STUDIES ON ACTIVE CHANGES IN DEFENSE RELATED ENZYMES IN PEPPER RESPONSES TO *HELICOTYLENCHUS MULTICINCTUM* AND *MELOIDOGYNE INCOGNITA* INFECTIONS

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SUMMARY

An experiment was conducted at the screen house of Niger State College of Agriculture, Mokwa to evaluated nine accessions and a variety of pepper *Capsicum* species for changes in activities of phenolic compounds accumulation in relation to resistance or susceptibility to combined parasitism of *Helicotylenchus multicinctum and Meloidogyne* incognita. The trial was laid out using completely randomized design with three replications. Each of the accessions and the variety was inoculated with approximately 2000 mixed population, 1000 each of *H. multicinctum and M. incognita* on the root and adhering soil. Root samples were collected a day before inoculation and at day 7, 14 and 21 post inoculation. Standard methods were employed to quantified phenolic content and tannins. Changes of pepper phenolic contents on total antioxidant capacity of peroxidase, polyphenol oxidase and tannins on their response to *H. multicinctum and M. incognita* infection were recorded. In comparison, extracts from the roots of five pepper accessions viz NGB00581, NGB00587, NGB00629, NGB00684 and NGB00574 had high concentrations of peroxidase, polyphenol oxidase and tannins as compared to the remaining varieties tested, indicating the strong level of their responses to combined *H. multicinctum and M. incognita* attack. Findings from this study suggest the potential of pepper phenolics in reducing nematode infection in pepper production. The result of the present study shows that five of the ten pepper accession/variety possess high quantities of natural antioxidants and can be further investigated for possible use in the management of pepper nematode infections.

Keywords: Accession, Antioxidant, Infection, Phenolic, Nematodes

PLANT-PARASITIC nematodes are important pest of crop. Abundant in nature, they are found in association with virtually all important agricultural crops and constitute a significant constraint to global food production (Gregory *et al*., 2017). Over the years, the relationship between plants and nematodes has led to the evolution of the plant-parasitic nematodes studies. As widely spread pathogens affecting vascular plants, huge losses in crop yields have been accredited to the incidence of plant parasitic nematodes (PPN). The complex relationship between plants and parasitic nematode has resulted in an "evolutionary arms race". Parasitic nematodes have developed strategies to suppress host plant immune responses for the growth of feeding sites. Therefore, plants have evolved specialized molecules to identify pathogens signaling the establishment of immune responses. Researches in alternative nematode control methods are gaining attention, as a result of decline in use of chemical pesticides. The use of nematode-resistant cultivars in crop breeding programs is an efficient nematode management strategy (Gregory *et al.*, 2017)

Pepper plant is composed of significant quantity of phenolic compounds that includes; phenol, tannin and antioxidants (Ogunlade *et al.*, 2012). Varieties of secondary metabolites, a biologically active substance, produced by plants take part in plants defense against insect pests and diseases (Pagare *et al.*, 2015). Phenolic compounds, alkaloids and terpenoids form the major categories of secondary metabolites. Of these three, phenolic compounds are often used to refer to a group of structurally different plant secondary metabolites (Mintel *et al.*, 2017). They have in common an aromatic ring containing single or multiple hydroxyl substituents. Mainly, the phenolic compounds are polyphenols and are classified into five groups: - phenolic acids and simple phenols, flavonoids, phenyl propanoids, quinone and tannins (Mintel *et al.*, 2017). The metabolism and activation of phenolics in plants, in responding to attack or injury by plant pathogens, like nematodes, fungi, bacteria and viruses, have been investigated and documented (Orhi and Pannu, 2010; Kihika *et al.*, 2017; Oliveira *et al.*, 2019). Natural resistance is considered by many when accessible, to be the best alternative for the management of plant- parasitic nematodes, for the reason that it is not only compatible with other management methods but it is cost-effective and eco-friendly (Molinari, 2011). Ravindra *et al.* (2014) reported differential host response of some pepper varieties to *M. incognita*. The objective of the present study was therefore, to evaluate the activity of polyphenol oxidase, peroxidase, Tannin and the phenolic content in pepper roots before and after inoculation with mixed populations of *H. multicinctum*and *M. incognita*

MATERIALS AND METHODS

Description of the Study Location

The experiment was conducted at the screen house of the Niger State College of Agriculture, Mokwa. Geographically, Mokwa is located on Latitude 9.3044° N and Longitude 5.06 6° E of the Equator, situated in the Southern Guinea Savanna agro - ecological zone of Nigeria. The trial was conducted during the 2018 cropping season. Mokwa has a mean annual rainfall of 1200 mm, which normally begins in April and ends in October. The temperature ranges between 35 and 37.5 °C, with relative humidity between 40 and 80% (Anon., 2018).

Sources of planting materials

Nine accessions and a variety of the red pepper seeds (*Capsicum* species) which belong to the *Capsicum frutescens*: sweet pepper (Atarodo) bird pepper (Ata wewe) and *Capsicum frutescens* (cayenne pepper) (Ata sombo) and *Capsicum annum* were used. Nine of the accessions: NGB00574, NGB00581, NGB00586, NGB00587, NGB00624, NGB00629, NGB00631, NGB00684 and NGB00702 were obtained from the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan and the local variety was purchased from Adalinci Agrochemical store, Mokwa, Niger State, Nigeria.

Raising of pepper seedlings in the nursery and transplanting

Nurseries for each of the accession/variety were raised separately in plastic pots of 14 cm 15 cm filled with 3 kg heat-sterilized soil as described by Paiko *et al*. (2019). Two seedlings each of the pepper accession/variety were transplanted at four weeks old into 3 kg plastic pots containing heat sterilized soil, shaded and watered as required.

Preparation of nematode inocula

Helicotylenchus multicinctum was isolated from pepper plants during a survey of plant parasitic nematodes in Niger state, Nigeria and cultured on plantain plants in IITA, Ibadan as described by Speijer and De Waele, 1997), and was used for the nematode treatment. Nematodes were collected from the plantain infected roots in distilled water using White head tray method and applied on the roots and adhering soils. For the *M. incognita,* infected galled roots were collected from pepper during a survey of plant parasitic nematodes in Niger state, Nigeria and cultured on tomato. The galled tomato roots were uprooted, washed, chopped and the egg mass extracted. Second-stage juveniles (J2) were recovered from hatched eggs by incubation in sterile distilled water at 28 ã 1¡C. The suspensions were made up with distilled water until 1000 juveniles/adults nematodes were counted for each.

Inoculation

A week after the establishment of the seedlings, each pot was inoculated with approximately 2000 mixed population, 1000 each of *H. multicinctum* and *M. incognita* nematode. The 20 ml aqueous nematode suspensions were poured into three holes, 2 - 4 cm deep around the roots of the plants. The plant in the control pots with no nematodes received 20 ml distilled water.The trial was laid in Completely Randomized Design (CRD) and replicated 3 times in a screen house with a photoperiod of 12 h and an ambient temperature of 24±4◦C, and irrigation done as required.

Treatments and samples collection

A week, after the establishment of the seedlings, treatment was applied to each pot with inoculation of 2000 mixed nematode population, 1000 each of *H. multicinctum* and *M. incognita*. The 20 ml aqueous nematode suspension (Juveniles/Adults) of the treatment was poured into three holes, 2 - 4 cm deep around the roots of the plant. Similarly, the soil in the control pots (a day before inoculation) received 20 ml of nematode-free water. The holes were created using pencil. Tagged root pieces from each accessions and the variety were removed, chopped, snap-frozen in liquid N_2

and stored at −80°C a day before inoculation or sample at 0 days. The plants were maintained in the screen house for another 7 days. At day 7, day 14 and day 21, one each of the remaining root pieces per plant was collected, chopped, snap-frozen in liquid N_2 and stored at −80°C.

Enzyme extraction and assay

Enzyme extraction and assay were done following the Aguilar *et al.* (2000) method. About 1 ml pre-cold 0.5 M Sodium acetate buffer of pH 5 was added to each 1 g root sample in 2 ml Eppendorf tube, after which 5 mg of polyvinyl pyrolidone was added. The mixture was centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was collected into new tubes and used for polyphenol oxidase (PPO), peroxidase (PO) and tannin enzymes assay.

Peroxidase (PO) Assay

The assay of Peroxidase was done by adding 0.67 ml of enzyme extract to 1 ml of reaction substrate containing 80 ml of 0.1M Sodium phosphate buffer of pH 6, 1 ml of 1mM H_2O_2 and20 ml guaiacol and incubated at 25°C till used. Spectrophotometer was used to record changes in absorbance at 470 nm at intervals of 3s per minute. Blank was prepared from reaction substrate without enzymes extract. The activity was expressed as changes in absorbance at 410 nm expressed as changes in the absorbance unit g^{-1} tissue according to the formula described by Kokkinakis and Brook (1979) as below:

$$\text{Unit g} - 1 \text{ tissue} = \frac{\text{optical density} \times \text{dilution factor}}{\text{g of tissue used in the assay}} \times 100$$

Polyphenol oxidase (PPO) activity

The activity of polyphenol oxidase was determined by observation in colour change intensity of pyrrol oxidation products. The reaction mixture consisted of a 100 âl of the enzyme extract of each sample added to 1.5 ml of 0.2 M sodium acetate buffer at pH 5 and temperature of 4°C, modified by replacing pyrogallol with catechol at 200 âl of 0.02 M. The activity was expressed as changes in absorbance at 410 nm expressed as changes in the absorbance unit g^{-1} tissue according to the formula described by Kokkinakis and Brook (1979) as below:

$$\text{Unit g} - 1 \text{ tissue} = \frac{\text{optical density} \times \text{dilution factor}}{\text{g of tissue used in the assay}} \times 100$$

Tannins Activity

The concentration of tannin was determined by adding 2 ml of the aqueous pepper extract to 2 ml of distilled water, and 2 drops of diluted ferric chloride solution was thereafter added. A blue-green or dark green coloration showed the presence of tannins

RESULTS

Qualitative phenol screening of pepper plants infected with H. multicinctum and M. incognita

The qualitative phenolic screening of the pepper root extracts studied (Table 1) revealed that Peroxidase (PO) at a day before inoculation, tested positive (showed presence of the enzymes) in only four (NGB00574, NGB00624, NGB0062 and NGB00684) of the nine accessions and a varieties evaluated, while at day 7, all tested negative (absence of the enzymes). However, at day 14 and 21, all tested positive (showed presence of the enzymes. Similarly, Polyphenol Oxidase (PPO) in the nine accessions and a varieties analyzed tested negative (absence of the enzymes) at both a day before, 7 and 14, except for NGB00631 that tested positive at the 14th day; they were all tested positive (showed presence of the enzymes) at day 21 in five of the nine accessions and a variety (NGB00586, NGB00624, NGB00629, NGB00631 an LV (Table 1)

However, qualitative phenolic screening showed that (Table 1) Tannins at a day before inoculation all tested negative (absence of the enzymes) with the exception of NGB00587, NGB00631 and NGB00684 and LV that were positive. At day 7, 14 and 21, the pepper root extracts tested positive (showed presence of the enzymes) for all the accessions and a variety (day 21).

Table 1: Phytochemical test of root extracts of nine accessions and a variety of Pepper inoculated with *H. multicinctum and M. incognita*

Pepper varieties	A day before inoculation	Peroxidase (PO)			0 Day	Polyphenol Oxidase (PPO)			0 Day	Tannins		
		Day 7	Day 14	Day 21		Day 7	Day 14	Day 21		Day 7	Day 14	Day 21
NGB00574	+	-	+	+	-	-	-	-	-	+	+	+
NGB00581	-	-	+	+	-	-	-	-	-	+	+	+
NGB00586	-	-	+	+	-	-	-	+	-	+	+	+
NGB00587	-	-	+	+	-	-	-	-	+	+	+	+
NGB00624	+	-	+	+	-	-	-	+	-	+	+	+
NGB00629	+	-	+	+	-	-	-	+	-	+	+	+
NGB00631	-	-	+	+	-	-	+	-	+	+	+	+
NGB00684	+	-	+	+	-	-	-	+	+	+	+	+
NGB00702	-	-	+	+	-	-	-	-	-	+	+	+
LV	-	-	+	+	-	-	-	+	+	+	+	+

Effect of mixed population of *H. multicinctum and M. incognita on* the activities of antioxidant enzymes (Tannins) in infected Pepper roots

The activities of Tannins in the infected roots of *Capsicum species* by *H. multicinctum* and *M. incognita combined* were evaluated, as they take part in defense responses of plants to nematodes infection. The result of the present study revealed that at a day before inoculation) the activities of Tannins in the pepper roots were almost the same (Figure 1) except for NGB00631 that exhibited lower enzymatic activities. At day-7, however, accession NGB00702 had higher enzyme activities followed closely by LV, NGB00574, and NGB00624 compared to others that were almost the same. At day-14; higher enzyme activities were recorded in LV and NGB00586 as compared to others that were almost the same. At day-21, however, Tannin activity declined in all the accessions/ variety except in LV, NGB00702, NGB00624 and NGB00574 that had remarkable increase.

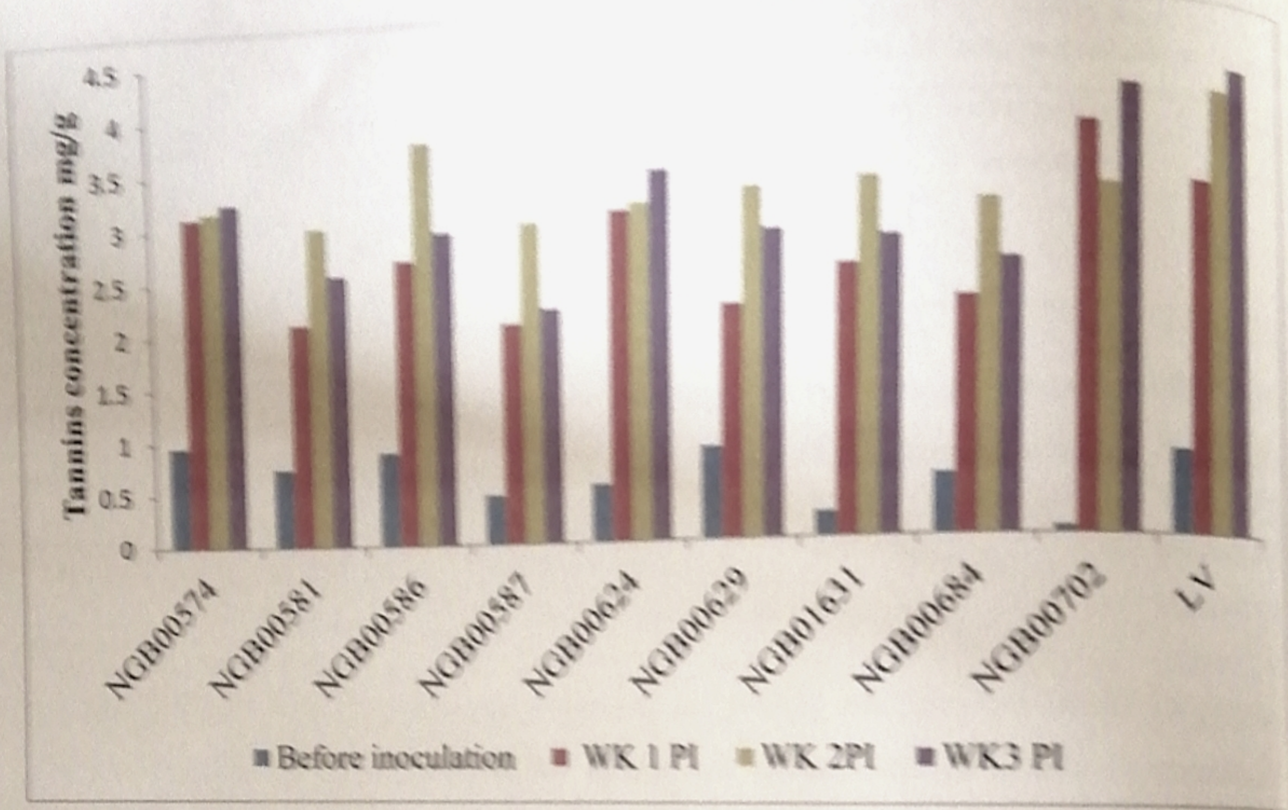

Figure 1: Tannins concentration from Pepper roots challenged with 2000 mixed population, 1000 each of *H. multicinctum and M. incognita*

Effect of mixed population of *H. multicinctum and M. incognita* on the activities of antioxidant enzymes (Polyphenol oxidase) in infected Pepper roots

The analysis of the activities of polyphenol oxidase in the infected roots of *Capsicum species* by mixed population of *H. multicinctum and M. incognita* revealed their presence and participation in pepper responses to infection. The results from this investigation showed increase in the activities of polyphenol oxidase of the pepper Accessions and a variety at a day before inoculation were almost similar, except for NGB00684 that showed higher enzymatic activities. However, day 7, 14 and 21 post inoculation, NGB00684 increased with increase in the number of days followed by NGB00624, while NGB00587 recorded the least enzymatic activities.

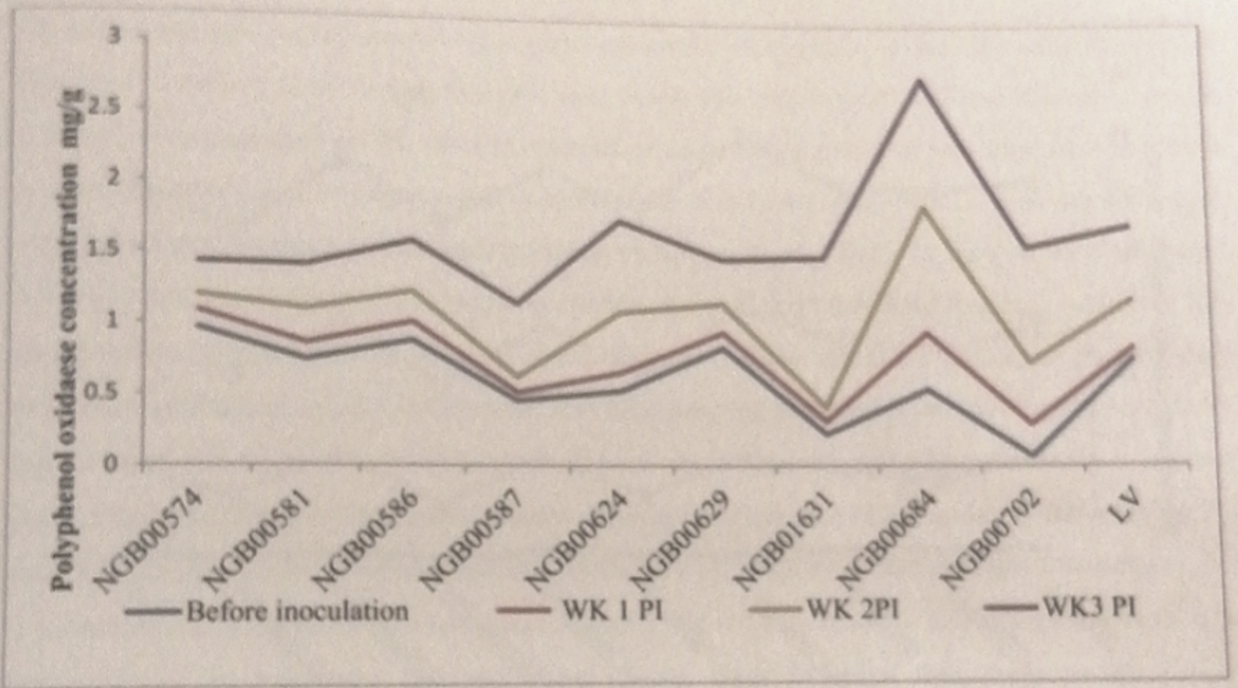

Figure 2: Polyphenol oxidase concentration from pepper roots challenged with 2000 mixed population, 1000 each of *H. multicinctum and M. incognita*

Effect of mixed population of *H. multicinctum and Meloidogyne* on the activities of antioxidant enzymes (Peroxidase) in infected Pepper roots

The concentration of peroxidase in the infected roots of Capsicum species by *H. multicinctum and M. incognita combined* were analysed. The results revealed that at a day before inoculation, the enzymatic concentration of the pepper accessions/variety were similar all through. However, at day 7, 14 and 21, NGB00587, NGB00629 and NGB00684 had higher enzymatic activities in defense responses of the plants against the nematode pathogens while the activities in other varieties were almost similar.

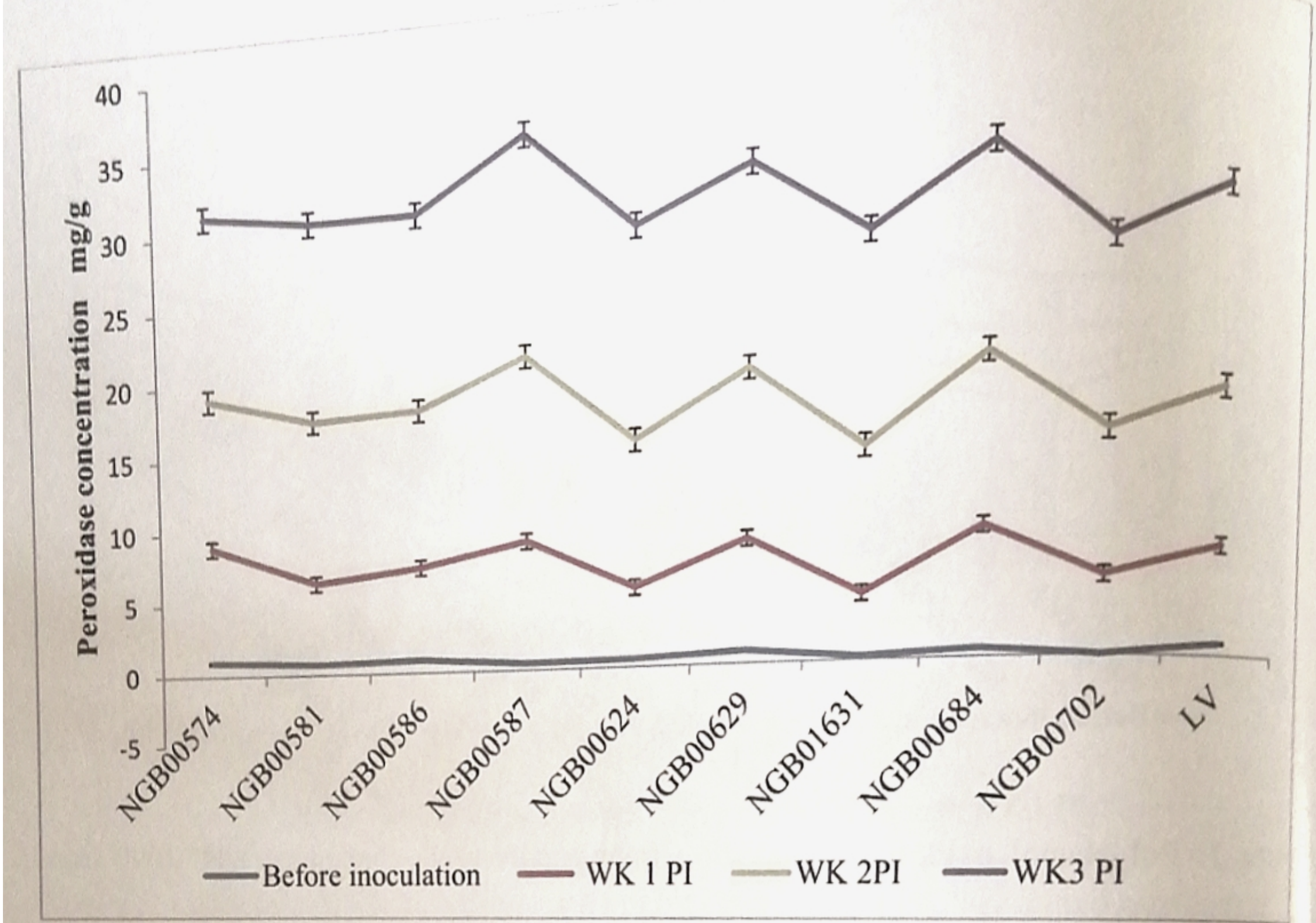

Figure 3: Peroxidase concentration from Pepper roots challenged with 2000 mixed populat[illegible]n, 1000 each of *H. multicinctum and M. incognita*

DISCUSSION

The involvement of widely distributed and natural occurring substances in plant resistance agai[illegible]st pathogen attack is a well-established phenomenon (Materu *et al*., 1995). The result of the prese[illegible]-day investigation evidently demonstrated that *H. multicinctum* and *M. incognita nematod*[illegible] infection of the pepper roots induced fast accumulation of phenolic compounds in the resistan[illegible] peppers compared to the susceptible ones that responded slowly to the compounds accumulation. The accumulation of phenolics at the pathogen infection sites is a common attribute of plant defense response to pathogenesis, which results in rapid cell death, retarding the development and penetration of the pathogen (Vaganan *et al*., 2014). Most of the phenolics compounds are found in plants in conjugated ester form with a sugar molecule attachment in one or two hydroxyls of phenolics. Looking from nematodes infection point, the wall-bound conjugated ester phenolic acids are very important as the esterification of phenols to the cell wall material. As a common biochemical process of resistance and accumulation of polymerized phenols, it is also known to occur as a rapid response to pathogen infection (Vaganan *et al*., 2014). In view of the findings of this study and reports from previous investigation, it could therefore hold that high activities and accumulation of phenolics in some of the accessions/variety evaluated constitute part of their resistance mechanism to *H. multicinctum* and *Meloidogyne* nematodes attack. Earlier reports by Aguilar *et al*. (2000) observed difference in PO activity in banana against Fusarium wilt. In another study, Bajaj and Bhatti (1984) found tomato cultivar infected with *M incognita* to show higher PO and PPO activity in resistance cultivar than in the susceptible). In nematode-plant interactions, PO is considered to be involved in resistance response as with increase in activity after infection to greater levels in resistance cultivars. The results seem to show that the resistant pepper accessions/variety responded well to the nematode infection through strong synthesis and deposition of phenolics. Induced phenolics and lignin accumulation in infected plants roots is established as one of the numerous plant defense responses against pathogens including plant parasitic nematodes (Collingborn *et al*., 2000). The remarkable increase of phenolic metabolites in wall bound fraction from roots of the resistance pepper plants upon the nematode's infection explains the role played by the metabolites in resistance to nematodes infection.

The existence of remarkable quantity of phenolics compounds in the roots of some of the evaluated pepper accessions and a variety in this study showed that, these five: NGB00581, NGB00587, NGB00629, NGB00684 and NGB00574 upon *H. multicinctum* and *M. incognita* infection, exhibited a metabolic initiatives and chemical defenses to pepper to provide resistance by biochemical means to infection by nematodes. Similarly, lower activities of phenolics recorded in the remaining accessions/variety, signifies their level of susceptibility to nematodes infection.

CONCLUSION

The present study has demonstrated that, of the investigated nine pepper accession/variety, five accessions - NGB00581, NGB00587, NGB00629, NGB00684 and NGB00574-possessed high content of phenolic compounds and they can be exploited for their use as bio nematicides in the management of mixed infections of *H. multicinctum* and *M. incognita;* and other nematodes by pepper producers. Response of these accessions and a variety may have been influenced by their genetic make-up and thus exhibited appreciable level of immunity and tolerance to the nematodes attack.

Monitoring and management of these nematodes therefore is fundamental to sustainable pepper production. Seeds of these five accessions should be subjected to further investigation by breeders using appropriate biotechnological tools. The result will enhance the development of cultivars with multiple resistances to economically important plan parasitic nematodes, which can be made available to poor resource farmers to enhance pepper productivity.

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