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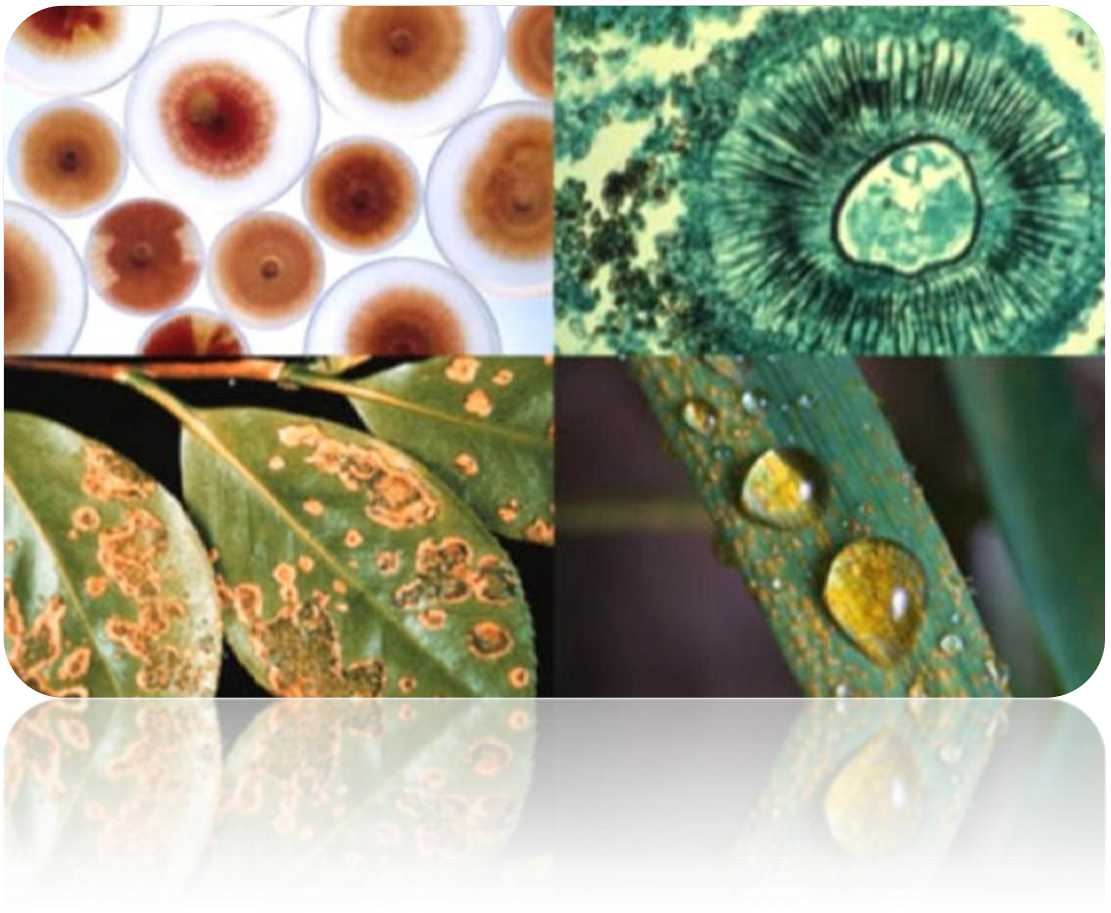

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EFFECTS OF FUNGAL DETERIORATION ON LIPID CONTENT OF SESAME SEEDS (*SESANUM* INDICUM L.)

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A B S T R A C T

Fungi associated with diseased seeds of *Sesamum indicum* L. from four markets in Jos and Okene central market were isolated to study the deteriorative changes in lipid content. In terms of number and fungal species abundance, Faringada market had the highest fungal occurrence, while *Aspergillus chevaliari* showed the highest percentage occurrence in that market. Visually healthy seeds of *Sesanum indicum* were inoculated with spores of each of the nine fungi isolated from diseased seeds and incubated at 25±2^{0}C for 7days. The healthy and fungal infected seeds were analysed for their lipid content. Significant decreases in lipid content were observed in the seeds inoculated with all the fungi except in seeds inoculated with *G. candidum*, which showed increase in lipid content. *A. chevalieri* was responsible for the maximum depletion of the lipid content of the seeds. The results clearly indicate that these fungal species are capable of depleting the lipid content in storage.

Keywords: *Sesamum indicum* L., lipids, sesame seeds.

INTRODUCTION

Sesanum indicum (family *Pedialiaceae*) is a high value ancient oil seed which is considered to be the oldest oil seed crop known to man for over 5000 years (Bedigian, 2012).Presently China, India and Myanmar are the leading producers of Sesame followed by Sudan, Nigeria, Pakistan, Bangladesh, Ethiopia, Thailand, Turkey and Mexico (F.A.O., 2004). Major producing areas in Nigeria are Benue, Gombe, Jigawa, Kano, Katsina, Kogi, Nassarawa, Plateau and Gombe states.

It is commonly called Beniseed in English, Ridi (Hausa), Ishwa (Tiv), Eeku (Yoruba), Igorigo (Ebira). The seeds are small, about 3 to 4mm long by 2mm inch and 1mm thick. They are ovate in shape, slightly flattered and usually range in colour from white, brown to black). Sesame seeds contain 40-60% oil content with a good stability due to the presence of antioxidants (F.A.O., 2010).The seeds are used extensively in manufacturing sesame oil used for cooking, perfumed oils and medicine purposes (Bedigian, 2010). They can be used to produce flour for baking and preparation of sweets and confectionaries such as cakes (Frederick, 2004). They are a rich source of protein, carbohydrate and nutrients like calcium and phosphorus and forms a valuable and nutritious feeds for mulch cattle (Tjai, 2006).

Although sesame is extensively used for numerous purposes, the crop has very low yielding capacity as compared to other plants due to various factors especially disease susceptibility (Ashri, 1998). Numerous deteriorative microorganisms (fungi) have constituted a problem to the production and storage of the seeds (Mbah and Akueshi, 2009). Previous works on sesame seeds have indicated the presence of A. *flavus* among other fungi (Mbah and Akueshi, 2001). These organisms on the seeds affects their palatability and germinability, thereby predisposing the seeds to other pathogens (McDonald, 1999). Fungi growing on stored grains reduce not only germination rate but also carbohydrate, protein, total oil content, increase moisture content and enhancing other biochemical changes (Chavan, 2011). Considering the above facts, emphasis is given on to study the mycoflora of the seeds in this environment and the effect of growth of fungi on the lipid content of the seeds.

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MATERIALS AND METHODS

Seeds of *Sesamum indicum* used in this study were obtained from Faringada, Terminus, Chobe, Rukuba Road Markets Jos and Okene central markets, Nigeria. The samples were preserved in plastic bags and stored in refrigerator until required.

The standard blotter and agar methods were used for the detection and isolation of fungi (Association, 1996). The seeds were surface sterilized with 1% Sodium hypochlorite solution and rinsed in 3 changes of sterile distilled water to remove surface contaminants. One hundred and fifty seeds were used for each location for the blotter method, the sterilized seeds were plated out on moistened filter paper (Whatman No.1) in Petri dishes at the rate of 10 seeds per plate. The plates were incubated at 25 ± 2^0c for 7 days. During the period, the incubated seeds were examined daily for evidence of fungal growth. At the end of the incubation period, the number of infested seeds were recorded as percentage incidence.

The following formula was used to record the percentage incidence.

$$\text{Incidence (\%)} = \frac{\text{No. of infected seeds}}{\text{Total no. of seeds}} \times 100$$

Results obtained were the mean for five replicates. Sampling from each location was done three times. The fungal colonies observed on the filter paper in the Petri dishes were transferred into sterilized Petri dishes containing freshly prepared Potato Dextrose Agar (PDA) to obtain pure cultures. The cultures were incubated for 7 days at 25 ± 2^0C, after which they were examined for fungal growth and pure cultures obtained for identification. Based on the morphological characteristics of vegetative hypae and spores, the fungal flora was identified after the reference of (Ellis and Ellis, 1987).

Fifty milliliters of sterile distilled water were aseptically poured into each of the pure culture plates of the fungi. A sterile glass road was used to dislodge the spores from the mycelia in the culture plates. The spore load for inoculation of the seeds was calculated with the aid of a haemocytometer. One milliliter of each inoculum (containing $2x10^6$ spores were used to inoculate potato dextrose agar) in Petri dishes. Five (5) apparently healthy surface disinfected seeds were introduced into the potato dextrose agar plates containing the spores of fungi. The plates were incubated at 25 ± 2^0C for 7 days. A re-isolation of the fungi was made from the sesame seeds. The similarities observed in the previous infested seeds and the fungi isolated were compared to that produced in the new experiment. This proved the pathogenicity of the fungi isolated.

Lipid Content Determination: This was estimated by the Standard Soxhlet method given by (A.O.A.C., 1980). The Lipid present in the seed was extracted in petroleum ether in Soxhlet extraction apparatus. Two (2) g of each sample (infested seeds and uninfected seeds) were placed separately in whatman filter paper (No.1) in a thimble, the mouth of the thimble was plugged with free absorbent cotton wool. Solvent was added in a dry250ml receiver flask from the Soxhlet assembly, just to reach the level of the neck. The thimble with sample was introduced into the Soxhlet. The apparatus was placed in a heating mantle with temperature controlling device. The extraction was carried out for eight (8) hours at 60 ^{0}C. When the extraction was over, thimble was removed from Soxhlet from the receiver flask. About 250ml solvent along with the extracted lipid was left in the receiver flask, the receiver flask was disconnected. The solvent was then transferred in a clean, previously weighed beaker. After drying in a hot air oven at 95 ^{0}C, it was then cooled in a dessicator and weighed. The amount of lipid was measured form extracted per 2g of the sample and amount of lipid as percent of dry matter (DM) was calculated. The above procedures were repeated for sesame seeds infested with each of the nine fungi and non-infested seeds which had previously been surface sterilized served as the control. The results obtained were the mean for 3 replicates. The lipid content of the infested and sterilized seeds were recorded in terms of mean value with Standard deviation.

RESULTS AND DISCUSSION

The results showed that nine (9) fungal species were associated with the diseased sesame seeds (Table 1). The results of this study showed that fungi were isolated from sesame seeds, indicating that the seed samples were highly infected with pathogens and could cause diseases in seeds. The presence of fungi on sesame seeds is in agreement with (Christensen and Kaufmann, 1974), who stated that fungi were the major cause of spoilage in stored grains and seeds. Species of *Aspergillus, Penicillium* and *Rhizopus* are reported to reduce the germination of seeds and damage the seeds in storage.

On a general note, Faringada area market had the highest percentage fungal occurrence of 44.3% (Table 2).

Table 1. Frequency of occurrence of fungi from each location.

Fungi Isolated	Location				
	A	B	C	D	E
Alternaria alternate	1.5	1.5	1.5	1.5	1.0
Aspergillus chevalieri	2.0	1.5	1.5	1.0	1.0
Aspergillus niger	1.0	2.0	1.5	1.5	1.5
Aspergillus oryzae	1.5	2.0	1.0	1.0	1.0
Aspergillus flavus	2.0	1.5	2.0	1.0	1.0
Aspergillus terreus	2.0	2.0	1.5	2.0	1.0
Cochliobolus Spp.	2.0	1.0	1.0	1.0	1.0
Geotrichum candidum	1.0	1.0	1.0	1.0	2.0
Phoma Spp.	2.0	1.5	1.0	1.0	1.0

Each value is a mean of three samples. A=Samples from Faringada; B=Samples from Terminus; C=Samples from Chobe; D=Samples from Rukuba Rd; E=Samples from Okene.

Scores based on a scale in which 1= absence of fungus and 2= presence of fungus; therefore any means score above 1 indicates presence of fungi (Ataga and Akueshi, 1986).

Table 2. Percentage incidence of fungi isolated from *Sesanum indicum.*

Fungi incidence	Location					
	A	B	C	D	E	Incidence (%)
Alternaria alternate	6.6	3.6	3.2	2.0	0.0	15.4
Aspergillus chevalieri	10.3	4.2	5.0	0.0	0.0	19.5
Aspergillus niger	0.0	6.1	7.4	2.6	5.2	21.3
Aspergillus oryzae	3.3	4.0	0.0	0.0	0.0	7.3
Aspergillus flavus	3.9	3.1	2.7	0.0	0.0	9.7
Aspergillus terreus	4.8	11.2	2.4	13.0	0.0	31.4
Cochliobolus Spp.	9.5	0.0	0.0	0.0	0.0	9.5
Geotrichum candidum	0.0	0.0	0.0	0.0	3.5	3.5
Phoma Spp.	5.9	2.0	0.0	0.0	0.0	7.9
Incidence/Location (%)	44.3	34.2	20.7	17.6	8.7	-

Each value is a mean of three samples. A=Samples from Faringada;B=Samples from Terminus;C=Samples from Chobe; D=Samples from Rukuba Rd; E=Samples from Okene.

From the result, *Aspergillus niger* had the highest percentage occurrence of 10.3% from the Faringada samples (Table 2). *A. niger* and *G. candidum* were isolated from samples from Okene market but absent from Faringada samples. All other isolated fungi were present in Faringada. Amongst the fungi isolated were *A. terreus, A. niger* and *A. oryzae*, which spoil seeds. These fungi were similarly isolated from groundnut, soybean, sesame and sunflower seeds (Chavan, 2011).

On the other hand, these fungi are known to produce mycotoxins which are harmful for human health. Fungi belonging to the genus *Aspergillus* commonly invade oil-rich seeds and grains, such as peanuts, corn and cottonseed, in which they produce the carcinogenic aflatoxins (Ghafoor and Khan, 1976). From the result, 8 of the 9 fungi caused a decrease in the lipid content of sesame seeds which showed that these fungi were associated with the deterioration of sesame seeds and cause changes in the lipid content of the seeds. (Table 3).This is in agreement with (Kakde and Chavan, 2011), who found that storage fungi were responsible for the decrease in fat content of oil seeds, as the fungi secrete enzymes necessary to degrade the lipid content of seeds.

The values obtained from changes in lipid content of sesame seeds in this work by *A. niger* (45.0%); *A. flavus* (48.70%); *A. oryzae* (43.50%) and *A. terreus* (45.45%) were within the range of other findings on the changes in oilseeds by *Aspergillus* Spp. (Chavan, 2011) reported 47.0%, 42.3%, 43.3% and 44.0% lipid content in sesame seeds infested with *A. niger, A. flavus, A. oryzae* and *A. terreus* respectively. However, the value for the control (50.0%) was higher than the 49.35% obtained for this work.

Table 3. Changes (%) in lipid content of *Sesanum indicum* due to fungi.

Fungi	Lipid content (%)
Aspergillus chevalieri	42.40
Aspergillus oryzae	43.50
Aspergillus niger	45.00
Aspergillus terreus	45.45
Alternaria alternata	47.80
Cochliobolus Spp	48.10
Aspergillus flavus	48.70
Phoma Spp.	49.15
Geotrichum candidum	49.40
Sum	419.5
Mean	46.62
Variance	5.95
S/deviation	±2.44

Values are expressed as mean± standard deviation (% mean ±SD)

The statistical analysis of the overall mean gave the upper and lower boundaries of 49.05 and 44.17 (95% confidence limit) as acceptable limit. A comparison of individual means shows that the lipid content of samples infested with *A. niger, A. terreus, A. flavus, A. alternata* and *Cochliobolus* with means of 45.0, 45.45, 47.80, 48.10, 48.70% respectively fall within confidence limit). The effect of infestation of the seeds by these fungi was not significantly different in terms of the lipid content. *A. chevalieri* was responsible for maximum depletion of the lipid content (6.95%) lower than the values obtained from the control.

The differences in lipid content of the fungal infected seeds could be mainly due to the influence of their pathways to use the lipid as energy source. This is due to fact that fungi utilize basic compounds of the seeds for their metabolism and growth. Thus there is a need to prevent fungal growth by employing various management techniques to ensure improvement of seed health which ultimately increase crop quality and human health.

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DISEASES OF PLANTATION FORESTRY TREES IN SOUTHERN GHANA

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A B S T R A C T

Ghana is on the verge of exhausting its timber resources and has embarked on reforestation. However, the establishment of pure stands of high value indigenous tree species such as Odum (*Milicia excelsa* and *M. regia*), Mahogany (*Khaya* and *Entandrophragma* species) and Kokrodua (*Pericopsis elata*) were not successful as a result of insect pest and disease problems. These failures, together with the need to establish plantations partly account for the widespread planting of teak (*Tectona grandis*), cedrela (*Cedrela odorata*) and other introduced tree species. Disease outbreaks in *Tectona grandis*, *Cedrela odorata* and *Ceiba pentandra* have been observed with the expansion of plantation estates. A study was therefore conducted to identify key diseases occurring in tree plantations, determine their distribution and evaluate management strategies to contain them. Diseases encountered include root rot caused by *Armillaria hemii* on *T. grandis* and *Cedrela odorata*, leaf spot and dieback on *Ceiba pentandra* seedlings and saplings caused by *Colletotrichum capsici, Fusarium solani* and *Lasiodiplodia theobromae*, stem canker on *Eucalyptus* spp. caused by *Botryosphaeria* sp. and leaf blight caused by a *Calonectria* species. Management of these diseases will require training of foresters on tree health issues, quarantine and silvicultural practices and initiation of sound breeding and selection programmes.

Keywords: *Armillaria hemii, Botryosphaeria sp., Lasiodiplodia theobromae,* indigenous and introduced trees, pure stands.

INTRODUCTION

Ghana has one of the highest rates of deforestation in West Africa (Benhin and Barbier, 2001). Between 1990 and 2000, the average estimated annual rate of deforestation was high (2%), covering approximately 135,000 hectares (FAO, 2010a). It was estimated by Ghana's Ministry of Environment and Science (2002) that less than 1% of the forest cover was found outside forest reserves, much of it being in small scattered patches in swamps and sacred groves. This prompted the Government to initiate a National Forest Plantation Development Programme which resulted in the establishment of 120,000 hectares of forest plantations by the end of 2007, to supplement timber from the natural forest and sustain its supply for domestic and international markets (Forest Commission, 2007). These plantations are predominantly made up of non-native species such as *Tectona grandis*, *Cedrela odorata*, *Gmelina arborea*, *Pinus caribaea* and *Eucalyptus* spp. with *T. grandis* alone constituting well over 70%. Native tree species included in the Plantation project were Mansonia *altissima*, *Terminalia superba*, *Terminalia ivorensis*, *Ceiba pentandra, Heritiera utilis*, *Entandrophragma angolense* and *Triplochiton scleroxylon* (Foli *et al*., 2009).

Establishment of many of the species was on a very small scale and limited to a narrow range of habitats and conditions. This reduced the chances of pest and disease outbreaks on these trees. However, with the establishment of pure stands over increasingly large areas the threat of disease problems has increased.

Some diseases hitherto unknown in Ghana have thus been observed recent times. The establishment of *Milicia excelsa* (Odum), for example, has been hindered by the attack of *Phytolyma lata*, which predisposes the plant to dieback (Wagner *et al*., 2008). Besides, *Lasiodiplodia theobromae* and

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Fusarium solani have been associated with dieback and decline of *M. excelsa* seedlings (Apetorgbor *et al*., 2003b). Attempts made in raising seedlings of *Ceiba pentandra* in nurseries and establishment in plantations have been hampered by leaf spot resulting in dieback. *Colletotrichum capsici* was isolated from the leaf spot while *Fusarium solani* and *Lasiodiplodia theobromae* were found associated with dieback of stems in both the nursery and field (Apetorgbor *et al*., 2003a). The establishment of *Khaya senegalensis* has been abandoned because of root rot by *Pseudophaeolus baudonii* which caused serious problems in mahogany plantations (Ofosu-Asiedu, 1988). *Pseudophaeolus baudonii* also caused appreciable losses to plantations of *Senna siamea*, *Eucalyptus citriodora*, *E. dagambae, Pinus caribaea* and *Tectona grandis* (Ofosu-Asiedu, 1988). During disease surveys of *Eucalyptus grandis*, *E. camaldulensis* and *E. dagambae* plantations in the Ashanti, Central and Eastern Regions of Ghana, stem cankers on mature *E. dagambae,* which resemble that caused by species of *Chrysoporthe* were observed in a single compartment near Kumasi (Roux and Apetorgbor, 2009).

Among the plantations established, disease problems started emerging on *Tectona grandis* (Teak) and *Cedrela odorata* (Cedrela) plantations. Plantation developers reported that disease problems in these plantations are increasing in the Anhwiaso South, Kwamisa, Opro, Tano Nimri, Mamiri and Afram Headwaters forest reserves (Apetorgbor and Bosu, 2009).

This paper presents new disease outbreaks and pathogens affecting tree plantations in Ghana. It also discusses management strategies undertaken to reduce the impact of these problems.

MATERIALS AND METHODS

Study Area: Field surveys were conducted randomly in 31 plantation situated in degraded forest reserves based on reports on disease incidents received from the National Forest Plantation Development Programme and small scale plantations. The reserves included the Afram Headwaters, Opro, Asubima, Mankrang, Yaya, Tain II, Worobong South and Volta Block I forest reserves in the Dry semi-deciduous forest zone. The others included Kwamisa, South Formangso, Asenanyo, Jimira, Anhwiaso South, Bosomkese, Esuboni, Birim and Pra Anum forest reserves in the Moist semi-deciduous forest zone and Mamiri and Tano Nimri in the Moist evergreen forest zone. Visits were also made to the African Plantations for Sustainable Development (APSD) Ghana Limited at Kwame Danso in the Guinea Savannah woodland (Figure 1).

Field survey: The surveys were on *Tectona grandis* and *Cedrela odorata* plantations in all the forest reserves, *Eucalyptus* species in APSD and *Ceiba pentandra* plantations and nurseries in the Afram Headwaters, Jimira and Pra Anum forest reserves from 2006 to 2012 (Figure 1, Table 1). Informal discussions were held with communities around these reserves prior to the field assessments to determine the status of diseases in the plantations. Background information on establishment of the plantations including planting techniques, land clearings and site history was documented. Hundred trees each per hectare were selected for sampling and inspected individually. Trees suffering from root diseases were identified based on observation of both above and below-ground symptoms. Trees with cracks on stems and branches, canker, wilt, dieback and leaf spots were examined. To determine the extent of damage to trees within the plantations, three replicates per hectare of each plantation were sampled out for intensive assessment. Infected trees were classified using a five-point damage rating:

(i) Healthy (No visible sign of disease)
(ii) Minor damage (New infections with symptoms evident on bole; however, tree shows no sign of damage)
(iii) Moderate damage (Infection on bole and spreading, most leaves are necrotic and/or drooping, sap flow)
(iv) Severe (Over 75% of infected trees completely defoliated, heavy flow of sap/cracks at base of stem)
(v) Death of trees.

Samples of diseased leaves, stems, twigs and roots were collected for laboratory examination.

Laboratory studies: Morphological studies of fungal pathogens were conducted macroscopically and microscopically. Ascomycetes fungi were isolated using 2.0% Malt extract agar (MEA) and Potato dextrose agar (PDA) supplemented with streptomycin sulphate (100ppm). For the isolation of Basidiomycetes, a selective medium containing benomyl and streptomycin sulphate was used.

Fungal colonies were isolated using identification keys (Brayford, 1997; Rivarden *et al*., 1994; Arora, 1986) as well as DNA sequence data (IGS and ITS gene regions).

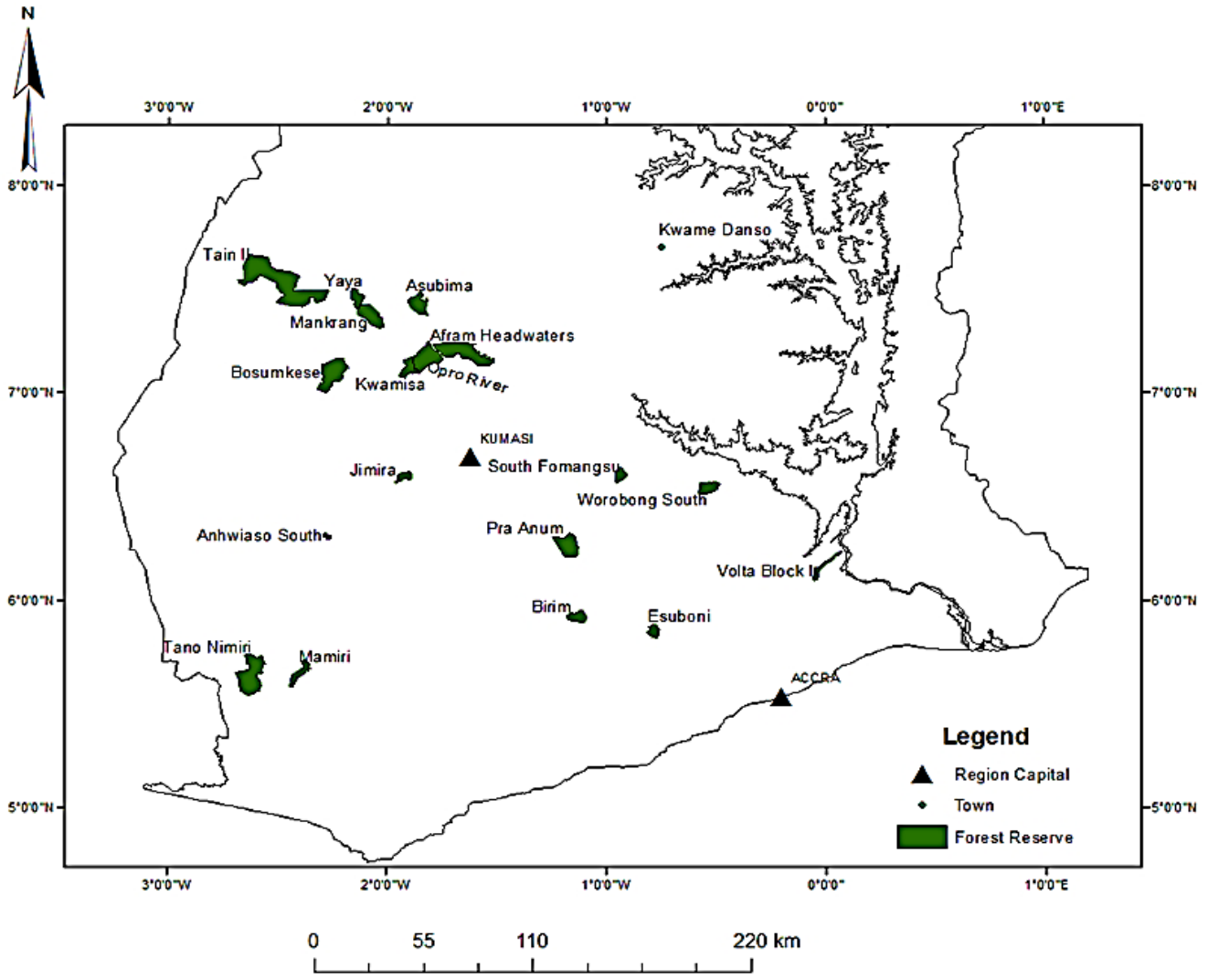

Figure 1. Distribution of plantations surveyed in forest reserves.

RESULTS

Disease incidence was variable depending on tree species, location and age.

Diseases of *Tectona grandis*: Ninety five percent of 17 teak plantations surveyed were free from diseases. However, teak mortality was observed in plantations around the Asubima, Afram Headwaters, Kwamisa, Mamiri and Opro forest reserves. Symptoms of poor tree health such as defoliation, dieback and stunted growth were recorded on trees as young as one year after planting while yellowing and dieback were observed ~~in~~ on pole-sized trees in the Yaya and Tain II forest reserves. Dying trees had their leaves yellowing from the crown and later died back. In Asubima and Kwamisa disease incidence was minor but moderate in pole-sized trees in the Afram Headwaters and Opro forest reserves.

Root rot was found in only three out of 17 plantations surveyed (Table 1). It was recorded in the Kwamisa, Mamiri and Tano Nimri plantations with mortality being severe in plantations in the Mamiri Forest reserve.

Distribution of disease was in isolated patches in all the plantations surveyed. For plantations in Asubima, Worobong South, Afram Headwaters and Opro, poor tree health was attributed to poor site factors. In Kwamisa, Mamiri and Tano Nimri the causes were as a result of root rot pathogen.

Symptoms above-ground were wilting, yellowing of leaves, bark cracking, defoliation and dieback. Severely infected trees shed their leaves and died eventually (Figure 2a). At the bases of tree trunks were rot which were advancing up the stem with white mycelia between the bark and wood of stem.

Table 1. Major disease outbreaks in forest reserves in Ghana (2006-2012).

Host	Region	Forest Reserve	Ecological Zone*	Age (Yrs.) of plantations	Disease Status	Disease Severity	Causative Agent	Disease Distribution
Tectona grandis	Ashanti	Asubima	DS	1-2	Mortality	Minor	Drought	Isolated
				1-2	Defoliation/Dieback	Moderate	Drought	Isolated
		Opro	DS	2-4	Defoliation/Dieback	Moderate	Drought/Hardpan	Scattered
		Afram Headwaters	DS	2-4	Mortality	Moderate	Drought/Hardpan	Scattered
		Kwamisa	MS	2-4	Dieback/Basal rot	Moderate	*Armillaria* sp.	Isolated
				6	Mortality/Basal rot	Minor	*Armillaria* sp.	Isolated
		Jimira	MS	6-8	Nil	Nil	Nil	Nil
		Mankrang	MS	1-2	Defoliation/Dieback	Moderate	Drought/Hardpan	Scattered
	Brong Ahafo	Yaya	DS	2-4	Defoliation/Dieback	Moderate	Drought/Hardpan	Scattered
		Tain II	DS	2-4	Defoliation/Dieback	Moderate	Drought/Hardpan	Scattered
	Western	Mamiri	ME-WE	9	Mortality/Basal rot	Severe	*Armillaria hemii*	Isolated
		Tano Nimiri	ME-WE	6	Mortality/Basal rot	Severe	*Armillaria hemii*	Scattered
		Anwhiaso South	MS	2-3	Dieback	Moderate	*Armillaria hemii*	Isolated
	Eastern	Pra Anum	MS	6-8	Nil	Nil	Nil	Nil
		Worobong South	DS	1	Stunting	Moderate	Drought/Hardpan	Isolated
		Birim	MS	6-8	Nil	Nil	Nil	Nil
		Volta Block I	DS-SM	8-10	Dieback	Minor	Drought	Drought
Cedrela Odorata	Ashanti	Asubim	DS	1-2	Mortality	Minor	Drought	Isolated
	Brong Ahafo	Yaya	MS	1-2	Defoliation	Moderate	Poor site	Scattered
					Stunting	Minor	Drought/Hardpan	Scattered
	Western	Mamiri	ME-WE	9	Basal rot	Severe	*Armillaria hemii*	Scattered
		Tano Nimiri	ME-WE	9	Basal rot	Severe	*Armillaria hemii*	Scattered
				8	Wind throwing	Minor	Drought/Hardpan	Isolated
Ceiba *pentandra*	Ashanti	Mankrang	MS	Nursery	Leaf spot	Severe	*Colletotrichum* sp.	Scattered
		Asenanyo	MS	1-2	Dieback	Severe	*Colletotrichum* sp.	Scattered
		FORIG	MS	Nursery	Leaf spot	Severe	*Colletotrichum* sp.	Scattered
	Eastern	Pra Anum	MS	3	Mortality	Moderate	*Colletotrichum* sp.	Scattered
Eucalyptus spp.	Brong Ahafo	Kwame Danso	DS		Cutting rot	Minor	*Calonectria* sp.	Isolated
					Leaf spot	Minor	*Mycosphaerella* sp.	Isolated
				Nursery	Leaf blight	Minor	*Cylindrocladium* sp.	Isolated
	Central	Yenku	SM	8-12	Nil	Nil	Nil	Nil

*DS: Dry Semi-deciduous; MS: Moist Semi-deciduous; ME-WE: Moist Evergreen-Wet Evergreen; SM: South Marginal

Figure 2a. Death of teak trees.

Figure 2b. Rot of stem with mycelia mat at base.

Figure 3a. White mycelia under bark of cedrela stem.

Figure 3b. Disease in *Cedrela odorata* plantations.

Table 2. Disease incidence in *Cedrela odorata* plantations.

Tree Health Status	Disease incidence (%) in *C. dorata* plantations in	
	Mamiri	Tano Nimiri
Healthy	31.7	13.0
Moderately diseased	14.5	4.1
Severely diseased	36.5	61.6
Death of trees	16.3	21.3

The incidence of disease was more severe in plantations in the Mamiri forest reserve with 1.2% of the trees infected with root rot. Macroscopic and DNA sequencing of isolates (fruit bodies) revealed *Armillaria hemii* complex as the pathogen (Figure 2b).

Diseases of *Cedrela odorata*: In plantations at the Tano Nimri and Mamiri forest reserves basal root rot was observed in 68.3% pole-sized trees. Disease was severe and scattered in the plantations. Bark of trees were cracked and decaying from the base up the stems, and leaves of newly attacked trees first turned yellowish and shed off as the infection advanced (Figure 3a). About 68% and 87% of plantations in Tano Nimri and Mamiri forest reserves, respectively were affected and cleared resulting in huge economic losses to the developers. *Armillaria hemii* was identified on the roots and base of affected trees as in the teak plantations (Figure 3).

Defoliation and dieback were observed in one to two-year-old trees in Yaya and Asubima respectively. Drought-related stunting appeared to be a common problem in the one to two-year-old plantations in Afram Headwaters, especially in areas with dry weather.

Diseases of *Ceiba pentandra*: In the nursery at

Mankrang, leaf spot and dieback were observed on seedlings at the onset of the rainy season in April. In Pra Anum and Asenanyo, dieback and mortality occurred in saplings of between one to four-year-olds. The severity of these infections resulted in 90-100% losses in the nursery depending on the provenances and 50-70% in plantations. Leaf spot was caused by *Colletotrichum capsici*, whereas *Fusarium solani* and *Lasiodiplodia theobromae* were associated with dieback of stems in both nursery and field.

Diseases of *Eucalyptus* species: Low levels of disease (<1.0%) were observed in Eucalyptus plantations; however, *Cylindrocladium* leaf blight was common on *Eucalyptus* trees in plantations of APSD Ghana Limited. A *Calonectria* species was also found causing rot in the nursery.

Figure 4. *Botryosphaeria* canker on trunk of Eucalyptus tree.

Isolated cases of disease caused by species of *Botryosphaeria* (Figure 4), *Pestalotiopsis* and *Phomopsis* (*Diaporthe*) were observed on branches and trunks of *Eucalyptus argophloia* and *E. grandis* hybrids. Species of *Mycosphaerella* and *Teratosphaeria* were isolated from leaf spot symptoms while *Quambalaria eucalypti* was found on a single *E. grandis* tree.

DISCUSSION

Defoliation, dieback and stunted growth were found in ten out of 17 teak plantations in one to two-year-old saplings as a result of drought during the dry season. This is similar to observations made in Indonesia by Widyastuti and Widyastuti (2002). Poor tree health was considered to have significant impact on plantation production, particularly during the early phase of establishment by tree growers (Mohd Farid *et al*., 2005).

Generally, majority of the plantations surveyed were free from root disease. Root disease was found in only two out of the twenty forest reserves surveyed. In these reserves basal root rot was present in both teak and cedrela plantations. Disease infection on teak was recorded seven years after planting in the Mamiri forest reserve. However, Mazia and Lee (1999) have reported teak trees aged two years and above were frequently infected by root disease in Malaysia.

In Ghana, signs and symptoms of pathogen attack were visible on stems and roots of cedrela and teak trees and were of high incidence and severity (68.3%) in cedrela and minor (1.5%) in teak). The first visible symptom of root disease was gradual yellowing of leaves followed by shedding of leaves and decay of most root system. Spread of disease to adjacent healthy trees was primarily through root contact. Earlier work by Tewari (1992) reported that this was the most common mode of disease spread in plantation-grown teak. Frequently, the source was infected old stumps and wood debris remaining in the soil or standing diseased trees.

Armillaria hemii was identified on the roots and root collars of affected teak and cedrela plantations in Mamiri and Tano Nimri forest reserves. Wilting and yellowing of tree crowns as well as occurrence of white mycelial fans under the bark of infected teak trees were common symptoms of *Armillaria* infections (Morrison *et al*., 1991). *Armillaria hemii* has been reported to have infected trees (*Eucalyptus*, *Pinus*, *Acacia*, *Grevillea* and *Cupressus* species) in several countries in south, central, east and western Africa (Coetzee *et al*., 2000; Mwenje and Ride, 1996; Gezahgne *et al*., 2003). Armillaria root rot exists naturally in dense rain forest zones where annual rainfall exceeds 1,300 mm. Clearing the forest breaks this natural equilibrium and causes pre-existing infection centres to spread in the new plantations. Thus the incidence of pathogens in young plantations depends on the previous density of inoculum in the forest (Nandris *et al*., 1987). Mohd Farid *et al*. (2005) indicated that root rot disease was found mostly in plantations with poor land preparation, where stumps and wood debris have been left on the ground to decay.

Outbreaks of leaf spot in *Ceiba pentandra* which resulted in dieback caused high mortality (70-100%) in nurseries established in Mankrang and Pra Anum. Reports have been made on leaf spot of *C. pentandra* in Assam, Madya Pradesh and Uttar Pradesh in India

where the disease was first detected in seedlings at the nursery. The disease was destructive and damaged as much as 40% of the leaf area (Mehrothra, 1989). *Colletotrichum capsici* was identified as the causal agent of leaf spot in the nursery whereas *Fusarium solani* and *Lasiodiplodia theobromae* were associated with dieback of stems in both nursery and field. However, in Nagpur, India, dieback and canker were caused by *Colletotrichum gloeosporioides* (syn. *Glomerella cingulata*) in established trees of *C. pentandra* (Charkrabarty *et al*., 1993).

On branches of *Eucalyptus argophloia* and *E. grandis* hybrid plantations in APSD, isolated cases of diseases caused by species of *Botryosphaeria* were observed. *Botryosphaeria* species are known to be saprophytes and opportunistic pathogens (Barnard *et al*., 1987; Shearer *et al*., 1987; Smith *et al.,* 1994) on Eucalyptus species. However, these are viewed as latent pathogens that occur on leaf and branch tissues of healthy woody plants which later cause disease when trees are stressed (Fisher *et al*., 1993; Smith *et al*. 1996) due to drought, frost, waterlogging and insect damage (Old *et al.,* 1990). In South Africa, wide-spread twig dieback and stem cankers caused by *Botryosphaeria dothidea* and *B. eucalyptorum* were observed on *E. grandis, E. nitens* and *E. smithii*, clones of *E. grandis*, hybrids of *E. grandis* with *E. camaldulensis*, as well as on *E. urophylla* (Smith *et al.,* 1994, 2001). Since the Eucalyptus plantations in Ghana are being developed on marginal soils where moisture is a limiting factor there is the need for careful matching of planting stock to site to avoid stress-induced damage.

Cylindrocladium leaf blight affected young trees in *Eucalyptus* plantations of APSD Ghana Limited. *Cylindrocladium* are common pathogens of *Eucalyptus* and *Acacia* species particularly in nurseries (Crous *et al*., 1991) causing damping off of young seedlings as well as leaf defoliation, shoot blight and cankers on the stems of young plants. In Kenya and Uganda young seedlings of Eucalyptus showed signs of *Cylindrocladium* infection, including damping off and stem cankers. Since *Cylindrocladium* infection is common on Eucalyptus trees in nurseries, it is a potential threat to plantation establishment in the country.

A species of *Mycosphaerella* was isolated from leaf spot on an *E. grandis* tree. Several *Mycosphaerella* species have been reported from southern and east Africa and found to display differences in host range and age with regard to infection of leaves (Crous, 1998; Hunter *et al*., 2004). Some *Mycosphaerella* species are capable of causing more serious disease on mature foliage in Australia and South East Asia (Crous, 1998; Crous *et al*., 1998; Wingfield *et al*., 1996) and every effort must be made to exclude them from African countries.

CONCLUSIONS

Although several forest tree plantations have been established under the National Forest Plantation Development Programme in the different forest districts of Ghana, survey was undertaken only in a limited number of plantations. The study found new records of exotic diseases with no serious management of the pathogens by plantation developers. Suggestions have been made for stump removal and trenching in areas affected with root rot, removal of infected trees, proper farm maintenance culture, sanitation and thinning followed by insecticide and fungicide application. Establishment of new plantations should take into account the presence of pathogens causing these diseases and also match planting stock to site to avoid stress. There is also the need to establish plantation health networks to monitor, protect and prevent introduction of new pathogens with the increase in new tree plantations.

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EFFECTS OF PH AND ECW ON GROWTH AND SPORULATION OF INDIGENOUS *TRICODERMA* SPP.

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A B S T R A C T

The influence of three levels of pH and degrees of ECw on the radial growth and sporulation of 34 local isolates of *Trichoderma* spp. were evaluated under laboratory conditions. The pH used were 4, 6 and 8 while, the ECw were 12.92, 14.30 and 17.8 Siemens per meter (ds/m) respectively. The data obtained showed that pH 6 was the optimum degree of growth parameters while pH 8 showed significant reduction ($p< 0.05$) in the growth parameters compared to pH 4. The results also revealed that ECw 17.8 ds/m significantly reduced ($p< 0.05$) the growth parameters compared to ECw 12.92 and 14.30 ds/m. Thus, the results obtained elucidated the importance of reduction in biocontrol activity of fungus in saline-alkali soil.

Keywords: Saline-alkali soil, pH alkaline, Electrical conductivity (ECw), *Trichoderma* isolates.

INTRODUCTION

The genus *Trichoderma* is a soil borne saprophytic fungus that is widely distributed in all soil types (Meraj-ul *et al.,* 2012; Kalaivani *et al.,* 2014; Wu *et al.,* 2014). *Trichoderma* isolates have different potential of antagonistic ability against plant soil borne pathogens and nematodes (Jabara, 2002). The chances of biological control success is still limited to their consistant performance, and one of the most important reasons for this problem is the environmental stress factors such as salinity and pH of the soil. The environmental stress factors of the soil have detrimental effects on *Trichoderma* isolate's growth, antagonistic activity and survival rate (Hafedh, 2001; Mohamed and Haggag 2010; Panahian *et al.,* 2012).

The cultivation of sensitive economic crops in saline soils has been a failure of crop cultivation. The numbers of economic crops resistant to salinity are still limited, and there were differences in their resistance to these conditions. Several studies have stated that the fungal genus *Trichoderma* are sensitive and not tolerant to high levels of pH (Mehrotra *et al.,* 1997; Hafedh, 2001) however, Harman (2000) and Mohamed and Haggag (2010) used protoplast fusion technique between two *T. harzianum* isolates and *T. koningii* and *T. reese* which had high specification tolerances under stress conditions, and obtained a new strain which was considered as highly antagonistic and more tolerant to stress conditions than their parental strains. *Trichoderma* isolates that are good saline-tolerant are more economical for crops cultures than the less tolerant to soil saline, for this reason the incessant search for a new isolates that have good ability to tolerate the soil saline is imperative. so, the objective of this study was to obtain the best antagonist of *Trichoderma* isolates that are more tolerant to soil saline and alkaline pH, the common characters of Iraqi soils.

MATERIAL AND METHODS

***Trichoderma* Isolates:** This study was conducted with 34 isolates of *Trichoderma*, 30 isolates were obtained from the department of biopesticide directorate of agricultural research, three isolates were obtained from the plant protection department college of agriculture University of Baghdad and one isolate was obtained from the public authority of Agricultural research-department of Agriculture.

Effect of pH on Growth Rate: In this assay we selected three levels of pH 4, 6 and 8, and study their biological effect on daily growth rate of (DGR) 34 different *Trichoderma* isolates. A 5 mm diameter discs from

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Trichoderma mycelium of all the isolates were individually applied in the middle of a 9 cm petri dish on potato dextrose agar (PDA). These plates were then incubated at (28±2 ^{0}C) and observed for fungal growth and sporulation over a period of five days, and the pH adjusted by using hydrochloric acid solution (HCL). The daily rate of growth was calculated according to the following equation:

$$DGR = \frac{(D2-D1)}{2(T2-T1)}$$ (Hafedh, 2001).

The sporulation ability of each isolate was calculated by adding 10 ml of distilled water to each plate, and spores collected by using glass rod, and prepared an appropriate dilution of three concentrations of spores for each isolate; and this was calculated using Haemacytometer as described by Hamdia and Kalaivani (2013).

Effect of ECw on Daily Growth Rate: To evaluate the effect of electrical conductivity ECw on the growth and sporulation of 34 *Trichoderma* isolates the medium used include the following components; (5 % of Dextrose, 0.5 % of K_2HPO_4, 0.1 % of $NaNO_3$, 0.2 % of Yeast extract, 0.1 % of $MgSO_4.7H_2O$ and 1.5 % of Agar). Also, the three concentrations of NaCl used were; 1g of NaCl (ECw. 12.92 ds/m) and 2g of NaCl (ECw. 14.30 s/m) and 5g of NaCl (ECw. 17.8 ds/m): The ECw of the salt medium (NaCl) was evaluated using a pH-meter (Handbook, 1954). The medium was sterilized at a temperature of 121 ^{0}C and pressure of 1.5 kg / cm^2 for 15 minutes, after that the medium was poured in petri dishes, and a 5 mm disc of mycelium from each *Trichoderma* isolates were placed in the middle of a 9 cm petri dish, each treatment triplicated.

And later incubated at a temperature of (28 ± 2 ^{0}C) in this test, daily growth rate and sporulation ability were calculated for each *Trichoderma* isolate as mentioned above. The data obtained from the observations were statistically analysis by using a completely randomized block design.

RESULTS

Effect of pH on the growth and sporulation of 34 *Trichoderma* Isolates: The results revealed that the tested isolates of *Trichoderma* spp. exhibited significant differences ($p<0.05$) in their response to tested pH levels (4, 6 and 8) Table 1. The laboratory experiment showed that *Trichoderma* isolates T.2, T.13, T.27 exhibited optimum growth rate at pH4, and isolates T.1, T.6, T.8, T.15 gave optimum growth rate at pH6, while isolates T.2, T.15, T.18, T.33 exhibited optimum growth rate at pH8 (Table 1). The results also showed that isolate T.30 gave the same response growth rate 1 cm at all pH tested Table 1. As for the sporulation the data shows in Table 2 that isolates T.3, T.5, T.13, T.20, T.23, T.26, T.27, T.33 exhibited optimum sporulation rate at pH4, and isolates T.1, T.4, T.8, T.14, T.21, T.22, T.31 revealed optimum sporulation rate at pH6, while isolates T.3, T.9, T.11, T.14, T.19, T.28 exhibited optimum sporulation rate at pH8.

Table 1. The Influence of pH on Daily Growth of 34 *Trichoderma* Isolates under Laboratory Conditions.

Isolates of *Trichoderma* spp.	* Daily Growth Rate * *Mean No. of Daily Growth (cm)		
	PH4	PH6	PH8
T.1	1.5	1.75	0.75
T.2	1.75	1.5	1.5
T.3	1.37	0.75	1
T.4	1.5	1.5	0.5
T.5	0.6	1.37	0.75
T.6	1.12	1.75	1.0
T.7	0.87	1.37	1.0
T.8	0.75	1.87	1.0
T.9	1.25	1.25	1.0
T.10	0.68	1.25	1.0
T.11	0.62	1.25	1.25
T.12	0.75	1.70	0.5
T.13	1.62	1.68	1.0
T.14	1.0	0.81	0.62
T.15	1.37	1.87	1.75
T.16	1.0	1.75	1.12
T.17	0.87	1.37	1.0
T.18	0.62	1.5	1.75
T.19	1.12	1.12	1.5
T.20	0.75	1.37	0.75
T.21	0.5	1.37	0.62
T.22	0.87	1.62	0.62
T.23	0.75	1.62	1.0
T.24	0.87	0.87	1.0
T.25	0.37	1.0	1.0
T.26	1.25	1.12	1.0
T.27	1.87	1.25	1.25
T.28	1.25	1.0	1.0
T.29	0.37	1.62	1.0
T.30	1.0	1.0	1.0
T.31	1.0	0.75	0.75
T.32	0.87	1.5	1.25
T.33	0.37	1.37	1.5
T.34	0.37	1.12	1.25
LSD (P < 0.05) of Growth and Sporulation			0.09
LSD (P < 0.05) of Isolates			0.32
LSD (P < 0.05) of Interaction			NS

*Three replicates of each isolate

* *Mean No. of Daily Growth Rate (DGR) was calculated as following (Hafedh, 2001).

Table 2. The Influence of pH on Sporulation of 34 *Trichoderma* Isolates under Laboratory Conditions.

Isolates of *Trichoderma* spp.	Sporulation *Mean No. of Sporulation × 10^8 spore/cm^3		
	PH4	PH6	PH8
T.1	30.08	2050	33.3
T.2	29.08	51.83	28
T.3	82.50	80.42	82.5
T.4	26.33	310.8	28.75
T.5	58.33	60.00	28.42
T.6	30.08	33.30	28.75
T.7	32.26	31.25	27.42
T.8	27.66	125.42	27.75
T.9	27.33	42.50	47.83
T.10	29.16	29.17	28.17
T.11	30.73	41.00	37.66
T.12	27.50	52.50	26.33
T.13	42.58	27.42	27.16
T.14	31.15	115.9	34.83
T.15	32.40	40.83	28.3
T.16	30.83	29.16	29.66
T.17	27.25	157.3	26.08
T.18	26.16	44.16	26.08
T.19	26.60	26.66	33.16
T.20	66.60	29.66	27.42
T.21	39.75	114.23	27.42
T.22	57.50	134.75	27.42
T.23	55.83	83.75	27.75
T.24	00.87	52.50	28.42
T.25	30.42	38.33	31.15
T.26	34.06	70.83	28.00
T.27	54.33	51.66	26.03
T.28	59.17	39.16	40.42
T.29	36.66	51.66	26.08
T.30	26.66	31.25	25.00
T.31	31.75	125.41	28.00
T.32	41.00	50.83	26.08
T.33	27.50	54.16	26.60
T.34	51.00	66.60	27.08
LSD (P < 0.05) of Sporulation			1.73
LSD (P < 0.05) of Isolates			4.1
LSD (P < 0.05) of Interaction			7.15

* Mean No. of three replicates of each isolate.

The effect of the degree of electrical conductivity (ECw) on the growth and sporulation of the 34 *Trichoderma* isolates: The results of the study showed significant differences ($p<0.05$) of the three degrees of ECw, 12.92, 14.30 and 17.8 ds/m on the growth and sporulation of the 34 *Trichoderma* isolates Table 3 and 4. Isolates T.7, T.15, T.18, T.21, and T.28 exhibited optimum growth rate at 12.92, and isolates T.9, T.13, T.18, T.19, T.28 gave optimum growth rate at 14.39, while isolates T.9, T.13, T.18, T.19, T.21, T.28 exhibited optimum growth rate at 17.69 Table 3.

Table 3. The Influence of ECw on Daily Growth of 34 *Trichoderma* Isolates under Laboratory Conditions.

Isolates	*Daily Growth Rate * *Mean No. of Daily Growth (cm)			
	Control	12.92 ds/m NaCl	14.30 ds/m NaCl	17.80 ds/mNaCl
T.1	0.34	0.57	0.61	0.62
T.2	0.41	0.50	0.68	0.73
T.3	0.32	0.35	0.59	0.67
T.4	0.40	0.44	0.61	0.61
T.5	0.36	0.43	0.60	0.62
T.6	0.32	0.35	0.67	0.69
T.7	0.31	0.36	0.78	1.00
T.8	0.40	0.48	0.68	0.75
T.9	0.58	0.69	0.73	0.95
T.10	0.49	0.51	0.68	0.70
T.11	0.38	0.59	0.69	0.73
T.12	0.48	0.57	0.70	0.80
T.13	0.56	0.65	0.72	0.81
T.14	0.52	0.59	0.73	0.83
T.15	0.54	0.62	0.76	0.80
T.16	0.55	0.62	0.66	0.82
T.17	0.37	0.45	0.57	0.58
T.18	0.61	0.67	0.77	0.78
T.19	0.64	0.68	0.72	0.78
T.20	0.41	0.46	0.71	0.76
T.21	0.65	0.71	0.75	0.83
T.22	0.20	0.66	0.73	0.75
T.23	0.39	0.41	0.48	0.78
T.24	0.42	0.71	0.61	0.82
T.25	0.07	0.65	0.70	0.81
T.26	0.22	0.34	0.73	0.86
T.27	0.36	0.47	0.67	0.72
T.28	0.62	0.71	0.77	0.84
T.29	0.49	0.69	0.68	0.82
T.30	0.41	0.47	0.41	0.74
T.31	0.31	0.36	0.72	0.75
T.32	0.37	0.42	0.65	0.69
T.33	0.40	0.63	0.69	0.67
T.34	0.53	0.63	0.73	0.79
LSD (P < 0.05) of Growth and Sporulation				0.009
LSD (P < 0.05) of Isolates				0.027
LSD (P < 0.05) of Interaction				0.054

*Three replicates of each isolate

**Mean No. of Daily Growth Rate (DGR) was calculated as mentioned in Table (1).

The results also showed that isolates T.30 gave the same response growth rate 1 cm at all pH tested Table 3. As for the sporulation the data shows that isolates T.10, T.17, T.20, T.28 exhibited optimum sporulation rate at 12.92, and isolates T.1, T.4, T.8, T.14, T.21, T.22, T.31 showed optimum sporulation rate at 14.39, while isolates T.3, T.9, T.11, T.14, T.19, T.28 exhibited optimum sporulation rate at 17.69 (Table 4).

Table 4. The Influence of ECw on Daily Growth of 34*Trichoderma* Isolates under Laboratory Conditions.

Isolates	Sporulation			
	*Mean No. of Sporulation × 10^8 spore/cm^3			
	Control	12.92 ds/mNaCl	14.30 ds/mNaCl	17.80 ds/mNaCl
T.1	511.33	82.33	30.00	12.50
T.2	122.00	113.00	22.50	21.83
T.3	245.00	51.50	9.50	7.17
T.4	142.33	117.50	29.67	7.00
T.5	450.00	51.33	45.00	14.00
T.6	143.67	27.50	15.00	11.50
T.7	220.50	32.50	25.67	12.50
T.8	160.50	102.00	35.00	13.67
T.9	151.50	62.33	38.67	20.00
T.10	198.33	197.00	40.50	20.00
T.11	622.33	33.67	32.50	27.00
T.12	623.67	30.83	30.00	15.83
T.13	114.00	40.00	32.17	28.50
T.14	222.67	35.00	24.00	23.67
T.15	123.67	75.00	22.67	13.00
T.16	209.00	35.83	28.33	12.33
T.17	571.00	222.33	31.50	27.67
T.18	111.50	98.67	31.00	22.67
T.19	63.33	74.00	27.67	19.67
T.20	640.50	310.83	42.33	28.50
T.21	594.83	38.33	36.33	28.67
T.22	282.33	82.67	24.00	12.00
T.23	322.00	22.50	21.00	18.00
T.24	90.83	65.83	37.67	28.67
T.25	60.50	37.50	25.83	23.33
T.26	144.83	60.00	24.00	20.33
T.27	282.00	42.50	22.33	18.33
T.28	294.50	284.83	31.00	29.00
T.29	345.17	44.67	31.67	12.00
T.30	303.50	42.50	36.50	23.67
T.31	832.33	106.67	34.00	28.00
T.32	615.83	50.50	40.67	15.50
T.33	145.17	92.67	40.67	17.83
T.34	137.33	82.67	33.33	14.00
LSD (P < 0.05) of Growth and Sporulation				0.56
LSD (P < 0.05) of Isolates				1.64
LSD (P < 0.05) of Interaction				3.28

DISCUSSION

The aim of this study was to evaluate the response of 34 *Trichoderma* isolates. Besides, the results of this study explained the variation of the *Trichoderma* isolates performance. The data Table 1 and 2 showed that pH 6 was the best for the growth and sporulation in most of the isolates studied. While the pH 8 showed high reduction of colony diameter growth rate and sporulation, compared to the pH 4 (Table 1). Table 1 and 2 also showed lack of correlation between the growth rates and the ability of sporulation, for example the isolate T.14 Table 1 showed the lowest rate of growth of diameter 0.81 cm when the pH was 6, while the average sporulation 115.9 × 10^8 spores / cm^3 Table 2 was high at the same pH 6. However, the isolate T.15 showed the highest daily growth rate of 1.87 when the pH was 6, but showed sporulation rate of 40.83 ×10^8 spores / cm^3 at the same pH of 6. On the other hand, some isolates such as T.1 Table 1 achieved the highest growth rate of 1.75 cm, and sporulation rate of 205 × 10^8 spores / cm^3 Table 2 at the same pH 6.

The data obtained from the present study agreed with some previous studies which found that pH of soil to had significant antagonistic effect on biological effectiveness of *T. harzianum*, and also indicated about 32% decreased in antagonistic effect of *T. harzianum* when bean seeds was treated with spores suspension at the pH 3.5. However, the antagonism increased to 65 % when the pH was 5.6 % (Marshall, 1982). Hafedh (2001) recorded high decline in the antagonistic ability of some *Trichoderma* isolates when used under field conditions.

The results of the study on the effect of the three degrees of ECw., 12.92, 14.30 and 17.8 s/m on the growth and sporulation of the 34 *Trichoderma* isolates Table 3 and 4 showed that degree of ECw12.92 s/m was the best for the growth and sporulation of most tested isolates, while the degree of ECw17.8 s/m, exhibited significant reduction in daily growth rates and sporulation Table 3 and 4 of most *Trichoderma* isolates as comparison to the degrees 12.92 and 14.30 s/m (Table 3). The results of this study also indicated the lack of correlation between growth rates and the ability of sporulation as denoted in (Table 3 and 4). The isolate T. 25 in Table 3 showed the lowest growth rate of 0.07 cm at the degree of ECw17.8 s/m, and showed high rate of sporulation of 23.33 × 10^8 spores / cm^3 Table 4, while T.19 isolate showed the highest rate of daily growth of 0.64 cm Table 3, and the

rate of sporulation was 19.67 × 10^8 spores / cm^3 Table 4 at that degree of ECw of 17.8 s/m.

Moreover, the present study documented on the low performance of *Trichoderma* spp. under environmental conditions even though this fungus has high capacity to grow in a wide range of soil as well as the biogenic properties of *Trichoderma* species. Furthermore, there are biotic conditions that play important role in reduction of the antimicrobial activity of this fungus (Danielson and Davey, 1973; Prabavathy *et al.,* 2006). On the other hand, the environmental conditions provide other soils suppressive which accentuates the effectiveness of *Trichoderma* spp. antagonism. The weak correlation between the daily growth of colony of each isolate and the ability to sporulation may be referred to the *Trichoderma* isolates behavior when provided the appropriate nutritional conditions leading to reduce sporulation ability of *Trichoderma* fungus. Additionally, the stress conditions encourage sporulation ability of *Trichoderma* isolates and reduce the normal growth rate in order to keep the species survive.

There is paucity of studies on *Trichoderma* isolates and their tolerance to the different concentrations of salt (NaCl). However, there were few studies carried out under field conditions, which indicated that some *Trichoderma* isolates had less activity in alkaline soils (Kumar, 1995). Besides he found that the accumulation of salts in the rhizospher area as a result of irrigation with saline water leads to the changes in the pH soil, as well as to produced large amounts of CO_2 which negatively affected the growth and sporulation of this fungus. Likewise, dissolving of the CO_2 in the water, resulted in the built up of bicarbonates (HCO_3) that played a role in changing the pH in the root zone (Macauley and Griffin, 1969), and all of these combined factors negatively affect the enzymatic activity of *Trichoderma* fungus which is considered one of the important antagonistic mechanisms of this fungus.

CONCLUSION

In this study, effect of pH and ECw on the daily growth rate and sporulation ability of the 34 *Trichoderma* isolates individually under laboratory conditions found that each isolate of *Trichoderma* varied in its inhibitory degree base on each pH levels and ECw degrees. Therefore the results showed that some *Trichoderma* isolates that had good ability to grow on salt media can be used successfully when a suitable formulation is used under field conditions as biocontrol agents.

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ASSESSMENT OF WILT INTENSITY AND IDENTIFICATION OF CAUSAL FUNGAL AND BACTERIAL PATHOGENS ON HOT PEPPER (*CAPSICUM ANNUUM* L.) IN BAKO TIBBE AND NONNO DISTRICTS OF WEST SHEWA ZONE, ETHIOPIA

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A B S T R A C T

Pepper wilt disease intensity was assessed on-farm in Bako Tibbe and Nonno districts of West Shewa Zone, Ethiopia during the main cropping season of October 2012. The wilt causing pathogens were identified from 50 diseased samples collected from the two districts. Of the 120 hot pepper fields surveyed, 116 fields were found to be infected with wilt disease. The overall percent prevalence and incidence of wilt disease was 96.7 and 86.4%, respectively. Identification and pathogenicity tests revealed that *Ralstonia solanacearum* and four fungal wilt pathogens (*Rhizoctonia solani*, *Fusarium spp*., *Phytophthora spp*. and *Verticillium spp*.) were detected in the surveyed fields. The percentage of occurrence of *Rhizoctonia solani, Fusarium spp., Phytophthora spp*. and *Verticillium spp*. were 45.0, 17.48, 12.59 and 11.89%, respectively; whereas, the frequency of *R. solanacearum* was 100%. Wilt disease in pepper in these two districts was caused by more than one wilt causing pathogen, thus management strategies should focus on these complex pathogens.

Keywords: *Rhizoctonia, Fusarium, Phytophthora, Verticillium, Ralstonia solanacearum,* pepper, wilt.

INTRODUCTION

Hot pepper (*Capsicum annuum* L.) is one of the most economically important vegetable crops used for consumption as vegetable, spice, and industrial raw material to produce oleoresin. The color and flavor extracts from hot pepper are used in both food and feed industries. It is a crop of high value in both domestic and export markets and generates employment to urban and rural workers. The main processed product, oleoresin, is exported to different countries and the spiced ground is supplied to local population. From 1992/93 to 2003/04, a total of 616.16 tons of oleoresin, which worth 106.6 million Birr, was exported to different countries by Ethiopian Spices Extracting Factory (ESEF, 2005). The estimate of area and yield of pepper for 2004-2005 in Ethiopia was 43,730 tons green pod from 4,627 ha, and 69,687 tons dry pod from 54,801ha (CSA, 2006). According to MOA (2011) the total area cultivated with pepper (green and red peppers) was 89,205.16 ha and the total production was estimated to 2, 676, 44.81tons. Different types of *Capsicum* spp. produced grow well under warm and humid conditions, but it requires dry weather at the time of maturity. It gives best green fruit yield and better seed set at 21 to 27^{0}C during the day and 15 to 20^{0}C at night. Hot pepper adapts well to sandy loam soil and well drained good clay loam (Lemma, 1998).

Ethiopian Export Promotion Agency has carried out a spice potential market study in three regional states in the country (Amhara, Oromiya and South nation nationality people regional states), and identified that pepper production accounts for 34% of the total spices production in these regions and generated an income of 122.80 million Birr for farmers in 2000/01 (EEPA, 2003). This value scaled up to 509.44 million Birr for smallholder farmers in 2004/05 (Seleshi, 2011) indicating that hot pepper is the important source of income to smallholder farmers and as exchange earning commodity in the country (Beyene and David, 2007).

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Despite its importance, the hot pepper production system has stayed as low input and low output with a national average yield of 7.6 tons/ha green pod and 1.6 tons/ha of dry pod, respectively (CSA, 2006). But the dry pod yield in experimental plot ranged between 2.5-3.0 tons/ha (Fekadu and Dandena, 2006) which is due to lack of adaptable varieties with the existing agro-ecology, poor agronomic practice, biotic and abiotic stresses, and shortage of water during dry seasons which can lead to flower abortion resulting in low productivity (EIAR, 2009). Among the production constraints, bacterial and fungal pathogens are known to be significantly important in all pepper growing areas of Ethiopia (Korobko *et al*., 1986; BARC, 1999).

Bako Tibbe and Nonno districts are the major hot pepper producing areas in West Shewa Zone. Production of hot pepper during 2003 was 5,600 tons from 3,600 ha in Bako Tibbe and 1,234.8 tons from 1,029 ha in Nonno district, respectively (WSZoARD, 2003). Wilt disease of hot pepper caused by *Rhizoctonia solani* and *Fusarium* spp. have been reported in Bako and Nejo areas (BARC, 1999/2000). In addition, pod rotting caused by *Phytophthora infestans,* frog eye leaf spot (*Cercospora capsici)* and pod bleaching (assumed to be caused by fungi and insect damage) were found to be important in the region (BARC, 2000). Now days, crop failure due to wilt diseases has been common in these districts and farmers are sometimes forced to abandon their production due to excessive infection pressure in the field. In addition, wilt disease could be caused by pathogens other than the already identified pathogens in the area. Despite this fact the identity and relative importance of wilt causing pathogens in the area has not been well profiled. Therefore, this study was carried out to assess the disease intensity and identify wilt causing bacterial and fungal pathogens and prioritizing them according to their frequency of occurrence.

MATERIALS AND METHODS

Description of the Study Areas: Bako Tibbe and Nonno districts are located in West Showa, Oromia Regional state at 270 km and 196 km from Addis Ababa, respectively. The average altitude of Bako Tibbe district is 1655m.a.s.l with mean annual rainfall of 920 mm and monthly minimum of 13.7^{0}C and maximum of 32.9^{0}C temperature. The average altitude of Nonno district is 1715 m.a.s.l. with mean minimum and maximum temperature of 16^{0}C and 24^{0}C, respectively. Mean annual rainfall of the district is 900 mm. These two districts are known for production of crops and rearing of cattle for income generation. Hot pepper is the major income source of the farmers in both low and mid altitude (WSZoARD, 2003).

Survey of Wilt Disease Intensity: Field survey was carried out in the main growing season from 1st to 3rd week of October, 2012. Purposive sampling (major pepper producing) was used to select ten localities from Bako Tibbe, and five localities from Nonno districts. Systematic sampling was applied to select farm field in each locality. Following all accessible roads, every hot pepper field 5-10 from the first spot/field was assessed. In the selected field, the assessment was made along the two diagonals (in an ''X'' fashion) using 1m^2 quadrants at approximately 20-50 m far from each other (Subbarao, 2003). Plants within the quadrant were counted and recorded as diseased/infected and healthy/non-infected. The incidence of wilt disease was calculated by using the number of infected plants and expressed as a percentage of the total number of plants assessed (Mehrotra and Aggarwal, 2003). For each surveyed field, supplementary information on place of collection, plant growth stage, pepper variety and soil type was collected. Global Positioning System (GPS) was used to record latitude of the area.

Sample Collection: Pepper plants showing wilt disease symptoms were uprooted and kept separately into polythene bags and placed inside the ice box of about 4^{0}C and brought to Ambo Plant Protection Research Center Laboratory for isolating disease causing bacterial and fungal pathogens.

Preparation of Culture Media

Bacteria: Three types of media were used for bacterial identification which includes Nutrient Agar (NA) medium (Beef extract 3.0 g, Peptone 5.0 g, Agar 15.0 g)/litter, King's B medium (Peptone 20.0 g, Glycerol 15.0 ml, K_2HPO_4 (anhydrous) 1.5 g, $MgSO_4 \times 7H_2O$, Agar 15.0 g)/litter and 2, 3, 5-triphenyltetrazolium chloride (TTC) media (Peptone 10.0 g, Casein hydrolysis 1.0 g, Glycerol 5 ml, and Agar 15.0 g)/litter. All the media were autoclaved for 20 minutes at 120 lb pressure. In TTC medium, 5 ml of 1%, 2, 3, 5 triphenyl tetrazolium chloride was added to the sterilized one liter medium before pouring.

Fungal: For identification of fungal pathogen, Potato Dextrose Agar (PDA) was pepared by diluting 39 g of PDA agar in 1000 ml of distilled water. This was autoclaved for 20 minutes at 120 lb pressure. To restrict

bacterial growth, PDA was amended with 100 mg/lt streptomycin, then poured into Petri plates.

Isolation and Identification

Bacteria: The stem and root of the wilted plants were cut into small pieces, surface sterilized with 70% alcohol and then rinsed in three changes with sterilized distilled water. Finally, the samples were macerated by pestel and mortar, then one loop full of the suspension was taken and streaking on the fore mentioned different media's and incubated in inverted position at 30^0C. After 24-36 hr, the bacterial colonies in NA and KB media were transferred to broth media amended with 20% glycerol and stored at 4^0C till further use (Dhanasekaran *et al*., 2005). To identify the bacterial species, isolates were subjected to gram reaction and different biochemical tests (Hayward, 1994; Hansen, 2006). TTC media was used for identification of *Ralistonia solanacearum*. Isolates that formed red color on TTC media indicated presence of *Ralstonia solanacearum* (Hugh and Leifson, 1953; Kelman *et al*., 1994; Zubeda and Hamid, 2011).

Biochemical Tests

Catalase Test: Catalyst test was carried out by mixing a loop full of a fresh bacterial culture with 2 drops of solution of ($3\%H_2O_2$) on the microscope slide according to method described by He *et al*. (1983). Presence of bubble indicated for catalase positive.

Oxidase Test: Oxidase activity was detected by the method of Kovacs (1956). Freshly grown (24 to 48 h) cultures from nutrient agar with 1% glucose were pathched onto a filter paper moistened with a fresh oxidase reagent (1% w.v aqueous solution of tetramethyl-para-phenylene diamine dihydrochloride) using a wood stick. A purple reaction in 30 s was recorded as oxidase positive (Sands, 1990).

Tween 80 Hydrolysis Test: 10 g of Peptone, 5 g of NaCl, 0.1 g $CaCl_2$, $2H_2O$ and 15 g of Agar were mixed with 1000 ml of distilled water in Erlenmeyer flask and heated to dissolve completely and 10 ml tween 80 also autoclaved separately then after, added to the medium then poured. From a fresh broth culture a loop full was taken and transferred on the agar medium by using spot inoculation method and incubated at 30^oC for up to seven days (Fahy and Hyward, 1983). An opaque zone of crystals around a colony was recorded as positive reaction for hydrolysis of Tween 80 positive (Sands, 1990).

Starch Hydrolysis Test: 5g of 2% soluble starch added in nutrient agar medium was melted and poured into the sterile Petri dishes and solidified. Then after by using sterile technique, it made single streaks inoculation of each bacterium at the center of its plate and incubated at 30^oC until heavy growth occurred an inverted position then flooded the surface of the plates with iodine solution with a dropper for 30 seconds. Finally, if a clear zone around a colony was recorded as positive reaction positive (Sands, 1990).

Fungal: Washed and sterilized infected roots and stem were placed in PDA medium. After five days of incubation at 25± 2°C, colonies of fungus were transferred to fresh PDA media. Sub culturing was done till distinct/pure culture was obtained. Identification of fungi was done based on the cultural characterstics and microscopic examination using the Standard manuals (Ellis, 1976; Bamett and Hunter, 1999). The procedure adopted by Ukeh *et al*. 2012 was used to determine the percentage of occurrence of different bacterial and fungal isolates in the culture.

$$\text{Percentage of occurrence} = \frac{X}{N} \times 100$$

where X**=** Total number of each organism in all the samples, N**=** Total number of the entire organisms in all the samples tested.

Pathogenicity Test: The test was carried out to confirm the ability of various isolated microorganisms to infect and cause wilting symptom apparently on healthy pepper seedling of cultivar, Mareko Fana. For the test, except *Rhizopus,* all the isolated microorganisms (*Ralstonia solanacearum*, *Fusarium spp., Verticillium spp.*, *Rhizoctonia solani* and *Phytophthora spp.*) were used for the reason that, species in these genera are reported to be wilt causing pathogens by Mimura and Yoshikawa (2009), Khalifa (1991), Bhat *et al*. (2003), Sadeghi *et al. (*2006) and Babadoost (2004), respectively. The experiment was carried out in the green house of Ambo Plant Protection Research Center which was maintained at a temperature of 25-30^oC. Five seedling of four weeks old (3-5 leave stages) were transplanted into 20x60 cm sized pot filled with the mixture of top soil, humus and sand at a ratio of 3:2:1, respectively (Mulgeta, 1996: Seleshi, 2011) The experiment was carried out in randomized complete block design (RCBD) with three replications. For further confirmations the same experiment was repeated twice. The pathogenicity test of the identified fungal and bacterial pathogens was done according to the procedures developed for the specific pathogens. In all the cases, un-inoculated pots

were used as a control. Plants which developed identical symptoms same like those which exhibited in field during field survey were subjected to re isolation and identification process to fulfill the Koch's postulate.

Ralstonia solanacearum: The Pathogenicity for *R. solancearum* was carried out according to the procedure developed by Mimura and Yoshikawa (2009). For this, infested soil was prepared by pouring 4mL of (4×10^8cfu/ml) the bacterial cell suspension into a planting hole**,** made at the center of a 20cm diameter plastic pot**.** The inoculated plants were observed for wilting symptoms for 6 weeks in the greenhouse. Plants having at least one wilted leaf were classified as wilted. The proportion of wilted plants (PW) was scored at weekly intervals for 6 weeks (Mimura and Yoshikawa, 2009).

Fusarium: The test was performed using the procedure developed by Khalifa (1991). Conidial suspensions of 6.4x106conidia/ml were used to infest the soil by pouring 4ml conidia in to the center of planting pot.

Verticillium: Pathogenicity of *Verticillium* was determined by dipping roots of four-week old seedlings of hot pepper cultivars in the conidial suspension of $8x10^8$conidia/ml for 30 min (Bhat *et al*., 2003). For the control, non-inoculated seedlings were dipped in sterile distilled water.

Phythophtora: Using the procedure developed by Babadoost (2004) suspension was used for the Pathogenicity test. The sporangial suspension was made in a known amount (100ml) of sterile distilled water. Spore concentration of $3x10^6$sporangia/ml was used for inoculation to prove the Pathogenicity. Root inoculation was done by pouring 4ml of sporangia per pot to the center of 20cm in diameter pot and mix well and watering still enough saturation of soil around the root zones of rooted seedlings for transplanting seedlings for the evaluation of resistance, each plant was scored for Phytophthora blight symptoms.

Rhizoctonia soloni**:** The procedure used by Sadeghi *et al.* (2006) with little modification was used for the test. Pieces of agar with actively growing hyphae of *R. soloni* were places in 25ml Erlenmeyer flasks that contain a presoaked and autoclaved (at 121^0C for 30 min at 15 1b) wheat seed. The flasks were incubated at 25 ^{0}C for nearly a month till full fungal mat developed. The infected medium in one flask was crushed thoroughly and place in a pot that contained the above soil mixture.

RESULTS AND DISCUSSION

Field Survey: Pepper wilt was observed in all the surveyed localities, which falls in altitude of 1575-1690 m.a.s.l., with varied disease intensity. During the survey, a total of 120 hot pepper fields were assessed, of which, 60% at full ripening stage, 35% of the crop was at pod setting to ripening stage, 5% at late flowering stage. The results of assessment indicated that the intensity of wilt disease varied across location and soil type. Pepper wilt was observed on 69 (98.57%) of the 70 hot pepper fields assessed in Bako Tibbe and 47 (94%) out of 50 hot pepper fields inspected in Nonno district (Table 1). The average disease incidence in Bako Tibbe district was 93.81%, while that in Nonno district was 78.66%. The difference in disease incidence might be due to the difference in the prevailing weather conditions in the two areas. Momol *et al*. (2008) and Joshi *et al*. (2012) reported that wilt disease is influenced by environmental conditions like soil temperature, soil moisture, soil type which influences soil microbial populations.

During the survey, the soil under cultivation was grouped into clay loam, sandy clay loam and sandy loam (Table 2). The highest disease prevalence (100%) and incidence (76.05%) were recorded in clay loam soil. The disease prevalence and incidence was 97.05% and 63.05% for sandy clay loam and 75% and 37.85% for sandy loam soil, respectively. Groenewald (2005) and Joshi *et al*. (2012) reported that, wilt diseases caused by different fungal and bacterial pathogens are among the major constraints of *solanaceous* crops (potato, tobacco, pepper and eggplant) production, but their intensity varied depending on the soil temperature, moisture and its water holding capacity. Therefore, the observed high disease intensity in the clay loam soil might be due to high water holding capacity that may indirectly favor wilt causing pathogens in the area.

Identification of Wilt Causing Bacterial and Fungal Pathogens

Bacterial Pathogen: From the diseased pepper sample culture in the Nutrient Agar and King B media, based on the gram reaction and different biochemical tests (Table4) only one wilt causing bacteria pathogen, *Ralistonia solancearum* was identified with 100% frequency of occurence. The same samples were also cultured in TTC media that uses to specifically identify *R. solancearum*. Thus, the observed colour change to red in all of the 50 TTC media indicated the presence of this pathogen in all the diseased pepper samples.

Table1. Hot pepper wilt prevalence and incidence across locations in 2012.

District	Altitude Range (m.a.s.l)	No. of fields inspected	No. of fields exhibited the disease	Prevalence (%)	Mean Incidence (%)
Nonno	1575-1626	50	47	94	78.66
Bako Tibbe	1602-1690	70	69	98.57	93.81
Total	1575-1690	120	116	96.7	86.4

Table 2. Prevalence and incidence of hot pepper wilt across soil types.

Soil type	No. of fields Assessed	No. of fields exhibited disease	Wilt Prevalence (%)	Wilt Incidence (%)
Clay loam	74	74	100	76.05
Sandy clay loam	34	33	97.05	63.05
Sandy loam	12	9	75	37.85

The pathogenicity tests that were carried out to confirm the ability of the isolated microorganism to infect and cause wilting symptom apparently on healthy pepper cultivar Mareko Fana confirmed that the bacterial isolate *R. solanacearum* caused wilt disease in pepper (Table 3). Hayward (1995) reported that *R. solanacearum* is the causal agent of bacterial wilt in many plants. Similarly French and Sequeria (1970) reported that, wilt caused by *R. solanacearum* is a major constraint in the production of several important crops, particularly *solanaceous crops* such as potato, tomato, eggplant, pepper and tobacco. In Ethiopia, bacterial wilt was recorded on potato, tomato and eggplant in many regions (Yaynu, 1989). Moreover, pepper plants infected with *R. solanacearum* was observed in Ethiopia. This disease was reported earlier around Bako area (BARC, 1999/2000). Fikire (2006) reported that pepper wilt was caused by *R. solanacearum* around Ziway. The finding of this study supports the importance of the disease (100% frequency) in these areas. The host range of the bacterium is exceptionally wide and many important crops as well as many weed hosts were recorded (Hyward, 1991). The pathogen is able to survive in the soil for long periods in the absence of host plant.

Table 3. Effect of pathogenicity test after inoculation of different fungi and bacteria in pepper in greenhouse.

Pathogens	Wilted plants (%)					
	Days after inoculation					
	25	32	39	46	53	60
Ralistonia solanacearum	6.67	46.67	73.00	73.33	73.33	73.33
Rhizoctonia solani	10.00	20.00	20.00	40.00	70.00	90.00
Fusarium spp.	13.33	26.67	53.33	53.33	46.67	80.00
Verticillium spp.	0.00	13.33	26.67	33.33	46.67	53.33
Phytophthora spp	0.00	0.00	0.00	46.67	46.67	66.67
Control (uninoculated)	0.00	0.00	0.00	0.00	0.00	0.00

Table 4. Gram staining and biochemical characterization of *Ralstonia solanacearum.*

S. No.	Gram/biochemical test	Reaction
1.	KOH Solubility	+ve
2.	Catalase	+ve
3.	Starch Hydrolysis	+ve
4.	Oxidase	+ve

Fungal Pathogens: The investigation revealed that from 50 wilted plant sample cultures in PDA media, a total of two hundred eighty six different fungal cultures were obtained. The fungi isolated were identified based on their typical colony characteristics (Figure1). The percentage frequency of occurence of *Rhizoctonia solani, Fusarium* spp., *Phytophthora* spp. and *Verticillium* spp. regardless of the districts were 45.0, 17.48, 12.59 and 11.89, respectively. The pathogenicity test confirmed that, the tested fungal isolates of the above identified pathogens were the causal agents of pepper wilt in the area (Table 3). BARC reported that wilt disease of hot pepper in Bako and Nejo areas was caused by *Rhizoctonia solani* and *Fusarium spp.* (BARC, 1999/2000). Similarly Babu *et al*. (2011) and Yoon *et al*. (1989) reported that wilt was also caused by

Phytophthora capsici, another devastating soilborne disease of *Capsicum* crops. *Verticillium* wilt is a serious disease of a large number of diverse plants including pepper. The causal agents, *Verticillium alboatrum* and *V. dahlia*, are ubiquitous, soilborne pathogens. The disease incidence and severity vary from year to year and from location to another. The disease significance also varies with host susceptibility, pathogen virulence, soil type, and environmental conditions (Goldberg, 2010).Thus it could be inferred that wilt disease in pepper in the two districts was caused by more than one pathogen, thus management strategies should focus on these fungal and bacterial pathogens. Host plant resistance is the best option to manage pepper wilt from its economic and environmental point of view. Thus, the currently available pepper cultivars should be tested for the identified wilt causing pathogens. Besides, the hot pepper improvement program has to focus on developing resistance cultivars strategy against the wilt causing complex pathogens.

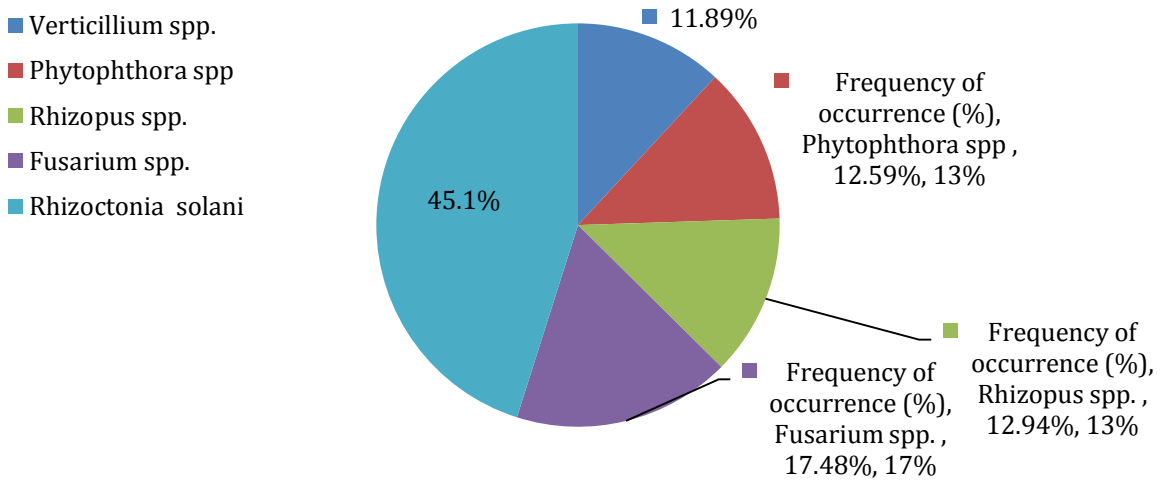

Figure 1. Frequency of fungi isolated from wilted pepper plants.

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IMPACTS OF SUNNHEMP AND PIEGON PEA ON PLANT-PARASITIC NEMATODES, *RADOPHOLUS SIMILIS* AND *MELOIDOGYNE* SPP., AND BENEFICIAL BACTERIVOROUS NEMATODES

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A B S T R A C T

Plant-parasitic nematodes such as burrowing nematode (*Radopholus similis*) and root-knot nematode (*Meloidogyne* spp.) are dominant in the banana, *Musa* spp., ecosystem. Beneficial nematodes such as bacterivores are also found in banana fields. A tropical cover crop, sunn hemp (*Crotalaria juncea*) (SH), can be used to suppress plant-parasitic nematodes and enhance beneficial bacterivorous nematodes. However, SH cultivation in Hawaii is under the threat of the flour beetle. Thus, two experiments: Trial-I and Trial-II were conducted to compare the effects of another tropical cover crop, pigeon pea (*Cajanus cajan*) (PP) with SH and no-cover crop control (CC) on *R. similis* and *Meloidogyne* spp. suppression and beneficial bacterivorous nematodes enhancement. In both experiments soils infested with *R. similis* and *Meloidogyne* were sampled and amended with cover crop treatments (SH or PP) or CC and kept for two weeks. At the end of each experiment, nematodes were extracted through the Baermann funnel technique. The results of Trial-I and Trial- II showed that SH and PP did not reduce *R. similis* number ($P > 0.05$). However, *Meloidogyne* numbers were reduced by SH and PP in Trial-I ($P < 0.05$). In Trial II, *Meloidogyne* was not found in SH and PP. In both experiments, SH consistently increased beneficial bacterivorous nematodes number ($P < 0.05$). Cover crop PP increased beneficial bacterivorous nematode numbers in Trial -I ($P < 0.05$), but not in Trial –II ($P > 0.05$). However, the trends associated with the numbers of beneficial nematodes were consistently higher in PP compared to CC. Farmers could choose PP as an alternate to SH, as a cover crop for *Meloidogyne* suppression and beneficial nematode enhancement.

Keywords: *Cajanus cajan, Crotalaria juncea,* free-living nematodes*, Radopholus similis,* root-knot nematode.

INTRODUCTION

The banana, *Musa* spp., is an important food crop in the world and a staple crop in many tropical regions (Sharrnock and Frison, 1999). Within the US, Hawaii ranks number one in banana production. However, the banana production of Hawaii has been declined since 2000 mainly because of the attack of *Banana bunchy top virus* (BBTV) and plant-parasitic nematodes (Wang and Hooks, 2009). Multiple plant-parasitic nematodes such as root-knot nematode, *Meloidogyne* spp. (Wang and Hooks, 2009) and burrowing nematode, *Radopholus similis* (Cobb) Thorne (McIntyre *et al.,* 2000; Wang and Hooks, 2009) are associated with the production decline of banana.

Plant-parasitic and beneficial nematodes such as bacterivores are closely associated on banana fields. In contrast to plant-parasitic nematodes, beneficial nematodes such as bacterivores and fungivores have a role in soil nutrient cycling and soil health improvement (Wang and McSorley, 2005). Higher number of bacterivorous nematodes is an indicator of a healthy soil (Wang and McSorley, 2005; Marahatta *et al.,* 2010). Nematode management for Hawaiian banana should include a strategy for suppressing *R. similis* and *Meloidogyne*, and improving the bacterivorous nematode population.

Tropical cover crop sunn hemp, *Crotalaria juncea* L., (SH) is a poor host or non-host of multiple plant-parasitic nematodes (Wang *et al.,* 2002) and SH helps to enhance beneficial bacterivorous nematodes (Wang *et al.,* 2002; Wang *et al.,* 2011; Marahatta *et al.,* 2012a). Furthermore, SH produces an allelopathic compound, monocrotaline, after incorporating its foliage in soil. This

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allelopathic compound, monocrotaline, is responsible for killing *Meloidogyne* and *R. similis* (Wang *et al.,* 2002). Thus, SH has been used in suppressing the plant-parasitic nematodes of banana and several other crops such as bitter melon (Marahatta *et al.,* 2010), pumpkin (Wang *et al.,* 2011) and pineapple (Marahatta *et al.,* 2012b) and enhance beneficial bacterivorous nematodes (Wang *et al.,* 2002; Wang *et al.,* 2011). However, a pest insect, flour beetle, can cause SH wilting and is a challenging problem of SH, if multiple cycles of SH are grown on the same field (K.-H. Wang, personal communication, 2012). Therefore, researchers have initiated to evaluate other leguminous cover crops such as pigeon pea, *Cajanus cajan* (L.) Millsp., (PP) for managing multiple plant-parasitic nematodes in the tropics. The cover crop PP is commonly used for soil conservation and is popular among organic farmers of Hawaii. Furthermore, PP has been documented as *Meloidogyne* suppressive plant (Reddy *et al.,* 1986). As a leguminous cover crop, PP produces a lot of foliage, which can be used as a soil amendment material and could enhance beneficial nematodes. Therefore, current research compares the effects of SH and PP on 1) *R. similis* and *Meloidogyne* spp. suppression and 2) beneficial bacterivorous nematode enhancement.

MATERIALS AND METHODS

Two laboratory experiments were conducted at Kauai Community College, Lihue, HI in Spring 2013 (Trial – I) and in Summer 2013 (Trial- II). In both experiments, Trial I and Trial II, soil infested with *Radopholus similis and Meloidogyne* spp. was collected from an established orchard planted with the tall variety of banana at S & F Takahashi Farm, Kalaheo, Kauai, HI, USA. The field soil collected from 15cm.-depth was composited and well-mixed in a large plastic bag. In each experiment, the final weight of composite soil sample was about 5 kg.

At starting of Trial I, soil nematodes were extracted using Baermann funnels (Walker and Wilson, 1960) and determined the initial nematode population (Pi). The Pi for *R. similis* and *Meloidogyne*/100 cm^3 soil, were 64 and 16 second stage juveniles (J2), respectively. After knowing the Pi, effects of sunn hemp (SH) and pigeon pea (PP) on *R. similis* and *Meloidogyne,* and bacterivorous nematodes were tested and results were evaluated in Trial I and Trial II.

In each experiment, *R. similis* and *Meloidogyne* infested field soil was potted into fifteen 7.62 cm-diameter planter pots. Each pot was filled with 300 cm^3 soil and immediately incorporated with (SH or PP) or without (CC) dry cover crop foliage powder at 1.0% (w/w). Cover crop foliage powder used for soil amendment was prepared with SH or PP plants grown at Kauai Community College Farm. These SH and PP were oven-dried at 72°C for three days to a constant weight and ground with a commercial blender (Winsted Conn, Waring Products Co., CT) into powder form (Marahatta *et al.,* 2012b). Each potted soil weight averaged 178.95g /pot. In each experiment, of the fifteen total pots, there were 5 of CC, SH and PP, respectively. Thus, the experiments were replicated five times and arranged in the randomized complete block design (RCBD).

The pots were left to incubate for 7 days before the Baermann funnel technique was used for nematode extraction. Fifty mL of water was used per Baermann funnel and left for additional 7 days to extract nematodes (Walker and Wilson, 1960). The extracted dominant plant-parasitic nematodes, *Meloidogyne* and *R. similis* and beneficial bacterivorous nematodes (Yeates *et al.,* 1993) such as Rhabditidae, *Cephalobus*, and *Eucephalobus* were identified whenever possible and counted using an inverted microscope (Fluovert, Leitz Wetzlar, Germany).

Statistical Analysis: Data were subjected to one-way analysis of variance (ANOVA) using the general linear model (GLM) procedure in Statistical Analysis System (SAS Institute, Cary, NC). Nematode abundance were log (x +1) -transformed accordingly to PROC UNIVARIATE in SAS prior to ANOVA. Untransformed arithmetic means are presented. Means were separated by Waller-Duncan k-ratio (k=100) t-test wherever appropriate.

RESULTS AND DISCUSSION

In Trial I and Trial II, the following effects of SH and PP were found at the termination of experiment (after 14 days from starting the experiment).

Effects on *Meloidogyne:* *Meloidogyne* was the most prevalent and damaging nematode at the soil sampled site. Before the beginning of the experiment, nematode population densities were not different among treatments (data not shown). In Trial I, compared to CC, SH and PP reduced *Meloidogyne* number (P < 0.05). In Trial II, *Meloidogyne* was not found in SH and PP (Figure 1).

In current experiment, compared to no cover crop amended treatment CC, SH and PP consistently reduced or showed a lower population trend of *Meloidogyne*. These results are consistent with the earlier findings where soil incorporated with SH 'Tropic Sun' (Wang *et al.,* 2011) and

PP 'FL81D' and 'Norman' (Reddy *et al.,* 1986) reduced soil population of *Meloidogyne* in comparison to bare ground fallow plots in Hawaii and Florida, respectively. *Meloidogyne* suppression results of SH and PP of current experiment shows the importance of allelopathic properties: yellow resinous microscopic secretions of PP (Rizvi and Rizvi, 1992) and monocrotaline of SH (Rodriguez-Kabana *et al.,* 1992; Wang *et al.,* 2001; Jourand *et al*., 2004) in the management of the most prevalent plant-parasitic nematode. Current results validate the *Meloidogyne* suppressive characteristics of SH and PP in banana grown soil. Furthermore, this result has demonstrated that powdered form of SH or PP could be used and effectively suppressed *Meloidogyne* population in absence of field grown cover crops.

Effects on *Radopholus similis:* At the soil sampled site, *R. similis* was the second most prevalent plant-parasitic nematode. Compared to no cover crop treatment CC, both cover crops, SH and PP, consistently did not reduce *R. similis* number ($P > 0.05$) at termination of Trial I and Trial II (Figure 2).

In contrast to the suppressive effects of SH and PP on *Meloidogyne*, there was no significant results in reducing the *R. similis* number in either of the two trials. However, the trends of *R. similis* number found in SH and PP in Trial I and Trial II are consistent with the findings of Chitamba *et al.* (2014) where banana and SH intercropped treatment suppressed *R. similis* in a glasshouse experiment.

Effects on Beneficial Bacterivorous Nematodes: In Trial I, higher numbers of bacterivorous nematodes were found in SH followed by PP ($P < 0.05$). This effect of SH ($P < 0.05$), not PP ($P > 0.05$), on bacterivorous nematodes number was consistent in Trial II (Figure 3). Rhabditidae was the most dominant bacterivorous nematode consistently found in Trial I and Trial II. Other bacterivorous nematodes found in both experiments were *Cephalobus*, and *Eucephalobus* (data not presented).

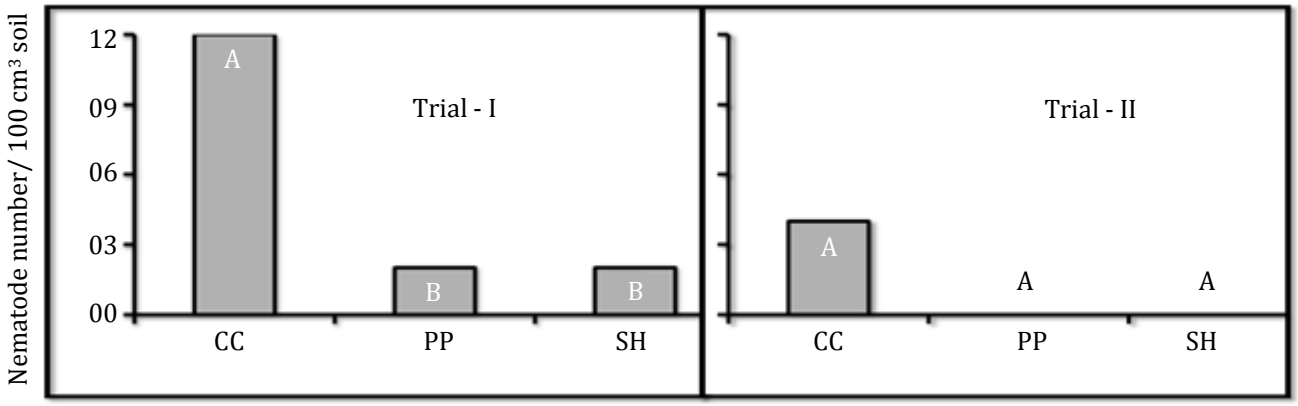

Figure 1. Effects of soil incorporated cover crops, sunn hemp (SH) and pigeon pea (PP), and no cover crop control (CC) on abundance of *Meloidogyne* spp. at termination of the experiment. Means are average of 5 replications. Means followed by same letter(s) do not differ according to Waller-Duncan *k*-ratio ($k = 100$) *t*-test based on log ($x+1$) transformed values.

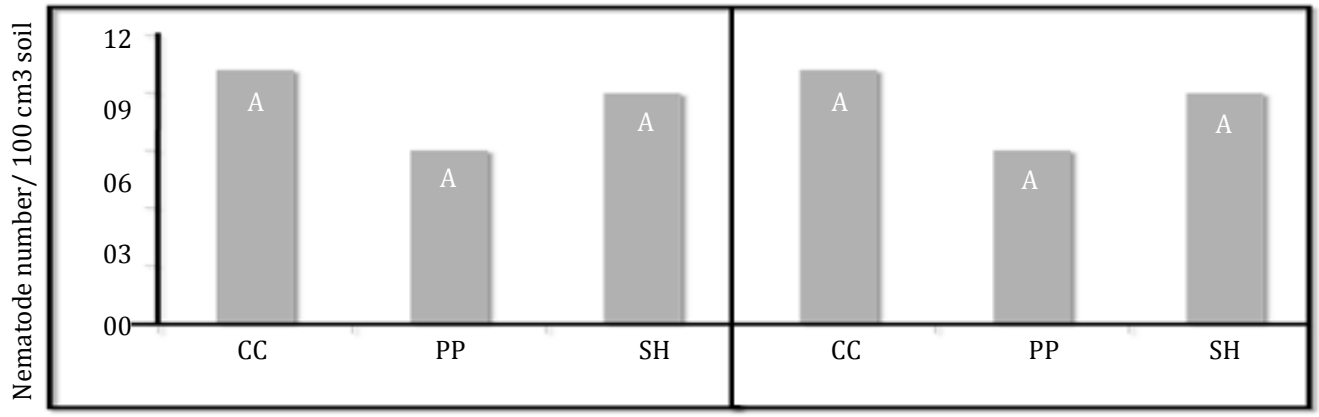

Figure 2. Effects of soil incorporated cover crops, sunn hemp (SH) and pigeon pea (PP), and no cover crop control (CC) on abundance of *Radopholus similis* at termination of the experiment. Means are average of 5 replications. Means followed by same letter(s) do not differ according to Waller-Duncan *k*-ratio ($k = 100$) *t*-test based on log ($x+1$) transformed values.

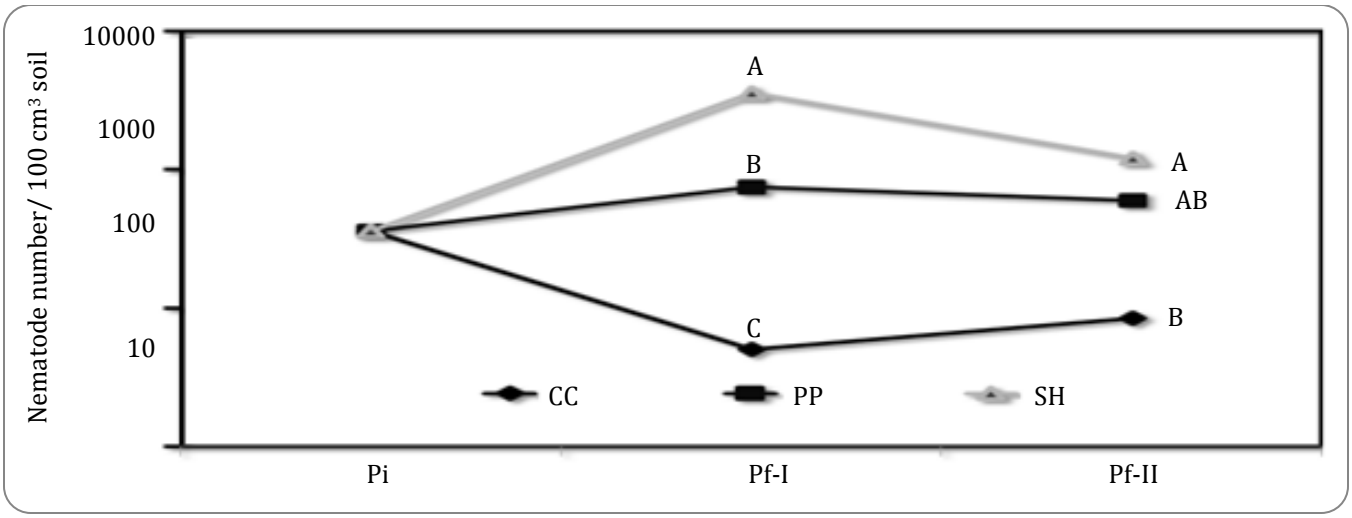

Figure 3. Effects of soil incorporated cover crops, sunn hemp (SH) and pigeon pea (PP), and no cover crop control (CC) on abundance of beneficial bacterivorous nematodes at starting (Pi) and termination (Pf- I and Pf- II for Trial-I and Trial-II, respectively) of the experiment. Means are average of 5 replications. Means followed by same letter(s) in each soil sampling date do not differ according to Waller-Duncan *k*-ratio (*k*=100) t-test based on log (*x*+1) transformed values.

Abundances of beneficial bacterivorous nematodes is often used as an indicator of soil nutrient enrichment (Bongers and Bongers, 1998; Ferris *et al.,* 2001). In the current experiment, SH followed by PP consistently increased the abundances or population trend of bacterivorous nematodes and demonstrated that cover crop incorporated treatments were enriched with nutrients. This result of SH is consistent with the previous findings where SH incorporated field plots consistently enhanced bacterivorous nematodes in strip-tilled SH cover cropping system (Marahatta *et al.,* 2010; Wang *et al.,* 2011). Additionally, the current experiment had demonstrated PP as a potential cover crop for enhancing soil nutritional status after SH, as indicated by beneficial nematode numbers. Thus, a higher population of bacterivorous nematodes, and a lower population of *Meloidogyne* on SH followed by PP incorporated pots indicate two simultaneous benefits of both tropical cover crops.

CONCLUSION

Both cover crops, SH and PP, did not suppress *Radopholus similis*, but suppress or show a trend of lower *Meloidogyne* number compared to CC. Compared to CC, PP enhanced beneficial nematodes in Trial I, and show a trend of higher number of beneficial nematodes in Trial II. Although SH had a greater effect in increasing beneficial nematode populations in both trials, farmers are recommended to use PP and SH alternately to protect the crop from flour beetles.

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SURVEY AND GENETIC DIVERSITY OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS-2 IN ALGERIA

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A B S T R A C T

Vineyards in western and center regions of Algeria were surveyed for the *Grapevine leafroll-associated virus 2* (GLRaV-2). Analyses by DAS-ELISA and Reverse Transcription Polymerase Chain Reaction (RT-PCR) reveal 15, 8% prevalence. The genetic diversity of the GLRaV-2 population was studied by phylogenetic analyses of the HSP70h gene region of seven samples sequenced in this study and other sequences downloaded from GenBank. Results reveal segregation of the GLRav-2 population into six distinct groups. An estimation of the ratio of non-synonymous substitutions per non-synonymous site to synonymous substitutions per synonymous site indicated that HSP70h gene evolve under positive selection. Similarity plot constructed with representative sequence from each group confirmed previous results. All Algerian isolates belong to group PN. As far as we know, this is the first characterization of GLRaV-2 isolates from Algeria.

Keywords: GLRaV-2, RT-PCR, genetic diversity, HSP70h gene.

INTRODUCTION

The grapevine leafroll disease represents the most widespread disease of *Vitis vinifera* worldwide (Martelli, 2014). It may be due to several virus belonging to the family of *Closteroviridae* which were designated as G*rapevine Leafroll-associated Viruses* (GLRaVs) and represented by GLRaV-1, -2, -3, -4, and -7 which were recognized as species and recently (Martelli *et al.*, 2012) indicate that GLRaV-5, -6, -9, GLRaV-Pr, GLRaV-De, and GLRaV-Car are strains of GLRaV-4, considered before as distinct species. All these viruses belong to the family *Closteroviridae*, with GLRaV-2 belonging to the genus *Closterovirus*, GLRaV-7 belongs to a newly proposed genus *Velarivirus* and the other GLRaVs to the genus *Ampelovirus*.

GLRaV-2 was described since 1984 and was associated with the grapevine leafroll disease (Gugerli *et al.*, 1984). Later, it was associated to graft-incompatibility (Greif *et al.*, 1995). The transmission vector for GLRaV-2 is unknown, although other members of the genus *Closterovirus* were transmitted by aphids (Karasev, 2000). GLRaV-2 is known to be transmitted by grafting with infected material. However, some isolates of GLRaV-2 were mechanically transmitted to herbaceous hosts like *Nicotiana benthamiana (*Goszczynski *et al., 1996)*.

GLRaV-2 presents flexuous and filamentous particles of about 1600 nm length and its genome is a single strand, positive sense RNA with about 16500nt organized into nine ORF encoding at least 11 proteins (Zhu *et al.*, 1998). Up to now, complete genome sequence was obtained for nine isolates (JX559644.1, NC_007448.1, DQ286725.2, AY881628.1, KF220376.1, FJ436234.1, JQ771955.1, AF314061.1 and NC_004724.1).

Several variants of GLRaV-2 have been described and characterized. (Zhu *et al.*, 1998) and (Abou-Ghanem *et al.*, 1998) described the first two variants from the varieties Pinot Noir and Semillon. Two years later, a new variant denoted GLRaV-2-H4 was discovered by (Ghanem-Sabanadzovic *et al.*, 2000) on *Vitis rupestris* St. George. Further study reveals the presence of virus associated to GLRaV-2 isolated from cv Redglobe and associated to graft incompatibility (Rowhani *et al.*,

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2000). Up to now, six variants were reported, the variant 'Pinot Noir, 93/955, H4, BD, RG and PV20 (Zhu *et al.*, 1998, Meng *et al.*, 2005, Ghanem-Sabanadzovic *et al.*, 2000, Rowhani *et al.*, 2002, Angelini *et al.*, 2004, Beuve *et al.*, 2007, Bertazzon *et al.*, 2010).

MATERIAL AND METHODS

Virus Source: The field study and sample collection were conducted in autumn 2010 and 2012 on table grape and wine grape collected in western (Aïn Témouchent and Mascara) and central (Algiers, Tizi-Ouzou and Boumerdes) regions of Algeria. A total 584 samples were collected from individual vines from different varieties (30 varieties) including commercials (10 varieties) (445 samples) autochthonous vineyard (two varieties) (110 samples) and autochthonous Grapevine germpalsm (18 varieties) (29 samples) of ITAF (Institute Technique de l'Arboriculture Fruitière et de la Vigne). Mature canes were randomly collected, one from each vine and stored at 4°C.

Virus detection by double antibody sandwich-enzyme-linked immunosorbent Assay (DAS-ELISA): All collected samples were tested by DAS-ELISA (Clark and Adams, 1977), for the presence of GLRaV-2 using specific commercial polyclonal antiserum (Agritest, Bari, Italy). The extracts were obtained by macerating phloem tissues in the PBS-buffer (V:V). Optical density was recorded at 405 nm using an automatic microplate reader (Multiskan Ascent, Labsystems, Waltham, MA, USA). Samples with Optical density readings exceeding or equal to three times that of the healthy samples were considered positive.

Virus detection by reverse transcription polymerase chain reaction (RT-PCR): All positive samples were analyzed by RT-PCR. Total nucleic acids were extracted from 0.2g of cortical scraping of dormant cutting cane, which was powdered using liquid nitrogen. The powder was homogenized in 1 ml of grinding buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate, 1 M potassium acetate, 0.025 mM EDTA, 25% PVP-40) mixed with 100µl NLS 10% and denaturized at 70°C. The solution was centrifuged and the supernatant was recovered. The Total Nucleic Acid was precipitated by Silica as described by (Svanella-Dumas *et al.*, 2000) with some modifications. Two step protocol was used for the reverse transcription (RT) and amplification (PCR) of target RNA. Reverse transcription was performed using 1 µl of Moloney murine leukaemia virus reverse transcriptase (M-MLV 200 units/ µl), 4 µl of 5× Fs M-MLV buffer, 2 µl of DTT (0.1 M) and 0.5 µl of dNTPs (10 mM). The mixture was incubated at 39°C for 1h and at 70°C for 10 min. PCR was carried out with primer pair L2 F (5′-ATAATTCGGCGTACATCCCCACTT-3′) and U2 R (5′-GCCCTCCGCGCAACTAATGACAG -3′) which encompass 331pb located in the HSP70h gene. The DNA amplifications were carried out in 12,5 µl total reaction volume. 1,25 µl of cDNA were mixed with 11.25 µl of the amplification mixture, consisting of 10 mM each dNTP, 20 µM each primer, 50 mM MgCl2, 5 U/µl TaqDNA polymerase (Promega) and 10x Taq buffer. PCR cycling conditions, in a thermocycler, include denaturation of cDNA at 95°C for 5 min, followed by 35 cycles of 94°C/ 30s, 58°C/45s and 72°C/60 s. To end with, final elongation step was carried at 72°C during 7 min. The PCR products with positive and negative control used were provided from Institut Nationale de la Recherche Agronomique (INRAT) collection and PCR markers of 100pb were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized using an UV-transilluminator.

Sequencing and sequence analyses: Amplified products of 331pb from HSP70h gene region of GLRaV-2 of seven isolates were randomly chosen for sequencing, they were purified using the ExoSAP-IT purification kit. Direct sequencing was performed with the same primers used for RT-PCR on 3730xl DNA analyzer (Applied Biosystems) automated sequencer.

To investigate the different variants of GLRaV-2 population, multiple alignments of nucleotide sequences were performed using CLUSTAL W (Thompson *et al.*, 1994), respectively, with default settings from MEGA6 software (Tamura *et al.*, 2013) and comparison at the nucleotide level for HSP70h gene regions of Algerian isolates and representative sequences of the different phylogenetic groups described up to now was conducted (Table 2).

Phylogenetic analysis: Nucleotide sequences of the Algerian isolates and those downloaded from GenBank were aligned and evolutionary relationships among GLRaV-2 sequences inferred using the neighbor joining method (NJ) (Saitou and Nei, 1987) with 1,000 bootstrap replications. All these analyses were conducted in MEGA6 software with GLRaV-3 isolate BR5 (KF417599.1) used as an outgroup.

Estimation of selection pressure and recombination analysis: Gene and site-specific selection pressures over the entire alignment of data set for HSP70h were analyzed using the Datamonkey online (*http://www.datamonkey.org/*).

Table 1. Percent identity between Algerian isolates and isolates from each group.

Seq->	ALG7	ALG9	ALG19	ALG22	ALG53	ALG55	ALG93	PV20	OR1	GRSLaV	BD	PN	93/955	GLRaV-2-SG
ALG7	ID	98%	99%	97%	98%	99%	99%	74%	98%	71%	75%	99%	86%	85%
ALG9	98%	ID	99%	98%	99%	98%	99%	74%	99%	72%	74%	99%	86%	85%
ALG19	99%	99%	ID	99%	99%	99%	100%	75%	99%	72%	75%	100%	87%	86%
ALG22	97%	98%	99%	ID	98%	98%	99%	74%	99%	72%	74%	99%	86%	86%
ALG53	98%	99%	99%	98%	ID	98%	99%	74%	99%	72%	75%	99%	86%	86%
ALG55	99%	98%	99%	98%	98%	ID	99%	75%	99%	71%	75%	99%	87%	85%
ALG93	99%	99%	100%	99%	99%	99%	ID	74%	100%	72%	75%	100%	86%	86%
PV20	74%	74%	75%	74%	74%	75%	74%	ID	74%	71%	74%	74%	77%	74%
OR1	98%	99%	99%	99%	99%	99%	100%	74%	ID	72%	74%	100%	86%	86%
GRSLaV	71%	72%	72%	72%	72%	71%	72%	71%	72%	ID	81%	72%	71%	72%
BD	75%	74%	75%	74%	75%	75%	75%	74%	74%	81%	ID	75%	74%	77%
PN	99%	99%	100%	99%	99%	99%	100%	74%	100%	72%	75%	ID	86%	86%
93/955	86%	86%	87%	86%	86%	87%	86%	77%	86%	71%	74%	86%	ID	85%
GLRaV-2-SG	85%	85%	86%	86%	86%	85%	86%	74%	86%	72%	77%	86%	85%	ID

The ratio of nonsynonymous substitutions per nonsynonymous site (dN) to synonymous substitutions per synonymous site (dS), which is considered as an indicator of natural selection was calculated using two methods for detecting sites under positive selection: single-likelihood ancestor counting (SLAC), random-effects likelihood (REL). Depending on dN/dS values, the selection pressure was considered negative or purifying ($dN/dS < 1$), neutral ($dN/dS = 1$), or diversifying or positive ($dN/dS > 1$).

Recombinant events analysis of GLRaV-2 population was performed using the GARD algorithm (Kosakovsky *et al.,* 2006) in the remote server Datamonkey (Delport *et al.,* 2010). A similarity plot was constructed with Simplot software (downloaded from; *http://sray.med.som.jhmi.edu/SCRoftware/simplot/*) using the isolate OR1 from group PN as reference sequence with a multiple sequence alignment of full genome sequences provided from the different representative groups (H4, BD, RG, and 93/955) except for PV20 group constructed with MEGA6 (Tamura *et al.*, 2013). Currently, there is no full-length sequence for groups PV20.

RESULTS

Prevalence of GLRaV-2: DAS-ELISA test reveals that the GLRaV-2 presents prevalence of 15,8 % in Algeria. The peak of prevalence is observed in Gros noir des Beni Abbes with 29%. The Alicante Bouchet presents 24,6%, King's Rubi (22,2%), Dattier de Beyrouth (18,7%), Valensi (14,5%), Chaouch Blanc and Muscat d'Alexandrie (10%), Cinsault (7,5%), Carignan (9,7%), Cardinal (6,4%) and autochthonous collection (6,9%). The GLRaV-2 is absent in Merseguerra and Chasselas. The autochthones germplasm show to be free of GLRaV-2.

Sequencing and Sequence Analysis: The 331-nt sequence fragment of the HSP70h Gene obtained by RT-PCR was cloned and sequenced for seven isolates of GLRaV-2 representing the first sequenced isolates provided from Algeria. Nucleotide sequences obtained were submitted to GeneBank (Table 2).

Table 2. Range of HSP70h nucleotide sequence identities within and between groups of GLRaV-2.

	Average distance within groups						Average distance between groups					
Isolates	BD	PN	H4	RG	93/955	PV20	BD	PN	H4	RG	PV20	93/955
Distance	98%	99%	95%	96%	100%	100%	72-78%	70-6%	71-6%	69-78%	69-74%	71-86%

In order to access phylogenetic relationship between these isolates, comparison at nucleotide level was conducted with representative sequences from each group described by (Jarugula *et al.*, 2010). The Algerian isolates present 97% to 100% identities. Comparison with the different groups reveal 98% to 100% identity with the isolates OR1 and PN , 74% to 75% identity with the isolates PV20 and BD, 71-72% identity with GRSLaV, 86-87% identity with isolate 93/955, and 85- 86% identity with isolate GLRaV-2-SG.

The genetic distance, transformed to the percent identity between and within groups was calculated using MEGA6 software with default parameters reveals that the group PN presents 99% identity. The group H4 presents 95% identity, 96%, 98% and 100% identity for RG, BD and 93/955. The between percent identity reveal that the group PN presents 70-86% with all the other groups, 72-78% for group BD, 71-86%, 69-78%, 69-74% and 71-86% respectively for H4, RG, PV20 and 93/95% (Table 2).

Phylogenetic Analysis: Phylogenetic tree constructed using the neighbor joining method (NJ) implemented in MEGA6 software reveals that GLRaV-2 population cluster into six groups. Each of these Lineages were assigned to a reference isolate as described by previous study to maintain a standardized nomenclature of GLRaV-2 sequence variant groups. The groups PN and H4 represent the most important group of this population. The RG group contains isolates provided from the variety Red Glob obtained in California. The PV20, BD and 93/955 groups were less represented. Analyses reveal that all Algerians isolates collected from different regions, vineyards and varieties belong to the group PN. Further prospections in local varieties were needed to get a great understanding of genetic variation of GLRaV-2 in Algeria (Figure 1).

Similarity plot comparison of representative isolates from each group was conducted. Results reveal that the group PN is distant to the other groups and the groups H4 and 93/955 are more and less related, the same result was obtained for BD and RG groups which were found to be closely related (Figure 2). Currently, there are no full-length sequences from representatives isolates of groups PV20.

Selection Pressure: Identifying evolutionary pressure represent a great deal because past environments that exerted these pressures can be different from present ones and these pressures are not unidirectional but rather the result of complex networks (Moury and Simon, 2011).

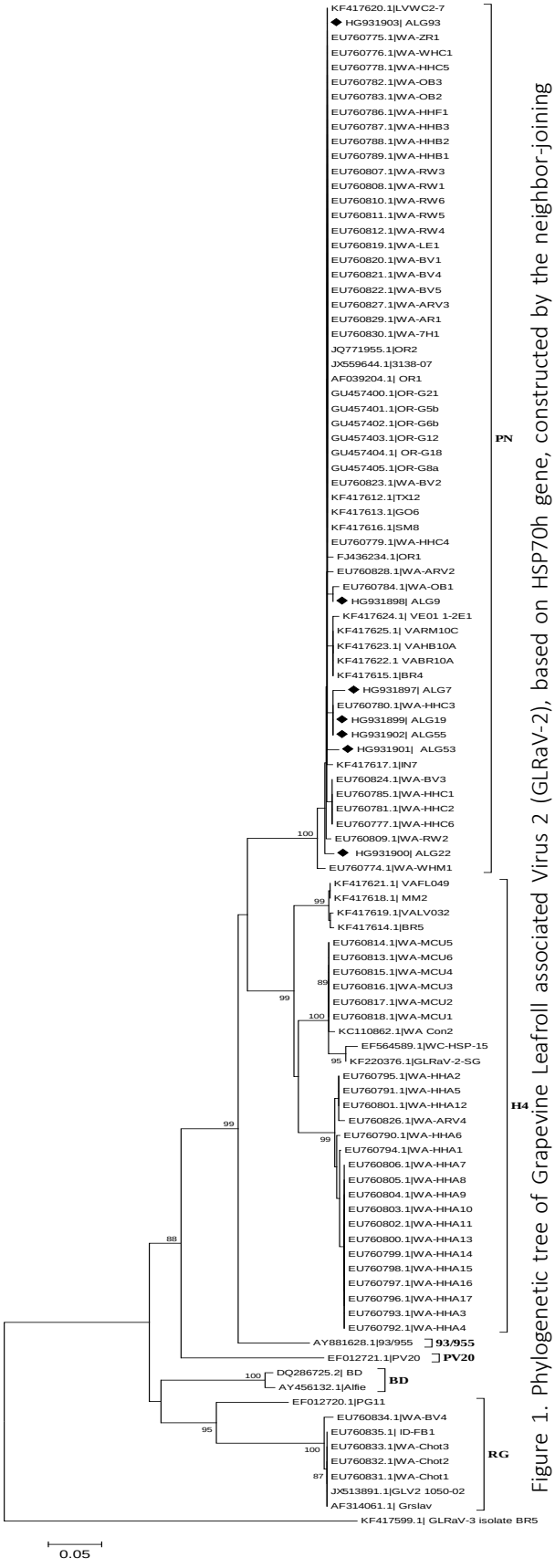

Figure 1. Phylogenetic tree of Grapevine Leafroll associated Virus 2 (GLRaV-2), based on HSP70h gene, constructed by the neighbor-joining method; The percentages of bootstrap support (≥ 75 %) from 1,000 replicates are shown at nodes.

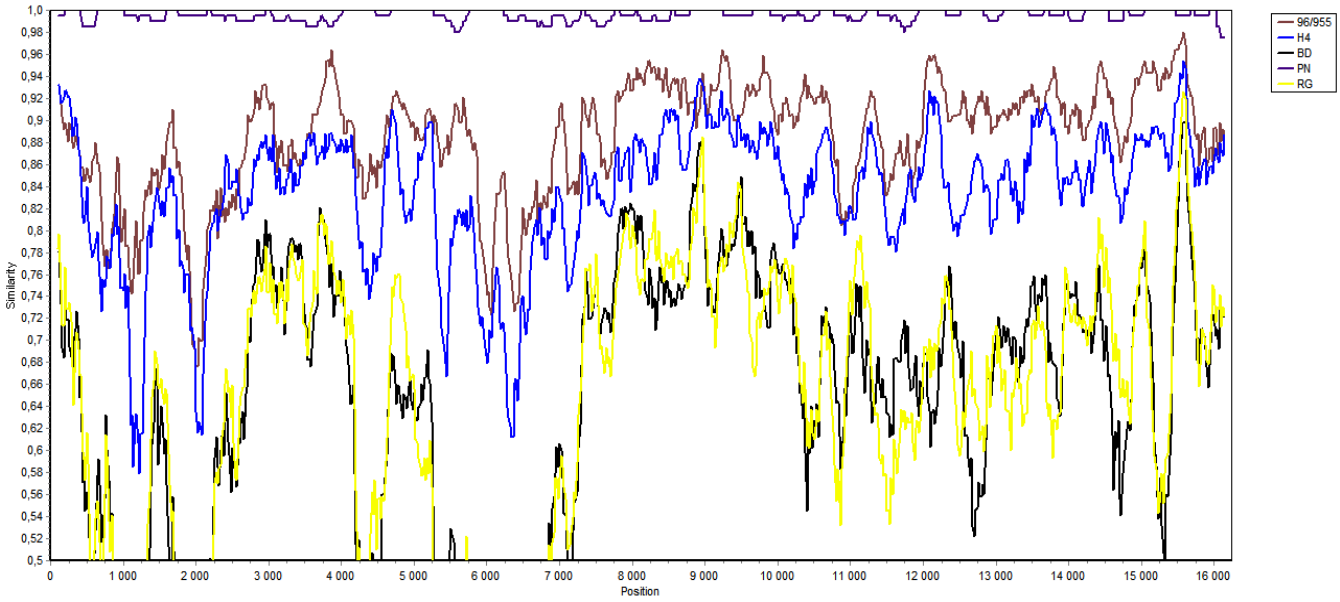

Figure 2. Similarity plot constructed from a multiple alignment of six full-length sequences representing five well-defined variant groups of GLRaV-2 using SimPlot 3.2.

In order to study selection pressure occurring in a GLRaV-2 population, an estimation of the ratio of nonsynonymous substitutions per nonsynonymous site (dN) to synonymous substitutions per synonymous site (dS) was performed. Indeed, dN/dS ratio indicates the magnitude of selective pressure on each codon. When the ratio dN/dS>1 on a certain codon it indicate that the site evolve under positive selection and when dN/dS<1 in another site it indicates that this site evolve under negative selection. The mean value of dN/dS ratio obtained with SLAC and REL methods is upper than unity suggesting the occurrence of positive selection. Indeed, SLAC reveal mean ratio of dn/ds>1 (1,81) with Codon site under positive selection and non-negative selected codon site were found. Approximately, same results were obtained by REL method with mean ratio dN/dS=3,71 with 6 codon under positive selection and 6 codon under negative selection. Plotting of SLAC established by dN-dS for each codon, indicating the negative or positive selection, reveal that the number of codon site under positive selection is more important comforting previous result obtained in this study (Figure 3). Detection of evidence of putative recombination events in the HSP70h sequences of the GLRaV-2 population was performed with the genetic algorithms for recombination detection (GARD) available on the Datamonkey webserver. GARD analysis reveals no recombinant events within a HSP70h gene in the GLRaV-2 population.

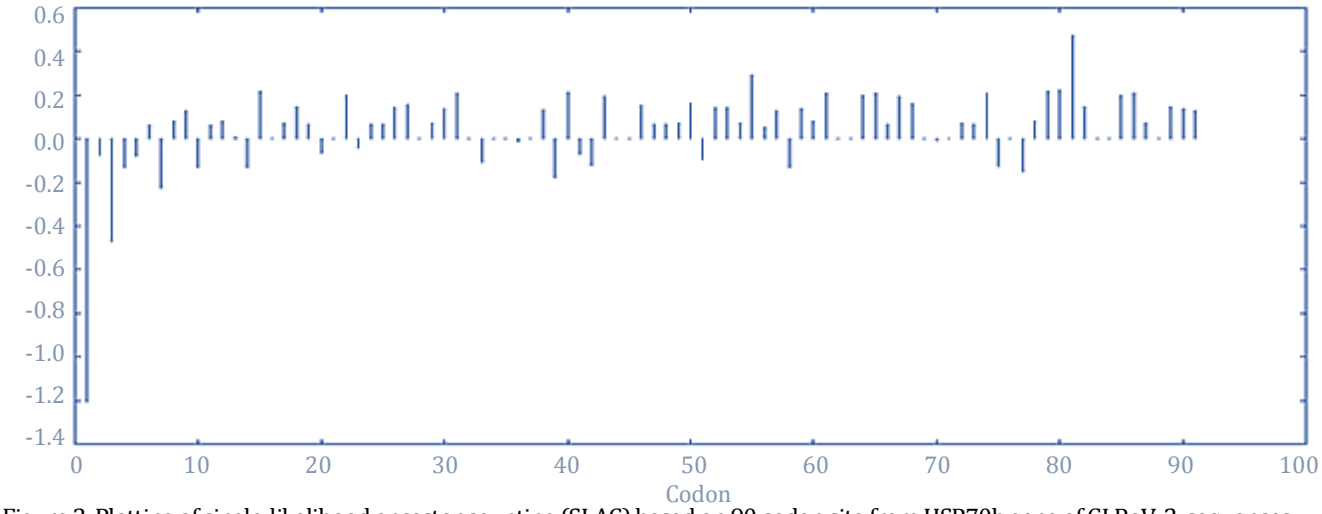

Figure 3. Plotting of single-likelihood ancestor counting (SLAC) based on 90 codon site from HSP70h gene of GLRaV-2 sequences.

DISCUSSION

Survey and genetic diversity of GLRaV-2 collected from different vineyards in the center and western regions of Algeria was established. This study represents the first comprehensive work on the prevalence and genetic diversity conducted in major grape-growing region of Algeria including an autochthonous germplasm collection with first sequencing of Algerian isolates. Phylogenetic analyses with isolates provided from different regions was conducted to make an update of molecular variability of GLRaV-2.

Grapevine leafroll associated virus 2 is present in all prospected regions. This may be due to the use of infected root stock which in majority provide from North America. Indeed, the vector transmission of GLRaV-2 was not described up to now (Martelli, 2014) and some grape nursery were found to present leafroll symptoms, allowing the assumption of infection by GLRaV-2 which is found almost associated with GLRaV-3 and propagation by infected shoot.

Lekikot *et al.* (2012) reported also the presence of GLRaV-2 in Algeria with less prevalence. The fact of this large propagation of GLRaV-2 may be due to the use of infected plant propagating materials. Indeed, observations made on some grape nursery during the prospection reveals the presence of leafroll symptoms. (Lekikot, 2012) reported that native varieties were more infected than the imported ones which may be due to large movements of infected material. Further studies on grape nursery were needed in order to understand the propagation of this virus in Algeria. Few studies were dedicated to the genetic diversity of GLRaV-2. Based on CP gene analyses, (Bertazzon *et al.*, 2010) reported five clades. In the same year, (Jarugula *et al.*, 2010) reported six lineages.

In this study, phylogenetic analyses of HSP70h gene performed reveal the presence of six lineages confirming previous studies. Indeed, comparison at the nucleotide level reveals that the described groups present less than 86% similarity between them and the similarity within groups reveal more than 95% identity. Furthermore, similarity plot reveals five distinct variants except for variant PV20 due to the absence of full length sequence. Our results were in concordance with those obtained by (Jarugula *et al.*, 2010) which reported an interlineage sequence identities between PN, 93/955, and H4 lineages of 83 to 86% and between 'PV20', BD, and RG lineages were 68 to 80%.

Phylogenetic tree reveals that all Algerians isolates provided from a distant region belong to the Group PN containing in general American isolates. Tree reveals the presence of isolates provided from a distant region and cluster in the same clade. The same result was obtained by several authors who rejected the hypothesis that the phylogeny of GLRaV-2 population depend on geographical origin. Thus, (Jarugula *et al.*, 2010) reported that isolates provided from distant vineyards show an important similarity and cluster in the same lineage. This observation may be due to the large movement of variants among root stock and cultivars. Indeed, a large survey on authoctonous and wild populations is needed for a better understanding of genetic variation of a GLRaV-2 population. However Results show that the groups PN and H4 were the most important group constituted in the majority of North American isolates, this result may be due to the large sequences providing from this region.

The high genetic stability of viruses can be attributed to negative or purifying selection to maintain the functional integrity of the viral genome. However, several genera were reported as evolving under positive selection (the genera Aureusvirus, Carmovirus, Dianthovirus, Necrovirus andTombuvirus). Thus (Jarugula *et al.*, 2010) reported that HSP70h evolve under negative selection with dN/dS <1. In our study, we found that this gene evolve under positive selection. Indeed, we obtained the mean ratio dN/dS greater than 1 (dN/dS= 1,81 for SLAC and dN/dS= 3,71). Results reveal also that some sites were under neutral selection and others under negative selection. Indeed, some sites in the HSP70h were exposed to mutation which were maintained in the population and other were under stability. To understand whether codon may be predisposed to mutation. The positive selection obtained may be explained by the changes on amino acid in some site of HSP70h gene, indicating specificity of each variant. It is interesting to know the effect of these mutations on plant virus interaction. Indeed, these mutations may explain the differences in virulence between the different variants. (Bertazzon *et al.*, 2010) reported that isolates from group BD were less virulent and were unable to induce graft incompatibility and it rarely caused leafroll symptom. In contrast, the RG isolates appeared to be more virulent.

The absence of recombination events may be explained by the fact that each vine may be infected by only one

variant limiting genome exchange between distant variants. Indeed, results reveal the presence of only one variant in Algeria and the absence of natural vector of GLRaV-2 limit the large distribution of variants. Further studies of variant distribution are necessary.

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A PIVOTAL ROLE OF REACTIVE OXYGEN SPECIES IN NON-HOST RESISTANCE MECHANISMS IN LEGUME AND CEREAL PLANTS TO THE INCOMPATIBLE PATHOGENS

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A B S T R A C T

Most of plants under normal conditions are resistant to most of the incompatible pathogens (viral, fungal and bacterial infections). This is called ″non-host resistance (NHR) phenomenon″. Till now it is not clear the non-host resistance mechanisms. As a result of inoculation of legume (pea and soybean) and cereal (barley and wheat) plants with compatible and incompatible pathogens, strong resistance symptoms were observed in the non-host/incompatible pathogen combinations as compared with host/compatible pathogen combinations which showed severe infection (susceptibility). Levels of reactive oxygen species (ROS) mainly hydrogen peroxide (H_2O_2) and superoxide (O_2.-) were significantly increased early 6, 12, 24 and 36 hours after inoculation (hai) in the non-host plants as compared with host plants. Interestingly enough that the activities of the antioxidant enzymes such as catalase (CAT), dehydroascorbate reductase (DHAR) and peroxidase (POX) were not significantly increased at the same early time 6 - 36 hai in the non-host plants. However, these enzymes were significantly increased later on 48, 72 and 96 dai in the non-host plants as compared with host plants. It seems that early accumulation of H_2O_2 and O_2.- could have a dual roles, first role is inhibiting or killing the pathogens early in the non-host plants, second immunization of the non-host plants by stimulating the activities of the antioxidant enzymes later on which thereby, neutralize the harmful effect of ROS and consequently suppressing disease symptoms. The author recommends giving more attention to these new mechanisms of non-host resistance particularly in relation to ROS levels and antioxidant activities which are very important for plant breeders and useful for finding alternative control strategies as well.

Keywords: Antioxidants, ROS, Non-host resistance, legumes, cereals.

INTRODUCTION

Non-host resistance (NHR) is a resistance showed by an entire plant species to all genetic variants of a non-adapted pathogen species. NHR refers to plant species immunity against the majority of microbial pathogens and represents the most healthy and strong form of plant resistance in nature (Yulin *et al.,* 2012; Lipka *et al.,* 2010). NHR phenomenon explains why most of plants are immune to the majority of pathogens and normally healthy. Mechanisms supporting NHR remain relatively unknown compared with the well-studied host resistance mediated by the products of plant resistance (R) genes, which establish pathogen race- or plant cultivar-specific resistance (Schulze-Lefert and Bieri 2005; Dangl and Jones 2001). NHR plants to pathogens mainly fungal, bacterial and viral infections can be defined as an innate non-specific resistance which is effective against all known isolates of several species of the pathogens (Király *et al.,* 2007; Thordal-Christensen, 2003). This NHR resistance is a strong and very effective type of plant immunity (Heath, 2000). On the other hand, appropriate pathogens escape defense reactions of the host by avoiding recognition or suppressing resistance of non-host or host but resistant plants (Schulze-Lefert and Panstruga, 2003). Researchers showed some experiments in relation to genetics of non-host type of resistance. However, only a few biochemical results are available as regards the formation of host cell wall appositions (papillae), local accumulation of autofluorogens and reactive oxygen species (ROS), such as

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hydrogen peroxide (H_2O_2) (Trujillo *et al.,* 2004; Hückelhoven *et al.,* 2001; Carver *et al.,* 1992).

Pea (*Pisum sativum* L.) is one of the most important legume vegetable crops grown in Egypt and many other countries all over the world. It has many nutritional values such as high content of protein, carbohydrates, phosphorus, iron, calcium and vitamins A and B (Watt and Merrill, 1963). Soybean (*Glycine max* L.) is one of the world's most important sources of oil and protein. It has the highest protein content among leguminous crops (Abdel-Monaim *et al.,* 2011). Barley (*Hordeum vulgar*e L.) ranks the fourth among the major cereal grians in terms of World and Egyptian production after maize, wheat and rice crops (Hafez *et al.,* 2014). Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world for both human food and animal feed (Abdelaal *et al.,* 2014; Chen *et al*., 2003). These important plants can be seriously damaged by *Botrytis cinerea*, *Alternaria solani*, *Blumeria graminis* f.sp. *hordei* (*Bgh*) and *Blumeria graminis* f.sp. *tritici* (*Bgt*). Due to the great and economic damages of these host/pathogen interactions, research into the functional and characterization of new resistance mechanisms are required. According to our knowledge, not too much experimental results have been achieved which would explain the question: what is arresting or killing the pathogens in the non-host resistant plants? However, some promising and preliminary results were obtained which indicated that ROS have a pivotal role in the arrest of pathogens in non-host plants. However, till now little is known about the nature of effective defense mechanisms in pea, soybean, barley and wheat plants to incompatible pathogens, especially pathogens with economic and biological importance.

The aim of this research study was to characterize the mode of action of the non-host resistance mechanisms in pea, soybean, barley and wheat to the *Papya ring spot virus* (*PRSV*), *B. cinerea*, *B. graminis* f. sp. *riticit* and *B. graminis* f. sp. *h*ordei respectively at the morphological, histological and biochemical levels which thereby, very useful for plant breeders and sustainable crop protection.

MATERIALS AND METHODS

Plant Materials: Pea (*Pisum sativum* L.) cultivar (cv.) Little Marvel and soybean (*Glycine max* L.) cv. Giza 111 seeds were obtained from Food Legumes Research Section, Sakha, Kafr El-Sheikh, Field Crops Research Institute (FCRI), Agricultural Research Station (ARC), Egypt. Barley (*Hordeum vulgar*e L.) cv. Giza 123 seeds obtained from Dept. of Barley, FCRI, ARC, Egypt. Wheat (*Triticum aestivum* L.) cv. Sakha 61 was obtained from Wheat Pathology Department, Sakha, Kafr El-Sheikh, ARC, Egypt. Seeds were sown in a 12-cm plastic pots containing soil mixed with peat moss (1:1) and grown in the greenhouse. Temperature was 18-23 ^{o}C, with 16 hours photoperiod per day using supplemental light with a light intensity of 160 µE m^{-2} s^{-1} and relative humidity 75-80%. These experiments were conducted in the laboratories, green houses and growth chambers of Botany Department, Faculty of Agriculture, Kafr-Elsheikh University, Egypt as well as Plant Pathophysiology Department, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary during the years 2012 -2014.

Plant Pathogens: The *Papaya ringspot virus* (PRSV) Egyptian isolate was obtained from Plant Pathology and Biotechnology Laboratory, Faculty of Agriculture, Kafr-Elsheikh University, Egypt. PRSV was maintained on the host susceptible squash (*Cucurbita pepo*). For mechanical virus inoculation, viral-infected leaves were homogenized in tap water. Carborundum was used as an abrasive for virus and mock inoculations.

Botrytis cinerea Pers., Bc-1 and *Alternaria solani* isolates were kindly supplied by Prof. László Vajna, Plant Protection Institute, Hungarian Academy of Sciences, Hungary. These pathogens were maintained on potato dextrose agar medium (PDA) under continuous fluorescent light. For inoculation, agar discs 5 mm in diameter were cut from 7-day-old cultures of the fungus *Botrytis cinerea* and 15-days old culture of the fungus *Alternaria solani* then, placed on the surface of pea and soybean leaves, respectively. Leaves were cut and put on wet filter paper in a Petri dish and held at 20°C in continuous light for at least 3 days for pea and 2-5 days for soybean (Hafez *et al.,* 2012).

Barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) and wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) Egyptian specimens were maintained under greenhouse conditions and were used for all inoculation experiments. Powdery mildew inocula were dispersed in the greenhouse atmosphere by placing plants of barley and wheat bearing sporulating colonies of both pathogens beneath ventilation fans of the greenhouse (Hafez and Kiraly, 2003).

Disease Severity Assessments: Disease severity percentage (%) of pea inoculated with *B. cinerea* (host)

and PRSV (non-host) as wel as soybean inoculated with *A. solani* (host) and *B. cinerea* (non-host) were measured as lesion diameter (cm^2) according to Hafez *et al.,* 2004. However, the disease severity percentage (%) of barley inoculated with barely powdery mildew (host) and inoculated with wheat powdery mildew (non-host) as well as wheat inoculated with wheat powdery mildew (host) and inoculated with barley powdery mildew (no-host) were determined as follows: Ten plants of each replicate were scored visually for percentage of leaf area covered by powdery mildew on a 0 (resistant) to 10 (susceptible) scale three times 3, 6 and 9 days after inoculation (dai) in each experiment. For analysis, disease scores were converted using the modified logarithmic scale of Horsfall–Barrett (Horsfall and Cowling, 1978) and Hafez et al., 2014. The scale was 0 = 0 %, 1 = 0–3 %, 2 = +3–6 %, 3 = +6–12 %, 4 = +12–25 %, 5 = +25–50 %, 6 = +50–75 %, 7 = +75–88 %, 8 = +88–94 %, 9 = +94–97 % and 10 = +97–100 %. Disease severity index (DSI) was calculated according to Kim *et al*., 2000 using the following formula:

$$\text{DSI} = \frac{\sum \text{R atings of each plant}}{10 \times \text{Number of plants rated}} \times 100$$

Histochemical Analysis of ROS: Histochemical staining for $O_2^{\cdot-}$ production in leaf tissue was based on the ability of $O_2^{\cdot-}$ to reduce nitro blue tetrazolium (NBT). $O_2^{\cdot-}$ was visualised as a purple coloration of NBT. Leaf discs (2 cm) of pea and soybean plants as well as hall barley and wheat leaves were vacuum infiltrated or injected (Hagborg, 1970) with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % NBT (Sigma–Aldrich, Germany) according to Ádám *et al.,* (1989). NBT-treated samples were incubated under daylight for 20 min and subsequently cleared in 0.15 % trichloroacetic acid (wt/vol) in ethanol: chloroform 4:1 (vol/vol). The solution was exchanged once during the next 48 h of incubation (Hückelhoven *et al.,* 1999). Subsequently, leaf discs and leaves were stored in 50% glycerol for evaluation.

For histochemical analysis of hydrogen peroxide (H_2O_2), leaf discs and hall leaves were infiltrated with 0.1% 3, 3-diaminobenzidine (DAB) in 10 mM Tris buffer (pH 7.8). Samples were incubated under daylight for two hours after the vacuum infiltration. Following staining, leaves were cleared as described above and the intensity of brown color was estimated (Hückelhoven *et al.,* 1999). Levels of $O_2^{\cdot-}$ and H_2O_2 were estimated 6, 12, 24, 36, and 48 hours after inoculation. These tests were repeated five times during six independent experiments.

Biochemical Assays of Antioxidant Enzymes: The tested antioxidant enzyme activities were measured on 5 weeks-old pea and soybean plants as well as 2 weeks-old barley and wheat plants. For enzyme assays in plants, 0.5 g detached leaves material from 5 and 2 weeks old plants, respectively, was homogenized at 0-4˚C in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA-Na_2 and 7.5% polyvinylpyrrolidone 1, 2, 3 and 4 days after inoculation. The homogenates were centrifuged (12,000 rpm, 20 min, 4˚C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant (Hafez, 2010, Hafez et al., 2014). All measurements were carried out at 25˚C, using the model UV-160A spectrophotometer (Shimadzu, Japan).

Activity of catalase (CAT) was determined spectrophotometrically according to Aebi (1984). Decomposition of H_2O_2 by catalase results in the decrease of the ultraviolet absorption of hydrogen peroxide at 240 nm. Enzyme activity can be calculated from this decrease. The reaction mixture contained, in a final volume of 2.15 ml, 2 ml 0.1 M Na-phosphate buffer (pH 6.5), 100 µl hydrogen peroxide and 50 µl leaf extract supernatant. The solution is mixed, and then the absorption change is registered for 3 min at 240 nm using a quartz cuvette.

Activity of dehydroascorbate reductase (DHAR) was determined spectrophotometrically according to Asada (1984). The reaction mixture contained, in a final volume of 2.3 ml, 50 mM sodium phosphate buffer (pH 6.5), 0.5 mM dehydroascorbate (DHA), 1.0 mM reduced glutathione (GSH), 0.1 mM EDTA and 0.1 ml supernatant. The assay was carried out in quartz cuvettes following the increase in absorbance at 265 nm due to the formation of ascorbate with extinction coefficient 14 mM^{-1} cm^{-1} (Klapheck *et al*., 1990). The reaction rate was corrected for the non-enzymatic reduction of dehydroascorbate by GSH.

Activity of peroxidase (POX) was directly determined of the crude enzyme extract according to a typical procedure proposed by Hammerschmidt *et al.,* (1982). Changes in absorbance at 470 nm were recorded every 30 sec intervals for 3min. Enzyme activity was expressed as increase in absorbance min^{-1} g^{-1} fresh weight.

Statistical Analysis: Six experiments were conducted in a completely randomized design with five replicates for each treatment. Data represent the mean ± SD. Student's t-test was used to determine

whether significant difference (P<0.05) existed between mean values according to O'Mahony (1986).

RESULTS AND DISCUSSION

Disease Severity and Disease Symptoms of Host and Non-host/Pathogen Combinations: In the "non-host" plants pea, soybean, barley and wheat showed resistance against *Papaya ringspot virus*, *B. cinerea*, *Blumeria graminis* f. sp. *tritici* and *Blumeria graminis* f. sp. *hordei*, respectively compared to the "host" plants pea, soybean, barley and wheat inoculated with *Botrytis cinerea*, *Alternaria solani*, *Blumeria graminis* f. sp. *hordei* and *Blumeria graminis* f. sp. *tritici*, respectively showed strong susceptibility. Disease severity percentage was significantly severe strongly in all the host/pathogen combinations as compared to the non-host/pathogen combinations (Figure 1 and Table 1).

Table 1. Reaction of host and non-host/pathogen combinations.

Plants	Host	Results	Non-host	Results
Pea	*Botrytis cinerea*	S	*Papaya ringspot virus*	R
Soybean	*Alternaria solani*	S	*Botrytis cinerea*	R
Barley	*Blumeria graminis* f. sp. *hordei*	S	*Blumeria graminis* f. sp. *tritici*	R
Wheat	*Blumeria graminis* f. sp. *tritici*	S	*Blumeria graminis* f. sp. *hordei*	R

S= susceptible, R= resistant

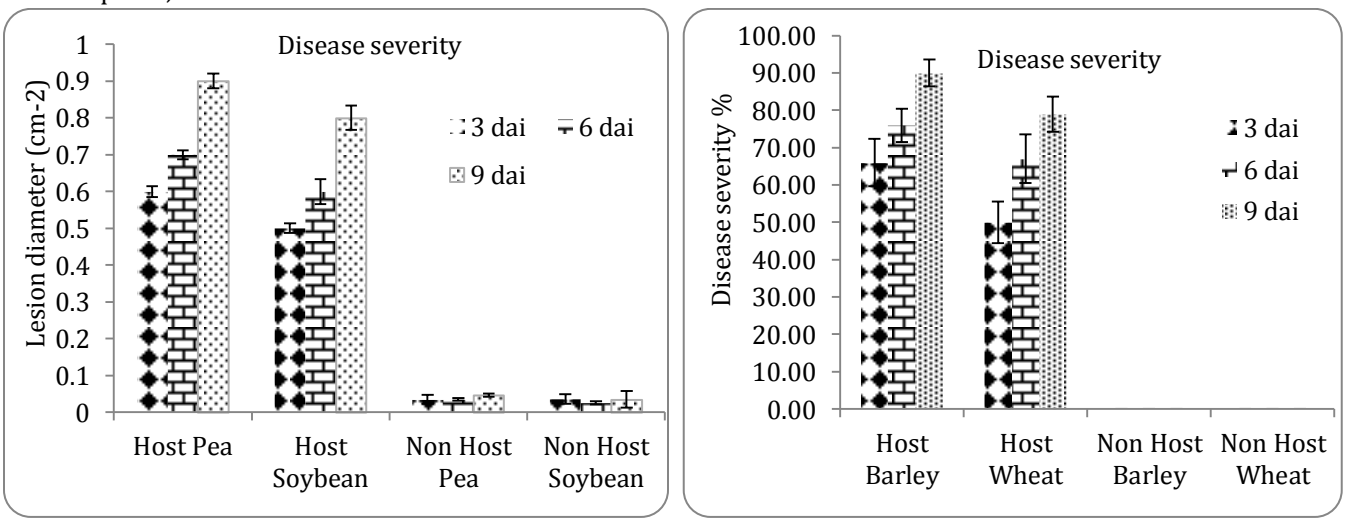

Figure 1. Disease severity percentage (%) of host and non-host/pathogen combinations 3, 6 and 9 days after inoculation (dai) of pea, soybean, barley and wheat plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani*. Non Host Pea: leaves inoculated with Papaya ringspot virus. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis f. sp. hordei* (Bgh). Non Host Barley: leaves inoculated with *Blumeria graminis f. sp. tritici* (Bgt). Host Wheat: leaves inoculated with Bgt. Non Host Wheat: leaves inoculated with Bgh.

Disease symptoms were also significantly appeared and visible in the host/pathogen combinations compared to the non-host which no symptoms appeared (Figure 2). Similar results have been obtained by Fabro *et al.* (2011) in which found that in the "non-host" plant *Brassica rapa* (turnip) which was more effectors of *Hyaloperonospora arabidopsidis* are recognized than in *Arabidopsis thaliana* which is a "host" of this oomycete pathogen. This could be a possible cause of the inability of *H. arabidopsidis* to grow in turnip.

In other words, the host plant cannot recognize a subset of effectors of its own pathogen which are recognized, and therefore induce an immune reaction in the non-host. However, it is still an unanswered question, how this immune reaction can inhibit pathogens in non-host plants? (Kiraly *et al.,* 2013).

Levels of Reactive Oxygen Species in Host and Non-host/Pathogen Combinations: Purple discoloration of superoxide ($O_2^{\cdot-}$) and brown discoloration of hydrogen peroxide (H_2O_2) reflect the intensivity of ROS levels in the leaves which cleared from chlorophyll (Figure 2). Levels of ROS mainly $O_2^{\cdot-}$ and H_2O_2 significantly accumulated early 6, 12, 24 and 36 hours after inoculation (hai) in all non-host/pathogen combinations in pea, soybean, barley and wheat plants (Figure 3, 4 and 5).

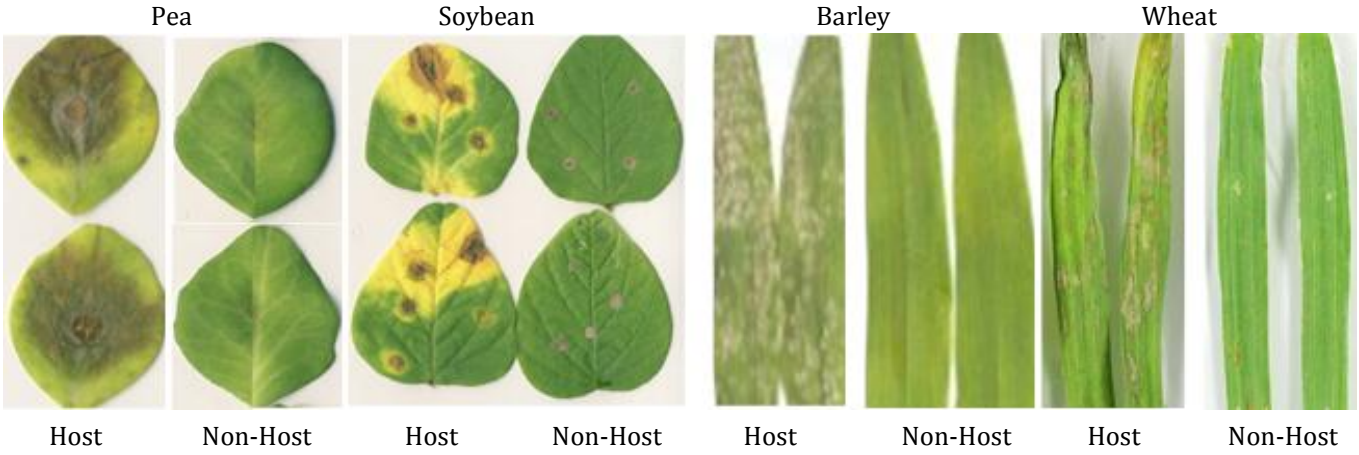

Figure 2. Disease symptoms of host and non-host/pathogen combinations 4 days after inoculation (dai) in pea and soybean as well as 2 dai in barley and wheat plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

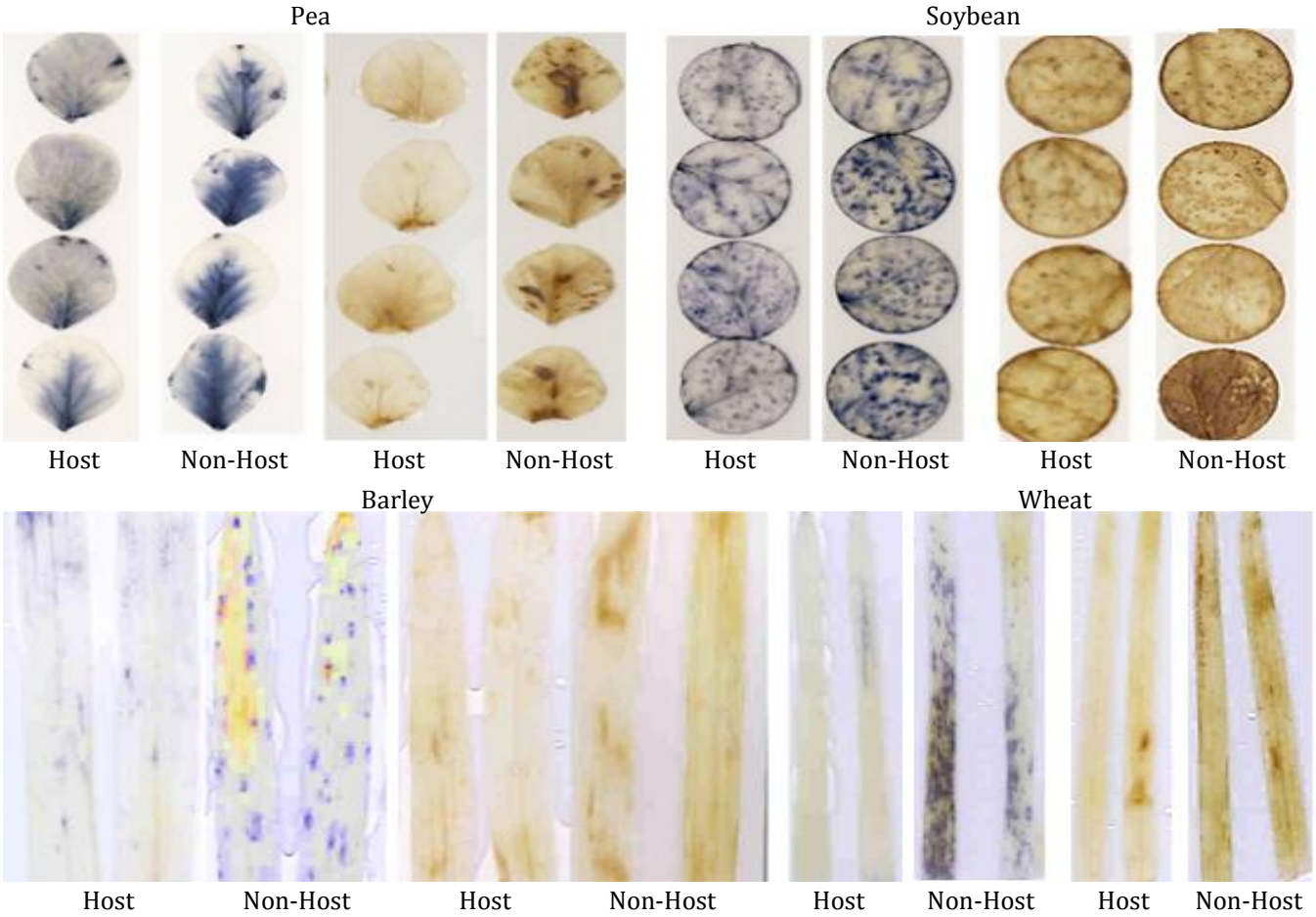

Figure 3. Purple discoloration of superoxide ($O_2^{\cdot-}$) and brown discoloration of hydrogen peroxide (H_2O_2) of host and non-host/pathogen combinations 12 hours after inoculation (dai) in pea and soybean as well as 6 hai in barley and wheat plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

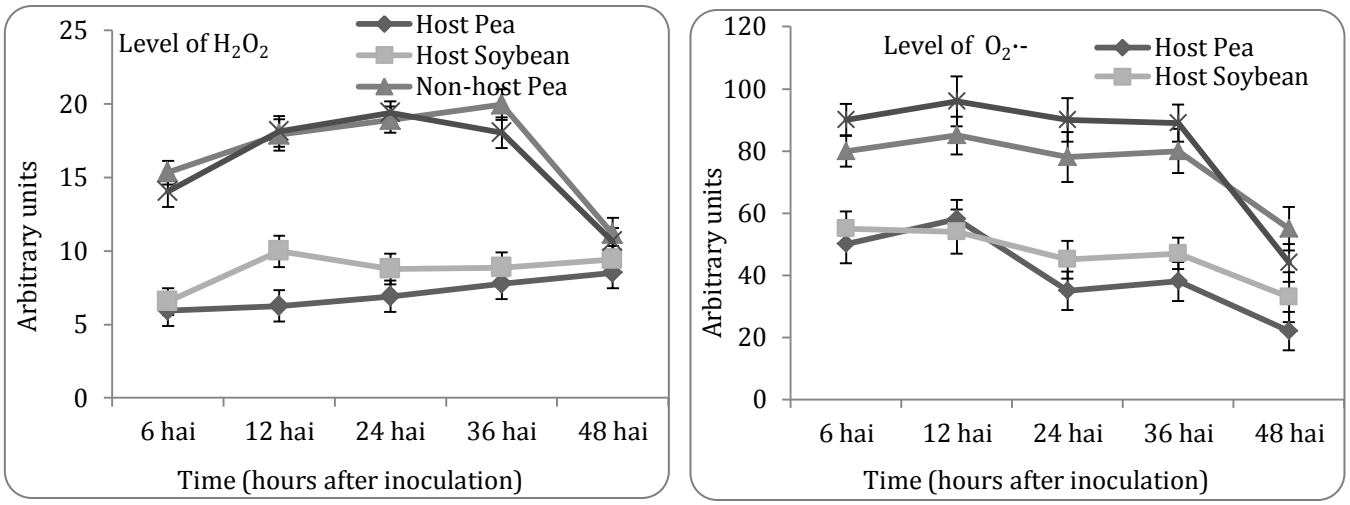

Figure 4. Levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) of host and non-host/pathogen combinations 6, 12, 24, 36 and 48 hours after inoculation (hai) in pea and soybean plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

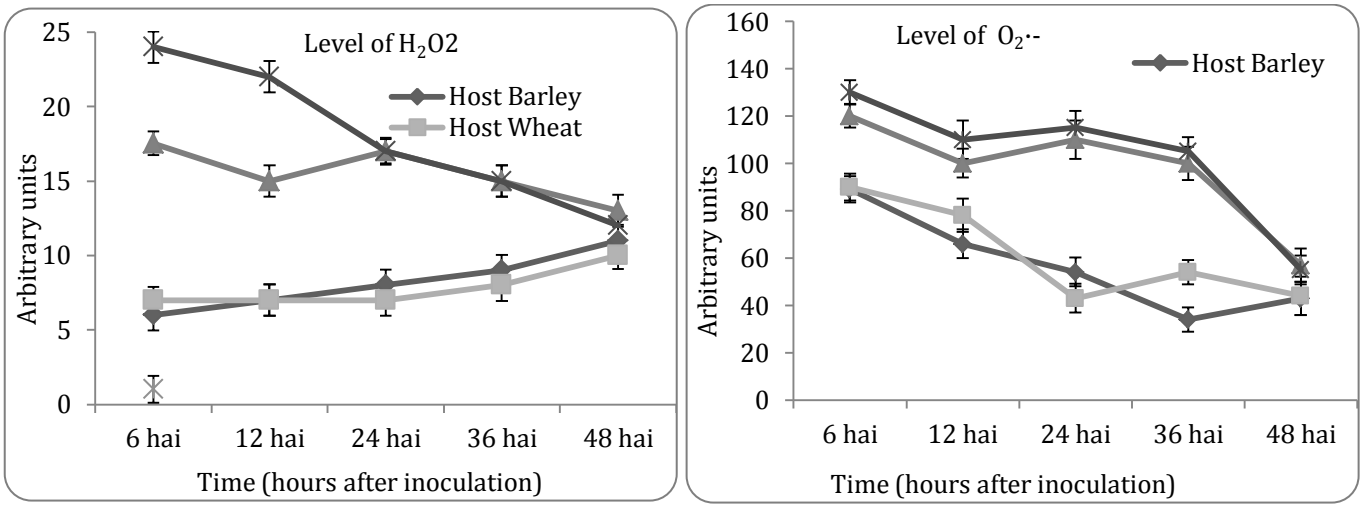

Figure 5. Levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) of host and non-host/pathogen combinations 6, 12, 24, 36 and 48 hours after inoculation (hai) in barley and wheat plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

Activities of Antioxidant Enzymes in Host and Non-host/Pathogen Combinations: Activities of antioxidant enzymes such as catalase (CAT), dehydroascorbate reductase (DHAR) and peroxidase (POX) were not changed or even increased early 6, 12, 24 and 36 hours after inoculation (hai) in all non-host/pathogen combinations in pea, soybean, barley and wheat plants, however, these enzymes were increased significantly a little bit later at 48, 72 and 96 hai (Figure 6 and 7).

One can say that in these non-host resistant pea, soybean, barley and wheat plants the early accumulation of $O_2^{\cdot-}$ and H_2O_2 not only inhibit or kill the incompatible pathogens 6-36 hai but also stimulate the activities of antioxidants CAT, DHAR and POX alter on 24-96 hai. Our results are supported by our previous results in which pointed out that under natural conditions the up-regulation of antioxidant defense systems seems to be a general response to oxidative stress (Hafez *et al.,* 2012).

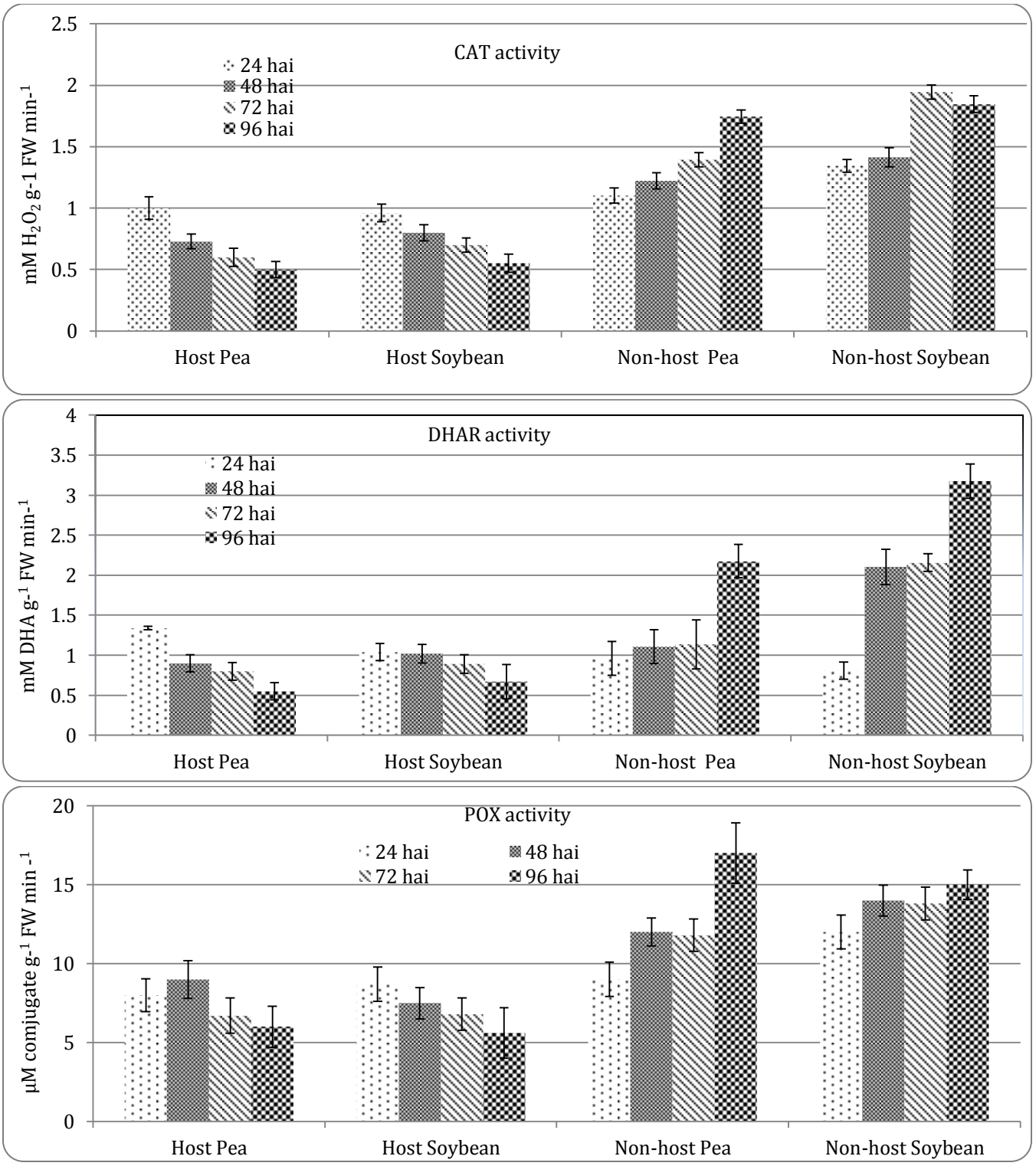

Figure 6. Activities of antioxidant enzymes catalase (CAT), dehydroascorbate reductase (DHAR) and peroxidase (POX) in host and non-host/pathogen combinations 24 48, 72 and 96 hours after inoculation (hai) in pea and soybean plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

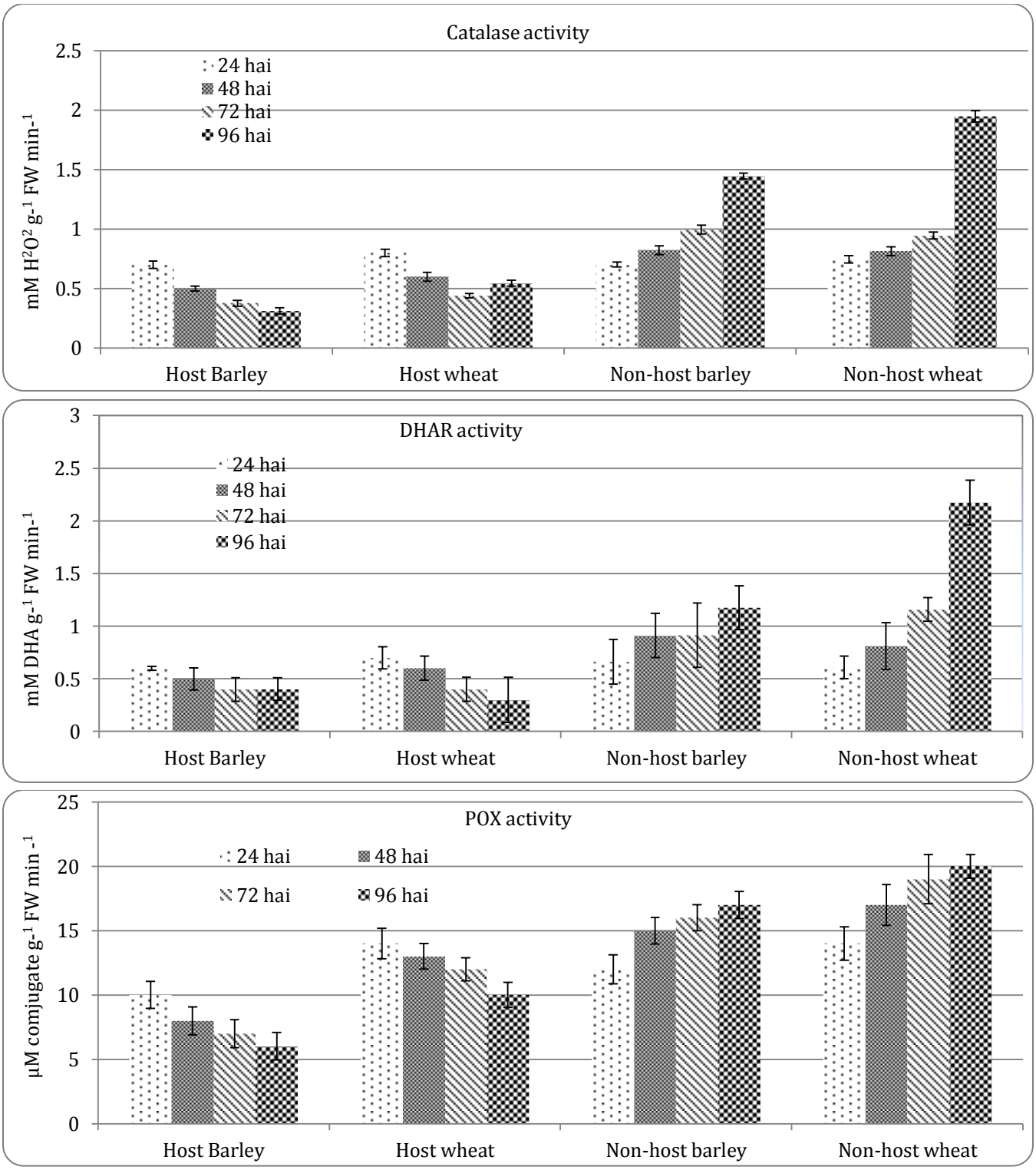

Figure 7. Activities of antioxidant enzymes catalase (CAT), dehydroascorbate reductase (DHAR) and peroxidase (POX) in host and non-host/pathogen combinations 24 48, 72 and 96 hours after inoculation (hai) in barley and wheat plants. Host Pea: leaves inoculated with *Botrytis cinerea*. Host soybean: leaves inoculated with *Alternaria solani.* Non Host Pea: leaves inoculated with *Papaya ringspot virus*. Non Host soybean: leaves inoculated with *B. cinerea*. Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *hordei (Bgh).* Non Host Barley: leaves inoculated with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Host Wheat: leaves inoculated with *Bgt.* Non Host Wheat: leaves inoculated with *Bgh.*

The early accumulation of ROS in the non-host resistant plants up-regulate the antioxidants go along with the results which proved that H_2O_2 as one of the most important reactive oxygen species associated with oxidative stress, can up-regulate antioxidant systems even at very low concentrations against abiotic stress (Gechev *et al.,* 2002) and also immunize plants, therefore, induces resistance to symptom development by suppressing pathogen-induced host cell and tissue necroses but not pathogen multiplication, while enhancing activities of at least three antioxidant enzymes such as catalase, guaiacol peroxidase and ascorbate peroxidase (Hafez *et al.,* 2012). It was pointed out that often the only evidence that oxidative stress has occurred in vivo may be it is the cause of up-regulation of antioxidant defense systems (Halliwell and Gutteridge, 1999). The high activities of enzymatic and nonenzymatic antioxidants neutralized the harmful effects of ROS (oxidative stress). Particularly, H_2O_2 seems to play a dual role by eliciting localized death of plant and pathogen cells and as a diffusible signal for the induction of antioxidant and pathogenesis-related genes in adjacent plant tissues (Hafez *et al.,* 2012; Wu *et al.,* 1997; Levine *et al.,* 1994).

It worth mentioning that the author use for this research four model important plants, two from legumes (pea and soybean) and two from cereals (barley and wheat) which support his new findings. It can be concluded that new histochemical and biochemical mechanisms of non-host resistance mechanisms were found by the author in this research article. ROS mainly superoxide and hydrogen peroxide accumulated early after inoculation which may be killing or inhibiting the pathogens in these non-host plants. This early accumulation of ROS stimulated the antioxidant enzymes activities later on which thereby could immunize plants by suppressing disease symptoms and neutralize the harmful effect of ROS against these incompatible pathogens. It is recommended to the researchers and plant breeders to give more attention to these interesting new results to find new strategies for future integrated control pest management practices and sustainable crop protection.

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