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Biological Control of *Fusarium* spp. Using Lactic Acid Bacteria

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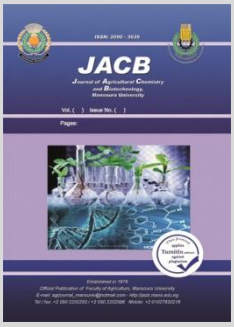

ABSTRACT

Biological control using lactic acid bacteria (LAB) is an environmentally friendly strategy for phytopathpgenic fungi management. This study contains bacterial isolates which were isolated from yoghurt and tested to antagonize *Fusarium* spp. usually attacks the germinated seeds and seedlings causing pre-and post-emergence damping-off. Therefore, the superior phytopathogenic fungi invade several plants as *Fusarium oxysporum* and *Fusarium solani* were chosen to evaluate LAB as a biocontrol agent for them. Of the five lactic acid bacterial isolates (LAB-1, LAB-2, LAB-3, LAB-4, and LAB-5), one isolate (LAB-3) recorded high inhibition linear growth for fusarium-pathogens (80.5% and 87.6% respectively). LAB-3 was chosen for the study of main characters, the results showed that the isolate was Gram-positive, non-sporulating, rod-shaped (bacilli), catalase-negative, microaerophilic, fermentated glucose, lactose, and other sugars. Therefore, this isolate (LAB-3) was identified as members of the Genus Lactobacillus. *In vitro*, these bacteria are very important because producing chitinases that are the main fungal cell wall structure. This enzyme produced by lactic acid bacteria can be safely and easily used in plant protection for the inhibition of fungal pathogens. In addition, the activity of antifungal metabolites is produced by LAB. *In vivo*, at the time of planting when treating plant seed with LAB-3 and soil with *Fusarium* showed increased seedling survival from 25 to 85% and from 40 to 80% respectively, in natural soil. This work indicated that this bacterium (LAB) is environmentally safe and can be used successfully to control phytopathogenic fungi.

Keywords: antagonism, lactic acid bacteria, phytopathogenic fungi

INTRODUCTION

Many soil-borne fungal pathogens attack plants, causing damping-off or root-rot. These soil-borne pathogens are among the most main factors limiting the yield of several economic plants, causing serious economic losses of crops (Thomasho 1996 & Afify and Ashour 2024). Many members of the Genus *Fusarium* cause root diseases in plants, including *Fusarium oxysporum* Schlectend and *Fusarium solani* which are the major fungal pathogens (Forbes *et al.* 1986). Biological control contains several living organisms called biocontrol agents such as beneficial insects, predators, and microorganisms (fungi, bacteria) which can reduce or mitigate damage caused by pests or pathogens (Ashour *et al.* 2021 & Ashour and Afify 2023) and viruses (Copping 2009). Recently, LAB has received much attention (Johan and Jesper 2005). Many studies have recorded the action by LAB in phytopathogens (Gupta and Srivastava 2014, Ouiddir *et al.* 2019 & Muhialdin *et al.* 2020). LAB act as biocontrol agents when they suppress fungal growth and produce antifungal metabolites, such as hydrogen peroxide and organic acids (Trias *et al.* 2008) fatty acids, and volatile compounds (Hirozawa *et al.* 2022). LAB are considered biocontrol agents for reducing the growth of pathogenic fungi (Sadiq *et al.* 2019). Interest in recent years, there has been another class of potentially useful lactic acid bacteria (LAB) that has increased their effects against phytopathogens (Jaffar *et al.* 2023). The antifungal metabolites of the bacterial strains mentioned were considered to contribute to the antagonistic activities of these bacteria (Afify 2024). Hamed *et. al.* (2011) introduced LAB as an efficient for the protection of plants by improving seed germination, as well as improving plant fresh weight and healthy against some phytopathogenic fungi. Under *in vivo*, LAB capability to act as plant growth-promoting bacteria and biocontrol agent against some phytopathogenic fungi. LAB also introduces more evidence that several strains are better for plant environments than others (Strafella *et al.*2021). Therefore, the aim of this study is lactic acid bacteria-based highlights successful application to isolate LAB and selected the bacterial isolate recorded highest inhibition linear growth of fuasrium-pathogen for identification. Moreover, to test the effect of LAB for protection the plant growth we evaluated antagonistic parameters produced by LAB as well as determined seedlings parameters such as pre-and post-emergence of seedlings damping-off (%) and survival plants (%).

MATERIALS AND METHODS

Source of bacteria (LAB)

Bacteria (LAB), used in this study, were isolated from yoghurt (Van den Berg *et al.* 1993). Fresh bacterial cultures are grown in Man Rogosa & Sharpe (MRS) medium. Further instead, the procedure presented in other paper (Sorascu *et al.* 2019) for the isolation and counting of *Lactobacillus* CFU has been applied.

Source of phytopathogenic fungi

High virulent isolates of fungal pathogens were taken from Plant Pathology Lab. Agriculture Research Center (ARC) Giza, Egypt. These fungal isolates (*Fusarium oxysporum* and *Fusarium solani*) were isolated from diseased root tomato plants and inoculated on potato dextrose agar (PDA).

***In vitro* experiments:**

Antagonistic activities

Antagonistic activity of each *Lactobacillus* spp. was performed according to Elkahoui *et al.* (2012). The method

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was conducted to test antagonism phytopathogenic fungi: *Fusarium oxysporum* and *Fusarium solani* by bacterial isolates. Control plates are fungal disc from each tested fungus was centered in PDA plate without bacteria. Data were expressed as growth inhibition (%) according to the formula proposed by Trivedi *et al.* (2008) as follows:

Antagonistic effect = A-B/A X 100

Where,

A is the diameter of mycelial growth of pathogenic fungus in control and B is the diameter of mycelial growth of pathogenic fungus with isolates of LAB

Characters of lactic acid bacteria isolate LAB-3

From antagonistic activities tests, the highest potent bacterial isolate, studied for phenotypic identification was performed by standard character examinations according to Bergey's Manual of Systematic Bacteriology (Garrity *et al.* 2009) and Jose *et al.* (2020).

Antagonistic parameters produced by LAB

Lytic enzymes detection

On the medium containing enzyme substrate, the activities of hydrolytic enzymes were detected by streaking antagonistic LAB-3 isolate individually (Basha and Ulaganathan 2002). Estimation of the enzyme was carried out according to Ngarajkumer *et al.* (2004). All treatments were carried out in triplicates.

Catalase production

The production of catalase was detected by flooding of 9-10 % solution of hydrogen peroxide to the bacterial biomass on 24 hr old slopes; the evolution of gas bubbles from the growth denoted the presence of catalase (Skerman, 1967).

Organic acids production

The pH of filtrate bacterial isolate (LAB-3) was measured by pH meter after centrifuged filtrate at 1000 rpm for 10 min was received. The titrable acidity was expressed by a volume of 0.01N NaOH consumed for each 5ml of culture filtrate by using few drops of Phenolphthalein indicator (Reena *et al.* 2013).

Volatile compounds production

a). Hydrogen Cyanide (HCN) production

The optical density (OD) at 625 nm was used to measure the detection of hydrogen cyanide production by LAB. This method was described by Castric (1975).

b). Ammonia (NH_3) production

For the production of ammonia, Nessler's reagent was added to the LAB culture peptone broth and recorded to change the color from brown to yellow (Cappuccino and Sherman, 2002).

c). CO_2 production

The analytic system presented by Kihal (1996). Evolved CO_2 by the culture in tubes was trapped and was measured by displacement of acidifying water in the burette.

***In vivo* experiments:**

Preparation of phytopathogenic fungi

The selected phytopathogenic fungi *Fusarium oxysporum* and *Fusarium solani* were mixed using sorghum seeds (Sorghum bicolord) moistened with water after sterilization inoculated with old fungal mycelium and incubated according to Paulitz and Schroeder (2005). Colonized sorghum seeds were used at a rate of 50 g in 500 g of soil potting mix.

Bacterial seed treatment

1.5 ml from bacterial isolate suspension grown in MRS broth ($1x10^6$cfu) was obtained for mixing at a rate of 5g of tomato seeds (UC97, 30 seed)/ 30 min for sown in pots (Mew and Rosales 1986).

Pot trials

Glasshouse experiment was conducted by using clay pots of 20 cm in diameter with used naturally clay soil (pH7.5 clay 62.1 %, E.C. 1.4 mmhos/cm) with four replications. The treatments of pots experiment were conducted as follows:

1- Inoculated with *Fusarium oxysporum* or *Fusarium solani*, prepared of soil with supplementation with LAB (seed treatment) one day before planting.
2- Un-inoculated as a control.
3- Inoculated with *Fusarium oxysporum* and *Fusarium solani*, prepared one day before planting, without supplementation with LAB (seed treatment).

During growing season 2022/2023 at Agricultural Research Center (ARC), Giza, Egypt. Percentage of pre- and post-emergence damping-off after 15 and 45 days as well as survival plants were calculated from planting as follows:

1.% of pre-emergence damping-off = (No. of sown seeds) x 100
2.% of post-emergence damping-off = (No. of killed seedlings) x 100
3.% of survival plants = (No. of un-deased plants/total No. of plants) x 100

Statistical analysis

The obtained data were subjected to analysis of variance (ANOVA) (Steel and Terrie 1960). Least significant differences (LSD) and for comparing means under study Duncan's multiple range test (DMRT) were applied (Duncan 1955).

RESULTS AND DISCUSSION

***In vitro* experiments:**

Screening of isolated Lactic acid bacteria (LAB)

Bacteria were isolated from several yoghurt samples that were taken from different markets. All isolated colonies from each sample were selected randomly and screened for fungal growth inhibition against *Fusarium oxysporum* and *Fusarium solani.* Five bacterial isolates recorded inhibition of fungal growth were LAB-1, LAB-2, LAB-3, LAB-4 and LAB-5, respectively. Different levels of inhibition were observed by all LAB isolates against both *F.oxysporum* and *F. solani*. On the other hand, one bacterial isolate (LAB-3) had highly inhibitory activity against both phytopathogens. Inhibited effects against *F. oxysporum* were 85.50% whereas *F. solani* was inhibited by 87.60%, respectively (Table 1). Therefore, from these results, bacterial isolate (LAB-3) was chosen for the following studies.

Table 1. Evaluation of Lactic acid bacteria isolates on the reduction (R%) of linear growth *F. oxysporum* and *F. solani in vitro*

LAB	*F. oxysporum*		*F. solani*	
isolates No.	LG "cm"	Reduction%	LG "cm"	Reduction%
LAB-1	4.00^{a}	53.04	4.07^{a}	52.76
LAB-2	4.03^{cd}	54.50	4.34^{c}	51.88
LAB-3	2.93^{f}	85.50	2.00^{e}	87.60
LAB-4	6.25^{b}	31.27	5.86^{b}	33.91
LAB-5	4.50^{c}	47.74	4.60c	49.44
Control (only fungus)	8.89^{a}	00.00	9.03^{a}	00.00

Values with different letters are significantly different LG: Linear growth of the pathogens (cm)

In the last few years, many studies published have reported the ability of LAB species to suppress the growth of several varieties of phytopathogenic and toxigenic fungi

(Lamont *et al.* 2017 & Shehata *et al.* 2019). The biocontrol agents as LAB could be used against phytopathogenic fungi were integrated (Jaffar *et al.* 2023).

Characters of lactic acid bacteria isolate LAB-3

Of the five lactic acid bacterial isolates, one isolate (LAB-3) was chosen for the study of main characters because the isolate showed the highest antagonism against both fungal pathogens (*F. oxysporum* and *F. solani*). The results showed that the selected isolate (LAB-3) was Gram-positive, non-sporulating, rod-shaped (bacilli), non capsulating, catalase-negative, microaerophilic and glucose fermentation (Table 2). The isolate LAB-3 was characterized and identified up to genus level based on morphological and biochemical properties especially temperature for growth as *Lactobacillus* sp. Most LAB are part of the Phylum Firmicutes, Class Bacilli, and Order Lactobacillales. LAB identification is based on the criteria originally stated by Orla-Jensen in 1919 (Champagne 1994), which contain characters of cell shape, type of lactic acid fermentation, ranges of temperature for growth, and utilization of carbohydrates according to the results of our study, isolate LAB-3 had a biochemical profile, typical for *Lactobacillus* sp. The probability (accuracy) of identification was 99% (Jose *et al*. 2020). At the same time, various sources for isolation of *Lactobacillus* such as processed fermented milk (yoghurt) fermented feed (silage), and living organs (digestive tract) (Lydfiani *et al.* 2021; Hirozawa *et al*. 2022 and Jaffar *et al.* 2023).

Table 2. Characters of lactic acid bacterial isolate LAB-3

Characters	LAB-3 isolate
Colony morphology	cream-white, circular
Cell shape	rod
Motility	non-motile
Gram-stain	positive
Spore-stain	non-sporeforming
Capsule stain	non-capsulated
Catalase test	negative
Oxidase test	negative
Urease test	negative
Oxygen requirement	microaerophilic
Fermentation of sugars:	
Glucose	positive
Galactose	positive
Sucrose	positive
Fructose	negative
Lactose	positive
Maltose	positive
Arabinose	negative
Citrate	negative
Nitrogen sources:	
Ammonia	negative
Peptides	positive
Reduce nitrates	negative
Temperature range	30 – 45ºC

Antifungal properties produced by LAB-3

Antifungal compounds produced by LAB-3 can originate from the metabolism, or bioconversion of extracellular compounds. Results of these compounds observed were presented in Table (3) showed that LAB-3 was positive with all except negative with catalase, ammonia, and CO_2. Similar results were reported by Sidhu and Dadarwal (2001) hydrolytic enzyme production has been described for the biocontrol activity.

Table 3. Production of some antagonistic compounds by LAB-3

Antagonistic compounds	LAB-3
Hydrolytic enzymes production	
a) Chitinase	+
b) ß-1,3 – glucanase	+
c) Glycoproteinse	+
d) Protease	+
e) Lipase	+
Catalase production	-
Organic acids production	+
Volatile compounds production	
a) Hydrogen Cyanide (HCN)	+
b) Ammonia	-
c) CO_2	-

***In vivo* experiment:**

Effect of inoculation with *Lactobacillus* sp. on damping-off and survival plants infected with *Fusarium* spp.

LAB-3 was highly effective *in vitro*, so it that selected to test their antagonistic activity in the glasshouse. Results in Tables (4&5) showed that LAB-3 decreased damping-off and increased healthy plants compared with the untreated. In Table (4) LAB-3 increased survival plants by 85% with *F. oxysporum*. While with *F. solani* (Table 5) LAB-3 increased survival plants by 80% compared with the control treatments of 25% and 40% respectively of both fungi.

In a study for the protection of tomato plants against some phytopathogenic fungi, LAB was reported as an efficient biocontrol agent, for improving seed germination as well as plant healthy (Hamed *et al.* 2011). Jaffar *et al*. (2023) in recent years, lactic acid bacteria have increased the high level of biosafety when they employ to stimulate plant growth. Same author reported that these lactic bacteria has the ability to serve as safe agriculture through the promotion of plant growth and the control of plant diseases.

Table 4. Evaluation of the efficacy of LAB-3 against *F. oxysporum* in controlling damping-off of tomato seedlings in glasshouse

Treatment	Damping-off (%)		Survival Plants (%)
	Pre-emergence 15 days	Post-emergence 45 days	
LAB-3 + *F. oxysporum*	9^b	15^b	85^a
Control (untreated)	30^a	55^a	45^b
Control (only *F. oxysporum*)	50^a	75^a	25^c

Values with different letters are significantly different

Table 5. Evaluation of the efficacy of LAB-3 against *F. solani* in controlling damping-off of tomato seedling in glasshouse

Treatment	Damping-off (%)		Survival plants (%)
	Pre-emergence 15 days	Post-emergence 45 days	
LAB-3 + *F. solani*	15^b	20^b	80^a
Control (untreated)	30^a	55^a	45^b
Control (only *F. solani*)	45^a	60^a	40^b

Values with different letters are significantly different

CONCLUSIONS

This study has focused on recent research concerning interactions between lactic acid bacteria (LAB) as biocontrol agents and *Fusarium* spp. causing seedlings damping off. The greatest interest has been concerned that LAB are environmentally safe and environmentally friendly strategy which able to control phytopathogenic fungi. Also, LAB are

widely regarded as an excellent biological resource for biocontrol agents which act by various mechanisms such as the production of lytic enzymes, organic acids, and volatile compounds. The utilization of LAB can be developed as another way to protect agricultural crops from phytopathogens. The conclusions of this study, LAB are environmentally safe, when able successfully to control phytopathogenic fungi.

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المقاومة الحيوية لفطر الفيوزاريوم بإستخدام بكتيريا حامض اللاكتيك

عا يده حافظ عفيفى[1] و عبد الودود زكى عاشور[2]

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الملخص

إن المقاومة الحيوية لفطريات أمراض النبات بإستخدام بكتيريا حامض اللاكتيك من أهم طرق المقاومة الصديقة للبيئة. فى هذه الدراسة تم عزل هذه البكتريا من مصدرها وهو اللبن الزبادى لماركات متعددة من محلات السوبر ماركت المختلفة ، حيث تم الحصول على خمسة عزلات من بكتيريا حامض اللاكتيك وكانت أرقام الكود لكل منها LAB-1, LAB-2, LAB-3, LAB-4 and LAB-5. وبناءاً على ذلك فقد أختبرت العزلات الخمسة فى المعمل لقدرتها على تضاد نوعين من الفطريات المسببة لأمراض موت البادرات لكثير من النباتات الإقتصادية الهامة. وبإجراء إختبار التضاد فى المعمل لهذه العزلات البكتيرية الخمسة ضد نوعين من فطر الفيوزاريوم (فيوزاريوم أوكسيسبورم وفيوزاريوم سولانى). أظهرت نتائج التضاد أن جميع العزلات البكتيرية أعطت مناطق تثبيط نمو لأنواع فطر الفيوزاريوم ولكن بنسب مئوية متفاوتة وكانت أعلى نسبة تثبيط فى نمو الفطرين مع العزلة البكتيرية الثالثة LAB-3 حيث بلغت النسبة المئوية للتثبيط لهذه العزلة البكتيرية 80.50 و 87.60% لكل من فطر فيوزاريوم أوكسيسبورم وفطر فيوزاريوم سولانى على الترتيب. أختيرت هذه العزلة البكتيرية LAB-3 لإجراء عليها الإختبارات المورفولوجية والبيوكيميائية للتأكد من أنها تتبع بكتيريا حامض اللاكتيك وقد سجلت العزلة LAB-3 أنها تمتلك فعلا خصائص بكتيريا حامض اللاكتيك وهى من العصويات الطويلة غير المتجرثمة والموجبة لصبغة جرام وغير متحركة ولاتكون كبسولة وسالبة للكتاليز كما أنها شحيحة فى إحتياجها للأكسجين وتنتج حموضة عند تخمرها لسكر الجلوكوز وبالتالى هذه العزلة نوع من جنس اللاكتوباسلس. وإستكمالاً لإختبارات المعمل على هذه العزلة البكتيرية تم الكشف على مواد التضاد التى تنتجها للفطريات وتبين أنها تنتج مواد تضاد عديدة لنمو الفطريات المسببة لأمراض النبات وكانت موجبة مع إنزيمات التحلل للجدار الخلوى للفطريات ولكنها سالبة مع إنزيم الجلوكونيز كما إنها تنتج أحماض عضوية بالإضافة للمواد السامه المتطايرة وجد أنها موجبة مع سيانيد الهيدروجين وسالبة فى إنتاج ثانى أكسيد الكربون والأمونيا. أما فى تجربة زراعة نبات الطماطم كأحد النباتات الإقتصادية الهامة التى تصاب بذورها وبادراتها بفطر الفيوزاريوم مما يسبب خسارة كبيرة فى المحصول فقد تم الزراعة فى أصص بالبيوت الزجاجية وعدوى التربة الطبيعية بالفطريات فيوزاريوم أوكسيسبورم وفطر فيوزاريوم سولانى كلا على حده وأخرى بدون عدوى (معاملة كنترول) للمقارنة مع عمل أربعة مكررات لكل معاملة وإضافة البكتيريا للبذرة قبل الزراعة. سجلت تجربة الزراعة هذه زيادة نسب إنبات البذور المعاملة بالبكتيريا بالمقارنة بمعاملة الكنترول وذلك بعد 15 يوم و 45 يوم من الزراعة. كما كان هناك زيادة فى النسبة المئوية للبادرات الباقية على قيد الحياة من 25 إلى 85% ومن 40 إلى 80% لكل من فطر فيوزاريوم أوكسيسبورم وفطر فيوزاريوم سولانى على الترتيب بالمقارنة بمعاملة الفطر بدون البكتيريا. هذه الدراسة تؤكد أن بكتيريا حامض اللاكتيك من عوامل المقاومة الحيوية الطبيعية الصديقة للبيئة وآمنه تماما لأنها معزولة من مصدر غذائى وقادرة على مقاومة الفطريات المسببة لأمراض النبات وبالتالى تزيد من كمية المحصول.