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Use of Polymerase Chain Reaction for Identification of the Pathogen and Management of Potato Soft Rot with Zinc Application

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Abstract

Effect of different Zinc doses was investigated against *Erwinia carotovora* ssp. *atroseptica*, the potato blackleg/soft rot causing organism, during 2009 and 2010 in Department of Plant Pathology and Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar-Pakistan. Out of 200 tested samples, 21 of them were proved to be *Eca*. However, these tentative *Eca* isolates showed some characteristics which were un-expected for *Eca*. We, therefore, decided to perform Polymerase Chain Reaction using *Eca*-specific primers, *Eca*1F and *Eca*2R for confirm identification. For disease management, at the time of sowing, pots containing 5 kg sterilized soil were applied with Zinc in four different treatments *i.e.* 8 mg, 10 mg, 12 mg and 14 mg along with one control. Results indicated that 12 mg (4.8 kg Zn ha^{-1}) were better doses in controlling the disease up to 73% and increasing the yield up to 117% as compared to control plants.

Keywords

***Erwinia carotovora*, Potato, PCR Identification, Soft Rot, Zinc**

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1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops of the world. Nutritionally, potatoes are best known for their carbohydrate content and can be grown in many different environments, but it is best adapted to temperate climates [1]. The climate of Pakistan, especially of northern areas, is very suitable for the production of potatoes. Still per hectare yield of the crop is considerably low. There are different reasons for this low yield of potato crop. The prevalence of bacterial disease is one of them.

In bacterial diseases Blackleg/soft rot is one of the most important diseases of potato in Pakistan causes 10% - 30% losses [2]. Causal agent of the disease is *Erwinia carotovora* ssp. *Atroseptica* (*Eca*). The pathogen is seed-borne and frequently remains undetected by the common detection methods. The pathogen is found in nearly all important potato growing areas of Pakistan. The traditional identification and detection of these bacterial pathogens in seed tubers are quite laborious and not really fool-proof. Polymerase chain reaction (PCR) that rapidly detects, identifies and characterizes microorganisms in a shorter time is a good alternate for the identification purposes.

On the other hand in controlling such disease, the use of chemicals is one the most effective and quick control measure. Chemical treatment is sometimes, the only option available but not popular among the researchers, because of their residual and hazardous effects on environment.

There are different components of an IDM program but the role of micronutrients is often neglected which is the relatively safer and cheaper than any other cultural or chemical method of control. In micronutrients, Zinc is an essential element for both plants and animals. It plays an important role in several plant metabolic processes; it activates enzymes and is involved in protein synthesis and carbohydrates, nucleic acid and lipid metabolism [3] [4]. Zinc may be supplemented as a single nutrient fertilizer or as an addition to the NPK fertilizer. Research has shown that all sources of Zn (except granular zinc oxide) have an equal effect on crop production [5]. Recommended dosage of per hectare zinc for most of the crops is in the range of 5 - 34 $kg \cdot ha^{-1}$ (pure zinc), but keeping in mind its availability factor as $ZnSO_4$ (with mono hydrate having 35% zinc and hepta hydrate having 22% zinc) can be used for field application. In current studies, efforts are made to test the effect of Zinc and its optimum dose in controlling the blackleg/soft rot disease of potato.

2. Materials and Methods

2.1. Isolation and Preservation of the Bacterial Isolates

Tubers and plant samples showing disease symptoms were collected from potato growing areas of the country, in laboratory samples were cleaned, surface-sterilized with 0.5% sodium hypochlorite (for a 10 seconds), washed with sterile distilled water, placed in sterile 0.85% saline solution and crushed using sterile mortar and pestle under aseptic conditions. The resulting suspension was left undisturbed for a few minutes. A loopful of this suspension was streaked on the surface of plates containing nutrient agar (NA: Bactoagar; 10 gm, NaCl; 5 gm, K_2PHO_4; 5 gm, KH_2PO_4; 2 gm, Bactopeptone; 1 gm), and incubated at 28˚C for 24 h. In order to obtain pure culture procedure was repeated several times with growing colonies re-inoculation on media.

2.2. Identification

Traditional biochemical, physiological and pathological tests were applied for identification but due to overlapping results it were decided to use *Eca*-specific primers in the polymerase chain reaction to accurately confirm the identity of the pathogen. Specific primers, *Eca*1F (5'-CGGCATCATAAAAACACG-3') and *Eca*2R (5'-GCACACTTCATCCAGCGA-3') as described by De Boer and Ward [6] were used for molecular identification.

For this purpose, *Eca* cells were grown in Luria-Bertani Broth medium (LB: tryptone at 10 g/L, yeast at 5 g/L, NaCl at 10 g/L; Bertani, [7]) at 27˚C for 15 h, DNA was extracted with commercially available DNAzol kit. The PCR master mix included 2 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ dNTPs, 1 $\mu mol \cdot L^{-1}$ each primer, *Taq* buffer (67 $mmol \cdot L^{-1}$ Tris HCl pH 8.8) and 5U of *Taq* DNA polymerase were added to each PCR tube with 3 µl of extracted DNA. DNA amplification was performed in a MJ mini thermocycler (Bio-rad, USA) for 40 cycles and temperature 94˚C for 30 sec denaturation, 47˚C for 30 sec primer annealing and 72˚C for 50 sec extension. After PCR amplification, 25 µl of the product was electrophoresed on a 2% (w/v) agarose gel as described by Sambrook *et al.* [7] [8].

2.3. Inoculation of Seed Potato

After successful identification bacteria was mass cultured on LB medium. Bacterial suspension $5x10^8$ cells mL^{-1} (A_{540} = 0.52) was prepared from the growing culture in 0.85% saline solution and potato tubers were washed and injured with needle and then dipped in bacterial suspension for 30 minutes and then grown.

2.4. Zinc Application

Inoculated potato tubers were sown in 15 inch diameter clay pots containing 5 kg sterilized (121˚C and 15 lb pressure for 30 mints) soil. At the time of sowing pots were applied with Zinc in four treatments *i.e.* 8 mg, 10 mg, 12 mg and 14 mg (*i.e.* 3.2, 4.0, 4.8 and 5.6 $kg{\cdot}ha^{-1}$ respectively) as soil application. A control with no zinc application was also included. The experiment was conducted in CR design with four replications each has 5 plants. Data was recorded for disease incidence, disease severity, yield/plant (g), fresh plant weight (g), % germination, plant height (cm), number of tubers per plant, tuber size and individual tuber weight (g). For disease severity, scale [9] was used as shown in **Table 1**.

3. Results

Identification of the Pathogen

A total of 200 suspected samples obtained from soft rotted tubers and black-legged potato plants were processed. Regarding the morphology of the bacterial colonies, on NA medium the colonies appeared to be transparent, circular, raised, shiny and creamy white after 48h incubation at 28˚C. Only 44 isolates were selected on morphological bases for Polymerase Chain Reaction (PCR) amplification with specific primers to confirm their identity. As clear from **Figure 1**, *Eca*-specific 690 bp band was amplified from 21 out of 44 isolates confirming that they were *Eca*, remaining 23 isolates might be *Ecc* or *Ech* according to the results of biochemical tests.

Significant differences were found among the different zinc concentrations for all the nine parameters on which the data were recorded. Over all 12 mg zinc was found to be the most effective chemical followed by 14 and 10 mg, (**Tables 2-4**). In most important parameter like incidence, severity and yield, 14 mg was found to be the most effective chemical allowing minimum disease incidence (22.74%) in other words incidence was decreased 75.92% over control where no zinc was applied statistically similar results obtained for 12 mg (30.55%) however this decrease was 67.65% over the control, while maximum disease incidence was recorded for 8 mg (70.19%), proved to be least effective treatment.

Table 1. Disease severity scale used for diseased (blackleg) plants.

Scale	Description
0	No blackleg/soft rot symptoms
1	Less than 50% of the plant had symptoms
2	More than 50% of the plant had symptoms
3	Plant completely dead

Table 2. Effect of zinc on the disease incidence.

Treat.	Incidence	% Decrease	Severity	% Decrease	Yield/plant	% Increase
8 mg	70.19 B	25.68*	2.25 AB	18.18	383.75 C	64.00*
10 mg	44.45 C	52.94*	1.50 BC	45.45*	452.50 B	93.38*
12 mg	30.55 D	67.65*	0.75 C	72.73*	507.50 A	116.88*
14 mg	22.74 D	75.92*	0.75 C	72.73*	483.50 AB	106.62*
Control	94.44 A	--	2.75 A	--	234.00 D	--
LSD	12.23		0.78		43.66	

Means followed by the same letters in columns are non-significant at $P \leq 0.05$. *Significant ($P \leq 0.05$), change over control.

Table 3. Effect of zinc application on potato germination and yield components.

Treat.	Germination	% Increase	No. of potato	% Increase	Height	% Increase
8 mg	59.75 C	30.60*	6.75 C	80.00*	50.75 A	20.12*
10 mg	75.50 B	65.03*	9.50 B	153.33*	53.50 A	26.63*
12 mg	77.50 AB	69.40*	12.5 A	233.33*	54.75 A	29.59*
14 mg	84.25 A	84.15*	6.25 C	66.67*	54.25 A	28.40*
Control	45.75 D	--	3.75 D	--	42.25 B	--
LSD	7.57		2.28		4.01	

Means followed by the same letters in columns are non-significant at $P \leq 0.05$. *Significant ($P \leq 0.05$), change over control.

Table 4. Effect of zinc on shoot and tuber production of potato.

Treat.	Shoot weight	% Increase	Tuber weight	% Increase	Tuber size	% Increase
8 mg	311.00 B	52.45*	50.50 CD	23.93	5.02 B	123.33*
10 mg	330.75 AB	62.13*	61.25 BC	50.31*	5.20 B	131.11*
12 mg	351.25 A	72.18*	79.50 A	95.09*	5.80 A	157.78*
14 mg	331.50 AB	62.50*	64.00 B	57.06*	5.57AB	147.78*
Control	204.00 C	--	40.75 D	--	2.25C	--
LSD	32.43		11.14		0.58	

Means followed by the same letters in columns are non-significant at $P \leq 0.05$. *Significant ($P \leq 0.05$), change over control.

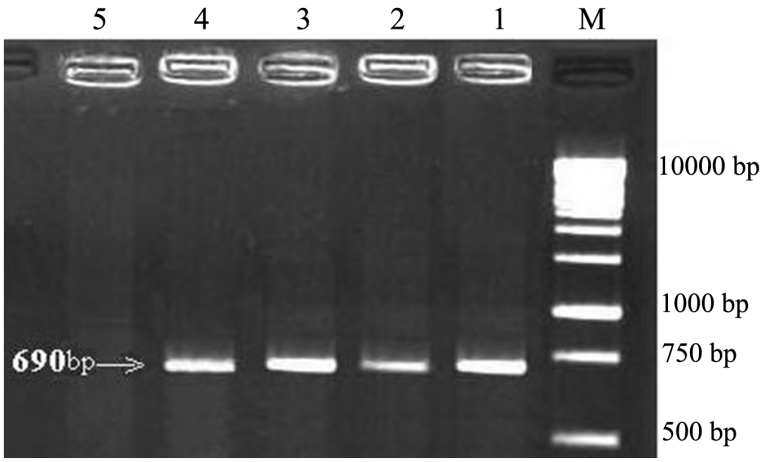

Figure 1. Amplification of *Eca*-specific 690 bp band.

In case of disease severity 12 and 14 mg (0.75) were found to be most effective treatments allowing the minimum disease severity as measured according to the scale and decrease in disease severity was 72.73% as compared to control. For 8 mg treatment the decrease over control was found to be non-significant and proved to be least effective treatment allowing maximum (2.25) amount of disease.

Plants treated with 12 mg zinc gave maximum yield (507.5 g/plant) followed by 14 mg zinc applied plants (483.5 g/plant) in both cases the increase in yield was more than 100% as compared to control plants. For other parameters shown in **Table 3** and **Table 4** like Germination, Numbers of potatoes/plant, shoot weight, Individual tuber weight, and Tuber size. Similar results were obtained *i.e.* 12 and 14 mg application of zinc was found to bring positive change and significant results were obtained over control plants. However, plant height was found

to be non-significant for all the treatment, shows that zinc has no effect on plant height.

4. Discussion

To control a plant disease effectively, accurate and timely diagnosis is a must. In case of plant bacterial diseases, a series of biochemical, physiological, pathogenic and other tests are done to accurately diagnose the causal organism. In the present studies, a large number of such tests were conducted to identify the pathogen but the actual causal organism *Erwinia carotovora* ssp. *atroseptica* was often misidentified because of the two other species of the pathogen *E. c. carotovora* and *E. chrysanthemi* although key diagnostic tests such as acid production from α-methyle glucoside, production of reducing substances from sucrose, sensitivity to erythromycin and growth at 36˚C were performed. But the results were quite conflicting, similar results were also obtained with Lelliot and Dicky [10] and Perombelon and Kelman [11]. But when our key diagnostic tests gave overlapping results regarding the identity of blackleg/soft rot causal organism an alternate quick, accurate and sophisticated approach was followed and that is Polymerase Chain Reaction (PCR) was performed (using *Eca*-specific primers) for all the isolates to confirm their identity. The 690 bp Eca-specific DNA band [7] was amplified from 21 isolates out of 44, confirming that they were Eca. The fact that we can successfully and accurately identify the potato-blackleg/soft rot causal organism using *Eca*-specific primers and our optimized PCR conditions has important implications for seed certifying agencies doing seed-potato indexing.

Management of a disease to bring yield and quality losses to a minimum possible level is a challenging job. Some diseases are especially difficult to control because each of the different available control measures contributes very little. Black leg/soft rot of potato is one of such hard-to-control diseases. A disease like potato black leg can only be successfully controlled if an integrated disease management program (IDM) is followed. There are different components of an IDM program but the role of micronutrients is often neglected which is the relatively safer and cheaper than any other cultural or chemical method of control. In micronutrients, zinc is involved in numerous aspects of cellular metabolism. It is required for the catalytic activity of approximately 100 enzymes [12] and it plays a role in immune function [13], DNA synthesis, protein synthesis, and cell division [14].

For this reason, it was decided to investigate the potential of zinc to control black leg/soft rot of potato caused by *Eca*. Finding a better chemical/nutrition to control a disease in not enough, it should also be known that what dose should be used. The dose studies indicated that 4.8 kg/ha (12 mg/kg soil) soil applied zinc was effective in controlling the *Eca* infection in potato plants and recommended for field application but its reaction with other macro and micronutrients should also be investigated. It is also suggested that a range of micronutrients should be tested to find which one can be used in to control plant bacterial diseases and at the same time avoid the easy development of chemical resistance in phyto-pathogenic bacteria.

5. Conclusion

From the above studies, it is concluded that zinc has a high potential to manage black leg/soft rot of potato caused by *Eca*. However, the effective dose for soil applied zinc should be 4.8 kg/ha (12 mg/kg soil) while managing the disease down to its minimum.

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