts against the test pathogens at the in vitro and in vivo levels compared with the control. Generally, the results show that the botanicals possess antifungal activity and have the potentials for exploitation and utilization as biocontrol agents in the fight against wet rot of tomato. Therefore, due to the fact that chemical control of disease is environmentally hazardous and very expensive, this inexpensive, non-hazardous and biodegrable plant material could be used as an alternative way of reducing and controlling rot disease by farmers to increase tomato production in many developing countries, where tomato is common vegetable crop.

Conflict of interest

The authors declare that they have no conflict of interest.

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