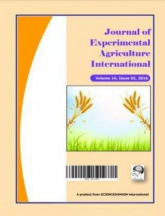

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Evaluation of the Phenol Production Potential in Maize (*Zea mays* L.) in Response to Infection Caused by *Fusariun verticillioides* (Niren.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author ILO conducted all the bench work and the literature search. Author AAS interpreted the statistical analysis and did the final editing of the manuscript. Author AOA did the data analysis and wrote the first draft of the manuscript. Author ACO designed the experiment as well as the overall supervision. All authors read and approved the final manuscript.

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ABSTRACT

This study investigated phenol production in five maize varieties in response to infection caused by *Fusarium verticillioides.* Pure culture of the pathogen was obtained from Plant Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The screen house experiment was laid out in a completely randomized design. Dual inoculation was done where soil was infected separately before planting and seedlings were infected separately two weeks after planting with two volumes (10ml and 20ml) containing 1.4×10^7 spores/ml suspension of *F. verticillioides.* The maize plants were harvested at 4th, 5th and 6th weeks after inoculation and the maize plants were dried at room temperature before determination of phenol content in each of the varieties. Data gathered on the agronomic parameters and phenol contents were subjected to analysis of variance (ANOVA) using SAS 9.1 statistical package.

All the maize varieties recorded more than 70% stalk rot incidence while the severity ranged from 19.01% in variety ART-98-SW1 to 25.21% in ART-98-SW6. ART-98-SW6 showed the most

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($p<0.05$) phenol content (21.28 mg/g). Soil inoculation produced the highest percentage stalk rot severity while seedling inoculation showed significantly higher phenol contents across the period of study. Similarly, disease severity increased with increasing inoculum levels with highest attained using 20 ml (23.99%) while higher phenol content was obtained at inoculum level 10 ml (18.61 mg/g) compared to results using 20 ml (15.63 mg/g) and control (7.88 mg/g). The maize variety; ART-98-SW6 with highest stalk rot severity also produced the highest phenol content. Overall, the rate of phenol production in maize corresponded with the extent of severity *F. verticillioides* infections.

Keywords: Maize varieties; phenol; Fusarium verticillioides; stalk rot incidence and severity.

1. INTRODUCTION

Maize (*Zea mays* L.) is one of the oldest and most productive cereals cultivated across the world [1,2]. The crop plays an important role in the diet of millions of African people due to its high yields per hectare, ease of cultivation and its adaptability to different ecological zones coupled with its versatile food uses and storage characteristics [3,4]. Maize has been well established in the farming system in Nigeria where it is grown as sole crop or as an intercrop with other crops such as root crops, grain legumes, cereals (sorghum, millet, rice) and even vegetables [5]. Despite the importance and wide cultivation of this cereal, its growth and productions are usually impaired by diseases caused by fungi, bacteria, viruses or nematodes which results in considerable yield loss and decreased grain quality [6].

Fungi were ranked as the second most important cause of maize diseases and the major genera commonly encountered on maize in tropical regions are *Fusarium, Aspergillus* and *Penicillium* (Ominski et al. 1994) [7]. *Fusarium* species are considered as the most devastating fungal menace of maize; *Fusarium verticillioides* is the prevalent specie causing root rot, stalk rot and ear rots in maize [8,9]. In addition to severe yield and economic losses in corn and other cereal crops worldwide, the potential occurrence of fumonisins and other mycotoxins in consequence of *F. verticillioides* infection are a matter of concern in current mycotoxicology [10].

In the recent times, the use of fungicides as a chemical control measure commonly employed in the management of diseases caused by fungi has been discouraged due to environmental and food contamination issues [11]. Hence, there is need to embrace a safer and novel biological approach to plant disease management. In order to improve plants' resistance to diseases, effort has been directed at the search for new anti-microbial materials from natural sources, which are mostly low-molecular weight secondary metabolites essential for plant disease resistance [12,13]. Accumulation of certain plant secondary metabolites such as phytoalexins is induced upon pathogen attack. Increased accumulation of phenolic phytoalexins in plants can promote host defense against pathogens [14,13]. More so, phenolic compounds with less complex structures, such as catechol and coumarin, have exhibited bactericidal and fungicidal activities [15]. Hence, the ability of plants to make and release phenols as a defense mechanism against infection by pathogens is of importance in determining resistance [16]. This study investigates production of phenolic compounds by five maize varieties in response to infection caused by *Fusarium verticillioides*

2. MATERIALS AND METHODS

Experimental Site: The experiment was conducted in the screen house of the Department of Botany University of Ibadan, Ibadan, Nigeria.

Sources of materials used: Seeds of maize varieties; SWAM1-SR, BR-9928-DMR-SR, ART-98-SW1, ART-98-SW6, BR-9943-DR-SR were obtained from maize germplasm of the Institute of Agricultural and Research Training (IAR&T), Apata, Ibadan. Pure culture of the characterised *Fusarium verticillioides* isolate was obtained from the Plant Pathology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Multiplication of the inoculum: *Fusarium verticillioides* isolate was sub-cultured into sterile Petri plates of Potato Dextrose Agar (PDA) using an inoculating needle and incubated at room temperature (28±2)ºC for 7 days to produce multiple cultures of the fungus. Each of the cultured plates was flooded with 2ml sterile distilled water. A sterile scalpel was used to

harvest the spores and mycelia growths by scraping them into a sterilized flask. The solution was adjusted with sterile distilled water, stirred and filtered with the use of muslin cloth. The resulting spore suspension was counted using a haemocytometer and adjusted to an inoculum load of 1.4×10^7 spores/ml.

Soil preparation and planting: Prior to the conduct of the experiment, the screenhouse was adequately sanitized. Agricultural soil of between 0 to 15 cm depth was collected from the experimental site of Botany Department, University of Ibadan, Ibadan. The soil was sterilized using electric soil sterilizer at 120ºC for 1 hour, and was transferred to a polythene bag at 5 kg per bag after cooling. Maize seeds were surface sterilized in a beaker containing 1% sodium hypochlorite for 30 seconds after which the seeds were dried in between layers of Whatmann filter paper for another 5 minutes. The seeds were planted at three seed per hole across the varieties.

Experimental Design: The experiment was laid out in a completely randomized design (CRD) in ten replications. Treatments across the five maize varieties consisted of two inoculation methods; soil and seedling inoculations. These were carried out at 0 ml, 10 ml and 20 ml volumes of the standardized (1.4×10^7 spores/ml) spore suspensions. The treatment with soil inoculation was conducted at the period of planting while seedling inoculation was carried out on the respective plots at 2 weeks after planting. The control experiments were treated with respective volumes of sterile distilled water.

Determination of disease incidence and severity: The percentage of disease incidence and severity were determined using the formulas:

Disease incidence (%) = {(number of infected maize plants/number of maize plants) ×100}

Disease severity (%) = {(area of plant tissue affected / total area) ×100}

Extraction of plant samples: Two replicates from each treatment across the varieties were carefully uprooted at the 4^{th}, 5^{th} and 6^{th} week after planting (WAP). Soil adhering to the root of maize plant was removed by agitation in water. The plants were then air dried at room temperature. Two gramme (2 g) of leaf sample per treatment was treated with 10 ml of 80% methanol in a tightly plugged flask. The treated leaf was allowed to stand for 3 minutes, after which the liquid fraction was filtered using muslin cloth. The step was repeated three times with 80% ethanol and the supernatants were collected into volumetric flasks. Final volume of the extracts was made to 50 ml with 80% ethanol and all the extracts from each sample were combined and centrifuged at 2000 revolutions per minutes for 20 minutes in a centrifuge.

Determination of phenol content: This was carried out using the Folin-Ciocalteu method as described by Singh et al. [17] using gallic acid as a standard and the total phenol is expressed as mg/g gallic acid equivalents (GAE). A total of 1 ml extract was mixed with 0.5 ml of Folin-ciocalteau reagent diluted with water in the 1:1 ratio with the aid of a sterile syringe and kept for 3 minutes. After which 2 ml of sodium carbonate (20%) was added, mixed thoroughly for 15 seconds and allowed to stand at 40ºC for 30 minutes after which blue colouration was observed. The tubes containing the supernatant was then placed in boiling water for 1minute and allowed to cool at room temperature. The absorbance was recorded at 765nm against a reagent blank using a spectrophotometer. The amount of total soluble phenol present in the sample was calculated according to Singh et al. [17] as phenol mg/g = Sample OD×Standard OD×Dilution factor.

Data collection and statistical analysis: Data were collected on plant height (cm), leaf number (cm), disease incidence (%) and disease severity (%) weekly for 9 weeks. All the data on growth, disease and phenol contents collected were subjected to ANOVA using SAS 9.1 statistical analysis software and Means were separated by Duncan Multiple Range System at 95% confidence interval.

3. RESULTS

The fitted model for the interactive effect of maize varieties, inoculation methods, inoculums' quantity and duration of experiment produced a significant ($p<0.01$) result on the growth and disease parameters measured. All the factors evaluated were significant with respect to plant height, number of leaves and disease severity, while only inoculum volume and duration of the experiment (WAP) was significant with disease incidence caused by *F. verticillioides* (Table 1).

Maize variety; BR-9928-DMR-SR followed by ART-98-SW6 recorded the most significant

(p<0.05) growth as measured by plant height and number of leaves across the varieties evaluated. This was followed by ART-98- SW1 and BR-9943-DR-SR which also recorded similar level of significance while SWAM 1-SR showed the least growth performances (Table 2).

All the maize varieties recorded more than 70% stalk rot infections with BR-9928-DMR-SR having highest rate of 73.11%. The stalk rot severity ranged from 19.01% in variety ART-98-SW1 to 25.21% in ART-98-SW6 (Fig. 1).

Table 3 shows the effect of different inoculation methods and inoculum volume on the growth and disease occurrence in maize plants. While no significant difference was recorded between the inoculation methods with respect to plant height and number of leaves, soil inoculation method produced a significant higher (p<0.05) disease incidence and severity compared to seedling inoculation which showed no significant difference from the control. Whereas, significant (p<0.05) reduction was recorded in the growth rate with increased inoculum volume. No significant result was obtained in disease incidence with respect to inoculum levels while at 20 ml, there was significant increase in stalk rot severity (Table 3).

There was a significant increase in plant height and leaf number with the increasing weeks after planting (Table 4).

Fig. 2 showed a linear relationship between the disease incidence caused by *F. verticillioides* in maize and the period of experiment. A consistent increase in the disease progression was observed until the 5th week when the infection reached its climax and maintained this position till the 9th week of experiment (Fig. 2).

The r^2 value of 0.8694 reflects the reliability of the result obtained for increasing severity of stalk rot over the period of the experiment. Having received *F. verticillioides* inoculation in the 2nd WAP, disease severity increased consistently from 3rd week (9.95%) to 9th week (36.29%) after planting (Fig. 3).

The model for the reaction of quantity of phenol produced was significant (p<0.01) through the period of experiment. The effect of maize varieties and inoculums volume also recorded significant result at the 4th, 5th and 6th (WAP). The method of inoculation used showed significant effects on phenol levels (p<0.01) at 4th and 5th WAP while it was significant at p<0.05 at 6th WAP (Table 5).

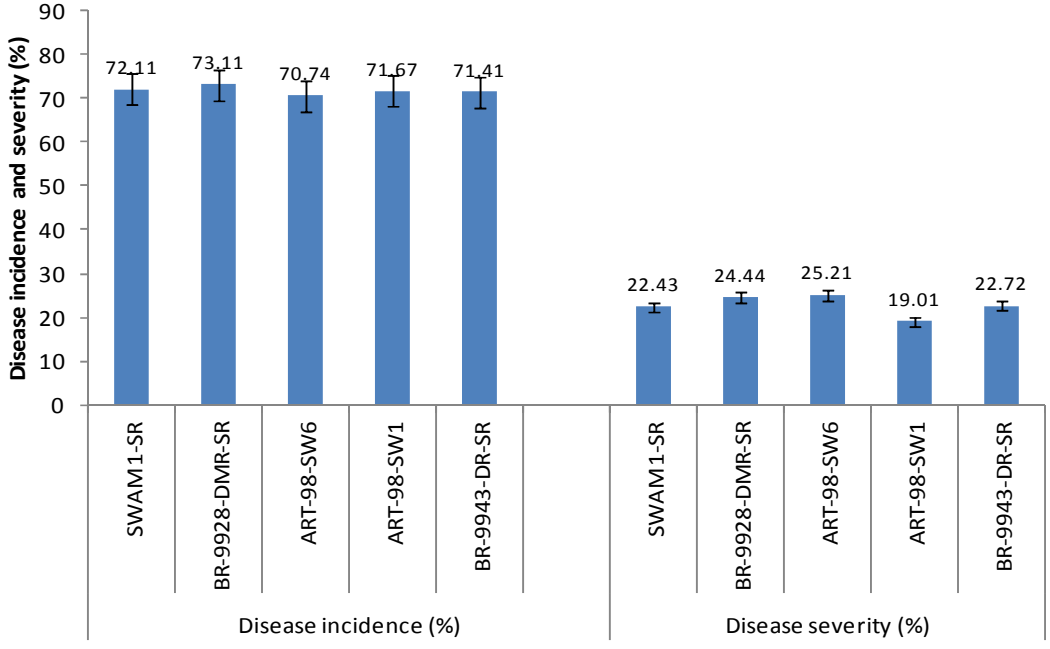

Fig. 1. Disease incidence (DI) and severity (DS) across the maize varieties caused by *F. verticillioides*

Table 1. ANOVA table of growth and disease occurrence in maize plants after inoculations with *Fusarium verticillioides*

Sources of variation	df	Plant height (cm)		Leaf number		Disease incidence		Disease severity	
		F value	P > F	F value	P > F	F value	P > F	F value	P > F
Model	21	162.46	0.0001**	157.48	0.0001**	210.05	0.0001**	72.74	0.0001**
Maize varieties	4	12.48	0.0001**	14.55	0.0001**	0.30	0.8765	8.82	0.0001**
Inoculum volume	2	43.49	0.0001**	39.33	0.0001**	11.00	0.0011**	34.64	0.0161*
Inoculation method	2	16.16	0.0001**	9.44	0.0001**	0.29	0.5882	5.81	0.0001**
Weeks After Planting	8	405.5	0.0001**	395.34	0.0001**	392.13	0.0001**	172.33	0.0001**
Replicates	9	1.56	0.1693	1.05	0.3864			0.42	0.8372
Error	1327								
Corrected Total	1348								

**Significant ** Highly Significant*

Table 2. Mean effect of *F. verticillioides* on plant heights and leaf number across the maize varieties

Varieties	Plant height (cm)	Number of leaves
SWAM 1-SR	43.27^{c}	4.53^{c}
BR-9928-DMR-SR	51.31^{a}	5.47^{a}
ART-98-SW6	54.56^{a}	5.68^{a}
ART-98- SW1	49.52^{b}	5.07^{b}
BR-9943-DR-SR	48.94^{b}	4.97^{b}
R^2	0.72	0.71

Mean with different letters are significantly different (p≤0.05)

Table 3. Mean effect of inoculation method and inoculum volume on growth and disease occurrence in maize plants after 9 weeks of planting

Parameters	Variables	Plant height (cm)	Number of leaves	Disease incidence (%)	Disease severity (%)
Inoculums' volume (ml)	Control	54.11a	5.63a	70.18a	21.51b
	10	48.88b	4.90b	71.78a	22.18ab
	20	41.58c	4.63c	72.65a	23.99a
Inoculation method	Control	52.99a	5.23a	69.56b	20.69b
	Seedling inoculation	46.72b	5.06a	70.18b	21.51b
	Soil inoculation	50.58b	5.17a	74.87a	25.59a
	R^2	0.72	0.71	0.94	0.53

Means with different letters are significantly different (p≤0.05)

Table 4. Mean effect of time (WAP) on the growth of treated maize plants

Weeks After Planting (WAP)	Plant height (cm)	Leaf number
1	2.08i	0.01i
2	11.78h	1.59h
3	24.61g	3.07g
4	39.63f	4.35f
5	54.69e	5.61e
6	66.92d	6.50d
7	75.94c	7.43c
8	82.57b	8.38b
9	87.65a	9.36a
R^2	0.72	0.71

Means with different letters in a column are significantly different

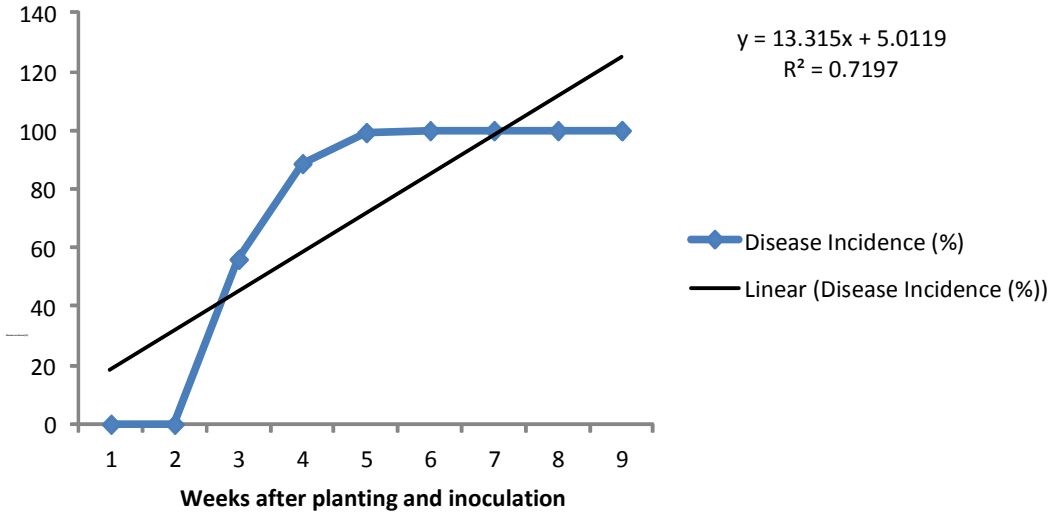

Fig. 2. Effect of time (WAP) on disease incidence caused by *F. verticillioides* in maize plants

Maize variety ART-98-SW6 showed the most significant ($p<0.05$) phenol content across the period of this study. This result was followed by BR-9928-DMR-SR while ART-98-SW1and BR-9943-DMR-SR showed no significant difference (Table 6).

The seedling inoculation method showed significantly higher phenol contents than soil inoculation, although the two methods produced results which were significantly higher than the control. More so, inoculum volume of 10 ml recorded a significantly higher phenol contents than at 20 ml (Table 7).

The inoculation methods were positively and significantly ($p<0.01$) correlated with inoculum volume ($r=0.64$). Also, inoculation method was

significantly associated with phenol production at week 4 (r=0.60), week 5 (r=0.68) and week 6 (r=0.71) after planting. Similarly, inoculum volume was significantly correlated with phenol content obtained at week 4 (r=0.81), week 5 (r=0.90) and week 6 (r=0.89) of the experiment (Table 8).

4. DISCUSSION

Virulence of *F. verticillioides* that ranged from 25.21% in ART-98-SW6 to 19.01% in ART-98-SW1 demonstrated small but significant variation in the severity of systemic infection on the host. While environmental factors also play a major in the activities of this cosmopolitan pathogen [18,19] variation recorded here in the growth rate and resistance of maize varieties to *F. verticillioides* infections is consistent with earlier findings in which host resistance was reported as a major determinant of variation in the activities of the pathogen [20,21].

Similar rate of maize growth observed in both seedling and soil inoculation methods is contrary to the results obtained by Khan et al. [22] in which a significant increase in the growth parameters of plants that received *Alternaria sp.* inocula through different methods was reported. However, higher incidence and severity of stalk rot recorded in the soil inoculation method conforms to earlier claims that attribute varying severities caused by artificial inoculation of *F. verticillioides* to different inoculation methods [23,24]. Furthermore, the effect of increasing inoculum quantity was consistent across both methods of inoculation, as has also been seen in another study [25].

The prevalence of stalk rot incidence observed to reach its peak at 5th week after planting affirmed that fungus colonizes maize stalks systemically without necessarily causing visible disease symptoms and that *F. verticillioides* strains can be vertically transmitted through seed-to-plant transmission and systemic stalk infection [26,18]. Meanwhile, the severity rate of 36.29% at 9th week after inoculation justifies the epidemiological claim that *Fusarium* stalk rot reduces output in maize by 10% typically and by 30–50% in severely affected areas [27].

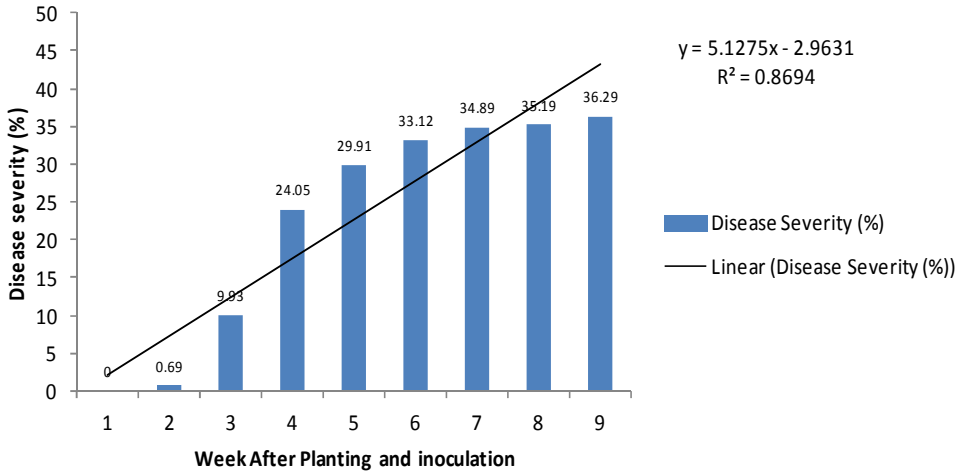

Fig. 3. Effect of time (WAP) on disease severity of *F. verticillioides* in maize plants

Table 5. ANOVA table for Phenol (mg/g) produced by the maize plants after days of infection

Source	df	4th WAP		5th WAP		6th WAP	
		F value	P>F	F value	P>F	F value	P>F
Model	7	90.24	0.0001**	227.49	0.0001**	310.19	0.0001**
Maize varieties	4	49.92	0.0001**	67.34	0.0001**	74.98	0.0001**
Inoculation methods	1	9.59	0.0065**	9.79	0.0061**	4.21	0.0560*
Inoculums volume	1	139.13	0.0001**	306.88	0.0001**	267.68	0.0001**
Error	17						
Corrected Total	24						

***=Highly significant *= Significant*

Table 6. Phenol (mg/g) content of the maize plants 4-6 weeks after infection (average of all 4 treatments?)

Varieties	4th WAP	5th WAP	6th WAP
SWAM1-BR-SR	7.40[d]	11.12[d]	14.04[d]
BR-9928-DMR-SR	13.80[b]	16.92[b]	20.96[b]
ART-98-SW6	16.16[a]	19.04[a]	21.28[a]
ART-98-SW1	11.20[c]	15.04[c]	19.22[c]
BR-9943-DMR-SR	10.52[c]	14.54[c]	18.74[c]
R^2	0.97	0.99	0.99

Means with different letters are significantly different (p≤0.05)

Table 7. Phenol (mg/g) content of the maize plants days after inoculation with different volumes of *Fusarium verticillioides*

Parameter	Variable	4th WAP	5th WAP	6th WAP
Inoculation method	Control	4.72[c]	5.94[c]	7.88[c]
	Seedling	14.32[a]	16.33[a]	17.58[a]
	Soil	12.86[b]	14.03[b]	15.48[b]
	R^2	0.97	0.99	0.99
Inoculum volume	Control	4.97[c]	5.94[c]	7.88[c]
	10 ml	13.37[a]	15.32[a]	18.61[a]
	20 ml	10.81[b]	13.04[b]	15.63[b]
	R^2	0.97	0.99	0.99

Means with different letters are significantly different (p≤0.05)

Table 8. Extent of association between the maize varieties, pathogen inoculation, and time with phenol content

Correlation	Maize varieties	Inoculation method	Inoculums' volume	Phenol contents		
				Week 4	Week 5	Week 6
Maize varieties						
Inoculation method	0.00					
Inoculum volume	0.00	0.64**				
Week 4	-0.13	0.60**	0.81**			
Week 5	-0.11	0.68**	0.90**	0.98**		
Week 6	-0.72	0.71**	0.89**	0.94**	0.99**	

** Significant, **Highly Significant*

The result of maize variety ART-98-SW6, followed by BR-9928-DMR-SR, ART-98-SW1, BR-9943-DMR-SR and SWAM1-BR-SR as the decreasing order of phenol production after inoculated with *F. verticillioides* was in agreement with some earlier reports that both constitutive and/or induced synthesis contribute to abundance and composition of phenolic compounds in cereal grains and this is highly variable depending on the species, variety and environmental conditions [28,29]. The observation of Reddy and Sireesha [30] that nutritional status and concentration of biochemical constituents in plants prior to infection determines the severity of disease possibly explains the reasons maize varieties ART-98-SW6 and BR-9928-DMR-SR with higher stalk rot severities also produced higher phenol contents than other varieties tested. This suggests that spread to more tissues leads to more defense response products, though not effective in blocking disease and further corroborated the reports that plants respond to pathogen invasion through the activation of complex defense strategies such as the accumulation of flavonoids, phytoalexins and phenolic compounds [31,32]. In this work, total phenol produced by the maize varieties acts as biochemical markers to analyse disease incidence and severity which conforms to the

work done by Singh et al. [17] on biochemical response and host-pathogen relationship of stalk rot fungi in early stages of maize (*Zea mays*).

Higher phenol contents produced in seedling inoculation method compare to soil inoculation could be associated with plants' reaction to direct inoculation which possibly resulted into a more effective delivery of *F. verticillioides* inoculums and more exposed tissue. Thus, the possibility of the pathogen inducing phenol production could be substantiated by the report that biochemical resistance, tolerance or susceptibility in plants against any disease depends mainly on preexisting, preformed or induced substances by the pathogen in the host [30]. In this study, the higher phenol production that occurred at lower inoculum volume was consistent with the findings of Perveen et al. [33] who reported a decrease in total phenol of the leaves of *M. arvensis* with increase in initial inoculum of *S. sclerotiorum*, a situation attributed to the altered rates of synthetic activity because of infection by pathogen [34].

The inoculation methods and inoculum volumes used in this study effectively delivered infective stalk rot inoculum dosage, the rate of which were found to be srongly correlated with phenol production in maize plants at 4^{th}, 5^{th} and 6^{th} week after inoculation. This, agreed with the claim that majority of phenolic compounds are a part of the preformed general defense system against potential pathogens [35].

5. CONCLUSION

Conclusively, phenol production in the maize varieties is associated with severity of *F. verticillioides* infections. Close association can be said to exist between stalk rot disease and maize plants' resistance to infection through phenol production. Researchers embarking on artificial infection of plants by *F. verticillioides* may as well bear this in mind as the resultant phenol production may tamper with their results.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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