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Efficacy of *Lactobacillus plantarum* C5 Cell and Their Supernatant Against *Colletotrichum gloeosporioides* on Germination Rate of Chilli Seeds

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Abstract: Lactic Acid Bacteria (LAB) were isolated from different fermented fruits and vegetables and screened for antifungal activity against chilli phytopathogen *Colletotrichum gloeosporioides*. Seven LAB showed antifungal activity as observed in the dual overlay assay method. These LAB grew between 10 and 45°C, pH 4.4 and 9.6 (except 1 isolate), 6.5 and 18% NaCl. Fungi infected seeds from four species of chilli were evaluated for germination rate with and without LAB treatments. LAB isolated C5 showed good fungal inhibition and allowed good seed germination and was identified as *Lactobacillus plantarum* using API CH50L kit. The results indicate that C5 has the potential to be used as a biological control for treatment of chilli seeds against *C. gloeosporioides* to replace the use of chemical fungicides.

Key words: *Colletotrichum gloeosporioides*, lactic acid bacteria, infected seeds, germination percentage, chilli

INTRODUCTION

Chilli (*Capsicum annuum* L.) is one of the important spice crop of the world and is widely cultivated throughout warm temperature, tropical and subtropical countries. It belongs to the family Solanaceae. The disease which is both seed borne and air borne affects seed germination and vigour to a great extent (Asalmol *et al*., 2001). Anthracnose is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. and is a serious disease in the chilli growing areas of Malaysia and affects many kinds of plants in tropical areas (Sangchote *et al*., 1998; Sangchote, 1999). It is also the main causal agent in tropical Asia (Kim *et al*., 1989). Usage of chemical fungicides would cause the development of resistance by the plant pathogenic fungi and also risk pollution of the environment. The use of biological control agents has been suggested as an alternative way of controlling plant diseases (Compant *et al*., 2005). Selected microorganisms such as Lactic Acid Bacteria (LAB) isolated from fresh fruits and vegetables showed inhibitory activities against phytopathogenic and spoilage bacteria and fungi and can be used as a biological control agents to protect seeds and seedlings from various pathogens (Trias *et al*., 2008). LAB are reported to improve the germination rate of seeds (Tsudam *et al*., 2006).

The disease appears as small circular spots that coalesce to form large elliptical spots on the fruits and leaves. Under severe conditions, defoliation of the affected plants occurs. Biological control using as antagonistic *Pseudomonas fluorescens* (bacteria) as seed treatment and as well as spray treatment were found to be effective against *C. capsici* (Srinivas *et al*., 2005). Trichoderma species (fungus) are able to effectively control *C. capsici* infection in chilli (Maymon *et al*., 2004). Other biological control agents that have been tested for efficacy against Colletotrichum include *Bacillus subtilis* and *Candida oleophila* (Wharton and Dieguez-Uribeondo, 2004). Also yeast isolated from Thai rambutan has been shown to suppress the fungal pathogen *C. capsici* in harvested chilli (Nantawanit *et al*., 2010).

LAB isolated from fresh fruits and vegetables were used as biocontrol against the phytopathogenic and spoilage bacteria and fungi *Xanthomonas campestris*, *Erwinia carotovora*, *Penicillium expansum*, *Monilinia laxa* and *Botrytis cinerea* (Trias *et al*., 2008). LAB strains isolated from wheat semolina were found to result in strong inhibitory activity against *Aspergillus niger*, *Penicillium roqueforti* and *Endomyces fibuliger* (Valerio *et al*., 2009). The biological control of plant pathogens by these lactic acid bacteria appears to involve multiple antagonistic mechanisms, including competition

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for space and nutrients (Janisiewicz *et al*., 2000). LAB have a long history of being safe to use to inhibit growth of spoilage bacteria and organisms (Lowe and Arendt, 2004). Additionally, LAB have the ability to produce antimicrobial compounds called bacteriocins and have potential usefulness as a natural substitute for chemical fungicides (Soomro *et al*., 2002).

During the past 50 years, many studies have reported about bacterial and fungal plant diseases as well as the application of different microorganisms as biocontrol agents. However, no information is available on the interactions of LAB with phytopathogenic fungi *Colletotrichum capsici* (Compant *et al*., 2005; Heydari and Pessarakli, 2010; Hamed *et al*., 2011).

The purpose of this study is to screen LAB isolated from different sources for antifungal activity against *C. gloeosporioides* which infects chilli seeds. This study will reduce the use of chemicals and instead use LAB as a biological control to reduce plant disease and increase the growth of seeds.

MATERIALS AND METHODS

Isolation and characterization of lactic acid bacteria: Lactic acid bacteria were isolated from fresh fruits and vegetables. The vegetables (1 g) were cut into small pieces and suspended in 9 mL MRS broth and the bags manually agitated. Turbid broth was serial diluted with peptone water (0.1% w/v) from 10^{-1} to 10^{-7} and spread-plated on modified MRS agar with 0.8% $CaCO_3$, 0.1% (w/v) cycloheximide to inhibit possible fungal contamination. All plates were incubated under anaerobic condition in an anaerobic jar at 30°C for 48 h or until the bacterial colonies were of sufficient size for colony formation. Colonies were tested for catalase activity with 4% H_2O_2 (Mallesha *et al*., 2010). All isolates were checked for catalase negative and gram positive reactions. Growth conditions were determined in MRS broth at different temperatures (10 and 45°C), pH (4.4 and 9.6) and with different salt (NaCl) concentrations of 6.5 and 18% incubated at 30°C for 48 h. Growth was determined in terms of turbidity (Sathe *et al*., 2007).

Screening of isolates for antifungal activity: Pure culture of *C. gloeosporioides* was obtained from the Faculty of Agriculture, University Putra Malaysia and maintained on Potato Dextrose Agar (PDA) plates and incubated at 28°C for 7 days. Selected LAB isolates were screened for antifungal activity against *C. gloeosporioides* by the overlay technique as described by Strom *et al*. (2002) on MRS agar plates. Briefly, overnight cultures of LAB were grown as 2 cm streaks on MRS agar plates followed by incubation at 30°C for 48 h under anaerobic conditions. These plates were overlaid with semisolid malt extract agar (0.7%) seeded with 10^4 spores mL^{-1} of *C. gloeosporioides* and incubated aerobically at 30°C for 24-72 h.

Identification of LAB by API 50 CHL kit: For species specific identification, selected LAB strains were preliminarily identified based on the phenotypic properties such as carbon dioxide production from glucose were subjected to API 50 CHL kit assay (Bio Merieux, l'Etoile, France) following the methods described by the manufacturer (Tamminen *et al*., 2004). The inoculated strips were incubated at 30°C and then monitored for changes in the colour of the medium after 1, 2 days. Change in colour was represented by a positive sign (+) while a negative sign (-) represented no change. Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

Chilli plant seeds experiment: Seeds from four varieties of chilli plants (CB, CP, KU and MC) were plated in 4 replicates of 100 seeds and each 25 seeds per plate were kept at 4°C before use. First the surfaces of the seeds were disinfected with ethanol (70%) for a minute and rinsed with distilled water 4 times to minimize microorganism development at the early stages of germination (Kurtar, 2010). The treatments included immersion of seeds for 10 min in LAB-C5 and LAB-C5S prepared on MRS Broth (10^8 cfu mL^{-1}) with 4 replicates of 25 seeds each. After immersion, the seeds were dried under laminar flow and then placed in Petri dishes before inoculation of the fungal spores by dropping spores of *C. gloeosporioides* (10^4 spores mL^{-1}).

Fungal spores were prepared by growing the fungus on PDA for 7 days at room temperature. The treated seeds were incubated at room temperature for 7 days and the germinated seeds were counted and expressed as germination percentage. For control the seeds were immersed in distilled water.

Statistical analyses: All data were analysed by One-way Analysis of Variance (ANOVA) and by the Tukey test, the statistical significance ($p \leq 0.05$) program from Minitab 16 software was used.

RESULTS

Characterization of lactic acid bacteria: Seven LAB were selected for further study. Six LAB isolates could grow at

Table 1: Characterization of the selected LAB that showed high antifungal activity against *Colletotrichum gloeosporioides*[a]

Strains	Source	Shape	Gram reaction	Catalase test	Temp. (°C) 10 and 45	pH 4.4 and 9.6	NaCl% 6.5 and 18
B3	Dragon	Cocci	+	-	++	++	++
D1	Dragon	Rod	+	-	++	++	++
D10	Starfruit	Rod	+	-	++	++	++
D11	Melon	Rod	+	-	++	++	++
G1	Guava	Rod	+	-	++	- -	++
C5	Durian	Rod	+	-	++	++	++
G7	Ginger	Rod	+	-	++	++	++

[a]Growth (+); No growth (-)

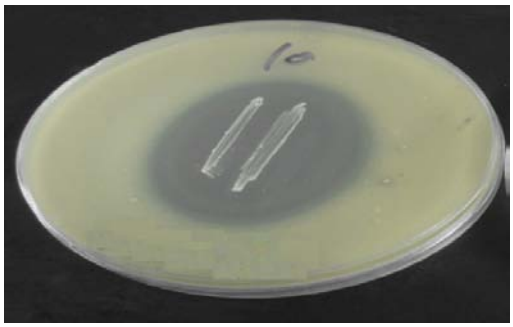

Fig. 1: Clear zone indicates growth inhibition of *Colletotrichum gloeosporioides* by lactic acid bacteria (C5) incubated at 30°C for 72 h by dual agar overlay method

10 and 45°C, pH 4.4 and 9.6 and at 6.5 and 18% NaCl, except isolate G1 strain which failed to grow at pH 4.4 and 9.6 (Table 1).

Screening for antifungal activity of LAB against *Colletotrichum glosporioeides* by overlay method: A total of 150 LAB were isolated from fresh fruits and vegetables; all the isolates were catalase negative and gram-positive bacteria. In total, 30 LAB isolates showed inhibitory activity against *C. gloeosporioides* by the dual agar overlay method (Fig. 1). The inhibition area per bacterial streak ranged between 10 to >20 mm of the Petri dish. LAB isolates D1, C5, G7, D10 and D11 had strong activity (+++ and ++++) against spoilage fungi *C. gloeosporioides*. Four isolates (Te, G1, D3 and B3) had moderate activity (inhibition area per bacterial streak was 6-10 mm of Petri dish) against *C. gloeosporioides* (Table 2).

Identification of LAB by API 50 CHL assay: Phenotyping of lactic acid bacteria using biochemical (API 50CHL) showed that six isolates as *Lactobacillus plantarum* and one isolate as *Lactobacillus parasasei* (Table 3).

Seeds germination: There were significant differences among the treatments in the germination percentage of chilli seeds (Table 4, Fig. 2). Seeds not infected with the fungi (control) showed the greatest number of germinated seeds. Both the cells and supernatant of C5 can be used together to inhibit the growth of *C. gloeosporioides*. Treating chilli seeds with C5 resulted in a high germination percentage for all four varieties of chilli plants namely, CB, CP, KU and MC which were 96, 96.5, 94.75 and 92.25%, respectively. A slight reduction in percentage germination was observed when seeds were treated with the supernatants. Seeds infected with the fungus *C. glosporioeides* resulted in a lower percentage germination at 35, 56, 64.75 and 58.75% on CB, CP, KU and MC, respectively.

Table 2: Selected lactic acid bacteria isolates showing inhibitory activity on *Colletotrichum gloeosporioides*, conidia germination after 48 h incubation at 30°C by dual agar overlay method

LAB	Antifungal activity[a]
C5, G7	++++
B1, C2, D1, G4, G5, D10, D11	+++
Te, G1, D3, B3	++
A2, P1, E8, C1, E1, D2, E2, Gr1, Gr2, W1, Bn3, W2, Bn2, Co, E3	+
P25, B2	-

[a]Antifungal activity: (-) = No growth; (+) = Inhibition zone of <6 mm; (++) = Inhibition zone of 6-10 mm; (+++) = Inhibition zone of 10-18 mm; (++++) = Inhibition zone of >20 mm

Table 3: Identification of LAB by API 50 CHL assay

LAB isolates	Percentage	Identification
D1	99.2	*Lactobacillus paracasei*
D10	99.9	*Lactobacillus plantarum*
D11	99.9	*Lactobacillus plantarum*
G1	99.9	*Lactobacillus plantarum*
B3	95.9	*Lactobacillus plantarum*
C5	95.9	*Lactobacillus plantarum*
G7	95.9	*Lactobacillus plantarum*

Table 4: Percent germination of infected chilli seeds with *C. gloeosporioides* treated with C5 cells and supernatant[a]

	Variety of chilli seeds			
Treatments	CB	CP	KU	MC
LAB-C5	96.0±0.81	96.5±1.91	94.75±3.30	92.25±2.62
LAB-C5S	84.75±1.25	85.5±1.91	85.75±0.95	86.75±0.95
C.g on LAB-C5	95.25±0.95	94.0±1.82	95.5±1.29	94.5±1.29
C.g on LAB-C5S	86.5±1.29	86.25±1.25	85.75±0.95	86.5±1.29
Seeds infected with C.g	35±7.48	56.0±8.08	64.75±6.23	58.75±3.5
Control	98.5±1.29	98±1.82	98.25±2.21	99.75±0.5

[a]CB, CP, KU, MC were four variety of chilli seeds used, LAB-C5 = Lactic Acid Bacteria cell; LAB-C5S = Lactic Acid Bacteria supernatant, C.g = *Colletotrichum gloeosporioides*; Results are mean values of four replicates determinations±SD

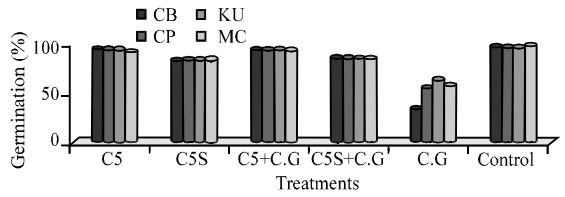

Fig. 2: Effect of seed treatment with *Colletotrichum gloeosporioides,* LAB and supernatant on seeds germination

DISCUSSION

Characterization of the LAB isolates could grow at 10 and 45°C, pH 4.4 and 9.6 and at 6.5 and 18% NaCl (Table 1) except the G1 strain which did not grow at pH 4.4 and 9.6. The results are in agreement with Sathe *et al.* (2007) who reported that the observed growth of LAB at extreme pH 4.4 and 9.6 and temperature (10 and 45°C) provides exciting properties for potential applications as a biocontrol against phytopathogenic fungi. The surface fungal infected seeds with *C. gloeosporioides* resulted in a low percentage germination. It was observed that seeds tissues were distorted which could be due to the activity of cellulolytic and pectionlytic enzymes produced by *C. capsici* (Sariah, 1980). Formation of the acervulus was initiated below the seed coat and also in the endosperm and emerged to the surface after disrupting the seed coat. Parenchymatous tissues were also distorted. The pathogen finally grows on the seed surface (Sariah and Nik, 1988), thus inhibiting seed germination. Anjorin and Mohammed (2009) reported that pathogenic seed borne fungi could seriously retard seed germination through softening and necrosis of tissues.

LAB with antifungal activity are well documented in food, meat and milk products as biopreservatives (Schnurer and Magnusson, 2005) while less attention has been paid to exploit the antifungal activity of LAB for biocontrol of phytopathogenic fungi. Very few *in vitro* studies have been reported about the efficacy of LAB against phytopathogenic fungi (Zulpa *et al.*, 2003; Trias *et al.*, 2008; Wang *et al.*, 2011). This study observed that five isolates namely D1 (*L. paracasasei*) and C5, G7, D10 and D11 (*L. plantarum*) showed good inhibition against phytopathogen *C. gloeosporioides* (Table 2, Fig. 1). It was also observed that both the cells and supernatant C5 can be used together to inhibit the growth of *C. gloeosporioides*. However, Laitila *et al.* (2002) and Lavermicocca *et al.* (2003) suggested that the antifungal activity of *L. plantarum* could be the results of many organic acids such as lactic acetic and phenyllactic acids. Another study suggested that some soluble compounds in culture supernatant may be responsible for the inhibition (Tang *et al.*, 2010). Sathe *et al.* (2007) reported that suspension of *L. plantarum* delayed the growth of *Aspergillus flavus*, *Fusirium graminearum*, *Rhizopus stolonifer* and *Botrytis cinerea* on cucumber. The inhibitory activity of *L. plantarum* in both the cells and supernatant against fungal *C. gloeosporioides* is in agreement with previous studies (Lavermicocca *et al.*, 2000; Magnusson *et al.*, 2003; Belal and Hassan, 2011). Also Carla *et al.* (2009) reported that the strains from LAB were able to inhibit the conidial germination and the mycelial growth. The conidia germination is the growth stage that is most sensitive to inhibition. In fact, the precise mechanism of antimicrobials can often not be defined because of a complex interaction between the different compounds produced during cell growth and the frequently synergistic effects among them (Legan, 1993). LAB isolated from fresh fruits and vegetables were reported to be effective against the phytopathogenic and spoilage bacteria and fungi, *Xanthomonas campestris*, *Erwinia carotovora*, *Monilinia laxa* and *Botrytis cinerea* on apple fruits (Trias *et al.*, 2008).

Recently, Hamed *et al.* (2011) reported that tomato seeds treated with lactic acid bacteria reduced the growth of *Fusarium oxysporium* and improved the growth of roots (Hoda *et al.*, 2011). Other microorganisms that were reported to be effective in controlling phytogenic fungi *F. oxysporum* were *Trichoderma harzianum*, *Pseudomonas chlororaphis* and *Streptomyces griseoviridis* (Rose *et al.*, 2003).

The antifungal activity of LAB against phytopathogenic fungi under *in vitro* assays may be attributed to the production of indol acetic acids and phenolic substances (Zulpa *et al.*, 2003), organic acids (Trias *et al.*, 2008) or proteinaceous compounds (Wang *et al.*, 2011; Belal, 2011). Another mechanism by which the yeast antagonist suppresses *C. capsici* on chilli fruits is the induction of resistance to the fungus (Nantawanit *et al.*, 2010).

CONCLUSION

The study indicates that if chilli seeds were treated with LAB either the cells or supernatant, they can resist the fungal infection as indicated by the good percentage germination of seeds. Further studies should be carried out to determine the potential of these LAB as a biocontrol against phytopathogenic fungi.

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