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Antagonistic Effects of Rhizobacteria Against Coffee Wilt Disease Caused by *Gibberella xylarioides*

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ABSTRACT

This study was designed to evaluate the antagonistic effects of rhizobacterial antagonists against Coffee Wilt Disease (CWD) caused by *Gibberella xylarioides* under *in vitro* and *in vivo* conditions. Arabica coffee is Ethiopia's main export crop. However, the production and productivity of coffee is being challenged primarily by coffee vascular disease (tracheomycosis).The greenhouse antagonism study was conducted with four antagonistic bacteria of one *Bacillus* (JU544) and three *Pseudomonas* spp. (JU941, JU13 and JU23). Out of 81 rhizobacterial antagonists tested on Half Strength King's B (HSKB) medium against *G. xylarioides*, 13.6% of them significantly ($p<0.0001$) reduced the radial mycelial growth of the pathogen. From 11 rhizobacterial isolates tested for their phytobeneficial traits, eight of them produced protease. Nevertheless, five of them produced Hydrogen Cyanide (HCN) and other lytic enzymes. The bio-control agents, time of applications and the interaction of the two were significantly ($p<0.0001$) reduced the CWD severity and incidence under greenhouse conditions. The CWD control efficiency was significant ($p<0.0001$) and the highest bio-control efficiency was 72.64% when the coffee seedlings were treated with *Bacillus* spp. (JU544) seven days before the pathogen. The bacterial antagonists, time of applications and interaction of the two significantly ($p<0.001$) reduced the progression of CWD incidence. The rhizobacterial antagonists especially *Bacillus* spp. (JU544) effectively reduced CWD severity and incidence under greenhouse condition and can be further evaluated under field condition to ascertain their future applicability for inoculum development.

Key words: Chitinase, protease, hydrogen cyanide (HCN), highland coffee; coffee wilt disease

INTRODUCTION

Coffee is the major category of Rubiaceae family and genus *Coffea* which is widely distributed throughout the tropical regions of the world including Ethiopia (Coste, 1992). Currently, Ethiopia is one of the leading Arabica coffee producers in Africa and the fifth largest Arabica coffee producer in the world next to Brazil, Vietnam, Indonesia and Colombia (ICO, 2011).

CWD caused by the fungal pathogen, *G. xylarioides* is a major constraint of coffee production in the major coffee producing areas of the country. CWD incidence was reported to be 60% in major Arabica coffee growing areas of southwestern Ethiopia, including Bebeka, Teppi, Jimma and Gera and the fungus is causing significant yield losses due to very severe damage and death of millions of trees (Girma *et al*., 2001). Due to the prevalence of CWD, Ethiopia losses ca. 3360 t of coffee annually; amounting to US$ 3.7 million in Ethiopia (ICO, 2003).

Several attempts have been made to manage CWD in Ethiopia as such using resistant coffee plants, plant and environmental management and synthetic copper based fungicides (Strange, 1993; Mulaw *et al.*, 2010). Nevertheless, resistance is being breaking since F-59 coffee cultivar was reported to be highly susceptible to *G. xylairoides* (Adugna *et al.*, 2005). Furthermore, the high cost of pesticides, consumer preference of chemical free products, emergence of fungicide resistant pathogen biotypes and other social and health related impacts of conventional agriculture on the environment have, however, recently led to an increased interest in agricultural sustainability and biodiversity conservation (Van der Vossen, 2005). Thus, there is a need for supplementary plant disease management options that provide effective management of the disease under question while minimizing cost and negative consequences to human health and the environment (Cook *et al.*, 1996; Muleta, 2007).

The use of beneficial soil microbes to control plant diseases which is a form of biological control, is an environment-friendly approach. Among the Plant Growth Promoting Rhizobacteria (PGPR), *Pseudomonas* and *Bacillus* spp. in particular have been utilized widely in biocontrol methods in low input agricultural production systems (Vassilev *et al.*, 2006; Muleta *et al.*, 2007).

Bacterial antagonism towards plant pathogenic fungi involves a diverse mode of actions such as the production of antibiotics, lytic enzymes, Hydrogen Cyanide (HCN), competition for nutrients, parasitism and emission of potent antifungal volatile organic compounds (Yoshihisa *et al.*, 1989; Muleta *et al.*, 2007).

Little is known about microorganisms associated with *C. arabica* regarding their antagonistic effect against coffee wilt diseases caused by noxious *Fusarium* spp. However, reports have revealed close associations of useful microorganisms with *C. arabica* including *Bacillus, Burkholderia, Pseudomonas,* members of the Enterobacteriaceae and others (Vega *et al.*, 2005; Muleta *et al.*, 2007). Despite the existence of useful antagonistic rhizobacteria in coffee and other crops rhizosphere, still there is scanty information with regard to rhzobacterial antagonists against CWD under *in vivo* conditions. Therefore, the major purposes of the current study were to evaluate antagonistic potential of the rhizobacterial isolates under *in vitro* conditions and to investigate the possible mechanisms of inhibition by assessing their ability to produce Hydrogen Cyanide (HCN) and some lytic enzymes. In addition, antagonistic effect of the rhizobacteria isolates against *G. xylarioides* at different time of applications was determined under *in vivo* conditions.

MATERIALS AND METHODES

Description of the study area: The study was conducted at Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), Jimma, Ethiopia. Laboratory activities were conducted in Plant Pathology Laboratory. Pot experiments were conducted in JUCAVM which is located at 7°42 N Latitude and 36°57' E Longitude and at an altitude 1710 m.a.s.l. The maximum and minimum temperature of the area is 26.8 and 11.8°C, respectively, with relative humidity of 91% and the mean rainfall of 1500 mm per annum (Abera *et al.*, 2011).

Experimental design: The experiment involved two factors (the rhizobacterial antagonist isolates and time of application). In this experiment one *Bacillus* spp. (JU544) and three *Pseudomonase* spp. (JU941, JU13 and JU23) were used under *in vivo* conditions. The JU544, JU13 and JU23 bacteria were isolated from Arabica coffee (*C. arabica* L.) while the JU941 bacterial isolate was recovered from Pea (*Pisum sativum* L.) rhizosphere (Demisse, 2012). These isolates showed the best performance in reduction of radial mycelial growth of the pathogen and were

characterized by morphological and biochemical characterization (Demisse, 2012). The three different inoculation times for antagonist were conducted at seven days interval (seven days before pathogen inoculation, after and at the same time with pathogen inoculation and antagonist treatment).The experiment was laid out in Complete Randomized Design (CRD) under laboratory and in Randomized Complete Block Design (RCBD) in the greenhouse condition with three replications.

***In vitro* antagonism study:** The laboratory antagonism study was carried out following the methods described in Muleta *et al*. (2007) with some modifications on dual culture experiment. On dual culture antagonism study, first the pathogen (GWO1 isolate which was randomly selected from all isolates) was cultured on Potato Dextrose Agar (PDA) medium for 5 to 7 days at 25°C from preserved stock culture. Then, a small fungal agar block (1×2 cm^2) from the leading margin of cultures propagated on PDA for at 5 to 7 at 25°C were centrally placed on pre-solidified half-strength King's B medium (KBHS). Thereafter, a loopful of actively grown (24-h-old) bacterial cultures were streaked as a broad band (making a straight short bar) approximately 3 cm away from the mycelial block at two opposite edges of triplicate Petri dishes (90 mm diameter). The plates inoculated with pathogen and without bacteria were used as control. Finally, plates were incubated at 25°C for 15 days and potent rhizobacterial isolates were selected depending on their degree of inhibition.

Fungal radial growth inhibition (a clear zone between the edges of fungal mycelia and bacterial colonies) was calculated as described in Montealegre *et al*. (2003):

$$\text{Inhibition}(\%)=\frac{\text{Control}-\text{Treatment}}{\text{Control}}\times 100$$

Where:

Treatment = Radial mycelial growth of fungus in plate with streaked bacteria
Control = Radial mycelial growth of fungus in plate without bacteria

***In vitro* phytobeneficial trait tests:** The rhizobacterial isolates showed antagonistic activity against radial growth of mycelium of fungal pathogen under *in vitro* conditions were further studied with different biochemical tests to determine the probable mechanisms of antimicrobial activities of the isolates.

Hydrogen cyanide production: Cyanide was detected qualitatively following the method described by Bakker and Schippers (1987).

Chitinase production: The chitinolytic activity of rhizobacterial isolates was tasted on medium containing fine powdered chitin (Renwick *et al*., 1991) (g L^{-1}): Chitin powder, 5.0; yeast extract, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; agar, 20.0. The pH of the medium was adjusted to 7.0 and the medium was sterilized by autoclaving at 121°C for 15 min. The bacterial isolate was spot inoculated individually onto solidified plates of medium. The un-inoculated plates were used as control in the experiment. The plates were incubated at 28°C for 72 to 96 h until zones of chitin clearing could be seen around the colonies.

Protease production: The Protease production was tasted using skim milk agar medium. A loop full of rhizobacterial cells was spot inoculated using heat sterilized inoculating loop and incubated

for two days at 28°C. Then, the proteolytic activities were identified by clear zone formation around the bacterial cells (Smibert and Krieg, 1994).

Lipase production: The rhizobacterial isolates were plated onto the surface of Luria Casei (LC) agar supplemented with 2.5 mL of 2% Tween-80 and the plates were incubated at 28°C for 5 days. The lipase activities of the isolates were proportional to the diameter of turbid zone around the grown bacterial colonies (Kravchenko *et al.*, 2002).

***In vivo* antagonism test**

Coffee seedlings rising: The *in vivo* antagonism test was done on *Catura rojo* coffee variety. *Catura rojo* is a susceptible variety to coffee wilt disease (*G. xylarioides)* (Girma, 2004). The seeds of this variety were obtained from Bebeka Coffee Plantation Share Company (Bebeka, Ethiopia) for the present antagonism study. The seeds were surface sterilized with 70% alcohol for 2 min, followed by rinsing with sterile water several times, soaked in distilled sterile water for about 48 h after removing the parchment. Then, the soaked seeds (20 seeds per pot) were sown into heat sterilized and moistened sandy soil in disinfected plastic pots. Water was applied every 2 days as needed to maintain optimum moisture for seed germination, emergence and growth of seedlings in the greenhouse (Girma, 2004).

Preparation of rhizobacterial and fungal inoculums for seedling inoculation: Four rhizobacterial isolates consisting of one *Bacillus* spp. (JU544) and three *Pseudomonas* spp. (JU941, JU13 and JU23) that showed promising results under *in vitro* condition were used for pot experiment when the seedlings reached at fully expanded cotyledon stage (75 days after sowing) under greenhouse conditions. The selected isolates from preserved stock culture were grown on KBHS medium for 2 days. The Colony Forming Unit (CFU) was counted using colony counter and then serial (1:10) dilution was made. Finally, CFU mL^{-1} was calculated and 10^9 CFU mL^{-1} bacterial cells were used for seedlings inoculation. On the other hand, the inoculum of pathogen (GWO1 isolate) was reproduced from the preserved stock culture. The stock culture of Gera (GWO1) isolate was used to initiate colony growth on to SNA medium for about a week under standard conditions as described before. The culture of GWO1 isolate, grown in plates for 7 days was flooded with 10 mL of sterile water and then rubbed gently from the agar surface to free the conidia. The spore suspension was uniformly stirred up with sterilized magnetic stirrer and then filtered into another sterile beaker through double layer of cheese clothes. Then the spore concentration was adjusted to 2.3×10^6 conidia mL^{-1} using Heamocytometer (Girma, 2004).

Seedlings inoculation: Based on the above *in vitro* antagonism tests, *Bacillus* spp. (JU544) and three *Pseudomonas* spp. (JU941, JU13 and JU23) were selected for further *in vivo* antagonism studies. When the coffee seedlings reached at cotyledon stage (75 days after sowing), they were inoculated with conidial suspension of the GWO1 isolate and rhizobacterial isolates by stem nicking procedures described in Pieters and van der Graaff (1980) with some modification. A sterile scalpel was first immersed into the inoculums suspension and then the stem of each seedling was gently wounded with the scalpel at about 2 cm from the soil level and a drop (nearly 1 mL) of fungal and bacterial inoculums was placed in the notch at seven day intervals. The bacterization of 15 seedlings was made 7 days before, simultaneously and 7 days after inoculation by the pathogen (*G. xylarioides*). The negative and positive controls were treated with sterile distilled

water and the pathogen, respectively. In order to create favorable conditions for both pathogens and antagonists, chambers were made for 10 days in greenhouse using transparent thick polythene sheet. The temperature in the chambers was measured three times a day and the mean temperature was between 22 and 26°C. The relative humidity of the chamber was maintained at above 85% by humidifier and it was measured using Psychrometer. Then, the number of healthy and wilting seedlings was counted based on the external symptom for 6 months starting from 30 days after inoculation (Girma, 2004). The percentage of disease incidence was calculated from cumulative number of dead over total number of seedlings (dead plus healthy). In addition, the disease severity was recorded (Musoli *et al.*, 2008) with some modifications on scales using a 0 to 4 scales (classes). Where 0 = no disease, 1 = curling leaves and stunted growth, 2 = leaf wilting and yellowing, 3 = leaf necrosis, leaf wilting and abscission and 4 = plants were dead. Disease index (DI) for each treatment was expressed as a percentage of the maximum possible infection using the equation used by Biratu (1995):

$$\text{Percentage disease index (\%DI)} = \frac{w + 2x + 3y + 4z}{4\,(\text{Higher rating value})\,(v + w + x + y + z)}$$

where, v is number of seedlings in class 0, w is number of seedlings in class 1, x is number of seedlings in class 2, y is number of seedlings in class 3, z is number of seedlings in class 4. Then, biological control efficacy was calculated using the formula described by Abbott (1925):

$$\text{Diease control (\%)} = \frac{\text{Disease index of control} - \text{Disease index of treatment}}{\text{Disease index of control}} \times 100$$

where, disease index of control is the disease index from only pathogen inoculated seedlings, diseases index of treatment is disease index from pathogen and bio-control agent inoculated seedlings.

The radial mycelial growth inhibition with rhizobacterial antagonists and its mode of action was recorded by studying their ability to produce Hydrogen cyanide and some lytic enzymes. The characteristic external symptoms, number of healthy and infected coffee seedlings per pot were recorded at 14 days intervals for 6 months starting a month after inoculation with *G. xylarioides* (Adugna and Huluka, 2000) and rhzobacterial isolates. The percentage of Disease Severity Index (%DSI) and biological control efficiency were computed. The area under disease progress curve (AUDPC) was calculated from percentage of disease incidence according to Tshilenge-Djim *et al.* (2004). This parameter was calculated using the formula:

$$\text{AUDPCi} = \Sigma[(X1+X2).0.05][t2-t1]$$

Where, X1 and X2 represent the coffee wilt incidence at time 1 and time 2, t2-t1: time interval between two observations.

Data analysis: The data were subjected to analysis of variance (ANOVA) using the General Linear Modeling (GLM) procedure of SAS-9.2 software (SAS, 2008) to compare different treatments. The greenhouse data were transformed to angular values before subjecting to ANOVA to normalize the data. Tukey's test was performed at 5% level of significance to denote significant differences between treatments.

RESULTS AND DISCUSION

***In vitro* antagonistic activities of rhizobacterial isolates against *G. xylarioides*:** Out of the 81 rhizobacterial isolates tested for *in vitro* antagonism against *G. xylarioides,* 11 (13.6%) were found to inhibit the radial mycelial growth of *G. xylarioides* with varying efficiencies (Table 1). The results of antagonism clearly demonstrated that rhizobacteria isolates exhibited significant ($p<0.0001$) inhibition against the radial growth of *G. xylarioides* (Table 1). The percentage of *in vitro* radial mycelial growth inhibition by the rhizobacterial isolates against *G. xylarioides* varied between 18.4% and 71.5% (Table 1). This differential sensitivity of the pathogen to the different isolates of antagonistic rhizobacteria might be due to the genetic potentialities of the pathogen to tolerate the different antimicrobial and the chemical substances produced from bacterial isolates. The mode of action exerted and/or the type of antifungal metabolite produced by the isolates may vary (Williams and Asher, 1996). The assay method also might influence the antifungal activity of rhizobacterial isolates (Fravel, 1988) against the pathogen. The antagonistic rhizobacteria isolates that caused significant radial mycelial growth inhibition was without physical contact with the pathogen (Fig. 1). This indicated that the rhizobacteria isolates released certain antifungal metabolites into the agar medium (Montealegre *et al.*, 2003).

Table 1: Inhibition of *G. xylarioides* radial growth by rhizobacterial isolates on half strength King's B medium

Bacterial isolates	Radial mycelial growth inhibition (%)
Bacillus spp. (JU544)	71.5[a]
Pseudomonas spp. (JU941)	66.2[b]
Pseudomonas spp. (JU13)	61.1[c]
Pseudomonas spp. (JU23)	54.4[d]
Micrococcus spp. (JU11)	48.8[e]
Pseudomonas spp. (JU31)	44.7[f]
Bacillus spp. (JU21)	41.8[g]
Burkholderia spp. (JU32)	40.7[g]
Flavobacter spp. (JU24)	35.5[h]
Pseudomonas spp. (JU44)	26.9[i]
Bacillus spp. (JU33)	18.4[j]
Control	0.0[k]

*Means followed with the same letter(s) in the same column are not significantly different at the probability level of ($p>0.05$) according to Tukey test

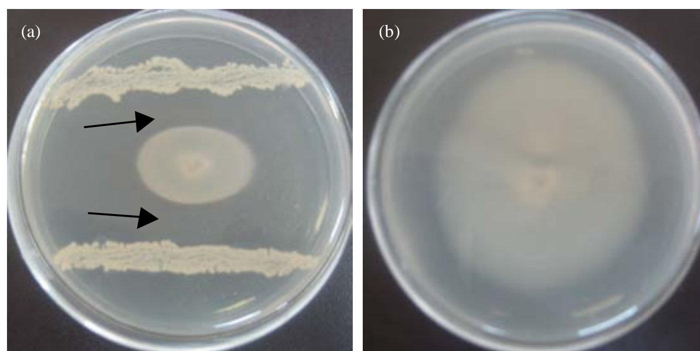

Fig. 1(a-b): Dual culture technique of *Bacillus* sp. (JU544) against *G. xylarioides* (a) *Bacillus* sp. (JU544) culture with *G. xylarioides* (b) Control of *G. xylarioides*. Arrows indicate the zones of inhibition

The maximum radial mycelial growth inhibition of *G. xylarioides* was caused by rhizobacterial isolates of *Bacillus* spp. (JU5444) (71.5%) followed by *Pseudomonas* spp. (JU941, JU13 and JU23, with 66.2, 61.1 and 54.4% radial mycelial growth inhibition, respectively (Table 1). The least radial mycelial growth inhibition of *G. xylarioides* was recorded with *Bacillus* spp. (JU21), *Burkholderia* spp. (JU32), *Flavobacter* spp. (JU24), *Pseudomonas* spp. (JU44) and *Bacillus* spp. (JU33). Although the radial mycelial growth of *G. xylarioides* significantly reduced by antagonistic bacteria, there was no significant (p>0)variation between rhizobacterial isolates of *Bacillus* spp. (JU21) and *Burkholderia* spp. (JU32). Control plates (not treated with the bacterial isolates) were completely covered with *G. xylarioides* showing no inhibition (Table 1 and Fig. 1). The *Bacillus* sp. (JU544) was the most effective by reducing the radial mycelial growth of *G. xylarioides*.

The present study is in agreement with the previous work of Muleta *et al.* (2007) who reported that 23 rhizobacterial isolates (13 *Pseudomonas* spp. and 10 *Bacillus* spp.) showed radial mycelial growth inhibition (>2.4 cm) against *G. xylarioides*. Similarly, Mark (2007) reported strong inhibition of fungal coffee pathogens including G. *xylarioides* by *Pseudomonas fluorescens*.

Some phytobeneficial traits of the rhizobacterial isolates: All of the 11 selected rhizobacterial antagonists produced at least one of the four tested phytobeneficial traits (Table 2). From 11 rhizobacterial isolates, three *Pseudomonas* spp. (JU941, JU13 and JU31), one *Bacillus* sp. (JU21) and one *Flavobacter* sp. (JU24) produced hydrogen cyanide (Table 2). Likewise, isolates JU544 (*Bacillus* sp.), JU13, JU23 and JU44 (*Pseudomonas* spp.) and JU32 (*Burkholderia* sp.) showed production of chitinase enzyme (Table 2). From the tested antagonistic rhizobacterial isolates, three *Bacillus* spp. (JU544, JU21 and JU33), four *Pseudomonas* spp. (JU941, JU23, JU31 and JU44) and one *Micrococcus* sp. (JU11) were positive for protease test (Table 2). In addition, two *Bacillus* spp. (JU544 and JU21), five *Pseudomonas* spp. (JU941, JU13, JU23, JU31 and JU44) produced lipase enzyme (Table 2).

Hydrogen cyanide (HCN) production: Among the effective rhizobacterial antagonists, five of them were able to synthesize hydrogen cyanide (Table 2). As described by Bakker and Schippers (1987), HCN producers formed orange brownish compound with sodium picrate and the intensity of the colour increased with the amount of HCN. In the present study, three *Pseudomonas* spp. (JU941, JU13 and JU331), one *Bacillus* sp. (JU21) and one *Flavobacter* sp. (JU24) produced HCN

Table 2: Rhizobacterial isolates with some phytobeneficial traits

Isolates	HCN	CHI	PRO	LIP
Bacillus spp. (JU544)	-	+	+	+
Pseudomonas spp. (JU941)	+	-	+	+
Pseudomonas spp. (JU13)	+	+	-	+
Pseudomonas spp. (JU23)	-	+	+	+
Micrococcus spp. (JU11)	-	-	+	-
Pseudomonas spp. (JU31)	+	-	+	+
Bacillus spp. (JU21)	+	-	+	+
Burkholderia spp. (JU32)	-	+	-	-
Flavobacter spp. (JU24)	+	-	-	-
Pseudomonas spp. (JU44)	-	+	+	+
Bacillus spp. (JU33)	-	-	+	-

*Rhizobacterial isolates were recovered from coffee and other crop rhizospheres, HCN: Hydrogen Cyanide, CHI: Chitinase, PRO: Protease, LIP: Lipase. +: Positive reaction, -: Negative reaction

that turned yellow colored filter strips to brownish color when compared to the untreated control. The remaining six isolates were not revealed the production of HCN since they did not develop orange brown color. Hence, the production of HCN by the rhizobacterial isolates might contribute to effective inhibition of radial mycelial growth of *G. xylarioides* under *in vitro* condition. This is due to the fact that HCN could effectively block the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The present study is in agreement with the previous research findings (Kremer and Souissi, 2001; Muleta *et al.,* 2007, Prashar *et al.*, 2012) who reported that over 30% of rhizobacterial isolates were cyanogenic.

Killani *et al.* (2011) reported that under *in vitro* conditions, *B. subtilis* inhibited the radial mycelial growth of soilborne fungal pathogens recovered from cowpea. The reason for the inhibitory activity of *Bacillus* spp. could be related to production of toxic metabolites such as HCN (Shobha and Kumudini, 2012).

Production of lytic enzymes: Out of the 11 isolates tested for the production of chitinase enzyme, five of them produced clear zones on chitin agar medium. Formation of a clear zone indicated that the isolates produced extracellular chitinase enzyme which is important for degradation of chitin. These chitinase producing rhizobacterial isolates significantly reduced radial mycelial growth of the pathogen. In the present study, one *Bacillus* sp. (JU544), three *Pseudomonas* spp. (JU13, JU23 and JU44) and one *Burkholderia* sp. (JU32) showed the ability to degrade chitin with the formation of clear zone around the bacterial cells under *in vitro* conditions.

Chet (1987) demonstrated that chitinolytic enzymes production by microbes that have the ability to degrade fungal cell walls which signify their considerable importance in the biological control of many plant pathogens. Likewise, Muleta *et al.* (2007) reported that from a total of 212 rhizobacterial isolates recovered from Arabica coffee rhizosphere, some isolates were able to produce chitinase enzyme and inhibited the radial mycelial growth of *F. xylarioides* under *in vitro* condition. Therefore, this could be considered the major mechanism involved in the inhibition of radial mycelial growth of *G. xylarioides* (Muleta *et al.,* 2007).

In the present study, the rhizobacterial isolates that were positive for chitinase activity had significant radial mycelial growth inhibition (>26.9%) against *G. xylarioides* (Table 2). Muleta *et al.*(2007) also reported that the rhizobacterial isolates that were positive for chitinase activity had remarkably large inhibition zones (>3 cm) when tested against *F. xylarioides*.

Four rhizobacterial isolates of *Pseudomonas* spp. (JU941, JU44, JU23 and JU31), three *Bacillus* spp. (JU544, JU21 and JU33) and one *Micrococcus* sp. (JU11) produced protease enzyme showing zone of clearance around the bacterial colony on skim milk agar medium. The remaining antagonistic rhizobacterials did not produce protease enzyme (Table 2). The antagonistic effect of *Stenotrophomonas maltophila* against *Pythium ultimum* in the rhizosphere of sugar beet was due to the production of extra cellular protease since this enzyme has a great role in degradation of pathogenic fungal cell wall (Dunne *et al.,* 1997; Muleta *et al.,* 2007) have also reported that from 23 rhizobacterial isolates (13 *Pseudomonas* spp. and 10 *Bacillus* spp.) recovered from Arabica coffee rhizosphere, all of them were able to produce protease enzyme.

According to the present study, rhizobacterial isolates of two *Bacillus* spp. (JU544, JU21) and five *Pseudomonas* spp. (JU941, JU13, JU23, JU31 and JU44) produced lipase enzyme (Table 2). The entire producers form turbid zone around inoculated bacterial cells on Luria Casei agar medium indicating the production of lipase enzyme which might be involved in lysing the fungal

Table 3: Interaction effects of bacterial antagonists and their time of application on suppression of *G. xylarioides* under greenhouse conditions

Bacterial isolates	Time	Severity (%)	Incidence (%)	CWD control efficiency (%)
Pseudomonas (JU941)	7DB	25.57 ± 4.3^{fg}	26.35 ± 4.8^{de}	64.14^{ab}
	AT	$40.95\pm9.3^{d\text{-}f}$	$41.66\pm9.9^{c\text{-}e}$	$48.45^{a\text{-}c}$
	7DA	$58.69\pm4.8^{b\text{-}d}$	59.03 ± 4.3^{bc}	$30.21^{c\text{-}e}$
Pseudomonas (JU23)	7DB	$27.69\pm13.3^{e\text{-}g}$	29.48 ± 16.1^{de}	62.12^{ab}
	AT	$48.54\pm3.7^{c\text{-}e}$	$49.55\pm5.9^{b\text{-}d}$	$40.70^{b\text{-}d}$
	7DA	$60.07\pm5.6^{a\text{-}d}$	61.92 ± 2.6^{bc}	$28.71^{c\text{-}e}$
Bacillus (JU544)	7DB	17.16 ± 14.2^{gh}	17.98 ± 3.7^{ef}	72.64^{a}
	AT	$57.57\pm8.4^{b\text{-}d}$	59.38 ± 7.9^{bc}	$31.39^{c\text{-}e}$
	7DA	71.82 ± 4.1^{ab}	72.87 ± 3.6^{ab}	15.95^{ef}
Pseudomonas (JU13)	7DB	$27.74\pm11.8^{e\text{-}g}$	29.82 ± 12.9^{de}	61.87^{ab}
	AT	$64.36\pm3.8^{a\text{-}c}$	$65.37\pm5.6^{a\text{-}c}$	$24.19^{c\text{-}f}$
	7DA	$64.29\pm6.8^{a\text{-}c}$	65.78 ± 8.3^{ab}	$24.01^{d\text{-}f}$
Control (N)	7DB	00.0 ± 0.0^{h}	0.0 ± 0.0^{f}	-
Control (P)	7DB	97.76 ± 0.0^{a}	100 ± 0.0^{a}	0 f
Mean		45.1 ± 7.0	47.2 ± 7.7	33.78 ± 7.9
CV		15.6	16.3	23.5

*Means with different letters are significantly different across columns at p = 0.05 according to the Tukey test. Control (P): Positive control (only *G. xylarioides* without bacterial antagonists), Control (N): Negative control (only sterile water inoculated seedlings), 7DB: Treating of coffee seedlings with biocontrol agent 7 days before the pathogen, AT: Treating of seedlings at the same time with pathogen, 7DA: Treating of coffee seedlings with biocontrol agent 7 days after the pathogen

cells. Muleta *et al.* (2007) reported that from 23 rhizobacterial isolates (13 *Pseudomonas* spp. and 10 *Bacillus* spp.) recovered from Arabica coffee rhizosphere, all of them were able to produce lipase enzyme.

Effects of rhizobacteria against cwd severity and incidence under greenhouse condition: Rhizobacterial antagonists, time of applications and their interaction effect showed a highly significant ($p<0.0001$) variation. The coffee wilt disease severity and incidence were reduced from 97.76% and 100% on positive control (*G. xylarioides* only) to 45.1 and 47.2%, respectively in the presence of the rhizobacterial antagonists (Table 3). However, the negative control (only sterile water inoculated seedlings) was free from the pathogen since no infection symptom was observed (Table 3).

Bacterization of the seedlings seven day before the pathogen, generally, revealed a remarkable reduction of CWD severity and incidence (Table 3). Hence, the least disease severity (17.16%) and incidence (17.98%) encountered when the seedlings were inoculated with *Bacilus* sp. (JU544) 7 days before the pathogen followed by *Pseudomonas* spp. (JU941, JU23 and JU13) compared to the positive control with CWD severity (97.76%) and incidence (100%) (Table 3). Nevertheless, there was no significant ($p>0$) variation in CWD severity between the two *Pseudomonas* spp. (JU23 and JU13). Similarly, there was no significant ($p>0$) variation in incidence of CWD among *Bacillus* sp. (JU544) and three *Pseudomonas* spp. (JU23, JU941 and JU13) when the seedlings were treated 7 days before the pathogen (Table 3). Although reduction of CWD severity and incidence were significant, the highest disease severity and incidence were recorded when the seedlings were inoculated with *Bacillus* sp. (JU544) followed by three *Pseudomonas* spp. (JU13, JU23 and JU941, respectively) (Table 3).

Under greenhouse condition, the four rhizobacterial isolates, time of application and the interaction of the two (bacterial treatment and time of applications) were highly significant ($p<0.0001$) in suppression of the fungal pathogen. When the antagonists were applied before, after and at the same time with the pathogen, the treatments gave a significant effect compared to infected control (Table 3). The highest percentage of biological control was obtained against *G. xylarioides* by using *Bacillus* sp. (JU544) followed by *Pseudomonas* spp. (JU941, JU23 and JU13) when the seedlings were inoculated 7 days before the pathogen. However, there was no significant ($p>0$) variation in control efficiencies among *Pseudomonas* spp. (JU941, JU23 and JU13) (Table 3).

Treatment of seedlings with *Pseudomonas* sp. (JU13) and *Bacillus* sp. (JU544) prior to the fungal pathogen showed a remarkable control efficiency ranging from 61.87 to 72.64%, respectively. Furthermore, control efficiency of at the same time inoculation with *Pseudomonas* spp.(JU13) and (JU941) ranged from 24.19 to 48.45%, respectively. Nevertheless, the least control efficiency was recorded when *Bacillus* sp. (JU544) and *Pseudomonas* sp.(JU941) were inoculated after the pathogen which ranged from 15.95 to 30.21%, respectively.

In the current study, rhizobacterial antagonists were found to reduce coffee wilt disease severity and incidence indicating that biological control has considerable promise for reducing *G. xylarioides*. A growing body of evidence shows that phytobeneficial soil microorganisms could adversely affect the population density of pathogens and their metabolic activities through competition, antagonism and hyperparasitism (Raaijmakers *et al.*, 2009). In addition, their diverse mode of action under *in vitro* condition might assist in reduction of CWD severity and incidence under greenhouse condition.

In Uganda, Mark (2007) found that the suppression of CWD incidence was 30% over a period of 90 days with inoculation of *Pseudomonas fluorescens*. This variation might be due to differences in environmental factors. Similarly, Larkin and Fravel (2002) reported that the basic environmental conditions, such as temperature, moisture, sunlight and relative humidity can greatly affect the pathogen and biocontrol organisms and these effects may influence efficacy of biological control. Other conditions related to coffee varietal differences, way of delivering bacterial and fungal inoculums to the seedlings, pathogen reaction to the introduced bacterial isolates and mechanism of antagonism of the different bacteria could influence biological control efficiency. The observed variation also could be due to the differences in taxonomical, morphological and genetic characteristics of the antagonistic rhizobacterial isolates (Williams and Asher, 1996). When the coffee seedlings inoculated seven days before the pathogen, the highest biocontrol efficiency (72.64%) was encountered with *Bacillus* sp. (JU544) followed by *Pseudomonas* spp. (JU941, JU23 and JU13) with their control efficiency of 64.14, 62. 12 and 61.87%, respectively.

This suggests that introduced bacteria could get opportunity to reproduce in stem tissue, move upward and downward from the point of application by colonizing the internal tissues which exclude the entry of a pathogen into the vascular tissue. Consequently, the entry of coffee pathogen (*G. xylarioides*) could be prevented from entering into the vascular tissue. Benhamou *et al.* (1998) reported that the entrance of *Fusarium* wilt pathogens into the vascular tissue prevented when the *Bacillus pumilus* inoculated prior to fungal inoculation and the mycelial growth restricted to the outer surface of the stem (dermal tissue). Their significant control efficiency also could be due to their greater ability to compete for nutrients such as organic carbon and/or for

infection sites than the pathogen. Alabouvette *et al.* (2006) reported that competition for nutrients, especially for carbon by *Bacillus* spp. is assumed to be responsible for the well known phenomenon of fungistasis by inhibition of fungal spore germination. Similarly, Haas and Defago (2005) reported that suppression of soilborne plant pathogens by *Pseudomonas* was through competition for niche and nutrients.

Furthermore, production of antifungal compounds and induction of systemic resistance by the introduced bacteria might be responsible for reduction of CWD symptom severity and incidence. Bent (2006) reported that the gram negative bacteria (*Pseudomonas* and *Serratia)* genera as well as gram-positive bacteria particularly *Bacillus* spp. were identified as inducers of systemic resistance to the host plant. Ferraris *et al.* (1987) reported that the levels of chitinase and glucanase increase in the plant tissue when the pathogen attack occurs which indicated that *Bacillus* spp. may activate defense systems in the host plant.

Effects of rhizobacterial isolates on progression of CWD incidence: The total Area under Disease Progress Curve (AUDPC) was calculated from the coffee wilt incidence for over a period of 5 months revealed that bio-controls, time of applications and their interactions had significant ($p<0.0001$) effect on progress of coffee wilt incidence under greenhouse conditions. The least AUDPCi was recorded when the Catura rojo coffee seedlings were treated with *Bacillus* (JU544), *Pseudomonas* (JU941), *Pseudomonas* (JU13), *Pseudomonas* (JU23) 7 days before the pathogen (Table 4).

The highest AUDPCi mean (8067) was obtained when the seedlings were treated with bacterial antagonists of *Pseudomonas* (JU13) 7 days after inoculation of pathogen (Table 4). Rhizobacterial isolates such as *Bacillus* (JU544), *Pseudomonas* (JU941) and *Pseudomonas* (JU23) also showed the actual mean AUDPCi of 7449, 6316.5 and 5782.3, respectively 7 days after inoculation of the pathogen. These results indicate that the bacterial biocontrol agents had significant effect on the progression of coffee wilt incidence over time. Particularly, treating of the

Table 4: Interaction effects of rhizobacteria and their time of application on the progress of coffee wilt disease incidence under greenhouse condition

	Mean AUDPCi (% day)		
	Time		
Biocontrol agents	7DB	AT	7DA
Bacillus (JU544)	(900) 1448.5^{gh}	(6150.3) 5518.1^{b-d}	(7449.7) 6470.6^{a-c}
Pseudomonas (JU941)	(1450.5) 2233^{fg}	(3867) 4040^{d-f}	(6316.5) 5645.7^{b-d}
Pseudomonas (JU23)	(2266.5) 2863.2^{e-g}	(4733.5) 4673.7^{c-e}	(5782.3) 5274.1^{b-d}
Pseudomonas (JU13)	(2100.5) 2767.3^{e-g}	(7514.7) 6451.2^{a-c}	(8067) 6847.7^{ab}
Control (P)	(9016) 8028.15^{a}	(9016) 8028.15^{a}	(9016) 8028.2^{a}
Control (N)	(0.0)109.35^{h}	(0.0) 109.35^{h}	(0.0) 109.35^{h}

*Means followed by the same letter (s) in the same column are not significantly different. The area under disease incidence progress curve (AUDPCi) was calculated from nine consecutive 15 days assessments of percentage of coffee wilt disease incidence, 7DB: Treating of coffee seedlings 7 days before the pathogen, AT: Inoculation of bacterial antagonists and pathogen simultaneously, 7DA: Treating of coffee seedlings with bacterial antagonists 7 days after the pathogen. Control (p) and Control (N) are seedlings inoculated by pathogen and sterile distilled water, respectively, Numbers outside the parenthesis are transformed data

seedlings 7 days prior to the pathogen by *Bacillus* (JU544) and *Pseudomonas* spp. (JU4941 and JU13) showed considerably greater potential for the management of *G. xylarioides* than the other treatments.

CONCLUSION

The results of antagonism study clearly demonstrated that rhizobacterial isolates exhibited inhibition of the radial mycelial growth of *G. xylarioides* under *in vitro* conditions. The fungal radial mycelial growth reduction could be occurred partly due to several modes of actions (HCN, chitinase, protease and lipase production). Under greenhouse conditions, treating of seedlings with antagonistic bacteria prior to the fungus generally showed a notable result in reducing CWD severity and incidence. The highest CWD control efficiency was recorded when the seedlings were treated with *Bacillus* sp. (JU544) and *Pseudomonas* sp. (JU13) 7 day before the pathogen application.

In general, the present rhizobacterial antagonists were found to reduce coffee wilt disease severity and incidence indicating that the rhizobacterial biological control has considerable promise in suppression of *G. xyliroides* populations. The *Bacillus* sp. (JU544) was found to be the most efficient in reducing the CWD severity and incidence compared to other bacterial biocontrol agents. Therefore, further field evaluation will be momentous to ascertain the antagonistic efficacy of those promising rhizobacterial isolates.

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