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Evaluation of bioagents and bactericides for controlling the Bacterial spot Disease in tomato

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Abstract

The bactericides, i.e., streptomycin, Ampicillin and two bioagents, Bacillus subtilis and Bacillus licheniformis were applied for controlling the Bacterial spot disease caused by Xanthomonas campestris pv. Vesicatoria in vitro and in field. In vitro, results showed, B. subtilis and B. licheniformis reduced the pecotlytic enzymes (PG and PME enzymes) and reduced the cellulolytic enzymes (CX). The tested materials were also powerful bactericide against the bacterial spot disease. Streptomycin, B. licheniformis and B. subtilis prevent the bacterial spot disease in daughter tomato tubers and increased the vegetative characters, plant height and number of leaf per plant. Results show that plant tubers yield and the average of tuber weight has been increased when the above bactericides were applied, compared with un-treated plants. Streptomycin and Ampicillin gave a moderate effect in reducing the incidence of bacterial spot disease, while a positive effect on tuber weight and plant tuber yield has been recorded than control. The incidence of bacterial spot disease and the weight loss in tomato tubers resulting from treated plants studied in storage.

Key words Xanthomonas campestris, tomato, Bacillus subtilis, Bacillus lichiniforms, Enzymes.

1. Introduction

Xanthomonas campestris is a major disease pathogen affecting tomato seed tuber pieces, after cultivation, during vegetative growth and on tomato tubers during storage. This particular disease also affects peppers and has been reported throughout the world whenever tomatoes and peppers are grown **(1-4)** biological control agents have been reported to be an effective method to control plant pathogen. Many group of micro organisms found in the soil are potential biological control agents, including several bacteria, such as *Bacillus subtilis*, *Bacillus sp*. *Streptomyes sp*. *Pseudononas spp*. When combined with herbicides (**4-7**) the maceration process involves the depolymerization of the pectin of plant cell walls and the middle lamella. Pectin is a hetero/polysaccharide with a backbone consisting of partially esterified galacturonic acid. The enzymes of pectinases secreted by plant pathogens of bacterial spot

bacterium xanthomonas *campestris*, as part of their strategy for penetrating the plant host cell walls. The production of pectinase (poly-lacturonase), the major virulence determinant of *xanthomonas campestris* is controlled by many regulatory factors. The antibiotics had a significant effect on the production and activity of cell wall degrading enzymes produced by plant pathogenic microorganisms (**8-10**) disease incidence of bacterial spot disease can be reduced by antibacterial treatments of seed tubers in field application. Treatment of tomatoes with bioagents before planting in soil infested with *xanthomonas campestris* reduced bacterial spot disease in daughter tomato tubers. The number and weight of tubers increased when tomato plants were treated with bioagents. A *bacillus* strains produced a natural biocontrol agents, which can be used as biopesticide against spoilage microorganisms (**11-15).** In this view evaluation of this work aimed to study the role of some bactericides and bioagents in decreasing the softening tubers in the field of production and in minimizing the existence of the initial inoculums potential of bacterial spot pathogens associated in tomato tubers pre storage.

2. Materials and Methods

2.1 Sample collection:

Diseased tomato samples showing typical bacterial spot symptoms were collected randomly from locations in the tomato growing rural areas of Hosur Taluk. From each field, tomato fruits showing typical bacterial symptoms were collected, placed in paper bags and brought to the laboratory for the isolation of target pathogen. From the collected samples microbial strains were isolated.

Isolation of Microorganism:

2.1.1 Method 1:

Tissue segments of about $2mm^2$ were excised from advancing lesion margins on symptomatic tomato fruits. The tissue segments were teased in a few drops of sterile distilled water, and allowed to stand for 10-15 minutes in a laminar airflow chamber. 0.5ml of the sample was spread onto plates of Trypticase Soy Agar (TSA) and incubated for 24hours at 25-28°C and observed for colony growth and further use.

2.1.2 Method 2:

Unwashed seed tubers were placed individually in plastic bag with 20ml of distilled water, sealed and incubated at 18°C for 10-15days. Bacterial spot symptoms were recorded. For isolation of bacterial spot pathogen *(Xanthomonas campestris),* nutrient Agar plates were prepared and the 15 days old seeds were ground using mortar and pestle and spread over the plate. It was then kept for incubation for 24 hours. The plate containing the pathogen symptoms were observed.

2.2 Identification of bacterial strain

Isolated colonies and reference strains of pathogens were identified using biochemical tests.

Antibacerial materials

Bactericides

Chloramphenicol was used.

Bioagents

Bacillus subtilis, Bacillus lichiniformis which proved to be highly antagonistic effect against phytopathogens.

2.2.1Invitro tests

The efficacy of bactericides and bioagents were tested at two concentrations against enzymatic activities and population of *Xanthomonas campestris* cultural medium. Streptomycin at concentrations of 25 ppm. *B. subtilis and B. lichiniformis* grown in NBM separately at 30°C for 48h, then it were tested. 50ml nutrient Agar medium was prepared for three Petri plates. Sterilized it and poured into Petri plates. After solidification, swab the two bioagents in appropriate plates. Make two wells, one was control and another was sample. To the well, 200µl of *Xanthomonas* pure culture were added. Kept it for incubation at 37°C for 48 hrs. The zone of inhibition against the bioagents was observed.

2.2.2 Enzyme Activity:

Pectinolytic and cellulolytic enzymes of *Xanthomonas campestris* pathogen were determined. The production of pectic enzymes; polygalacturonase (PG) and pectin methylesterase (PME) were carried out using the nutrient broth. Flasks contained 50ml of the broth were autoclaved. This 50ml is also used for celluloytic enzyme Assay. The flask was inoculated with 0.5ml of *Xanthomonas campestris* suspension. After incubation at 30°C for 72 hours, the supernatants were obtained by centrifugation at 5000rpm for 20mins, and then the supernatants (Crude enzyme preparations) were used for enzymatic assay.

2.2.2.1 PG Assay:

PG activity was assayed by estimating the loss viscosity of 1.2% citrus solution after incubation at 30°C. Reaction mixture consisted of 1ml crude enzyme + 1ml of 1.2% pectin solution buffered at pH 4.5 with phosphate buffer. Boiled crude enzymes were used for control.

2.2.2.2 PME Assay:

PME activity was determined by the titration method using 0.01N NaOH solution after incubation for 24hours at 30°C. Reaction mixture consisted of 1ml crude enzyme + 1ml of 1.5% pectin solution (pH 7.0). Activity was expressed as milliliters of NaOH solution required to neutralize the carboxylic groups.

2.2.2.3 Cx Assay:

Cx activity was determined by measuring the loss in viscosity of 1% carboxymethyl cellulose solution, after incubation for 3 hour at 30°C. Reaction mixture (1ml of crude enzyme + 1ml of 1% CMC, pH 5.0) were used.

Boiled crude enzyme was used as control.

2.3 Field Experiment:

The experiment was designed in a randomized complete block, three lines in each block were used as a replicates for each treatment, where each line include 12 pits and one seed piece was sown in each pit. Irrigation and fertilization was carried out.

The efficacy of bioagents of *Bacillus subtilis* and *Bacillus lichiniformis* which proved to be highly antagonistic against phytopathogens, were used as seed pieces dressing against bacterial spot pathogen in field application. Mixture of each bioagent suspension was mixed, separately, with seed pieces for 5mins. Then, treated seed pieces were shown.

3. Results and discussion

The bacterial black spot pathogen which isolated from tomato fruit and seeds (Figure 1). The plant pathogen infected black spot organism was isolated and plated on TSA medium. The isolated organism was characteristics based on morphological and biochemical methods were shown in Table 1. The identified bacterial *Xanthomonas* was determined survival of thermal temperature above 80 C (Table 2). Thermal death time of exposure of cells for above 30 mins (Table 3). The bioagents were used in this study, *Bacillus subtilis*, *Bacillus lichiniformis* obtained and maintained in nutrient broth. The invitro study with bioagents treated against *Xanthomonas campestris* , the inhibition of zones were indicated that induction of plant pathogens by bioagent. The TSA broth containing bioagents, antibiotics and plant pathogen were kept under incubation. The extracted samples were carried on activity assay of PME, PG and CX were tabulated (Table 4). In this yield experiment of infected seeded plants were determine the vegetative growth of stem and weight (Figure 2 &Table 5).

Table 1- Biochemical Tests

Tests	Reaction
Soft rod on tomato slices	Positive
Yellow colonies on YDC medium	Negative
Fluorescent pigment on King's medium	Negative
Deep pits on CVP medium	Positive
Anaerobic growth	Positive
Gelatin liquefaction	Positive
Growth atNacl 5%	Positive
Growth at 37 C	Positive
Sensitivity to Chloramphenicol	
Acid form	Positive
Arabinose	Positive
Trehalose	Positive
Glucose	Positive
Lactose	Positive
Mannitol	Positive

Salicin	Positive
Starch	Positive
Gas from glucose	Positive

Table – 2-Thermal Temperature

Microorganism	Temperature regime (°C)					TDP
	4°C	25°C	37°C	45°C	80°C	
Xanthomonas campestris	+	+	+	+	+	Above 80°C

Highest mortality (%dead cells) will be corresponded with that of incubation times, hence maximum TDP will be recorded at above 80°C in *Xanthomonas campestris.*

Table – 3-Thermal Death Time

Organism	Time of exposure of cells / conidis (min) at 60°C											Thermal Death Time (mins)
	0	3	6	9	12	15	18	21	24	27	30	
Xanthomonas campestris	+	+	+	+	+	+	+	+	+	+	+	Above 30 min

Affer proper incubation observes the plates for the growth of test org^{m} in each sector by comparing with '0' (control) sector, hence maximum TDT will be recorded at above 30 mins, in *Xanthomonas campestris.*

Table – 4 -Pectolytic and cellulolytic enzyme activities of *Xanthomonas campestris* resulting as reaction to bactericides and bioagents (in vitro)

Treatments	Concentration	Incubation Time : 24 hours		
		PG	PME	Cx
Control		0.96	0.76	0.86
Antibiotic + Bioagent 1	25ppm + 1%	0.97	0.86	0.98
Antibiotic + Bioagent 2	25ppm + 1%	0.99	0.88	0.99

Table – 5 -Effect of bactericide and bioagent treatment in vegetative growth of tomato plant

Treatment	Vegetative characters	
	Stem height	Weight
In Pot experiment		
Control	8 cm	0.26
Bioagent I	9 cm	0.25
Bioagent II	10 cm	0.45

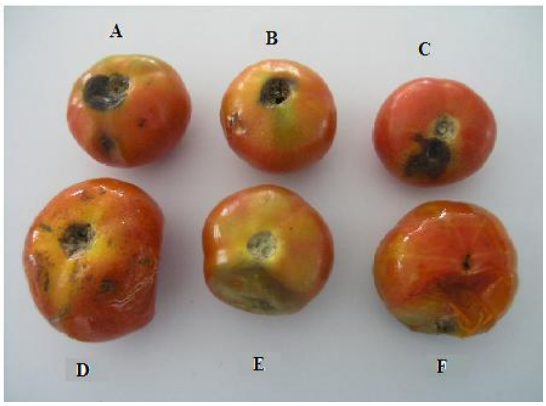

Fig 1- Black spot disease caused Tomato samples collected from different region

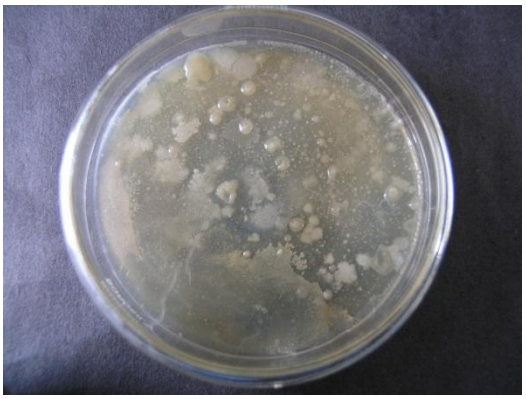

Fig 2 - Black spot disease causing organism plated in Trypticase Soy Agar (TSA)

Fig 3 Unwashed seed tubers were placed individually in plastic bag with 20ml of distilled water (Before incubation)

Fig 4 Unwashed seed tubers were placed individually in plastic bag with 20ml of distilled water (After incubation)

Fig 5 -In bag treatments the bactericides and bioagent applied in field experiment the length of stem was increased in treated plants. It was clear that applied chloramphenicol. *B. lichiniformis* gave the highest value of plant height comparing with untreated plants

3.1 Bacterial black spot pathogen:

In this study the bacterial isolate which isolated from tomato seed, fruit pieces, and were pathogenic to tomato tubers under artificial infection conditions. The morphological characters of bacterial isolates were gram negative and short rods. The cultural character of bacterial colonies was yellow pigmented, circular, convex and smooth. The isolated bacteria identified as *Xanthomonas campestris* according to morphological, cultural and biochemical characters. Earlier *E.caratovora* sub sp. isolates from potato seed tubers. This bacterial isolate grow at anaerobic growth condition. Sodium chloride 5% temperature of 37°C, gelatin liquefaction and sensitive to erythromycin. The isolation produced acid only from arabinose, maltose, lactose and mannitol (**16**). Therefore, it was very important to reduce the bacterial count of black spot pathogen on tomato seed, fruit surfaces.

3.2 Invitrotest: The efficacy of bactericides and bioagents on:

3.2.1 Pectolytic and cellulolytic enzyme activities:

The efficiency of tested concentration of bactericides chloramphenicol (25ppm) and bioagents **(*Bacillus subtilis* *and Bacillus lichiniformis*)** on the ability *Xanthomonas campestris* to secrete PG, PME and Cx enzymes in invitro tests were assayed and calculated in table. Activity of PG enzyme was assayed by spectrophotometric estimation. PG enzyme yield of *Xanthomonas campestris* in treated culture medium *Bacillus subtilis* was less than *Bacillus lichiniformis* culture. The inhibitory effect of bactericide and bioagent treatments on PG enzyme activity of *Xanthomonas campestris* was increased by increase in 25ppm concentration of tested material. There were significant differences observed between the inhibitory effects of tested treatments, between the effects of concentration. The strongest inhibition of PG enzyme secretion was obtained with chloramphenicol followed by *Bacillus lichiniformis*. In earlier, the best PG enzyme inhibitory effect obtained with *T.harzinum*, starner, streptomycin sulfate, *B.subtilis* at the first concentration, respectively. The values of relative loss in viscosity were 12.5, 14.0, 15.0, 15.3 and 17.0%, while the values of enzyme reduction were 33.9, 25.9, 20.6, 19.5 and 10.1%, respectively. At the second concentration, the highest inhibition of PG enzyme yield obtained with starner, followed by *B.Subtilis, T.harzianum* streptomycin sulfate and micronite soreil, respectively. In this study, PME enzyme activity of *Xanthomonas campestris* at tested concentration of bactericide and bioagent treatments, the PME enzyme activity comparing the control treatment. There were no significant differences observed between the inhibitory effect of bactericide and bioagent treatments, the PME enzyme activity comparing the control treatment. There were no significant differences observed between the inhibitory effect of bactericide and bioagent treatments. The highest inhibition of PME enzyme activity obtained with Chloramphenicol in *Bacillus lichiniformis* culture. In earlier studies, the *T.houzianum*, *B.subtilis,* Micronite Soreil, starner and streptomycin sulfate reduced the PME activity, where the values of NaOH solution were 0.8, 1.8, 1.9, 1.9 and 2.2ml, while the enzyme reduction (%) were 73.0, 40.0, 36.7, 36.7, and 26.7 comparing the control, respectively. At 2^{ed} concentration, the starner, *T.harzianum*, *B.subtilis*, Micronite soreil and strepto-mycin sulfate reduced the PME enzyme yield comparing the control,

respectively. In this study, Cx enzyme activity, the efficiency of bactericide and bioagent treatment and the ability of *Xanthomonas campestris* to secrete the Cx enzyme (**17-19**). The most reduction enzyme activity obtained with chloramphenicol in *B.lichiniformis* culture. It is revealed that these materials play an important role in controlling the bacterial black spot disease.

3.3 Field Experiment:

3.3.1 Black spot incidence:At hearvest:

The field application of chloramphenicol, *B.lichiniformis*, *B. subtilis* protected the daughter tomato tuber and fruit in treated plants against black spot disease, where, the percentage of softening tubers were zero, comparing with control plants. In earlier it is obvious that the treatment of seed pieces, as pre saving applications, with streptomycin, *T.harzianum* and *B. subtilis* may significantly contribute to soft rot diseases suppression during plant production, (20).

3.3.2 Effect of vegetative growth:

In bag treatments the bactericides and bioagent applied in experiment the length of stem was increased in treated plants. It was clear that applied chloramphenicol. *B. lichiniformis* gave the highest value of plant height comparing with untreated plants.In pot treatments the bactericides and bioagent applied in experiment the length of stem was increased in treated plants. It was clear that applied chloramphenicol, *B. lichiniformis* gave the highest value of plant height comparing with untreated plants. These results suggest that the used treatments increase the plant growth expressed as plant height and numbers of leaves.

4.0 Conclusion

The predominant recovery of *Xanthomonas campestris* strains from tomato fields reported here may be indicative that this species is becoming an important component of a bacterial spot complex and is no longer as rare in nature. Therefore, attempts were made to identify the pathogen species present in this plants region. The biocontrol activity of *Xanthomonas* was treated with *Bacillus subtilis* and *Bacillus lichiniformis*. It has moderate effect in reducing the incidence of bacterial spot disease.

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