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Isolation of probiotic *Lactobacillus SP* from raw cow milk and its usage as plant growth promoting bacteria

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Abstract
Chilli plant leaf spot is caused by bacteria belonging to several species in the genus *Xanthomonas* especialy *Xanthomonas campestris.* Infected chilli plant were collected from local area of Coimbatore and botanical name was confirmed as *Capsicum annum* and were processed for isolation of *Xanthomonas.* Among the processed samples colonies with typical characteristics namely smooth, round (1.5cm-2cm) with entire margins were picked and were subjected to morphological and biochemical characteristics. Isolates are reacted negatively to gram staining under a light microscope. *Xanthomonas campestris* were able to utilize citrate, gelatin is liquefied, indole is produced, catalase is positive, nitrate is reduced, starch and casein is hydrolysed, oxidase is positive. *Xanthomonas campestris* can able to grow on nutrient agar with 5% glucose. *Lactobacillus* sp is a probiotics bacteria found mainly associated with milk products. Raw cow milk sample were collected from the local area of Coimbatore and processed for isolation of *Lactobacillus.* Among the processed samples colonies with typical characteristics pure, white, small (2-3mm diameter) with entire margins were picked and were subjected to morphological and biochemical characteristics. The isolates were identified as *Lactobacillus acidophilus* and *Lactobacillus sporogenes*. The isolates reacted positively to gram staining under a light microscope. *Lactobacillus* sp not able to reduce nitrates, gelatin is not liquefied, indole is not produced, acidic and non acidic end products are not produced and are catalase negative. The cultures supernatant obtained from the bacteriocin producer strain were tested for the antibacterial activity against isolates of *Xanthomonas campestris* by agar well diffusion assay. Inhibitory zones shows that the strain *Lactobacillus acidophilus* show strong antimicrobial activity.
For the *in vitro* efficacy of LAB as biocontrol and Plant Growth Promoting Bacteria, chilli seeds from two varieties (CO1, K2) were treated with LAB strain and treated seeds showed inhibitory activity against *Xanthomona campestris* strain. They can resist the bacterial infection and showed good percentage of increase in shoot length and root length in the seeds treated with *Lactobacillus acidophilus*. The use of chemicals and bacteriocides in agriculture as well as the environmental pollution would be avoided by LAB as a promising PGPB and Biocontrol agent.

Keywords: Chilli, Xanthomona campestris, Lactobacillus SP, PGPB and Biocontrol Agent

Introduction
According to human history more than 7000 species of plants were found to be a definite food for the survival and development of human populations throughout the globe in spite of pests, disease, climate change and other environmental hazards.
Chilli under *Solonaceae* family is one of the important spice/ vegetable/ cash crop grown in India. It is an ancient essential ingredient of Indian home as it provides colour, flavor and aroma. Chilli is a good source of vitamin and have small quantity of protein, fats and carbohydrate. Chilli extracts are used in wide range of medicines against tonsillitis, diphtheria, loss of appetite, rheumatism, sore throat, swelling and hardened tumors.
India being a largest producer, consumer and exporter of chilli in the world (Asalmol *et al*., 2001) [2] is facing a major negativity in growth, development and cultivation because of bacterial (Soum Sanogo *et al*., 2008) [29] and fungal infections. The bacterial disease caused by *X. campesteris* causing leaf spot with lesions covering 80% of the leaf area which persist atleast 4 months and 10 months in seeds has reduced the development of the plant. Many physical and chemical methods were tried on the plant to control the disease.
Physical control includes tillage to control pests (Haridson, 1976) [13]. On other hand tillage is

energy expensive (Phillips *et al*., 1980) [23] and contributes soil erosion. Open field burning contributes air pollution. The use of biological control agents has been suggested as an alternative way of controlling plant diseases (Compant *et al*., 2005) [7]. Biological control using antagonistic *Pseudomonas fluorescens* (bacteria) for seed treatment and as well as spray treatment were found to be effective against *C.capsici* (Srinivas *et al*., 2005) [31]. *Trichoderma* species (fungus) are able to effectively control *C.capsici* infection in chilli (Maymon *et al*., 2004) [20].

Experts in the *Lactobacillus* Pafi Techno Resources Corporation in Cebu, Philippines extensively made a scientific study on Lactoplant as the viable replacement of the old conventional method of farming.

Lactoplant is an effective growth enhancer that could boost production in a maximum level, harmless to living being and restores the fertility of the soil for life. Probiotic microorganisms in Lactoplant are extremely important to every agricultural system because it will substantially provide much fertility of the soil.

In our present study probiotics Lactic acid Bacteria (LAB) was isolated from the natural sources like raw cow milk and were applied to determine their efficiency to control the infections caused by *Xanthomonas* sp in chilli plant. This study will reduce the continous use of chemical fertilizer and bacteriocides. The use of biocontrol agent might promote rapid plant growth.

In our present studies the following objectives was undertaken;

- *Xanthomonas campestris* from infected chilli leaf was isolated and characterized
- LAB from raw cow milk was isolated, identified and characterized
- Determination of *In vitro* Antagonistic activity against *Xanthomonas campestris* using LAB
- *In vitro* and *In vivo* access of the efficacy of LAB to act as Biocontrol, Plant growth promoting bacteria

Materials and Methods

Collection of sample

Infected chilli plant sample (*Capsicum annum* L) were collected from the local area in Coimbatore, Tamilnadu, due their wide cultivation in Coimbatore (Fig 1) and botanical name was confirmed from TNAU. Two varieties of chilli seeds (CO1, K2) were collected from TNAU. Immediately after collection, the sample were stored for further study

Fig 1: Infected Chilli plant

Raw cow milk sample were collected from the local area in Coimbatore, Tamilnadu due to their wide acceptance among the consumers of Coimbatore. Immediately after collection, the sample were stored aseptically in low temperature (4^0C), to protect it from contamination and deterioration.

Media

The bacteria *Xanthomonas campestris* was isolated from infected chillli plant leaves by using special media nutrient agar with 5% glucose media and nutrient broth with 5% glucose (Aneja, 1996).

The bacteria *Lactobacillus* sp was isolated from raw cow milk sample by using MRS broth and MRS agar media.

Isolation and identification of bacteria

Isolation and Identification of *Xanthomonas campestris*

Xanthomonas campestris were isolated from infected chilli leaves and biochemical charaterisation were performed. (Dye, 1980; Sands, 1990) [11, 24]: Gram's stain; oxidative / fermentative metabolism; catalase, oxidase activities; nitrate reduction; hydrogen sulfide production; starch, gelatin and casein hydrolysis; growth on nutrient agar with 5% glucose (Bradbury, 1984) [6]; indole production from Tryptone; citrate utilization (Aneja, 1996) [1].

Isolation and Identification of *Lactobacillus* sp

Raw cow milk sample was serially diluted and plated on to a De Man Rogosa Sharpe (MRS) medium for *Lactobacillus* isolation and incubated at 37^0C for 48-72 hrs. Well isolated colonies with typical characteristics namely pure white, small (2-3 mm diameter) with entire margins were picked from each plate and transferred to MRS broth. Around two colonies were picked and were designated as LAB1 and LAB2. Further identification of the Lactobacilli was performed according to their colony morphological, cultural, physiological and biochemical characteristics (Sharpe, 1979; Kandler and Weiss, 1986) [28, 15]. The isolates were identified as *Lactobacillus acidophilus* and *Lactobacillus sporogenes*.

Detection of antagonistic activity

Agar spot assay test and agar well diffusion test

Agar spot assay test of the isolates against the indicator organism was performed and and the appearance of inhibitory zones were observed. Inhibition was scored positive if the zone was wider than 2mm in diameter (Kilic *et al.*, 1996) [16]. The positive isolates were subjected to agar well diffusion method (Toba *et al*., 1991) [32]. Inhibitory activity was performed against Gram positive (LAB) and Gram negative organism (*Xanthomonas campestris*).

In vitro and *in vivo* efficacy of *lactobacillus* sp as biocontrol, plant growth promoting bacteria Chilli plant seeds experiment

Seeds from two varieties of chilli plants such as CO1 and K2, were placed in two replicates of 50 seeds. Each 25 seeds per plate were kept at 4^0C before use. First the surfaces of the seeds were disinfected with ethanol (70%) for a minute and rinsed with distilled water four times to minimize microorganism development at the early stages of germination. The treatments included immersion of seeds for 10 minutes in LAB1 and LAB2 prepared on MRS broth with two replicates of 25 seeds each per microbial isolates. After immersion, the seeds were dried under laminar flow and then placed in petridishes after the inoculation of *Xanthomona campestriss* broth (Asma *et al.*, 2012) [3].

In vitro and *in vivo* germination design

In vitro trials

Experiments were performed under *in vitro* condition. First the LAB treated infected chilli seeds from each variety was inoculated in the MS medium in aseptic under *in vitro* condition. Non- treated infected chilli seeds were inoculated as control. After 30 days, expression of healthy seedling were observed.

***In vivo* trials**

In vivo trials were performed in pot (30cm diameter) filled with unsterilized natural soil. First the LAB treated infected chilli a seed from each variety was inoculated in different pots, at *in vivo* condition. Non treated infected chilli seeds were used as control. After 30 days, expression of healthy seedling were noted (Hoda *et al*., 2011) [14].

Germination and Statistical method

After one month's growth, chilli plants were harvested under both *in vitro* and *in vivo* condition. To reveal the effect of LAB on the growth characteristics, each plant was measured for shoot length and root length. Statistical analysis data were analyzed using SPSS for windows (SPSS Inc.) by means of a one-way ANOVA and subsequently differences between treatments and control were determined using least significant differences LSD at α0.05 (Hoda *et al*., 2011) [14].

Results and Discussion

Isolation and identification of bacteria

Isolation and Identification of *Xanthomonas campestris*

Xanthomonas campestris with typical characteristics namely smooth, round (1.5cm-2cm) with entire margins were picked and were subjected to morphological and biochemical characteristics. The isolate was identified as *Xanthomonas campestris*, based on their morphological and biochemical characters (Dye, *et al*., 1980) [11], (Table 4.1). *Xanthomonas campestris* were able to utilize citrate, gelatin is liquefied, indole is produced, catalase is positive, nitrate is reduced, starch and casein is hydrolysed, oxidase is positive. *Xanthamonas campestris* can able to grow on nutrient agar with 5% glucose Isolate of *Xanthomonas campestris* were gram negative (Schaad, 1988) [26]. Bradbury, (1984) reported isolates of *Xanthomonas campestris* grow on Nutrient agar with 5% Glucose. *Xanthomonas campestris*showed gelatin liquefaction, Nitrate reduction (Dickey and Kelman, 1988) [10], starch hydrolysis, Casein hydrolysis, Indole production, and Hydrogen sulphide production (Aneja, 1996) [1], oxidase and catalase characters are shown.

Isolation and Identification of *Lactobacillus* sp

Pure white, small (2-3mm diameter) with entire margins colonies were picked and subjected to morphological and biochemical characteristics. Two isolates described reacted positively to gram staining under a light microscope. Lactobacilli are generally long rods, some times they are short rods. *Lactobacillus* sp do not possess flagella and do not create endospores, nitrates are not reduced, gelatin is not liquefied. Indole is not produced, acidic and non-acid end products are produced and catalase negative (Table 4.2). Ozlem Erdo and Feryal, (2006) [22] has stated that *Lactobacillus bulgaricus* and *Lactobacillus casei* isolated from yoghurt, different kinds of cheese and a traditional food named 'tarhana' (a fermented food made of a mixture of cereal, yoghurt and thyme).

All isolates of *Lactobacillus* sp were able to grow at 15ºC and 24% of the isolates were not able to grow at 45ºC. De Man *et al*., (1960) [9] stated that Lactobacilli are generally isolated on rich media such as MRS, which is routinely used for the isolation and counting of Lactobacilli for most fermented food products. Kandler and Weiss, (1986) [15] have classified Lactobacillus sp isolates from temperate regions according to their morphology, physiology and molecular characteristics. Few strains were able to utilize citrate and were found to be non-motile, catalase, indole, VP negative, nitrates are not reduced and gelatin was not liquefied. Sphelhaug and Hardlender, (1989) [30] reported that MRS agar was suitable for bacteriocin assay of lactobacilli. Coppola *et al.* (2000) [8] studied the morphological characters of lactobacilli from raw milk, nature whey starter and cheese.

Azizpour, (2009) [4] stated that LAB isolated from rainbow trout of west Azarbaijan, Iran were Gram positive, catalase positive bacilli, were able to grow at 15ºC and 45ºC. The identified showed 80% or more similarly to the MTCC type cultures and showed variations in their sugar fermentation pattern.

Mallesha *et al*. (2010) [19] isolated Lactic acid bacteria from raw and fermented products like milk, curd, idli batter and pickle, out of 44 isolates 18.75% isolates are *Lactobacilli.*

Detection of antagonistic activity of *Lactobacillus* sp against against *Xanthomonas campestris*

Agar spot assay and Agar Well Diffusion Assay

The culture supernatants obtained from *Lactobacillus* sp tested for antibacterial activity against the same group of *Lactobacilli*. This has shown clear zone of inhibition against the indictor organism and they were selected as potential bacteriocin producers. All the isolates were able to inhibit the indicator organism. The cultures supernatant obtained from the bacteriocin producer strains was tested for antibacterial activity against isolates of gram negative bacteria, *Xanthomonas campestris*. Bacteriocins obtained from the isolates showed inhibitory activity against all the tested strains in the form of zone of inhibition (mm in diameter). The antibacterial activity of *Lactobacillus acidophilus* against *Xanthomonas campestris* was 23 mm in diameter. The antibacterial activity of *Lactobacillus acidophilus* against *Xanthomonas campestris* was 16.5 mm in diameter. *Lactobacillus acidophilus* strongly inhibited the organism when compared to the other isolate (Table 4.3). The LAB isolate was screened for bacteriocin producers by Agar spot assay test (Kilic *et al*., 1996) [16]. Nowroozil *et al*. (2004) [21] has stated that antibacterial activities were done by an agar spot in which only 14.3% of strains made known to produce bacteriocin. Trias *et al.* (2011) [33] reported LAB isolated from fresh fruits and vegetables effective against the phytopathogenic and spoilage bacteria and fungi, *Xanthomonas campestris, Erwinia carotovora, Monilinia laxa* and *Botrytis cinerea* on apple fruits. Isolates of LAB5 and LAB1 showed higher antibacterial activity under *in vitro* agar well diffusion method.

Bacteriocins form the pores in the membrane of sensitive cells and deplete the trans membrane potential and/or the pH gradient, resulting in the leakage of cellular materials. The inhibitory effect was assumed to be bacteriocin and not due to H_2O_2 since there was no oxidizing effect on bacterial cells which destroy the molecular structure of cell protenis (Zsolt Zalan *et al*., 2005) [34].

***In vitro* and *In vivo* efficacy of *Lactobacillus* sp as Biocontrol, Plant growth promoting bacteria**

Chilli plant seeds experiment

Seeds of two varieties of chilli plants such as CO1 and K2 were treated with strains of *Lactobacillus* (LAB1 and LAB2). Treated seeds are showed activity against the strain of *Xanthomonas campestris*. CO1 seeds were strongly inhibited when compared to K2. LAB1 inhibited the seeds CO1 (8.8 mm in diameter) and K2 (7.6 mm in diameter) at a very stronger rate when compared to other isolates. LAB2 showed inhibition 8.2 mm in CO1 and 7.2 mm in K2 against

Xanthomonas campestris (Table 4.4).

***In vitro* and *In vivo* germination trail**

***In vitro* trails**

In vitro trails were performed in MS medium at *in vitro* condition using LAB treated seeds CO1, K2. LAB treated seed revealed their ability to enhance plant growth when compared with control which was processed after one month. Plant growth characteristics significantly differed in response to different LAB strains. Seeds not treated with LAB (control) showed 8.25cm shoot length and 5.5cm root length. LAB1 treated seeds germinated than other seeds and their shoot length was 8.125cm and root length was 8.25cm. But no rapid increase in K2 variety when compared with control. Treated plantlet doesn't show any symptoms of disease (Table 4.5).

***In vivo* trails**

In vivo trails were performed on pot at *in vivo* condition using LAB treated seeds such as CO1, K2. LAB applied as seed treatment show their ability to enhance plant growth compare with control. LAB1 treated seeds showed better germination when compared to the other LAB treated seeds. The shoot length was 6.6cm and the root length was 8.66cm found to be strongest enhancement than other treated seeds. K2 variety doesn't show any obvious increase. Treated plantlet doesn't show any symptoms of disease (Table 4.6).

These result reveal the capability of LAB to be considered as Plant Growth Promoting Bacteria and Biocontrol agent.

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) [33].

LAB with antifungal activity are well documented in food, meat and milk products as biopreservatives (Schnurer and Magnusson, 2005) [27] while less attention has been paid to exploit the antifungal activity of LAB for biocontrol of phytopathogenic fungi.

Sathe *et al*. (2007) [25] reported that supension of *Lactobacillusplantarum* delayed the growth of *Aspergillus flavus, Fusirium graminearum, Rhizopus stolonifer* and *Botrytis cinerea* on cucumber. The inhibitory activity of *Lactobacillusplantarum* 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) [17, 18, 5].

Recently, Hamed *et al*. (2011) [12] reported that tomato seeds treated with lactic acid bacteria reduced the growth of *Fusarium oxysporium* andimproved the growth of roots (Hoda *et al.,* 2011) [14].

Table 1: Biochemical characterization of *Xanthomonas campestris* isolated from infected chilli leaf

S. No	Isolate	Morphology	Gram Reaction	Growth on Nutient agar with 5% Glucose	Gelatin liquefaction	Starch Hydrolysis	Casein Hydrolysis	Nitrate Reduction	Indole Production	Oxidase	Catalase	Citrate Utilization	Species Identified
1	Organism isolated from infected leaf	Rods	-	+	+	+	+	+	+	+	+	+	*Xanthomonas capestris*

Table 2: Biochemical characterization of *Lactobacillus* sp isolated from raw cow milk

S.No	Isolates	Gram Reaction	Morphology	Indole	MR	VP	Citrate	Catalase	Nitrate Reduction	Gelatin	Species identified
1	LAB1	+	Rod	-	-	-	-	-	+	-	*Lactobacillus acidophilus*
2	LAB2	+	Rod	-	-	-	-	-	-	-	*Lactobacillus sporogens*

Table 3: Antibacterial activity of LAB against *Xanthomonas campestris*

S. NO	Isolates of *X. campestris*	Zones of inhibition (mm in diameter)	
		Isolates of *Lactobacillus* sp	
		LAB 1	LAB 2
1	*X. campestris*	23mm	16.5mm

Table 4: Chilli plant seeds experiment

Sl. No	Isolates of *Lactobacillus* sp	Zone of inhibition (mm in diameter)	
		CO1	K2
1	LAB1	8.8mm	7.6mm
2	LAB2	8.2mm	7.2mm

Table 4.5: Growth and Statistical analysis
In vitro germination

Treatments	Shoot length	Root length
Control	8.25±1.08	5.5±0.55
LAB1	8.125±1.05	8.25±0.25
LAB2	7±0.95	5.8±0.83

Results are mean values of three replicates determination ±SD

Table 4.6: In vivo germination

Treatments	Shoot length	Root length
Control	11± 17.6	5 ±0.083
LAB1	6.66±1.05	8.66±0.25
LAB2	6.60±0.93	7.5±0.25

Results are mean values of three replicates determination ±SD

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