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BIOLOGICAL CONTROL OF FUSARIUM WILT OF CUCUMBER (*Cucumis sativus*) BY ANTAGONISTIC LACTIC ACID BACTERIA ISOLATED FROM RHIZOSPHERE OF FIVE MEDICINAL PLANTS

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ABSTRACT

Fusarium wilt is one of the important diseases of cucumber and causes economic loss to farmers. The present study was undertaken to evaluate the potential of rhizosphere lactic acid bacteria as biocontrol agents of *Fusarium* wilt of cucumber. Lactic acid bacteria (LAB) were isolated and identified from the rhizosphere of five medicinal plants. The *in-vitro* antagonistic activity of LAB strains on *F. oxysporum* f.sp. *cucumerinum* was evaluated by dual culture method. The screen house experiment was then conducted to assess the effect of antagonistic LAB isolates on *Fusarium* wilt disease incidence in cucumber plants. The antagonistic LAB strains were further characterized using 16S rRNA gene sequencing technique. The total LAB counts of rhizospheric soil samples ranged from 7.0×10^5 cfu/g to 15.0×10^5 cfu/g. The LAB isolates were identified as strains of *Lactobacillus acidophilus* (21.4%), *L. plantarum* (35.7%), *L. fermentum* (28.6%), *L. alimentarius* (7.1%) and *L. brevis* (7.1%). Treatment of cucumber seeds with antagonistic LAB strains significantly reduced *Fusarium* wilt of cucumber incidence from 95% to 48%. *Lactobacillus fermentum* isolated from the rhizosphere of *A. indica* exhibited strong disease suppression (49.5%). The study therefore revealed that the rhizospheric-LAB could be applied to reduce the manifestation of *Fusarium* wilt in cucumber.

Keywords: Biocontrol, Cucumber, *Fusarium* wilt, Lactic acid bacteria, Medicinal plants, Rhizosphere

INTRODUCTION

Cucumber (*Cucumis sativus* L.), which belongs to family *Cucurbitaceae*, is one of the most widely grown vegetable crops in the world. The crop is grown either in the open field or under protected houses (Ahmed, 2010). In Nigeria, it is mainly cultivated in Jos, Plateau State and some other states of the federation (Wilcox *et al*., 2015). However, this crop can be infected with many bacterial and fungal diseases in which Fusarium wilt is included.

Fusarium species are the major soil-borne and seed-borne pathogens that cause wilt and rot diseases in more than 80 plant species (Mahfooz *et al*., 2011). The pathogens could cause up to 100% yield loss worldwide

(Shanthi and Vittal, 2013). *Fusarium oxysporum* f. sp. *cucumerinum,* which survives in soil and seed in the form of chlamydospores, is the frequent pathogen of cucumber (Farrag and Moharam, 2012). The mycelia of this pathogen enter the epidermal tissues through roots, extend to the vascular bundles and form spores in plants (Chehri *et al.,* 2010). The pathogen causes seed abortion and rot, vascular wilting, yellowing, foliar necrosis, reduction or elimination of germination capacity as well as plant damage at later stages of plant growth resulting in development of the disease as systemic or local infection (Khanzada *et al.,* 2002). The Fusarium wilt can be controlled by using resistant cultivars, chemical fungicides as well as fumigants (Fravel *et al.,* 2005) and biological control agents (Idris *et al.,* 2007).

Chemical pesticides are extensively used to prevent or control plant diseases but associated with harmful effects. Hence, there is a need to search for alternative approaches of pest control in order to reduce over-dependence on chemical pesticides (Alwathnani and Perveen, 2012). Biological control is therefore being considered as an alternative approach of minimizing the use of chemical inputs in agriculture (Oloyede *et al*., 2017).

Biocontrol is the use of the disease-suppressive microorganisms or their metabolites, keeping the level of deleterious microorganisms under control or below a threshold limit (Maji and Chakrabartty, 2014). Commonly used biocontrol agents with antagonistic activities against plant pathogens include fungi and bacteria such as species of *Trichoderma, Pseudomonas, Bacillus* and *Streptomyces*.

Though lactic acid bacteria (LAB) with antifungal and antibacterial activity are well utilized in food, meat and milk products as biopreservatives, but less attention has been paid to exploit the antifungal activity of LAB for biological control of phytopathogenic fungi. Therefore, the present study was designed to characterize lactic acid bacteria isolated from the rhizosphere of some medicinal plants and to evaluate their ability to suppress *Fusarium* wilt of cucumber under screen house conditions.

MATERIALS AND METHODS

Collection of rhizospheric soil samples

Soil samples were collected aseptically from the rhizosphere (0 – 15 cm depth) of five medicinal plants (*Moringa oleifera*, *Azadirachta indica, Vernonia amygdalina, Ocimum gratissimum* and *Aloe vera*) into sterile containers. Intact root systems were dug out and the soil samples were carefully taken. The rhizospheric soil samples were collected in triplicates for each of the plant variety and transported to the laboratory in ice-box. In the laboratory, extraneous materials were removed and the soil samples from each plant species were thoroughly mixed as a composite sample.

Isolation and characterization of Lactic acid bacteria

The lactic acid bacteria (LAB) were isolated from the soil samples by direct plating procedure described by Ekundayo (2014) with little modifications. The soil samples were inoculated on De Mann Rogosa and Sharpe (MRS) agar plates and incubated at 35^{o}C for 48 hours under anaerobic conditions. The number of LAB colonies on the agar plates were counted and expressed as Colony forming unit per gram (CFU/g) of soil. Phenotypic characterization was carried out by observing their colonial characteristics, Gram staining and series of biochemical tests.

In-vitro antagonistic activity of Lactic

acid bacterial strains on Fusarium oxysporum f. sp. cucumerinum

An *in-vitro* study was conducted to determine the antagonistic potentials of the lactic acid bacterial isolates on *Fusarium oxysporum* f.sp *cucumerinum* (causing Fusarium wilt of cucumber) using dual plate culture technique described by Nawangsih and Purba (2013) and Dinesh *et al*. (2015) with little modifications. *Fusarium oxysporum* f.sp. *cucumerinum,* was collected from the Department of Crop Protection, Federal University of Agriculture, Abeokuta and confirmed at the Department of Microbiology.

The test pathogen, *F. oxysporum* f. sp. *cucumerinum,* was inoculated on potato dextrose agar plates and incubated at room temperature (28±2°C) for seven days. The mycelia and agar were cut into pieces using 5.00 mm (in diameter) sterile cork borer, and each piece of mycelia was placed on one side of PDA plate while the LAB strain was streaked vertically on the opposite side of the fungus. The control plates were prepared by replacing the LAB isolate with sterile distilled water. The plates were then incubated at 28±2°C for 7 days. The radial mycelia growths of test pathogen in treated and control plates were measured and the percentage inhibition of growth over untreated control was estimated using the formula below:

$$I = [D_1 - D_2/D_1] \times 100$$

where I is the percentage inhibition and D_1 and D_2 are the radial mycelia growths of the pathogen in control and treatment, respectively.

In-vivo biocontrol study of Lactic acid bacterial strains on Fusarium wilt of cucumber

The screen house experiment was conducted at Federal University of Agriculture, Abeokuta. Cucumber seeds were surface-sterilized with 5% Sodium hypochlorite solution for 2 minutes, washed three times in sterile distilled water and then air dried at room temperature (25±2°C). The inocula of antagonistic lactic acid bacteria were prepared by growing each strain in sterile De Mann Rogosa and Sharpe (MRS) broth at 30°C for 48 hours under anaerobic conditions. Each LAB culture was centrifuged at 10,000 rpm for 10 minutes to pellet the cells and the cells were washed twice with sterile distilled water. The bacterial cells were then suspended in a sterile 0.1 M Phosphate buffer (pH 7.0) and made to 1.5×10^8cfu/ml. Dry seeds were then immersed in each LAB suspension and stirred frequently for one hour. The treated seeds were air-dried overnight at room temperature. The seeds used for control experiments were treated with sterile distilled water. Similarly, the fungal inoculum was prepared by growing *Fusarium oxysporum* f.sp *cucumerinum* on PDA plates at room temperature for 7 days. The spores were carefully scrapped, mixed in sterile distilled water and then adjusted to 1.0×10^6 spores/ml.

The screen house experiment was conducted in a completely randomized design with six treatments and four replicates. The treatments consisted of T_1: neither LAB nor the pathogen was applied, T_2: cucumber plants infected only with *Fusarium oxysporum* f.sp *cucumerinum*, T_3: cucumber plants infected with *F. oxysporum* f.sp *cucumerinum* and treated with antagonistic LAB4, T_4: cucumber plants infected with *F. oxysporum* f.sp *cucumerinum*

and treated with antagonistic LAB7; T_5: cucumber plants infected with *F. oxysporum* f.sp *cucumerinum* and treated with antagonistic LAB8 and T_6: cucumber plants infected with *F. oxysporum* f.sp *cucumerinum* and treated with antagonistic LAB14.

The LAB-treated and control seeds were sown and grown for 7 days in 18.0 cm diameter pots containing sterilized soil. The seedlings were then inoculated with 1.0 ml of fungal spore suspension per pot, by adding the inoculum around the experimental plants after removing soil, except the seedlings in T_1 treatment which were inoculated with 1.0 ml of sterile distilled each. After inoculation, all pots were covered with polyethylene bags for 24 h to maintain high humidity. The plants were then kept in the experimental screen house with a 12-hour day light and temperature of 25 to 30°C. The plants were watered regularly with sterile water and the seedlings were observed after 4 weeks. Disease severity was recorded by visual observation of the disease symptoms with reference to the untreated infected controls where no LAB but the pathogen was applied. Disease index data were obtained and recorded according to the scale ranged from 0 to 4 (Saha *et al*., 2012). Symptom severity was graded into five disease classes as follows: 0 (No disease or wilt), 1 (1 - 25% of leaves withered), 2 (26 - 50% of leaves withered/traces of stem rot), 3 (51 - 75% of leaves withered/stunted growth/ stem rot) and 4 (76-100% of leaves withered/ damping off/ wilting/ seedling death). Based on the classes, the percentage disease incidences (PDI) and Disease reduction percentage (DRP) were calculated as follows:

$$\text{Disease incidence} = \frac{(\text{Disease index} \times \text{number of diseased plants in this index}) \times 100\%}{\text{Total number of plants investigated} \times \text{Highest disease index}}$$

$$\text{Disease reduction percentage (DRP)} = \frac{(\text{Disease incidence in untreated control} - \text{Disease incidence in treated plants}) \times 100\%}{\text{Disease incidence in untreated control}}$$

(Xue *et al*., 2009; Ahmed *et al*., 2010)

Molecular characterization of antagonistic Lactic acid bacterial strains

The two lactic acid bacterial isolates (LB4 and LB8) that showed maximum reduction of Fusarium wilt incidence were further characterized using 16S rRNA gene sequencing method. Extraction of genomic DNA of the isolates was performed using Bacterial genomic DNA extraction kit (Norgen Biotek Corporation, Canada) following the manufacturer's instructions. The 16S rRNA genes of the LAB strains were amplified by polymerase chain reaction using a pair of 16S rRNA universal primers designated as 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') for forward and 1492R (ACG GCT ACC TTG TTA CGA CTT-3') for reverse (Jiang *et al*., 2006; Abdul Hamid *et al*., 2012). The PCR fragments were purified and sequenced. Gene sequences were compared with GenBank database of National Centre for Biotechnology Information (NCBI) using BLASTn search tool to identify the strains.

Statistical analysis

Data collected were subjected to One-way Analysis of Variance (ANOVA). Means were separated using the Duncan Multiple Range Test (DMRT) at $P \leq 0.05$.

RESULTS

Isolation of Lactic acid bacteria from rhizosphere of medicinal plants

Figure 1 shows the total LAB counts of soil samples from the rhizosphere of *Moringa oleifera*, *Aloe vera*, *Vernonia amygdalina*, *Ocimum gratissimum* and *Azadirachta indica*. The total LAB counts ranged from 7.0×10^5 cfu/g (*Aloe vera*) to 15.0×10^5 cfu/g (*Moringa oleifera*).

From this study, a total of fourteen (14) lactic acid bacterial isolates were obtained and identified by phenotypic methods as strains of *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus fermentum* and *Lactobacillus alimentarius* (as shown in Table 1).

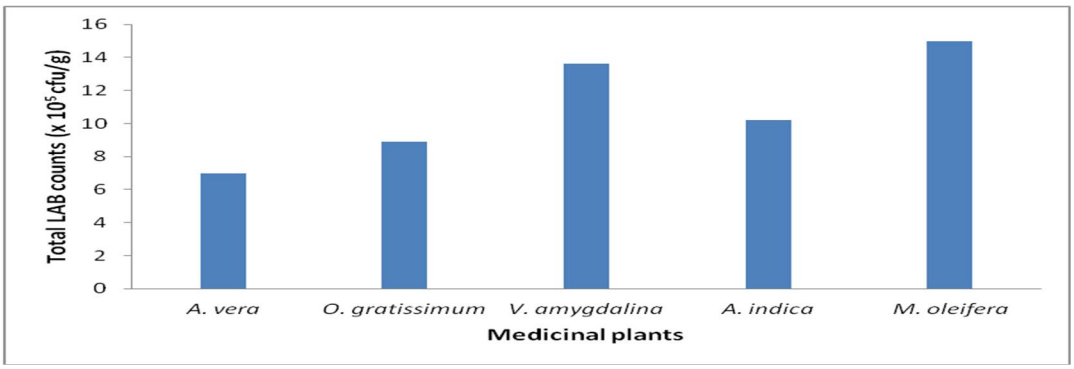

Figure 1: Total lactic acid bacterial counts of soil samples from the rhizosphere of selected medicinal plants

Table 1: Lactic acid bacteria isolated from the rhizosphere of selected medicinal plants

LAB isolate	Source	Phenotypic identification
LB1	*Moringa oleifera*	*Lactobacillus acidophilus*
LB2	*Aloe vera*	*Lactobacillus acidophilus*
LB3	*Aloe vera*	*Lactobacillus plantarum*
LB4	*Azadirachta indica*	*Lactobacillus fermentum*
LB5	*Vernonia amygdalina*	*Lactobacillus fermentum*
LB6	*Vernonia amygdalina*	*Lactobacillus alimentarius*
LB7	*Moringa oleifera*	*Lactobacillus acidophilus*
LB8	*Ocimum gratissimum*	*Lactobacillus plantarum*
LB9	*Moringa oleifera*	*Lactobacillus plantarum*
LB10	*Moringa oleifera*	*Lactobacillus fermentum*
LB11	*Moringa oleifera*	*Lactobacillus brevis*
LB12	*Vernonia amygdalina*	*Lactobacillus plantarum*
LB13	*Moringa oleifera*	*Lactobacillus plantarum*
LB14	*Ocimum gratissimum*	*Lactobacillus fermentum*

In-vitro antagonistic activity of Lactic acid bacterial strains on F. oxysporum f. sp. cucumerinum

Figure 2 shows the *in-vitro* antagonistic activity of lactic acid bacterial isolates on *Fusarium oxysporum* f. sp. *cucmerinum*. *Lactobacillus fermentum* isolated from the rhizosphere of *Azadirachta indica* had the highest antagonistic activity (68.7%) while no inhibitory effect was recorded for some lactic acid bacterial strains.

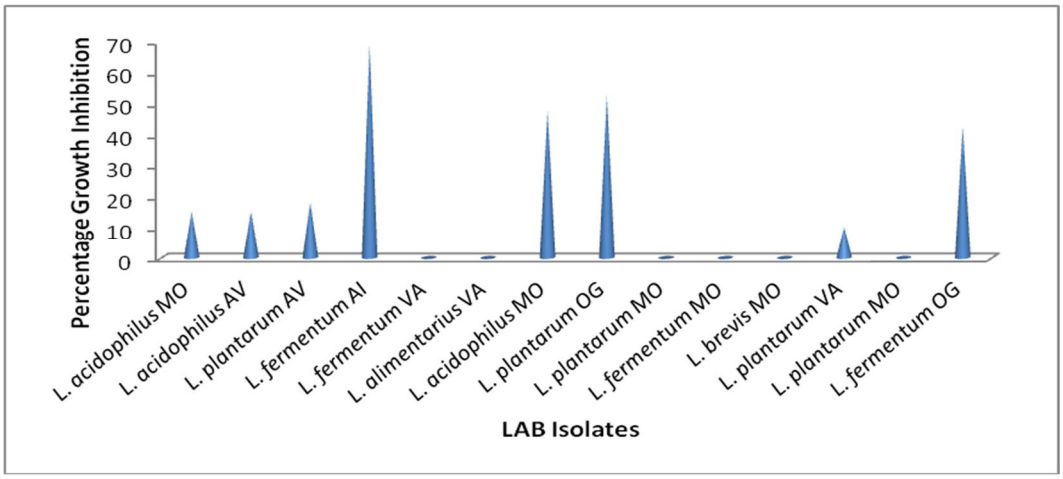

Figure 2: *In-vitro* antagonistic activity of lactic acid bacterial strains against *Fusarium oxysporum* f. sp. *cucumerinum*

In vivo biocontrol study of antagonistic Lactic acid bacterial strains on Fusarium wilt of cucumber

The screen house experiments revealed that two of the selected antagonistic LAB strains (LB4 and LB8) significantly suppressed Fusarium wilt incidence compared to other LAB strains (LB7 and LB14) and untreated control (Table 2). LB4 showed highest disease reduction percentage (49.5%).

Table 2: Suppression of Fusarium wilt of cucumber by antagonistic LAB strains

Treatments	Disease incidence (%±S.D)	Disease reduction (%±S.D)
LB4	48.0 ± 0.8^{c}	49.5 ± 1.1^{a}
LB7	77.3 ± 1.2^{b}	18.6 ± 0.06^{b}
LB8	52.7 ± 0.5^{c}	44.5 ± 0.09^{a}
LB14	72.2 ± 1.1^{b}	24.4 ± 0.1^{b}
Untreated control	95.0 ± 1.8^{a}	0.0 ± 0.0^{c}

Note: Means with different letters along the columns are significantly different at $P < 0.05$

Molecular characterization of antagonistic lactic acid bacteria

Amplification of 16S rRNA genes of two most effective antagonistic LAB strains (LB4 and LB8) by PCR resulted in products estimated as 1,500bp in size (Figure 3). Sequencing of the PCR products followed by BLASTn searches at GenBank of the NCBI

library revealed that LB4 and LB8 showed 96% and 100% similarity to *Lactobacillus fermentum* IFO3956 and *L. plantarum* WCFS1 respectively. The results of the molecular characterization were consistent with the phenotypic traits of the antagonistic LAB isolates.

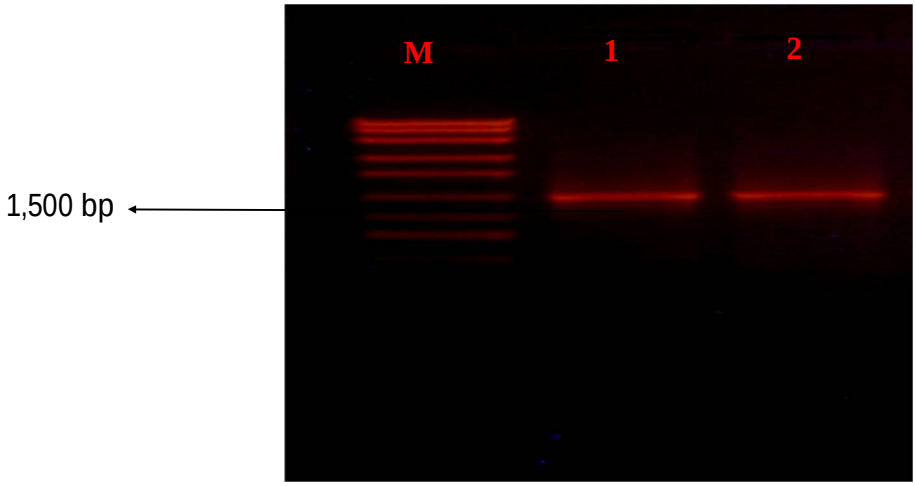

Figure 3: 16S rRNA gene amplicons of antagonistic lactic acid bacteria isolated from the rhizosphere of selected medicinal plants

M: 1Kb DNA ladder (Mid Ranger), 1: LB4, 2: LB8

DISCUSSION

Lactic acid bacteria (LAB) are usually isolated from fermented products of animal and plant origins. However, low numbers of this group of bacteria have been found naturally in some environments such as floor of poultry house, rhizosphere of fruit trees, and around horse barn (Chen *et al*., 2005; Yanagida *et al*., 2005). The present study revealed the presence of lactic acid bacteria in the rhizosphere of medicinal plants which seems to be influenced by the abundance and nature of root exudates. In this study, the LAB populations (7.0×10^5cfu/g to 15.0×10^5 cfu/g) of the rhizospheric soil samples of *M. oleifera* , *O.gratissimum, A. indica, A. vera* and *V. Amygdalina* were lower than the densities obtained from the rhizospheric soil samples of mango, guava and banana by Ekundayo (2014). The lower densities of LAB observed in this study could be due to the chemical composition of root exudates of the selected medicinal plants. The lactic acid bacteria isolated from the rhizosphere of these plants are strains of *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus acidophilus, Lactobacillus alimentarius* and *Lactobacillus brevis.* Similar LAB strains were also obtained from the rhizosphere of olive trees and desert truffles of Tunisia by Fhoula *et al*. (2013) and the rhizosphere of mango, guava and banana plants by Ekundayo (2014).

However, the *in-vitro* antagonistic assay of these LAB strains on *Fusarium oxysporum* f. sp. *cucmerinum* revealed that only few of these strains had antagonistic activities on this pathogen. No inhibitory effects were observed in some LAB strains whereas, the

pathogen was fairly inhibited by *Lactobacillus plantarum* (21.43%) and *Lactobacillus fermentum* (17.3%) isolated from the rhizosphere of *Ocimum gratissimum*, while the highest inhibitory effect was observed in *Lactobacillus fermentum* isolated from *A. indica*. The results of this study corroborate with the studies of Trias *et al.* (2008) who reported that LAB isolated from fresh fruits and vegetables produced organic acid substances that affected some phytopathogenic and spoilage bacteria and fungi such as *Xanthomonas campestris, Erwinia carotovora, Penicillium expansum, Monilinia laxa,* and *Botrytis cinerea.* Similar results were also obtained by Fhoula *et al.* (2013).

Furthermore, the study showed that the selected antagonistic lactic acid bacterial strains provided some levels of reduction in Fusarium wilt disease of cucumber as compared to the infected controls. *Lactobacillus fermentum* and *L. plantarum* isolated from rhizosphere of *Azadirachta indica* and *Ocimum gratissimum* respectively provided highest percentage disease suppression (49.5% and 44.5% respectively) and they might be effective in controlling the disease than other strains. The results are similar to the findings of Sathe *et al.* (2007) who found suspension of *Lactobacillus plantarum* to delay the growth of *Aspergillus flavus, F. graminearum, R. stolonifer* and *B. cinerea* on cucumber. Wang *et al.* (2011) also found that metabolites of *Lactobacillus plantarum* IMAU10014 possessed high inhibitory activity against some plant pathogenic fungi.

As it has been reported by Hamed *et al*. (2011) and El-Mabrok *et al*. (2012), the combination of different substances such as antibiotics especially nisin, organic acids, hydrogen peroxide, cyclic dipeptides, phenolic and proteinaceous compounds could be responsible for the detected suppression activity exhibited by the antagonistic LAB strains. Moreover, since LAB could attack and colonize plant roots, other mechanisms such as competition between the LAB strains and *F. oxysporum* f. sp. *cucmerinum* for space and nutrients as well as competitive exclusion of the pathogen from entry sites in the roots of cucumber seedlings preventing the pathogen to colonize the roots could also be responsible for the growth inhibition of the pathogen.

CONCLUSION

Since *Lactobacillus fermentum* and *L. plantarum* isolated from the rhizosphere of medicinal plants exhibited Fusarium wilt disease suppressing activity, they may be applied in the development of new, safe and effective cucumber seed treatments as an alternative to chemical fungicides. However, further studies concerning the field applications of these antagonistic LAB strains as effective biocontrol agents need to be conducted.

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