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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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ABSTRACT

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 annum* 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°C during the day and 15 to 20°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°C and maximum of 32.9°C temperature. The average altitude of Nonno district is 1715 m.a.s.l. with mean minimum and maximum temperature of 16°C and 24°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 1m² 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°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, K₂HPO₄ (anhydrous) 1.5 g, MgSO₄ × 7H₂O, 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 prepared 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 pestle 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°C. 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°C 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 *Ralstonia 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% H₂O₂) 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 patched 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 CaCl₂, 2H₂O 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°C 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°C 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 characteristics and microscopic examination using the Standard manuals (Ellis, 1976; Bameett 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°C. 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. solanacearum* was carried out according to the procedure developed by Mimura and Yoshikawa (2009). For this, infested soil was prepared by pouring 4mL of (4×10^8 cfu/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 6.4×10^6 conidia/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 8×10^8 conidia/ml for 30 min (Bhat *et al.*, 2003). For the control, non-inoculated seedlings were dipped in sterile distilled water.

***Phytophthora*:** 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 3×10^6 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 solani*:** 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. solani* were placed in 25ml Erlenmeyer flasks that contain a presoaked and autoclaved (at 121°C for 30 min at 15 lb) wheat seed. The flasks were incubated at 25 °C for nearly a month till full fungal mat developed. The infected medium in one flask was crushed thoroughly and placed 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 (Table 4) only one wilt causing bacteria pathogen, *Ralstonia solanacearum* was identified with 100% frequency of occurrence. The same samples were also cultured in TTC media that uses to specifically identify *R. solanacearum*. 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
<i>Ralstonia solanacearum</i>	6.67	46.67	73.00	73.33	73.33	73.33
<i>Rhizoctonia solani</i>	10.00	20.00	20.00	40.00	70.00	90.00
<i>Fusarium spp.</i>	13.33	26.67	53.33	53.33	46.67	80.00
<i>Verticillium spp.</i>	0.00	13.33	26.67	33.33	46.67	53.33
<i>Phytophthora spp</i>	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 occurrence 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 albo-atrum* 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.

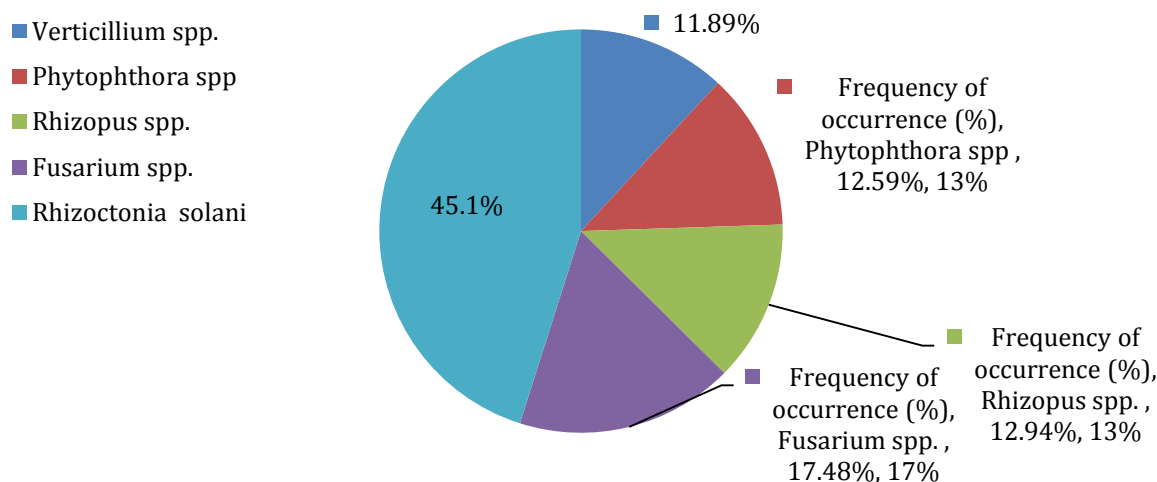


Figure 1. Frequency of fungi isolated from wilted pepper plants.

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