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Characterization and Efficacy of *Lactobacillus* Species as Bio control Agent against Latent Fungal Endophyte in Beans

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Abstract: **Latent fungal endophytes are responsible for major crop losses in Kenya and worldwide in general. Therefore, there is need for control to maintain the level of yield produced both quantitatively and qualitatively. In the past, farmers have been relying heavily on use of fungicide to control fungal plant diseases. However, these pesticides have many detrimental effects on the environment due to over-use. Alternative methods to the use of synthetic chemicals for disease control are increasingly being sought such as use of bio-control agents (BCA). Lactic acid bacteria isolated from sour milk (cow's milk) and yoghurt and a lyophilized lactobacillus were tested for their effectiveness against latent fungal endophytes in protection of bean plant. *In vitro* and *in vivo* assay for antifungal activity of LAB against latent fungal endophyte was determined. Results showed improved plant growth and increased total fresh weight over control which confirms potential of LAB to act as bio-control agent against latent fungal endophytes, therefore reliance of fungicide can be avoided using LAB as a promising bio-control agent. The assays were performed in three replicates leading to proper control and data analyzed for significant differences using Minitab 17 by means of one way ANOVA.**

Keywords: **Bio-control, Lactic acid bacteria, Latent fungal endophyte, Phytopathogen.**

1. INTRODUCTION

Beans (*Phaseolus vulgaris*), belong to the family Fabaceae, and is one of the important leguminous crops in the world and is a yearly crop that needs warm temperatures to grow. They are staple crop in Kenya and are rated second after maize. Beans are grown in almost all areas in Kenya but the major counties include; Bomet, Kericho, Homabay, Uasin Gishu, Kakamega and Embu. They are the main source of plant proteins and are rich in vitamins such as acid lysine, tryptophan, methionine, vitamin B, nicotine acid and iron (1). This crop is however vulnerable to plant pathogens (2) which affects yield and quality of grain.

Fungi constitute the largest number of plant pathogens that are responsible for a range of serious plant diseases, resulting in economic losses (3). The use of chemicals such as fungicide to control fungi and other microorganisms has been a subject of public concern due to harmful effects on non target organisms and some possible carcinogenicity of some chemicals (4). Other major problems include development of resistance by plants pathogenic fungi and also demand for products free of chemicals; therefore the use of biological control agents is being suggested as the alternative way of controlling plant diseases (5, 6, 7, 8 and 9). Biological control is the inhibition of growth, infection or reproduction of one organism using another organism. Antagonist in biological control of plant pathogens are biological agents with potential to interfere in the life process of plant pathogens (2).

Different species of bacteria and fungi are being used as control agents against phytopathogenic fungi (10). Most recently many studies on the use of lactic acid bacteria (LAB) for biological control are being undertaken (11). Use of LAB present

great interest because these microorganisms are generally recognized as safe (GRAS) and thus can be applied to food crops without any harmful effect on human health and it is also environmentally safe (12).

LAB have been studied and have been known to produce a variety of antimicrobial compounds and substances such as lactic acid, acetic acid, probionic acids, antibiotics, bacteriocins as well as hydrogen peroxide and carbon dioxide (13). The mode of action of these biological control agents can only be hypothesized due to complex and synergistic interactions between different compounds but it can be by predation, induced resistance, antibiosis, cross protection and hyper parasitism (11). The biological control of plants pathogens by LAB is said to be through competition for nutrients and also by production of antimicrobial compounds (14).

Previously, studies have been done on fungal plant diseases as well as application of different microorganisms as biological control agents. Such studies includes diseases of leaves and flowers such as powdery mildew, diseases of fruits and vegetables such as borty and other fungal diseases in soil (15). At the moment, there is limited information on interaction of LAB with latent pathogenic endophytes. The aim of this study is to promote the use of biological control agents such as LAB for plant disease management of latent pathogenic endophytes.

2. MATERIALS AND METHODS

Samples collection of latent fungal endophyte and LAB:

Latent pathogenic fungal endophyte infected bean plant roots were collected from KALRO, Nairobi and maintained on potato dextrose agar (PDA) at 4 ºC. Lyophilized strain of *lactobacillus acidophilus* was obtained commercially from Ultralab E.A limited, while other lactic acid bacteria were isolated from yoghurt and sour cow's milk (Fresh milk let to stay overnight for two days to ferment). The strains were kept on MRS (de Men RogosaSharpe) agar. Fresh cultures were grown in MRS broth at 30 ºC for 24 hours before being used in the assays.

Isolation and identification of lactic acid bacteria:

Yoghurt and fresh milk samples were collected under aseptic condition using sterile plastic bags and then homogenized in 90ml of sterile saline solution (0.85%, pH 7). The homogenate was diluted three times then inoculated using MRS agar (16).

Identification of lactic acid bacteria was done per their colony morphology (observed microscopically after gram staining), physiological and biochemical characteristics - (i.e. the production of catalase, fermentation of sugars, growth at 10ºC to 45ºC in MRS agar and motility) (17).

Morphological characterization:

Gram stain:

This was done as described by Moyes *et al.,* (18) whereby, crystal violet stain was added onto a fixed culture for 60 seconds; the stain was poured and rinsed in gentle running water. Iodine solution was added onto the smear for 60 seconds then rinsed. Drops of ethyl alcohol (decolorizer) were then added and rinsed after 5 seconds. Fixed culture was then counterstained with fuchsine solution for 60 seconds, and then washed gently and finally air dried.

Biochemical characterization:

Catalase test:

This was done as described by Bailey and Scott (19), whereby inoculums from a pure culture was taken and streaked on MRS agar. The inoculated plate was incubated at 35 ºC ± 2 for 24 hours. Growths from the plate were smeared on a microscope slide and a drop of hydrogen peroxide placed on the smear.

Carbohydrate test:

This was done as described by James and Shermon (20), whereby; tubes containing broth and test sugars (glucose, lactose and maltose) were labeled then, wire loop used to aseptically inoculate each labeled tube with the bacteria. Un-inoculated tubes were also used as a control. The tubes were incubated for 24 hours at 37 ºC.

Motility test:

This was done as described by James *et al.*, (21), whereby, a semisolid agar called sulfide indole motility medium (SIM medium) was used to test the motility of bacteria. The medium was aseptically inoculated using a loop zig-zag needle in which bacteria was stabbed in the center of the medium to a depth of half an inch. Inoculated medium was then incubated for 24 hours at 37 ºC then checked for turbidity.

Isolation and identification of latent pathogenic endophyte:

The infected plant root tissues were cut into small pieces of 5mm then surface sterilized for 1 minute with 0.5% sodium hypochlorite and rinsed in sterile water and dried. The pieces were placed on PDA medium containing 50mg/L of streptomycin sulfate and maintained at 25ºC for 48-72 hours to grow. Fungal hyphae from developing colonies were then transferred to PDA plates. Colonies with typical characteristics were randomly picked using sterile needle and transferred to a slide and covered with a cover slip, then observed microscopically (22).

***In vitro* antifungal test:**

The *in vitro* trial was carried out using the agar-well diffusion methods as described by Elbadry, (23) whereby PDA was dispensed into the sterile Petri dishes. After the media solidification, four wells of 7mm diameter were bored in each plate. An aliquot of 100µL of cell free culture of LAB was pipetted into the wells and plates kept at low temperatures (10 ºC) for 6 hours to allow diffusion of the antimicrobial substances. An agar plug of 7mm was removed from latent pathogenic endophyte culture and placed at the center of PDA plates and incubated until fungal growth in control plates fill at least the edge of the wells (24). The radius (cm) of the growth in the treatment and the control were measured and the results recorded. The antifungal activity of LAB was calculated using the following formula (24).

% FI = (Rc- Rt/ Rc) x 100

Where

% FI = % Fungal inhibition

Rc = Radius of growth zone in the control

Rt = Radius of growth zone in the treatment

***In vivo* experimental design:**

In vivo experiments were performed in containers measuring 12 cm in diameter with sterilized natural soil obtained within Kenyatta University, main campus. All containers had soil inoculated with latent fungal endophyte at a rate of 10mL homogenized culture per container, prepared a day prior to planting. Two treatments were performed: first, the bean seeds were pre-soaked in culture broth of LAB, and resultant seeds placed in containers inoculated with latent endophyte, without supplementation of soil with LAB (seed treatment). Secondly, non soaked seeds were used; seeds were placed in containers in presence of latent fungal endophyte with supplementation of soil with LAB, at a rate of 10mL culture broth per container and the control contained non soaked seeds. All experiments and control were replicated three times. Seeds were grown for 30 days between 25 ºC and 38 ºC. *In vivo* test for shoot and root length, number of secondary roots and total fresh weight of plants were performed after two months (24).

Molecular characterization:

DNA extraction of latent fungal endophyte and LAB:

Latent fungal endophyte mycelia and LAB was both harvested using sterile spatula under aseptic conditions and their genomic DNA extracted using the Isolate II DNA extraction kit (Bioline, London, UK), following the manufactures protocol. The resultant DNA was eluted in 50 µl of buffer and its quality checked using Nanodrop 2000/2000c spectrophotometer (Thermo scientific).

DNA amplification of latent fungal endophyte by PCR using ITS 4 and 5 primers:

Amplification were carried out for the DNA region of the latent fungal endophyte isolate using the ITS primers (25). Typical circle conditions were as follows: initial denaturation at 95°c for 1 minute, followed by 35 circles of denaturation at 95°c for 30 seconds, annealing at 59°c for 40 seconds, and primer elongation at 72°c for 1 minute followed by a final

extension at 72°c for 10 minutes. The PCR was carried out in a total volume of 20µl containing 0.2µm of each primer, (ITS 5; 5' GGAAGTAAAAGTCGTAACAAGG 3' and ITS 4; 5' TCCTCCGCTTATTGATATGC 3' respectively). 5X My *Taq* Reaction buffer (Bioline), 1.24Mm $MgCl_2$, 1 unit My *Taq* DNA polymerase (Bioline) and 15ng/µl of DNA template. This gave a target region of approximately 600bp. The PCR was done in Mastercycler nexus gradient (Thermo scientific).

Amplification of the bacterial 16S SSU rRNA gene region

Polymerase chain reaction (PCR) was done to amplify the 16S region to explore the bacteria diversity using the 27F 5'-AGAGTTTGATCMTGGCTCAG -3' and 1492r 5'- TACCTTGTTACGACTT -3' (26) primers. A product of approximately 1400 base pairs (bp) was amplified in a final reaction volume of 20µl containing 5X My *Taq* reaction buffer, 10µm of each primer, 25mM $MgCl_2$, 0.125 µl My *Taq* DNA polymerase and 15ng/µl of DNA template. This reaction was set up in the Master cycler Nexus gradient (Thermo scientific) using the following cycling condition: initial denaturation for 2 minutes at 95 °C, followed by 40 cycles of 30 seconds at 95°c, 45 seconds at annealing temperature of 51.9 °C and 1 minute at 72 °C, then final elongation step of 10 minutes at 72 °C.

Detection and analysis of PCR reaction products:

All the amplified PCR products were resolved through a 1% agarose gel sustained with ethidium bromide (10mg/ml) and subjected to electrophoresis at 100 volts for 1 hour. DNA bands on the gel were visualized and documented under ultra violet (U.V) trans-illuminator using the KETA GL imaging system (Wealtec corp)

Sequencing of gene of interest:

The successfully amplified ITS and 16S rRNA region from the samples were gel excised and purified then sent for bio-directional sequencing at Macrogen Inc, Europe Laboratories. The PCR products were purified using the Isolate II PCR and Gel Kit (Bioline, UK) following the manufactures instruction.

Data analysis:

In vitro antifungal activities were calculated using the following formula (24) %FI = (Rc-Rt/Rc) x 100. *In vivo* analysis was done by harvesting bean plant after two months growth. To reveal the effect of LAB on the growth characteristics, each plant was measured for shoot and root length, number of secondary roots and total fresh weight of plants (24).

Nucleotide sequences were edited using Geneious, version 8.1.9 and Chromas lite, version 2.1. The edited nucleotide sequences were queried at the National Center for Biotechnology Information (NCBI) using a nucleotide BLAST (Basic Local Alignment Search Tool) search. This search was carried out for comparison of the sequences obtained with all the sequences available in the GenBank. A species was identified if its sequences closely matched one in the GenBank giving the highest percentage sequence similarity and lowest e-value possible.

The assays were performed in three replicates leading to proper control. Data were analyzed using Minitab 17 by means of one way ANOVA. Comparison of mean was performed using least significant differences (LSD) at $P \leq 0.05$ (24)

3. RESULTS AND DISCUSSIONS

Latent fungal endophytes were subjected to morphological and molecular characterization. LAB isolates reacted positively to gram staining as observed under a light microscope; appearing as rod shaped. Biochemical characterization showed LAB to have reacted positively to carbohydrate and motility tests while was negative to the catalase test. Amplification of latent fungal endophytes and LAB genes were carried out on the extracted genomic DNA and this was followed by agarose gel electrophoresis for confirmation of the amplification. The latent fungal endophyte and LAB amplification gave the expected band size of approximately 600bp and 1400bp respectively. Results for *in vitro* efficacy of LAB against latent fungal endophyte were average. *In vivo* seed and soil treatment results gave crops with enhanced plant growth as compared with the control.

Morphological characteristics of latent endophyte:

Ten samples of infected bean plant (root) were collected from bean field in KALRO, Kangemi and colonies with typical characteristics were identified and observed under a microscope (100x) (27), as illustrated by figure 1

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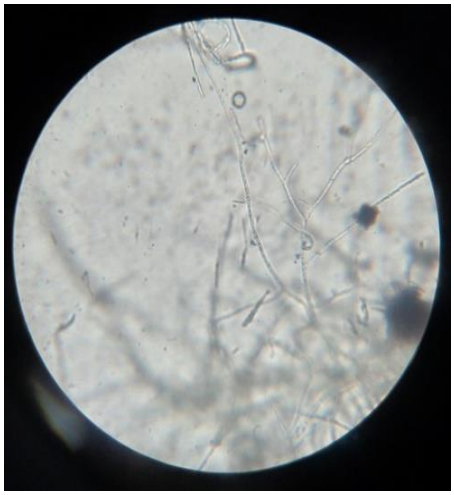
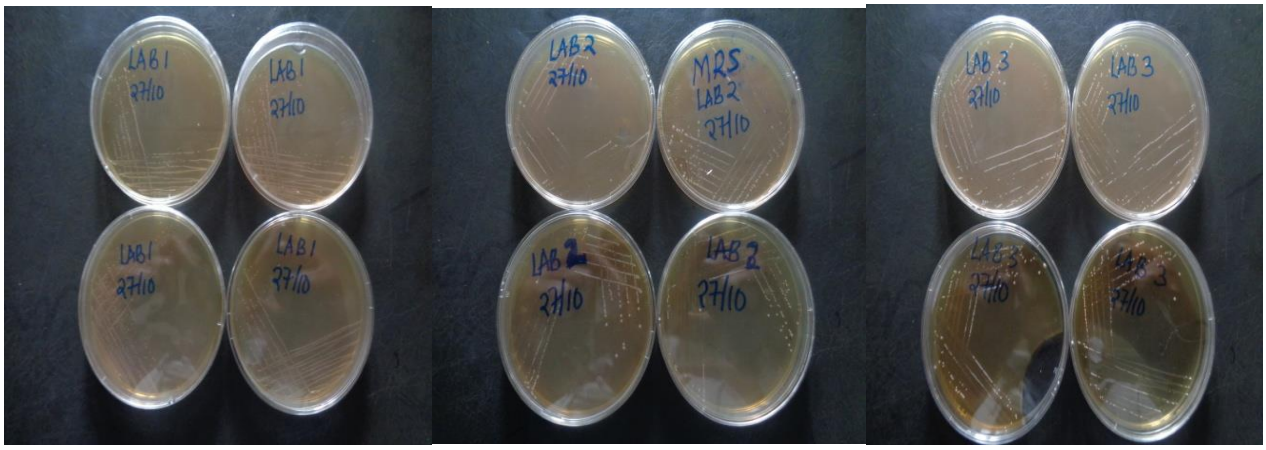


Figure 1: Microscopic aspect of hyphae of latent fungal endophyte isolated from infected roots of a bean plant.

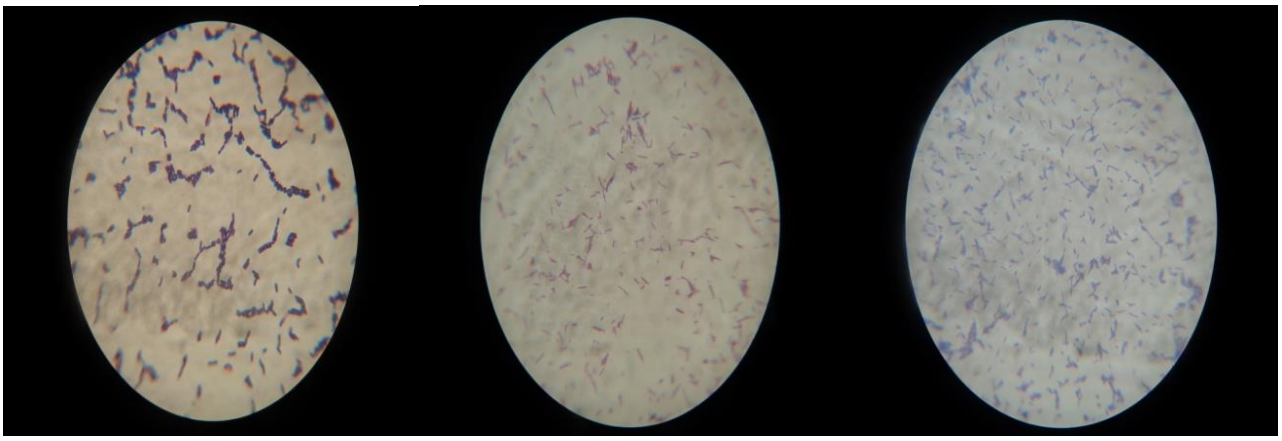
Morphological characterization of LAB:

LAB from yoghurt, sour milk and a lyhophilized *L. acidophilus* were subcultured on MRS (de Men RogosaSharpe) agar at 30 ºC until a pure colony were established (Figure 2) and morphological characteristics of isolated colonies of LAB were observed microscopically after gram staining (Figure 3).



LAB 1 **LAB 2** **LAB 3**

Figure 2: LAB isolated colonies on MRS agar



LAB 1 **LAB 2** **LAB 3**

Figure 3: Microscopic observation of LAB isolates (Magnification x100)

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Biochemical identification of LAB:

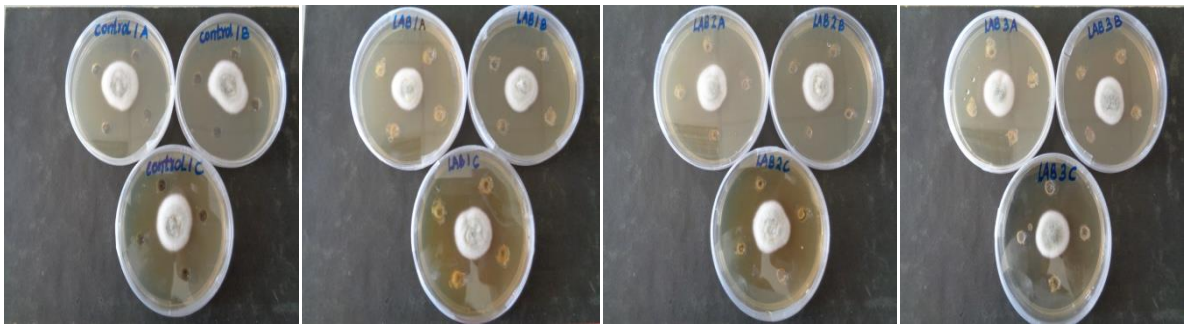
The three LAB isolates studied- were rod shaped and were positive for gram test, sugar fermentation, motility test and grew at 45 °C. They were also negative for catalase test and did not grow at low temperature (10 °C). (Table 1)

Table 1: Biochemical characterization of lyophilized bacteria (LAB 1), LAB isolated from yoghurt (LAB 2) and cow's milk (LAB 3) were as shown below.

Isolates	Gram test	Morphology	Catalase	Sugar fermentation	Growth at 10°c	Growth at 45°c	Motility
LAB 1	+	Rod	-	+	-	+	+
LAB 2	+	Rod	-	+	-	+	+
LAB 3	+	Rod	-	+	-	+	+

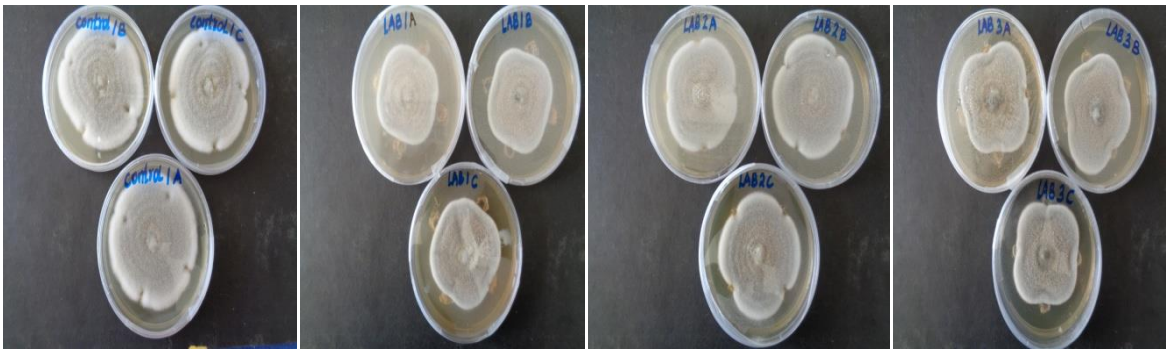
***In vitro* inhibition of latent fungal endophyte against LAB:**

In vitro fungal inhibition was done using agar well diffusion method. Four wells in each plate had LAB except the control and latent fungal endophyte placed at the center and incubated until the fungi on control filled the plate as shown in figure 4, figure 5 and figure 6 respectively



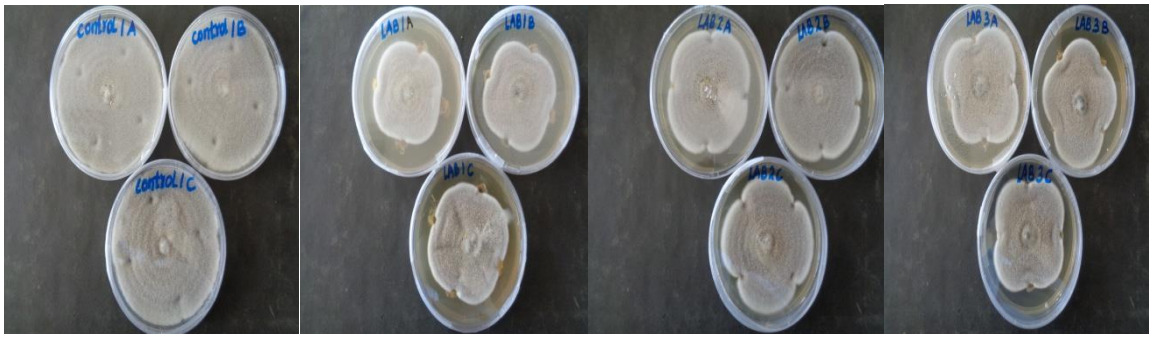
CONTROL **LAB 1** **LAB 2** **LAB 3**

Figure 4: *In vitro* inhibition of LAB 1, LAB 2 and LAB 3 on day 3



CONTROL **LAB 1** **LAB 2** **LAB 3**

Figure 5: *In vitro* inhibition of LAB 1, LAB 2 and LAB 3 on day 7



CONTROL **LAB 1** **LAB 2** **LAB 3**

Figure 6: *In vitro* inhibition of LAB 1, LAB 2 and LAB 3 on day 10

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Efficacy of *in vitro* inhibition of LAB against latent fungal endophyte:

In vitro efficacy of LAB against latent fungal endophyte on day 3 (using mean of replicates), were as follows; LAB 1 showed higher inhibition of 14.44%, followed by LAB 3 and LAB 2 at 13.33% and 12.33% respectively. On day 7, LAB showed better inhibition, with LAB 1 having higher inhibition of 28.89%, followed by LAB 3 and LAB 2 at 22.86% and 19.59% respectively. On day 10, LAB 1 still showed better inhibition of 30.19%, followed by LAB 3 and LAB 2 at 21.18% and 18.43% respectively. Findings showed that LAB 2 exhibited lower inhibition on day 3, day 7and day 10 while LAB 1 inhibition double on day 7 as compared with day 3. All isolates showed decreased inhibition between day 7 and day 10 except LAB 1 which showed continuous increased inhibition from day 3 to day 10. Shown in figure 7 below

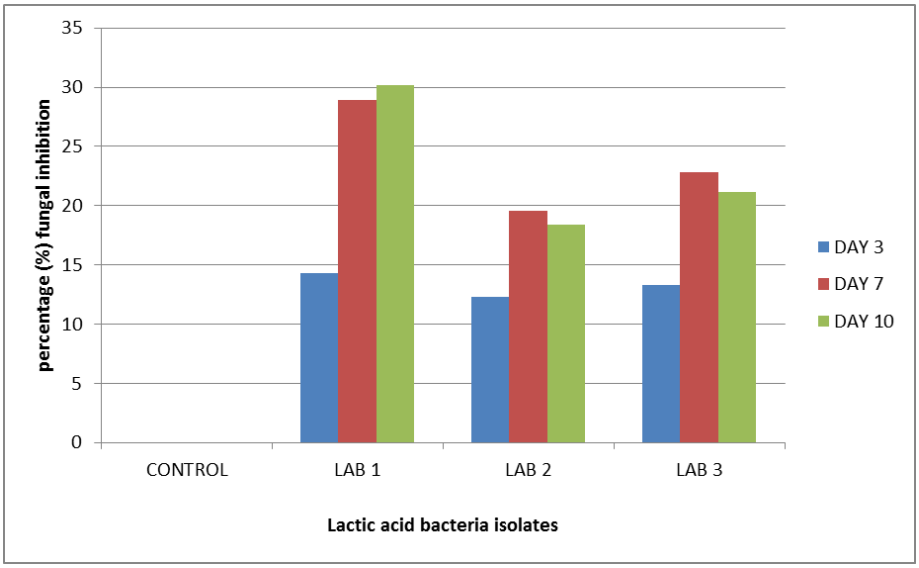

Figure 7: *In vitro* percentage (%) fungal inhibition of latent fungal endophyte by lactic acid bacteria isolates LAB 1, LAB 2 and LAB 3

One-way ANOVA for *in vitro* inhibitory effect of LAB against latent fungal endophyte:

In vitro inhibitory effect of LAB against latent fungal endophyte was moderate. On day 3, fungal inhibition was low with LAB 1(at 14.44±1.93) showing better inhibition than other isolates, and then was followed by LAB 3 and LAB 2 respectively (at 13.33±6.67 and 12.22±5.09).All isolate showed significance difference at $P \leq 0.05$

Fungal inhibition on day 7 was moderate, with LAB 1(at 28.94±0.81) showing better inhibition than other isolates, and then was followed by LAB 3 and LAB 2 respectively (at 22.86±1.40 and 19.59±1.62). All isolate showed significance difference at $P \leq 0.05$. On day 10 fungal inhibitions was still moderate, with LAB 1(at 30.19±1.36) still showing better inhibition, and all isolates were significant to the control at $P \leq 0.05$. Results are shown on the table 2 below.

Table 2: Mean standard deviation, significance difference, confidence intervals, F-value and P-value of *in vitro* inhibitory effect of LAB against latent fungal endophtye after day 3, day 7 and day 10

	DAY 3				DAY 7				DAY 10			
ISOLATES	Mean±sd	95% CI	F-value	P-value	Mean±sd	95% CI	F-value	P-value	Mean±sd	95% CI	F-value	P-value
CONTROL	0.00±0.00	-05.73, 05.73	07.33	0.01	0.00±0.00	-1.52, 1.52	358.15	0.00	0.00±0.00	-1.50, 1,50	380.66	0.00
LAB 1	14.44±01.93	08.71, 20.17			28.94±0.81	27.41, 30.46			30.19±1.36	28.70, 31.69		
LAB 2	12.22±05.09	06.49, 17.95			19.59±1.62	18.06, 21.11			18.43±1.36	16.94, 19.93		
LAB 3	13.33±06.67	07.60, 19.06			22.86±1.40	21.35, 24.39			21.18±1.18	19.68, 22.67		

***In vivo* effect of LAB against latent fungal endophyte:**

In vivo inhibition was done, whereby bean seeds were planted and harvested after 2 months. To reveal effects of LAB on growth characteristics, each plant were measured for shoot and root length, number of secondary roots and total fresh weight of bean plant. Seed and soil treatment effects were as shown below by figure 8 and figure 9 respectively.

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CONTROL LAB 1 LAB 2 LAB 3

Figure 8: Protective effect of LAB against latent fungal endophyte as a seed treatment.

CONTROL LAB 1 LAB 2 LAB 3

Figure 9: Protective effects of LAB against latent fungal endophyte as a soil treatment.

***In vivo* efficacy of LAB as seed treatment:**

In vivo treatment involved bean seeds which were pre-soaked in culture broth of LAB and placed in containers inoculated with fungi without supplementing with LAB. Results for shoot length and total fresh weigth (g) showed LAB 1, LAB 2 and LAB 3 having longer shoot length and more weigth than the control. Root length for LAB 1 and LAB 2 were longer while LAB 3 was shorter compared with the control. Number of secondary roots were more in LAB 1 and LAB 2 and equal in LAB 3 with control. Efficacy of LAB as seed treatment is shown by figure 10 below.

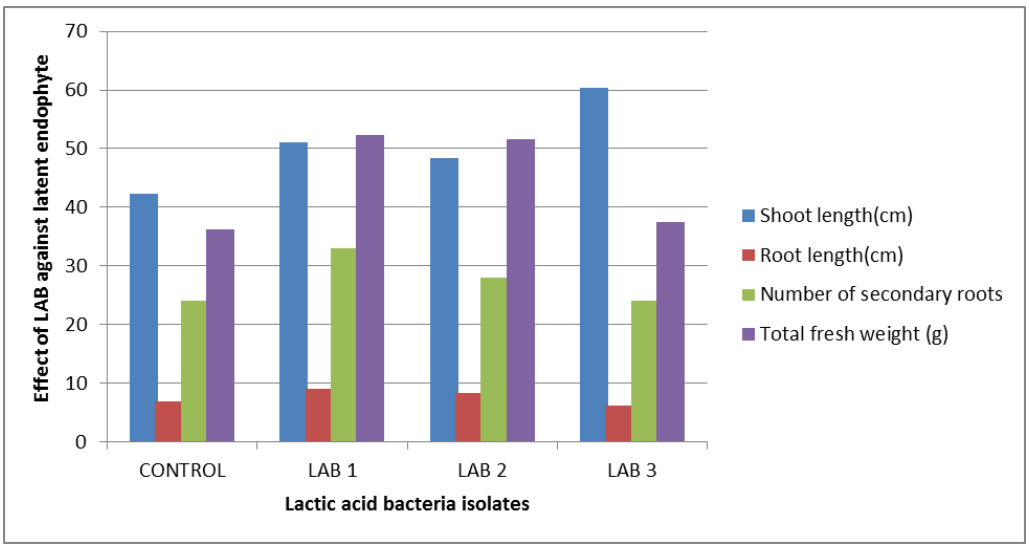

Figure 10: Mean of the three replicates showing shoot and root length(cm), number of secondary roots and total fresh weight (g) for seed treatment

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One-way ANOVA for seed treatment:

Shoot length for LAB 1, LAB 2 and LAB 3 (at 51.00±09.54, 48.33±10.41 and 60.30±24.00 respectively) were longer than the control (at 50.00±2.65) but had no significance difference at $P \leq 0.05$. Root length of control (at 06.90±02.19) was longer than LAB 3 (at 06.27±01.80) and shorter against LAB 1 and LAB 2 (at 09.10±03.61 and 08.30±00.56 respectively) but had no significance difference at $P \leq 0.05$. The control (at 24.33±04.73) had less number of secondary roots than LAB 1 and LAB 2 (at 33.00±02.00 and 27.67±07.02 respectively) and equal number of secondary roots with LAB 3 (at 24.33±03.21) but had no significance difference at $P \leq 0.05$. Total fresh weigth (g) for LAB 1, LAB 2 and LAB 3 (at 52.30±17.80, 51.57±04.01 and 37.50±09.18 respectively) were heavier than control (at 36.27±04.40) but had no significance differences at $P \leq 0.05$. (Table 3)

Table 3: Mean standard deviation, significance difference, confidence intervals, F-value and P-value of shoot and root length, number of secondary roots and total fresh weight of bean seeds pre-soaked in culture broth of LAB and placed in containers inoculated with latent endophyte without being supplemented with LAB (Seed treatment)

TREATMENT	N	SHOOT LENGTH				ROOT LENGTH			
		Mean±sd	95%CI	F-value	P-value	Mean±sd	95%CI	F-value	P-value
CONTROL	3	42.33±02.52	23.72, 60.95	0.86	0.500	06.90±02.19	03.82, 09.98	0.94	0.467
LAB 1	3	51.00±09.54	32.38, 69.62			09.10±03.61	06.02, 12.18		
LAB 2	3	48.33±10.41	29.72, 66.95			08.30±00.56	05.22, 11.38		
LAB 3	3	60.30±24.00	41.70, 78.90			06.27±01.80	03.19, 09.34		

TREATMENT	N	NUMBER OF SECONDARY ROOTS				TOTAL FRESH WEIGHT (G)			
		Mean±sd	95%CI	F-value	P-value	Mean±sd	95%CI	F-value	P-value
CONTROL	3	24.33±04.73	18.16, 30.15	2.34	0.150	36.27±04.40	22.37, 50.17	2.09	0.180
LAB 1	3	33.00±02.00	26.83, 39.17			52.30±17.80	38.40, 66.20		
LAB 2	3	27.67±07.02	21.49, 33.84			51.57±04.01	37.67, 65.47		
LAB 3	3	24.33±03.21	18.16, 30.51			37.50±09.18	23.60, 51.40		

***In vivo* efficacy of LAB as soil treatment:**

Treatment involved bean seed placed in containers in presence of latent fungal endophyte and soil supplemented with LAB (soil treatment). Results for shoot length showed LAB 2 and LAB 3 being longer and LAB 1 being shorter than the control. Root length and total fresh weigth (g) for all isolates were longer and weigth more than control. Number of secondary roots for LAB 2 and LAB 3 were more and LAB 1 had equal number with control. *In vivo* efficacy of LAB as soil treatment is shown in figure 11 below.

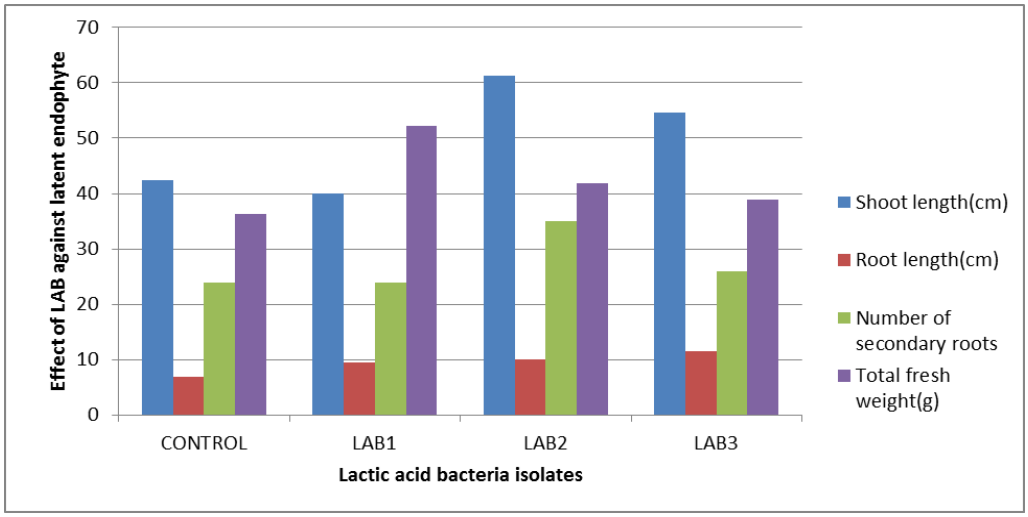

Figure 11: Mean of three replicates showing shoot and root length, number of secondary roots and total fresh weight for soil treatment

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One-way ANOVA for soil treatment:

LAB 1 had shorter shoot length (at 40.00±01.00), while LAB 2 and LAB 3 had longer shoot length (at 61.30 ± 23.00 and 54.67 ± 02.52 respectively) than the control(at 42.33±02.52) and had no significance difference at P≤0.05. The root length of LAB 1, LAB 2 and LAB 3 were longer (at 09.57±01.16, 10.13±01.90 and 11.60±00.46 respectively) than the control (at 06.90±02.19) and had significance difference of 0.037 at P≤0.05. Number of secondary roots for LAB 2 and LAB 3 (at 35.00±09.17 and 26.33±12.50 respectively) were more and LAB 1(at 24.00±06.08) had equal number with control (at24.33±04.73) and had no significance difference at P≤0.05. Total fresh weigth (g) for LAB 1, LAB 2 and LAB 3 (at 52.17±10.72, 41.93±06.06, 38.90±05.90 respectively) were heavier than control (at 36.27±04.40) and had no significance difference at P≤0.05. (Table 4)

Table 4: Mean standard deviation, significance difference, confidence intervals, F-valeu and P-value for shoot and root length, number of secondary roots and total fresh weight of bean seeds placed in containers in presence of latent fungal endophyte and soil supplemented with LAB (Soil treatment)

TREATMENT	N	SHOOT LENGTH				ROOT LENGTH			
		Mean±sd	95%CI	F-value	P-value	Mean±sd	95%CI	F-value	P-value
CONTROL	3	42.33±02.52	26.82, 57.85	2.27	0.157	06.90±02.19	04.80, 09.00	4.64	0.037
LAB 1	3	40.00±01.00	24.49, 55.51			09.57±01.16	07.46, 11.67		
LAB 2	3	61.30±23.00	45.80, 76.80			10.13±01.90	08.03, 12.24		
LAB 3	3	54.67±02.52	39.15, 70.18			11.60±00.46	09.50, 13.70		

TREATMENT	N	NUMBER OF SECONDARY ROOTS				TOTAL FRESH WEIGTH (G)			
		Mean±sd	95%CI	F-value	P-value	Mean±sd	95%CI	F-value	P-value
CONTROL	3	24.33±04.73	12.81, 35.86	1.07	0.416	36.27±04.40	26.71, 45.82	2.82	0.107
LAB 1	3	24.00±06.08	12.48, 35.52			52.17±10.72	42.61, 61.72		
LAB 2	3	35.00±09.17	23.48, 46.52			41.93±06.06	32.38, 51.49		
LAB 3	3	26.33±12.50	14.81, 37.86			38.90±05.90	29.35, 48.45		

PCR and agarose gel electrophoresis:

Amplification of latent fungal endophyte and lactic acid bacteria genes were carried out on an extracted genomic DNA and this was followed by an agarose gel electrophoresis for confirmation of the amplification

Amplification product of latent fungal endophyte:

As shown in figure 12, four samples (lane 3-6) had their DNA extracted from pure culture and amplified using ITS 4 and 5 primers and this was followed by an agarose gel electrophoresis for confirmation of the amplification. The latent fungal endophyte gave expected band size of approximately 600bp.

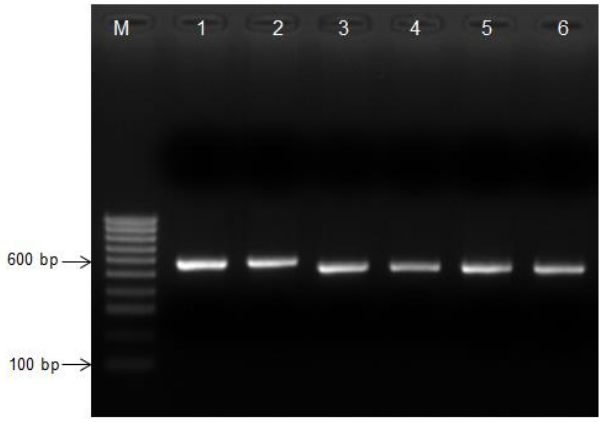

Figure 12: PCR amplification of ITS region using primers ITS 4 and 5 followed by 1% Agarose gel electrophoresis for latent fungal endophyte samples. M: 100bp DNA ladder (bioline, UK). Lane 1-2: Positive control, Lane 3-6: Latent fungal endophyte

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Amplification product of Lactic acid bacteria:

As shown in figure 13, three samples had their DNA extracted in duplicate from pure culture and amplified using 27F 5'-AGAGTTTGATCMTGGCTCAG -3' and 1492r 5'- TACCTTGTTACGACTT -3' primers and this was followed by an agarose gel electrophoresis for confirmation of the amplification. LAB gave expected band size of approximately 1400bp.

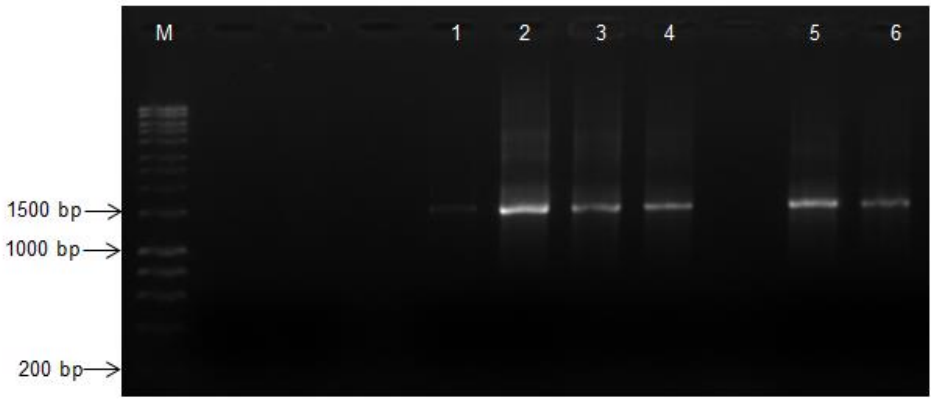

Figure 13: PCR amplification of 16S region of LAB using primers 27F and 1492r followed by 1% Agarose gel electrophoresis for LAB samples. M- 1kb DNA ladder (Bioline, UK). Lane 1-2: LAB 1, Lane 3-4: LAB 2, Lane 5-6: LAB 3

Sequence analysis:

Nucleotide sequences obtained from each sample were edited using Geneious, version 8.1.9 and Chromas lite, version 2.1. A consensus sequence was obtained by assembling sequences from both the forward and reverse sequences and eliminating the primer sequences. Each of the consensus sequences was subjected to BLAST search in GenBank at NCBI. Nucleotide sequences matches gave identity of the species as shown by table 5 below.

Table 5: A nucleotide sequence BLAST search results for the amplified latent fungal endophytes and LAB samples, the nucleotide sequences of samples matched with those in database and their respective similarities are stated below.

Sampling source	Sample name	ID from GenBank, NCBI	Accession no.	Query %	E-value	%ID
Bean roots	RS	Amphelomyces sp.	AY513943.1	100	0	100
Lyophilized lactobacillus acidophilus	LAB 1	lactobacillus acidophilus	CP000033.3	100	0	100
Yoghurt	LAB 2	Lactobacillus acidophilus	CP000033.3	100	0	100
Vendors milk	LAB 3	Lactobacillus acidophilus	CP000033.3	100	0	100

4. DISCUSSION

Biochemical test for LAB was not definitive for species, because of different species within a genus but was used to demontrate the same fermentation pattern and growth requirements (28). Latent fungal endophyte had only a hypal structure and therefore could not be identified to any taxonomic level due to lack of spores producing structures and both the sexual and asexual spores (29). Many literature reports have concluded that the properties and characteristics of a given strain must be well defined and studies on even closely related strains can not be generalized to all strains of that species(29). The study also applied molecular markers as a method of accurate identification of latent fungal endophyte and LAB over biochemical method. Amplification of DNA gene region of latent fungal endophyte was by use of ITS primers (25). These primers amplify a range of fungal targets and works well to analyse DNA isolated from individual organism (30). Amplification of the rRNA gene region for LAB was via by 27F and 1492r primers (26) and we chose this primer set because of its overall amplification efficiency and specificity (31). Molecular identification gave us specific species as opposed to biochemical identification, therefore it holds a promise for sensitive and specific identification within a short time. Biochemical and molecular identifications gave effective results, therefore both are important tools to identify micro organisms. However to avoid false positive results, all biochemical results should be confirmed by means of molecular method.

Results for *in vitro* fungal inhibition showed that LAB had the ability to inhibit latent fungal endophyte. LAB with antifungal effects have been shown in foods such as milk and meat as preservatives (32) while few studies such as use of lactic acid bacteria against *fusaruim oxysporum* in tomato plant (24), have been channeled to exploit potential of LAB to act as a bio-control agent against phytopathogenic fungi. Rosalia et al., (33) found that LAB isolated from fruits produced antimicrobial substances such as organic acid and lactic acid which affected some phytopathogenic fungi. Rosista et al., (34) also found that traditional dairy products produced promising antifungal activities which could be used as bio-protective antifungal adjuvant. This study showed that growth inhibition of latent fungal endophyte by LAB under *in vitro* test was observed even under conditions favoring growth of fungi. The inhibitory effect of LAB under *in vitro* test may have been due to production of metabolites such as antibiotics. This study showed reduced inhibition in some isolates during day 10, and could be attributed to some cells dying. The moderate inhibition of latent fungal endophyte by LAB isolates under *in vitro* test may be elucidated by an assumption that the concentration of LAB may not have been high enough to better inhibit the fungi.

Results for *in vivo* test with LAB isolates being used as seed treatments and soil treatment revealed an effect in enhancing plant growth as compared with the control. These results do show the capability of LAB to act as a bio-control agent. Protection of bean plant through seed and soil treatment with LAB has helped in improving plant growth given the low concentration of LAB used. A possible mechanism for improved plant growth and increasing total fresh weight of plant by LAB is due to efficient transfer of nutrients in soil to plant, attributing to protective nature of LAB at roots. Furthermore, increased plant growth and weight could be attributed to metabolites such as antibiotics produced by LAB against latent fungal endophyte. Studies by Stephane et al., (35) showed that treatment of soil with LAB triggered a mechanism called systemic acquired resistance (SAR) which occurred when plant activated their defense against a pathogen causing the plant to synthesize plant defense chemicals which supported growth of plant.

Use of LAB as bio-control agent against fungi has offered an opportunity for disease management. Mechanism of LAB for *in vivo* disease management is difficult to explain due to complex and synergistic interaction of compounds (36). However, in this study the mechanism of diseases management was by antifungal compounds produced by LAB mainly antibiotics.

5. CONCLUSION

Morphological, biochemical and molecular characterization of latent fungal endophyte and LAB in this study showed that these are effective tools for identification however molecular approach gave higher accuracy within a short time due to its sensitivity and specificity.

The study has also demonstrated the bio-protection of bean plant by LAB isolated from sour cow's milk, yoghurt and lyophilized *lactobacillus acidophilus*. It has shown that LAB has the potential to inhibit latent fungal endophyte in bean plant under *in vitro* test. The effect of LAB against latent fungal endophyte under *in vivo* test was also seen in both seed and soil treatments with trials having increased plant growth over control despite low concentrations used. Therefore over reliance on fungicides for agricultural use and consequential pollution of environment can be avoided by LAB as a promising bio-control agent.

6. RECOMMENDATIONS

Morphological, biochemical and molecular characterization are both effective tools for identification however; morphological and biochemical identification should be confirmed by molecular approach to avoid uncertainty since it is the most effective.

Further studies should also be done to determine mechanisms used by LAB to inhibit latent fungal endophyte and other inhibitory substances produced.

ABBREVIATIONS:

BCA- Biocontrol agent, LAB- lactic acid bacteria, PDA- potato dextrose agar, GRAS- generally recognized as safe, KALRO- Kenya Agricultural Livestock and Research Organization, MRS- de Men RogosaSharpe, DNA- deoxyribonucleic acid, ITS- internal transcribed spacer, PCR- polymerase chain reaction, SSU- small subunit, NCBI- national centre for biotechnology information, BLAST- basic local alignment search tool, bp- base pair, SAR- systemic acquired resistance

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