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ORIGINAL RESEARCH

Characterization of bacteriocin from lactic acid bacteria and its antibacterial activity against *Ralstonia solanacearum* causing tomato wilt

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is a major disease in solanaceous plants. It is one of the most destructive bacterial diseases of tomato and other economically important crops. One hundred isolates of *R. solanacearum* were obtained from rhizosphere soil of tomato fields in different parts of Karnataka. Forty strains of lactic acid bacteria (LAB) were isolated from soil samples. Among 40 LABs, isolate 35showed a maximum zone of inhibition (22–26 mm) against *R. solanacearum* by agar overlay method and it was identified as *Lactobacillus paracasei* by 16S rRNA analysis. The bacteriocin from the culture supernatant was found to be proteinaceous in nature. It was 60%stable at temperature range of 30-32 °C for 30 days. It showed maximum antagonistic activity at a pH range of 4-10. Antibacterial activity increased with up to 1% surfactants and decreased at low concentration of metal ions at 0.5mg/l. The antimicrobial substance was sensitive to the proteolytic action of trypsin. Seed germination and seedling vigour enhanced upon treatment with bacteriocin. Under greenhouse conditions, bacteriocin treatment increased the plant growth promotion and reduced wilt by 57.48% and 54.77% by seed treatment and soil drench methods respectively. In the present study, it is the first report that bacteriocin exhibits antagonistic activity and increases the plant growth promotion against *R. solanacearum in vitro* and *invivo.* These important results recommend that soil may be a common source for the isolation of bacteriocin producing lactic acid bacteria and can be used as biocontrol agent.

Keywords: *Lactobacillus paracasei;* Bacteriocin; *Ralstonia solanacearum*; bacterial wilt; plant growth promotion.

Introduction

Tomato (*Solanum lycopersicum*) is an important crop susceptible to several pathogens. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) is the most significant disease of tomato (Hayward 1991). Bacterial wilt is a destructive and prevalent soil borne disease that limits tomato production in the tropics and subtropics. The pathogen has an extremely wide host range, including more than 200 species in 50 families (Genin et al. 2012). Strains of *R. solanacearum* have been classified into five biovars and five races (Bin Li et al. 2010). Race1-*R. solanacearum* strains can be found in humid areas throughout the world and attack many solanaceous crops i.e., pepper, tomato, and eggplant and Biovar 3 of *R. solanacearum* in tomato growing areas. Losses incurred by *R. solanacearum* in tomato crops vary extensively according to the host, cultivar, climate, soil type, cropping pattern, and infecting strain, from 0 to 91% (Yuliar et al. 2015) and in India the yield loss may vary between 10.8 and 90.6% (Kishun 1987).

Biological control of bacterial wilt is still in its research stage, with few studies reported by Messiha (2007). Biological control not only increases yield and decreases disease severity, but also avoids environmental pollution by chemicals. Among plant pathogens, soil borne pathogens are considered to be more limiting than seed borne or air borne pathogens in the production of numerous crops and reported for 10–20% of yield losses yearly (Yuliar et al. 2015). Management of bacterial wilt in tomato has been difficult and it still threatens commercial tomato production (Coll and Valls 2013).The environmental pollution caused by extreme usage of agrochemicals (Fertilizers Pesticides) as well as the development of resistant pathogenic strains has promoted the search for alternative approaches, i.e. the use of microorganisms or their metabolites (Montesinos 2003). It has been established that different species of bacteria and fungi such as *Trichoderma, Pseudomonas* and *Bacillus* are used as antimicrobial agents against phytopathogenic bacteria. Recently, LAB has received much attention. Application of LAB in traditional food, feed fermentation and preservation is well documented (Johan et al. 2005). LAB are generally classified into two groups: homofermentative and heterofermentative; homofermentative LAB produce lactic acid alone through the Embden-Meyerhof pathway, heterofermentative LAB produce supplementary byproducts, as well as ethanol, acetic acid, and carbon dioxide (Moon et al. 2012).

A lactic acid bacterium is a known to improve human and animal health (probiotics) and LAB excretes lactic acid as main end product and they are generally recognized as safe (GRAS) organisms (Stiles 1996). The antibacterial effect has been attributed to the production of antibiotics or antibiotic like substances such as acidophilin, lactocidin, lactolin, nisin etc., and a variety of inhibitory compounds such as lactic, acetic, probionic acidsand bacteriocins as well as hydrogen peroxide and carbon dioxide (Kleanthous 2010, Szala et al. 2012). Bacteriocin production has been reported in different strains such as *Bacillus thuringiensis* NEB17, *Pseudomonas fluorescens Pf-5,B. licheniformis, Xanthomonas campestris* pv. *Glycines, B. thuringiensis* subsp. *kurstaki strain BUPM4,Bacillus cereus* BC7 and *Pseudomonas aeruginosa* (Annabel et al. 2005, Heu et al.

2001, Gray et al. 2006, Kamoun et al. 2005). The plant growth promoting potency and antibacterial activity of LAB against *X. campestris,* bacterial wilt and bacterial soft rot is a well-established fact (Shrestha et al. 2009, Anupama et al. 2014). Very few *in vitro* studies have been reported about the efficacy of LAB against phytopathogenic fungi (Wang et al. 2011). Hence, in the present study, production, purification and characterization of bacteriocin from *Lactobacillus paracasei* has been extensively studied. In addition to this, purified bacteriocin was tested for its antimicrobial activity, plant growth promotion against *R. solanacearum in vitro* and *in vivo*.

Materials and methods

Isolation and identification of *R. solanacearum*

Symptomatic tomato plants and soil samples were collected from different tomato growing regions of Karnataka. The rhizosphere soil solution and surface sterilized plant samples were plated onto TZC (2, 3, 5-triphenyl tetrazolium chloride) media (Kelman 1954). Isolates of *R. solanacearum* were identified by morphological, biochemical and molecular characteristics and pathogenicity on tomato seedlings, confirmed in previous work was used in this study (Narasimha Murthy et al. 2012). The 16 S rRNA gene amplified PCR products were sequenced and a phylogenetic tree was constructed by the BLAST analysis and the sequences were deposited in NCBI Gen Bank.

Isolation and identification lactic acid bacteria

Lactic acid bacteria were isolated from soil by standard pour plate method onto De Man, Rogosa and Sharpe agar (MRS). One gram of soil samples were placed into 9ml of sterile distilled water. Nine- fold serial dilutions were then made from the mixed solution and 1ml from the last 3 dilutions (10^{-7} , 10^{-8} , 10^{-9}) were pipetted and plated on De Man Rogosa and Sharpe (MRS) agar plates by pour plate method (Awan and Rahman 2005). The plates were then incubated at 35°C for 48 hours. The selected LAB was identified by morphological, biochemical and molecular methods. 16S rDNA gene sequence of the isolated strain was amplified and compared with the GenBank database through BLAST sequence analysis.

Screening of LAB against *R. solanacearum*

Forty isolates of LAB were screened for antagonistic activity against ten virulent strains of *R. solanacearum* by agar overlay method (Jacobsen et al. 1999). Strains of LAB were spot inoculated on MRS agar, incubated at 35°C for 24h. *R. solanacearum* suspension (1×10^{8}cfu/ml) was inoculated into tryptone soya soft agar and overlaid on spot inoculated LAB. Plates were incubated at 35°C for 18-22h and zone of inhibition was measured in mm and LAB which showed maximum inhibition was used further study (Alvarado et al. 2006).

Screening of bacteriocin from LAB against *R. solanacearum*

Cell free purified bacteriocin from selected LAB was serially diluted with sterile distilled water and each 10 µl of the purified bacteriocin was placed in wells in plates previously inoculated with *R. solanacearum* (1×10^{8}cfu/ml). Plates were then incubated at 30°C for 22h and an inhibition halo was measured after incubation (Schillinger et al. 1989). The activity was defined as the highest dilution showing inhibition of the indicator lawn and was expressed as units per ml (AU ml^{-1}) (Rammelsberg et al. 1990).

Bacteriocins isolation and partial purification from LAB

The selected strain LAB-35 was grown for 12 h in MRS medium, at its optimum temperature previously determined for bacteriocin production. After incubation, the cells were removed by centrifugation (10,000×g for 15 min, 4°C). The cell free culture supernatant was neutralized and precipitated with ammonium sulphate (40 % saturation), overnight, with gentle shakeup. The suspended pellicle formed was collected after centrifugation (10,000*g*, 10 min, 4 °C), dissolved in 5 MM potassium phosphate buffer (pH 6.5) and then extracted with 15 volumes of a mixture of chloroform/methanol (2/1, v/v). After l h at 4 °C, the white precipitate formed in the organic phase was centrifuged for 15 min at 13000×g and resuspended in sterile distilled water and used for further study (Ohmomo *et al*., 2000; Grosu-Tudor *et al*., 2014).

Characterization of bacteriocin from *Lactobacillus paracasei*

The concentrated purified bacteriocin was used for these tests (Sivaramasamy et al. 2014).The proteinaceous nature of the inhibitory compound was confirmed by testing their sensitivity to proteolytic enzymes (trypsin). Aliquots of the purified bacteriocin were treated with the following enzymes (1 mg/ml) and incubated for 2 h at 30 °C. To find out the effect of pH on bacteriocin activity it was tested by incubating at various pH by adjusting the range from 4.0 to 10.0. The effect of temperature on activity of the purified bacteriocin was tested by incubating at various temperatures between 20, 30, 60, 90 and 120°C for 15min. The effect of surfactants on bacteriocin activity was tested by adding 1% sodium dodecyl sulfate (SDS), Tween 20, Tween 80with the purified bacteriocin separately. The effect of storage and metal ions on activity of the purified bacteriocin was also tested. After the purified bacteriocin was tested for antibacterial activity by using the agar well diffusion method, *R. solanacearum* was spread on tryptone soya agar before boring the wells; samples were loaded into the wells and incubated at 35°C for 24h. After incubation the zone of inhibition was measured (Malini *et al*., 2012).

Effect of bacteriocin on tomato seed germination and seedling vigor index

Effect of bacteriocin on tomato seed germination and vigor index of seedlings were evaluated under laboratory conditions. Tomato cultivar Arka Megali (wilt susceptible) was procured from IIHR Bangalore, India. The germination test was conducted according to the paper towel method (ISTA, 2005).The rolled towels were incubated for 14 days at 24 ± 1^{o}C.After incubation paper towels were unrolled, number of germinated seeds were counted and seedling vigor index was calculated. The experiment consisted of four replicates of 100 seeds each and was repeated thrice. The vigor index was calculated by using the formula VI (vigor index) = (Mean root length + Mean shoot length) x Germination percentage (Abdul et al. 1973).

Greenhouse studies

Inoculum of *R. solanacearum* was prepared by culturing it in Casamino acid Peptone Glucose (CPG) broth (Kelman, 1954). Cultures were centrifuged at 12000 g for 10 min at 4°C.The pellet was resuspended in distilled water and suspensions were spectrophotometrically adjusted to O.D 600nm=0.1 (approximately 1×10^{8}cfu/ml (Ran et al. 2005).Under

greenhouse conditions,21 day old seedlings were treated with 50 ml fresh bacteriocin from LAB by soil drench method. Along with untreated controls the treated seedlings were sown separately in pots filled with sterilized potting soil (soil, sand and coconut pith compost), watered when required with no additional fertilization. After one week after transplantation, the bacteriocin treated seedlings were challenge inoculated with *R. solanacearum* (1×10^8cfu/ml). Following 30 days of incubation, seedling emergence and plant growth promotion (mean shoot length, root length, fresh weight and disease incidence) was recorded. Each treatment consisted of 4 replicates (10seedlings/pot) and was repeated three times (Neelu et al. 2012).

Plant growth promotion assessment

The bacteriocin from LAB was tested for their ability to promote plant growth. At the end of the experiment (45 days after challenge inoculation) plants were harvested and its fresh weight, dry weight, shoot length, root length and disease incidence was measured. Healthy plants were counted; growth promotion was measured and compared with the untreated control (Lim et al. 1997). Percentage of wilt incidence was recorded using the formula.

$$\text{Percent Disease Incidence (PDI)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

Control of wilt under field conditions

The field trial was conducted in a farm of Chintamani, Karnataka, India during 2013 - 2014. Treatments were dispersed in a complete randomized block design, the individual trial plot area was 25 m^2 containing fourteen rows, each row was 3.5 meter long and space between rows was 50 cm and each plot contained 140 plants. At the time of transplanting, bacteriocin solution was mixed with 10 kg of vermicompost and was applied to the soil near the root zone of plants at 10g/plant (Sivakumar and Swamy 1998). At 20 days after transplanting, all tomato seedlings were challenged with *R. solanacearum* by soil inoculation technique (Winstead and Kelman, 1952), except in uninoculated control treatment plot. The numbers of wilted plants were recorded up to 90 days after pathogen inoculation.

The percentage (%) wilt incidence was calculated by using the formula

$$\%\ \text{Wilt incidence} = \frac{\text{No. of plants wilted in each treatment}}{\text{Total no. of plants receiving the treatment}} \times 100$$

Wilt incidence was measured up to 60 days after challenge inoculation and plant height, stem growth, No. of fruits/plant, yield/plant (kg), were measured. Yields were recorded and in order to evaluate the efficacy of bacteriocin to control tomato wilt, yield per plot was calculated as quintals per hectare (Wydra et al. 2005).The experiment was repeated twice.

Statistical analysis

Data from laboratory and greenhouse studies were analyzed separately for each experiment and were subjected to Analysis of Variance (ANOVA) (SPSS Software). Significant effects of treatments were determined by the F values (P=0.05). Treatment means were analyzed using Scheffe post hoc test.

Results

Isolation and identification *R. solanacearum*

Around hundred *R. Solanacearum* strains were isolated and among hundred isolates, ten isolates were highly virulent based on pathogenicity assay.

Table 1. Effect of temperature on stability of bacteriocin against *R. solanacearum.*

Time in min	Temperature						
	Zone of inhibition in mm						
	15 °C	30 °C	45 °C	60 °C	75 °C	90 °C	100 °C
15	12.56±0.33^{ab}	12.0±0.66^{ab}	11.80±0.57^{ac}	9.86±0.88^{bc}	8.33± 0.33^{a}	7.33±0.15^{ab}	6.57±0.33^{ab}
30	12.23±1.16^{d}	12.30±0.88^{ac}	11.70±1.15^{ab}	9.80±0.57^{bd}	8.26±0.33^{ab}	7.30±0.57^{acd}	6.50±0.21^{abc}
45	12.10±1.15^{bc}	12.0±1.16^{ab}	11.83±0.66^{ac}	9.06±0.66^{ab}	7.87±0.57^{bcd}	6.97± 0.2^{ab}	6.0±0.33^{b}
60	12.5±0.57^{acd}	12.0±0.88^{ab}	12.0±1.15^{abc}	8.90± 0.21^{ac}	7.80± 0.57^{ab}	6.97±0.33^{ab}	3.90±0.17^{bd}

Scheffe post hoc test. Means sharing different alphabetical (a, b, c, d) superscripts in a column significantly different (P≤0.05). Means of the groups homogenous subsets are displaced.

Table 2. Effect of seed treatment with bacteriocin on tomato seed germination and seedling vigour under laboratory conditions.

Treatment	Germination (%)	MRL(cm)	MSL(cm)	Fresh weight	Dry weight	VI
Control	92.66±5.75^{ad}	5.40±0.66^{ac}	9.36±1.12^{cd}	1.08±0.01^{bc}	0.28±0.025^{a}	1368.43±15.66^{bd}
RS1	34.0±1.52^{bc}	2.95±0.043^{bc}	3.32±0.025^{ad}	0.40 ±0.033^{a}	0.12±0.003^{ab}	213.41±5.77^{bc}
RS2	35.0±1.15^{bd}	2.59^{ab}±0.011^{ab}	3.16±0.057^{ac}	0.37±0.011^{ad}	0.13±0.006^{ac}	201.57±6.35^{ad}
RS3	32.66±1.2^{a}	2.75±0.057^{c}	3.26±0.025^{b}	0.37±0.05^{ac}	0.12±0.002^{a}	196.64±3.46^{ac}
RS4	35.33±1.15^{ab}	2.65±0.011^{ab}	3.11±0.033^{b}	0.40±0.057^{c}	0.12±0.001^{ac}	203.67±5.77^{ad}
RS5	34.33±0.88^{ad}	2.40±0.011^{abc}	3.17±0.057^{ac}	0.34±0.033^{ad}	0.12±0.003^{d}	191.59±6.57^{ac}
RS6	35.66±1.12^{ac}	2.57±0.033^{ab}	3.17±0.012^{a}	0.35±0.066^{ac}	0.12±0.003^{ad}	204.86±5.19^{ac}
RS7	34.0±1.86^{ac}	2.50±0.028^{ad}	3.19±0.045^{ac}	0.45±0.025^{ac}	0.12±0.005^{b}	193.60±4.61^{c}
RS8	35.0±1.7^{ab}	2.85±0.057^{ab}	3.18±0.066^{ac}	0.40±0.011^{bd}	0.12±0.006^{bd}	211.35±3.46^{ab}
RS9	34.66±1.12^{ac}	2.68±0.011^{a}	3.15±0.033^{c}	0.36±0.021^{bc}	0.12±0.005^{d}	202.41±4.61^{ab}
RS10	35.33±1.57^{d}	2.67±0.033^{ab}	3.24±0.057^{d}	0.36±0.011^{c}	0.12±0.003^{bc}	209.11±6.92^{abc}
Bacteriocin	94.0±3.46^{ac}	5.66±0.57^{bc}	9.53±1.6^{bd}	1.36±0.11a^{cd}	0.38±0.03^{cd}	1427.86±20.33^{bc}

MRL-Mean Root Length; MSL-Mean Shoot Length; VI-Vigour Index. Scheffe post hoc test. Means sharing different alphabetical (a, b, c, d) superscripts in a column significantly different (P≤0.05).

The *R. solanacearum* isolates were identified by Biochemical, morphological and physiological characteristics (Narasimha Murthy et al. 2012) and the amplified PCR products were sequenced and a phylogenetic tree was constructed by the blast analysis and multiple sequence alignment data (Fig. 1). The sequences were deposited in NCBI GenBank and accession numbers are KF924739–KF924748.

Isolation and identification of lactic acid bacteria

Among 35 lactic acid bacteria, one isolate (LAB-35) showed the highest area of inhibition against ten isolates of *R. solanacearum* and area of inhibition was22–26 mm in diameter.

The LAB-35 strain was determined to be a gram-positive, spore-forming bacterium, with a positive catalase reaction, biochemical characteristics. LAB-35 was identified based on 16S rDNA gene sequencing and confirmed as *Lactobacillus paracasei* (Malini et al. 2012).

Table 3. Effect of bacteriocin from *L. paracasei* ssp. *paracasei* on wilt disease in tomato by seed treatment.

Treatments	Seed treatment					
	Plant height (cm)	MSL (cm)	MRL (cm)	MFW (gm)	Dry Weight (gm)	DI (%)
Control	27.65±3.33^{cd}	14.23±0.57^{abc}	8.76±0.66^{cd}	11.56±0.3^{bc}	2.72±0.68^{ad}	0.00^{a}
RS1	6.5±0.57^{ab}	3.45±0.33^{ac}	3.1±0.15^{b}	5.17±0.57^{ad}	2.12±0.057^{cd}	85.13±5.57^{ab}
RS2	6.25±0.3^{b}	3.34±0.12^{abc}	2.8±0.12^{ad}	4.23±0.66^{acd}	2.11±0.019^{bc}	84.41±3.66^{acd}
RS3	6.46± 0.33^{ad}	3.23±0.3^{acd}	2.6±0.12^{bc}	5.67±0.27^{c}	2.14±0.07^{ab}	83.68±4.57^{d}
RS4	7.25±0.23^{ab}	3.27±0.21^{cd}	2.6±0.013^{cd}	5.61±0.66^{ad}	2.16±0.07^{abc}	81.23±3.67^{ab}
RS5	6.89±0.45^{ab}	3.57±0.15^{ac}	3.11±0.15^{ab}	5.32±0.57^{ac}	1.52±0.011^{abc}	80.44±4.75^{c}
RS6	6.62±0.6^{ac}	3.61±0.13^{cd}	2.76±0.13^{ab}	4.6±0.57^{acd}	2.27±0.03^{ad}	83.23±4.57^{bcd}
RS7	6.56±0.32^{d}	3.43±1.15^{bc}	2.64±0.15^{acd}	4.79±0.3^{ab}	1.74±0.016^{a}	82.45±5.66^{ac}
RS8	6.74±0.57^{ac}	3.29±0.21^{bd}	3.32±0.15^{cd}	5.11±0.3^{ad}	2.21±0.03^{abc}	81.36±4.56^{ab}
RS9	7.11±0.3^{cd}	3.28±0.15^{cb}	3.31±0.12^{ab}	4.22±0.57^{bc}	2.18±0.057^{ab}	80.11±2.45^{cd}
RS10	6.56±0.15^{ac}	3.31±0.3^{c}	2.92±0.11^{acd}	4.82±0.5^{bd}	1.84±0.06^{cd}	82.37±4.57^{bd}
Bacteriocin	28.70±2.1^{cd}	15.13±1.12^{cd}	9.73±0.88^{cd}	13.48±0.33^{bc}	1.83±0.15^{ac}	0.00^{a}
R. solanacearum + Bacteriocin	20.76±1.66^{ab}	9.50±0.57^{bc}	6.80±0.2^{bc}	8.66±0.57^{bd}	2.47±0.09^{bcd}	34.78±3.2^{bc}

MSL: Mean Shoot Length, MRL: Mean Root Length, MFW: Mean Fresh Weight, DI: Disease Incidence of tomato plants treated by bacteriocin and infested with *R. solanacearum* (RS) by seed treatment. Scheffe Post Hoc Test. Means sharing different alphabetical (a, b, c, d, e) superscripts in a column significantly different (P≤0.05).

Table 4. Effect of bacteriocin from *L. paracasei* ssp. *paracasei* on wilt disease in tomato by soil drench.

Treatments	Soil Drench					
	Plant height (cm)	MSL (cm)	MRL (cm)	MFW (gm)	Dry Weight (gm)	DI (%)
Control	30.13±2.57^{ad}	15.20±1.33^{bc}	7.20±0.66^{abd}	12.80±0.57^{cd}	3.68±0.11^{ac}	0.00^{a}
RS1	7.43±0.66^{abc}	4.56±0.57^{acd}	4.23±0.21^{ab}	6.11±0.33^{cd}	2.14±0.057^{ab}	86.45±4.32^{d}
RS2	7.56±0.43^{bc}	4.43±0.3^{ab}	4.16±0.11^{ac}	5.56±0.57^{cd}	1.37±0.019^{bcd}	85.56±4.57^{ad}
RS3	8.32± 0.5^{abc}	4.67±0.6^{ac}	3.65±0.15^{bc}	5.45±0.21^{cd}	2.16±0.07^{bc}	81.43±3.33^{cd}
RS4	7.87±0.67^{b}	4.78±0.57^{cd}	3.81±0.13^{ad}	5.76±0.17^{ab}	1.82±0.07^{ac}	80.71±4.12^{abc}
RS5	8.65±0.35^{bc}	4.48±0.21^{ad}	4.13±0.17^{ab}	6.75±0.33^{cd}	2.17±0.014^{abc}	82.37±3.57^{d}
RS6	7.45±0.3^{abd}	4.91±0.25^{ad}	4.12±0.3^{bc}	5.66±0.66^{ad}	1.98±0.03^{abc}	81.66±5.43^{bd}
RS7	8.73±0.42^{ad}	4.54±1.21^{cd}	3.56±0.21^{ac}	5.91±0.33^{bc}	1.94±0.016^{ab}	80.91±4.21^{ac}
RS8	7.89±0.57^{ab}	4.78±0.18^{bc}	4.17±0.19^{cd}	4.98±0.33^{ad}	2.1±0.03^{bc}	84.02±6.3^{bc}
RS9	8.15±0.5^{ac}	4.63±0.3^{bd}	4.13±0.16^{c}	6.12±0.66^{cd}	1.78±0.03^{c}	83.30±3.41^{acd}
RS10	7.85±0.25^{bd}	4.65±0.17^{ab}	4.14±0.21^{cd}	6.17±0.57^{bcd}	2.16±0.06^{ad}	83.43±5.66^{cd}
Bacteriocin	33.63±3.43^{ad}	16.26±1.12^{bc}	7.66±0.23^{cd}	13.87±0.57^{ab}	3.93±0.088^{ab}	0.00^{a}
R. solanacearum + bacteriocin	20.73±2.15^{cd}	11.03±0.88^{ac}	6.86±0.33^{cd}	6.86±0.33^{cd}	2.80±0.033^{ab}	31.68±2.33^{d}

MSL: Mean Shoot Length, MRL: Mean Root Length, MFW: Mean Fresh Weight, DI: Disease Incidence of tomato plants treated by bacteriocin and infested with *R. solanacearum* (RS) by soil drench. Scheffe post hoc test. Means sharing different alphabetical (a, b, c, d) superscripts in a column significantly different (P≤0.05).

Characterization of bacteriocin *Lactobacillus paracasei*

Purified bacteriocin fromLAB-35 was found to be sensitive to trypsin indicating that the inhibitory substance was proteinaceous in nature. The inhibitory activity was unchanged up to 100°C for 60min and 70% of activity retained after heat treatment at 120°C for 15min (Table 1). The antibacterial bacteriocin was stable at pH range of 4-10 (Fig. 2A). Surfactants were showed less activity and more stable at storage temperature at -20°C and 4°C for 30 days (Fig. 2B). Increase metal ion concentration showed reciprocal effect on the activity of bacteriocin (Fig. 2C). Based on the above characteristics of the compound was confirmed as bacteriocin from *L. paracasei*.

Table 5. Effect of *L. paracasei* ssp. *paracasei* on plant growth and tomato yield under field conditions.

Treatments	Plant height(cm)	Fresh weight(g)	Dry weight(g)	Stem growth(cm)	Fruits/plant	Yield Q/ha	Wilt Incidence (%)
Control	65.56±3.57^{cde}	36.58±2.57^{bcd}	4.74±0.57^{bcd}	1.30±0.07^{abc}	42.32±4.66^{abd}	181.3±7.66^{ade}	0±00^{a}
RS1	34.47±2.57^{acd}	19.65±1.11^{acd}	3.26±0.6^{abc}	0.53±0.05^{cde}	15.54±3.21^{ab}	61.4±4.33^{abc}	92.6±5.33^{cde}
RS2	35.89±3.33^{ae}	19.37±1.3^{ab}	2.74±0.33^{ad}	0.49±0.03^{bf}	13.54±2.57^{bd}	62.5±2.57^{c}	94.7±4.6^{abd}
RS3	37.64±2.6^{abc}	19.32±1.57^{ac}	3.21±0.15^{e}	0.51±0.07^{adf}	9.76±1.57^{abc}	61.7±5.8^{cd}	92.4±6.21^{acd}
RS4	34.53±2.57^{ade}	19.45±1.49^{de}	3.15±0.12^{abc}	0.6±0.06^{ae}	13.78±3.33^{cde}	60.3±2.9^{def}	94.6±4.3^{cd}
RS5	38.34±3.45^{bc}	18.78±1.66^{ab}	3.17±0.14^{cd}	0.52±0.05^{bde}	13.56±2.6^{ab}	61.7±5.21^{bcd}	91.9±4.2^{ef}
RS6	35.67±3.76^{de}	19.34±1.33^{d}	2.79±0.21^{be}	0.52±0.05^{ade}	12.87±2.11^{ad}	59.3±3.54^{abd}	94.4±3.4^{adf}
RS7	34.78±3.73^{bcd}	17.56±1.7^{bc}	3.22±0.3^{cde}	0.59±0.06^{abc}	14.52±2.24^{bcd}	61.4±4.56^{abc}	91.6±4.57^{bcd}
RS8	36.64±2.57^{bc}	18.6±2.86^{c}	3.12±0.21^{acd}	0.47±0.07^{e}	15.34±2.21^{aef}	60.9±3.55^{ef}	90.3±4.3^{abd}
RS9	38.2±3.66^{abc}	19.45±2.62^{bcd}	2.75±0.3^{ae}	0.52±0.02^{ade}	13.56±1.87^{abc}	61.8±4.43^{cd}	93.5±2.4^{acd}
RS10	33.32±2.66^{de}	18.56±1.48^{de}	3.13±0.12^{af}	0.58±0.05^{bdc}	15.61±1.3^{cd}	60.4±2.47^{ace}	93.3±5.3^{cde}
LAB	68±2.55^{bd}	40.87±1.15^{ef}	6.12±0.66^{abc}	1.42±0.05^{def}	50.67±3.56^{abd}	204.4±8.78^{ce}	0±00^{a}
Bacteriocin	68±4.3^{bc}	41.15±3.33^{abc}	6.91±0.7^{abd}	1.84±0.08^{ad}	51.78±4.23^{cde}	205.3±6.87^{ac}	0±00^{a}
R.solanacearum + Bacteriocin	58±3.57b^{de}	29.79±4.45^{bd}	4.56±0.41^{abc}	0.78±0.09^{bde}	35.55±2.57^{cde}	152.5±5.57^{ab}	38.6±2.57^{bcd}

Means ± SE (standard error) followed by the same letter do not differ significantly according to Scheffe post hoc test. Means sharing different alphabetical (a, b, c, d, e, f) superscripts in a column significantly different (P≤0.05).Distilled water treated seeds served as control.

Effect of bacteriocin on tomato seed germination and seedling vigor index

The tomato seed germination and seedling vigor index improved upon bacteriocin treatment and treatment with *R. solanacearum* showed reduction. The bacteriocin treatment improved the vigor index when compared to control (Fig. 3A; Table 2).

Plant growth promotion assessment

The bacteriocin treatment increased plant growth as compared to the untreated control plants (Fig. 3B).

It increased the fresh weight, dry weight, shoots length, root length and reduced the wilt incidence in bacteriocin treated plants. The wilt percentage was decreased to 57.48% and 54.77% in plants treated with bacteriocin by

seed treatment and soil drench methods respectively (Table 3).

Biocontrol under field conditions

In field trials, plots treated with bacteriocin recorded significantly lower wilt when compared to untreated control treatments. In the present study, the control plots yielded an average of 181.3 quintals/ha. The plot treated with only *R. solanacearum* yielded an average of 59.3 quintals/ha and with bacteriocin gave an average of 205.3 quintals/ha tomato yield. As compared to the control the bacteriocin increased the yield by 13.5% (29 quintals/ha). Seedlings treated with both *R. solanacearum* and bacteriocin yielded an average of 152.5 quintals/ha.

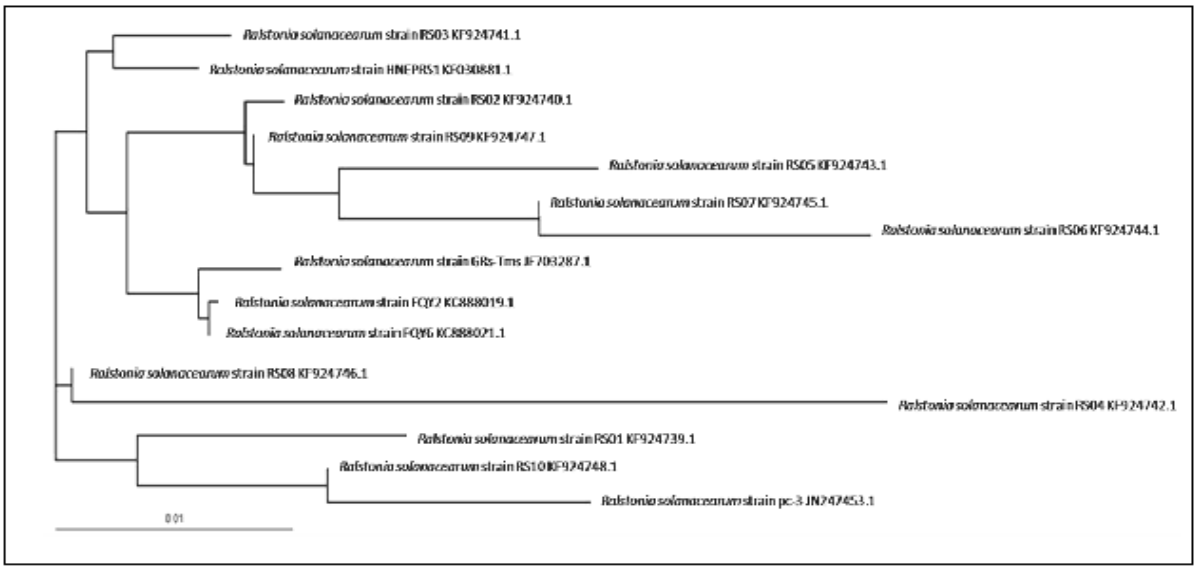

Fig. 1. Phylogenetic relationships of *R. solanacearum* isolates inferred by neighbor-Joining (NJ) bootstrap tree analysis of 16S rRNA sequences.

The outcome of wilt reduction after treatment with bacteriocin is recorded as 56.1% (Table 5).

Discussion

In the present study, bacteriocin from *L. paracasei* has shown antagonism against *R. solanacearum*, increased plant growth and reduced wilt disease severity. The inhibitory activities of LAB against pathogens have been generally shown to be due to the bactericidal effect of protease sensitive bacteriocins. However, the antagonistic effects of LAB towards pathogens could be related to the production of organic acids and hydrogen peroxide also (Ito et al. 2003). Biocontrol of tomato wilt by LAB was found to be not only by their inhibition capacity but also their persistence in the soil under durable conditions. Information of bacteriocin regulation produced by rhizospheric strains is pertinent due to their latent application as biocontrol agents or biofertilizers. Bacteriocin applications have extended during the last decades, for their use as biocontrol agent against many plant pathogens and to enhance inoculant competence (Holtsmark et al. 2008). Bacteriocins that inhibit phytopathogenic bacteria have been reported from bacteria associated with plants (Parret et al. 2005). However, until now there has been no account

of a bacteriocin involved directly in increasing plant growth and germination (Prithiviraj et al. 2003). A major benefit of using LAB as biocontrol agents is that they are considered GRAS and usually fulfill with all endorsements for food products. Moreover, LAB is natural colonizers of fresh fruit and has been previously described as good antagonists of several bacteria and fungi in different food products (Batish et al. 1997).

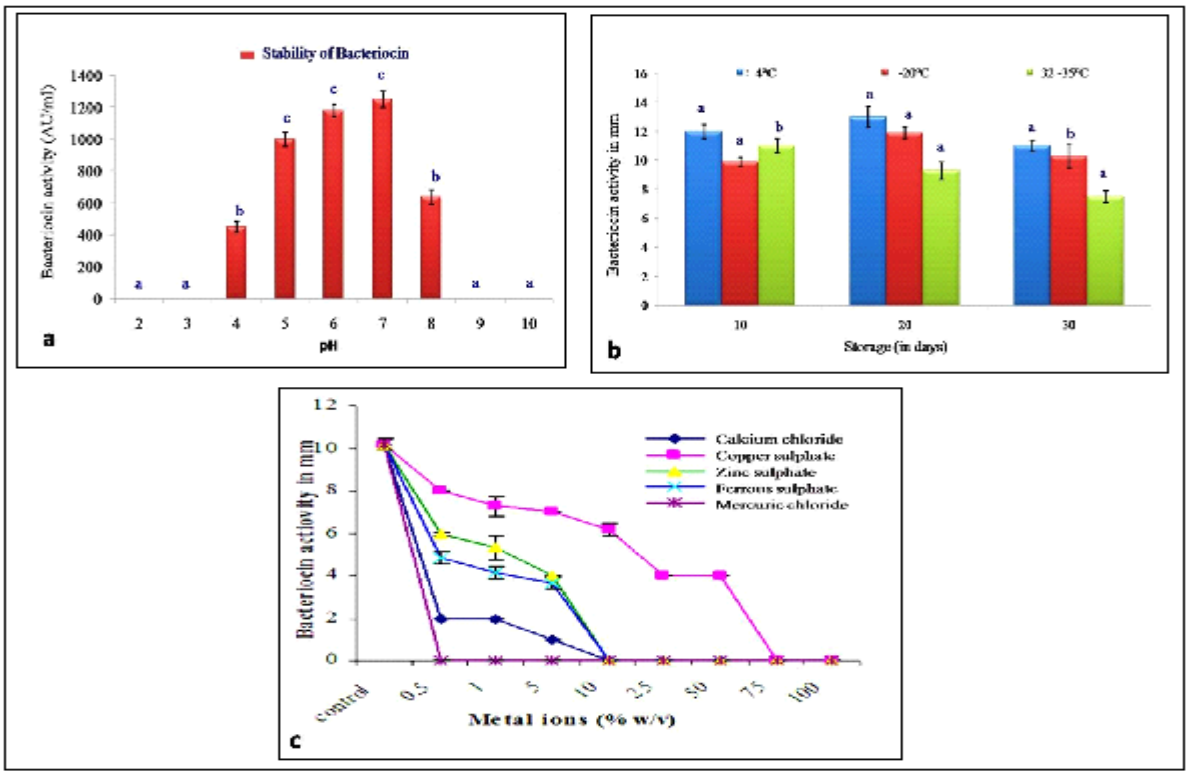

Fig. 2. Effect of pH, storage and metal ions on stability of bacteriocin.Scheffe post hoc test.Means sharing different alphabetical (a, b, c) superscripts in a column significantly different (P≤0.05).

The results of *in vitro* antagonism studies revealed that the LAB and its bacteriocin inhibited *R. solanacearum*. Reports have suggested a high quantity of the isolates, able to suppress *R. solanacearum* growth in a confrontation tests. These results also designate that soil is a novel source for LAB strains for the management of soil borne pathogens and post-harvest diseases (Lutz et al. 2012). Under laboratory conditions and *invivo* pot trials, the bacteriocin increased seed germination and exhibited highly effective antagonism against the *R. solanacearum* respectively. LAB isolate was used in this study improved germination percentage and extremely high amount of seedling vigour without negatively affecting plant growth.

Under greenhouse conditions both the seed and soil drench methods of bacteriocin treatments reduced wilt incidence. The above results indicate LAB as a novel attractive aspirant group for the biological control of soil borne pathogens. The mechanisms of enhancing plant growth by LAB may be due to the effectiveness in nutrient transfer from soil and production of bacteriocin. Somers et al. (2007) also reported *L. lactis* isolated from organic agricultural soil

exhibited plant growth promoting activity in cabbage considerably and some LABs confirmed growth promoting effects on cucumber and tomato seedlings (Lutz et al. 2012).

Under field conditions infected seedlings treated with bacteriocin increased the average tomato yield compared to control. These results indicate that LABs may be used as effective biocontrol agents in the control of bacterial wilt of tomato. Bacteriocin treatments also reduced wilt incidence in tomato. The results of this study showed that the bacteriocins may play an important role in inter specific competition and its broad spectrum activity against wilt pathogen. Also this study recommends LAB for its great efficiency for improving the growth and yield of tomato plant; however field trials are necessary to study the mechanisms behind this plant growth promoting effects (Anupama et al. 2014).

Fig. 3. a. Effect of bacteriocins on tomato seed germination of tomato seeds, A-Bacteriocin treated, B, C-Controls, D-*R.solanacearum* treated. **b**. Plant growth promotion effect of bacteriocins on tomato. A-Control, B- purified bacteriocin treatment, C-*R. solanacearum* treated.

Therefore, we conclude that the LAB is a promising bacterium to suppress soil borne pathogens and promote plant growth. The use of chemicals in agriculture as well as the environmental pollution can be avoided by LAB as a promising biocontrol agent. Therefore, the usage of LABs can be further developed as a stable move towards controlling *R. solanacearum* caused wilt.

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References

Abdul Baki AA, Anderson JD. 1973.Vigour determination in soybean seed by multiple criteria.Crop Sciences **13,** 630–633.

Alvarado C, Garcia ABE, Martin SE, Regalado C. 2006. Food associated lactic acid bacteria with antimicrobial potential

from traditional Mexican foods. Microbiologia **48,** 3–4.

Annabel HAP, Koen T, De Mot R. 2005. Novel lectin-like bacteriocins of biocontrol strain *Pseudomonas fluorescens* Pf-5. Applied Environmental Microbiology **71,** 5197–5207. doi: 10.1128/AEM.71.9.5197-5207.

Anupama S, Beom SK, Duck HP. 2014. Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. Biocontrol Science and Technology **24,** 763–779. Doi.org/10.1080/ 09583157.2014.894495.

Awan JA, Rahman SU. 2005. Microbiology Manual. Unitech Communications, Faisalabad Pakistan, 49-51 p.

Batish VK, Roy U, Lal R, Grover S. 1997. Antifungal attributes of lactic acid bacteria—a review. Critical Reviews in Biotechnology **17**, 209-225. doi: 10.3109/07388559709146614.

Bin Li, Ting Su, Rongrong Yu, Zhongyun Tao, Zhiyi Wu, Soad AE, Algam, Guanlin Xie, Yanli Wang, Guochang Sun. 2010. Inhibitory activity of *Paenibacillus macerans* and *Paenibacillus polymyxa* against *Ralstonia solanacearum*. African Journal of Microbiology Research **4,** 2048-2054.

Coll NS, Marc Valls. 2013. Current knowledge on the *Ralstonia solanacearum* type III secretion system. Microbial Biotechnology **6,** 614-620. doi: 10.1111/1751-7915.12056.

Genin S, Denny, TP. 2012. Pathogenomics of the *Ralstonia solanacearum* species complex. Annual Review of Phytopathology **50,** 67–89. doi: 10.1146/annurev-phyto-081211-173000.

Gray EJ, Lee KD, Souleimanov A, Di Falco MR, Zhou X, Ly A. 2006. A novel bacteriocin, thuricin 17, produced by PGPR strain *Bacillus thuringiensis* NEB17: isolation and classification. Journal of Applied Microbiology **100,** 545–554. doi: 10.1111/j.1365-2672.2006.02822.x.

Grosu-Tudor S, Stancu M, Pelinescu D. *et al*., World Journal of Microbiol Biotechnol. 2014. **30,** 2459. doi: 10.1007/s11274-014-1671-7.

Hayward AC. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytopathology **29,** 65–87.

Heu S, Oh J, Kang Y, Ryu S, Cho SK, Cho Y. 2001. *gly* Gene cloning and expression and purification of glycinecin A, a bacteriocins produced by *Xanthomonas campestris* pv. *glycines* 8ra. Applied and Environmental Microbiology **67,** 4105–4110. PMID: 11526012.

Holtsmark I, Eijsink VG, Brurberg MB. 2008. Mini review. Bacteriocins from plant pathogenic bacteria. FEMS Microbiology Letters 280:17. doi: 10.1111/j.1574-6968.2007.01010.x.

International Seed Testing Association (ISTA). 2005. Proceedings of the International Seed Testing Association. International rules of seed testing. Seed Science and Technology 15 A, 1–9.

Ito A, Sato Y, Kudo S, Sato S, Nakajima H, Toba T. 2003. The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogen. Current Microbiology **47,** 231-236.

Jacobsen CV, Rosenfeldt V, Hayford AE, Moller PL, Michaelsen, Paerregaard KF. 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *In vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Journal of Applied Microbiology **11,** 4949–56.

Johan S, Jesper M. 2005. Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science and Technology **1,** 70–78.

Kamoun F, Mejdoub H, Aouissaoui H, Reinbolt J, Hammami A, Jaoua S. 2005. Purification, amino acid sequence and characterization of Bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis* J Appl Microbiol, **98,** 881–888. doi: 10.1111/j.1365-2672.2004.02513.x.

Kelman A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology **44,** 693–695.

Kishun R. 1987. Effect of bacterial wilt on yield of tomato. Indian Phytopathology **38,** 606.

Kleanthous C. 2010.Swimming against the tide: progress and challenges in our understanding of colicintrans location. Nature Reviews Microbiology **8,** 843–848. doi: 10.1038/nrmicro2454.

Lim H, Kim S. 1997. Role of siderophores in biocontrol of *Fusarium solani* and enhanced growth response of bean by *Pseudomonas fluorescens* GL20. Journal of Microbiology and Biotechnology **7,** 13–20.

Lutz MP, Michel V, Martinez C, Camps C. 2012. Lactic acid bacteria as biocontrol agents of soil-borne pathogens. Biological Control of Fungal and Bacterial Plant Pathogens IOBC-WPRS Bulletin **78,** 285–288.

Malini M, Savitha J. 2012.Detection of heat stable bacteriocin from *Lactobacillus acidophilus* NCIM5426 by liquid chromatography/mass spectrometry. Indian Journal of Science and Technology **5,** 3.

Messiha NAS, van Diepeningen AD, Farag NS, Abdallah SA, Janse JD, van Bruggen AHC. 2007. *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. European Journal of Plant Pathology **118,** 211–225. doi: 10.1007/s10658-007-9136-6.

Montesinos E. 2003. Development, registration and commercialization of microbial pesticides for plant protection. International Microbiology **6,** 245–252. doi: 10.1007/s10123-003-0144-x.

Narasimha Murthy K, Srinivas C. 2012. *Invitro* screening of bioantagonistic agents and plant extracts to control bacterial wilt (*Ralstonia solanacearum*) of tomato (*Lycopersicon esculentum*). Journal of Agricultural Technology, **8,** 999–1015.

Neelu S, Siddiqui A. 2012. Inoculation of Tomato with *Ralstonia solanacearum, Xanthomonas campestris*, and *meloidogyne javanica.* International Journal of Vegetable Science **18,** 78–86. doi: 10.1080/19315260.2011.579232.

Ohmomo S, Murata S, Katayama N, Nitisinprasart S, Kobayashi M, Nakajima T *et al*., 2000.Purification and some characteristics of enterocin ON–157, a bacteriocin produced by *Enterococcus faecium* NIAI 157. Journal of Applied Microbiology **88,** 81–89.

Parret AHA, Temmerman K, De Mot R. 2005. Novel lectin-like bacteriocins of biocontrol strain *Pseudomonas fluorescensPf-5*. Applied and Environmental Microbiology **71,** 5197–5207. doi: 10.1128/AEM.71.9.5197–5207.

Prithiviraj B, Zhou X, Souleimanov A, Wajahatullah MK, Smith DL. 2003. A host specific bacteria-to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. Planta **216,** 437–445. doi: 10.1007/s00425-002-0928-9

Rammelsberg M, Radler F. 1990. Antibacterial polypeptides of *Lactobacillus* species. Journal of Applied Microbiology **69,** 177–184. doi: 10.1111/j.1365-2672.1990.tb01507.x.

Ran LX, Liu CY, Wu GJ, van Loon LC, Bakker PAHM. 2005. Suppression of bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. in Chinese Journal of Biological Control **32,** 111–120.

Schillinger U, Lucke FK. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology **55,** 1901–1906.

Moon SK, Wee YJ, Choi GW. 2012. A novel lactic acid bacterium for the production of high purity L-lactic acid, *Lactobacillus paracasei* subsp. *paracasei* CHB2121.Journal of Bioscience and Bioengineering **114,** 155–159. doi: 10.1016/j.jbiosc.2012.03.016

Shrestha A, Choi K, Lim KC, Hur HJ, Cho S. 2009. Antagonistic effect of *Lactobacillus*sp strain KLF01 against soil borne plant pathogen *Ralstonia solanacearum*. The Korean Journal of Pesticide Science **13,** 45–53.

Sivakumara G, Narayanaswami NT. 1998. Biological control of sheath blight of rice with *Pseudomonas fluorescens*. *Oryza* **35,** 57–60.

Sivaramasamy E, Neelamegam A, Packiyam M, Thangavel B. 2014. Production, purification and characterization of bacteriocin from *Lactobacillus murinus* AU06 and its broad antibacterial spectrum. Asian Pacific Journal of Tropical Biomedicine. **4,** S305–S311. doi: 10.12980/APJTB. 4. 2014C537.

Somers E, Amke A, Croonenborghs A, Overbeek LS Van, Vanderleyden J. 2007. Lactic acid bacteria in organic agricultural soils. Montpellier: Plant Research International. Montpellier, France, August 26-31.

Stiles ME.1996. Biopreservation by lactic acid bacteria.Antonie Van Leeuwenhoek. **70,** 331–345.

Szala B, Paluszak Z, Motyl I. 2012. Antagonistic effect of Lactic Acid Bacteria on *Salmonella Senftenberg* in mixed cultures. Polish Journal of Environmental Studies **21,** 1399–1403.

Wang H, Yan H, Shin J. 2011. Activity against plant pathogenic fungi of *Lactobacillus plantarum* IMAU10014 isolated from Xinjiang Koumiss. Annals of Microbiology **61,** 879–885. doi: 10.1007/s13213-011-0209-6.

Winstead NN, Kelman A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology **42,** 628–634.

Wydra K, Semraum J. 2005. Phenotypic and molecular characterization of the interaction of antagonistic bacteria with *Ralstonia solanacearum* causing tomato bacterial wilt. In: Zeller, W., Ulrich, C. (eds). 1st International Symposium on Biological Control of Bacterial Plant Diseases 112–118. Darmstadt, Germany.

Yuliar, Yanetri AN, Koki T. 2015. Recent Trends in Control Methods for Bacterial Wilt Diseases Caused by *Ralstonia solanacearum.* Microbes Environment **30,** 1–11. doi: 10.1264/jsme2.ME14144.