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# INDUCTION OF DEFENSE ENZYMES AND SUPPRESSION OF *MACROPHOMINA PHASEOLINA* : A ROOT ROT PATHOGEN, INCITING BLACK GRAM BY MICRONUTRIENT ENRICHED *TRICHODERMA VIRIDE*

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## Abstract

The experiments were conducted in pot culture to find out the efficacy of the newly formulated *Trichoderma viride* talc formulation against *Macrophomina phaseolina* causing root rot of black gram. Among the treatments, it was observed that application of zinc and boron (combination) enriched *T. viride* significantly suppressed the soil borne pathogens and increased the shoot length, root length, DMP, zinc and boron uptake, vigour index and yield. The incidence of root rot was reduced to 12.50 per cent as against the inoculated control, which respectively recorded 73.54 per cent incidence. The zinc and boron uptake was maximum (7.45, 4.54) respectively in soil application of zinc sulphate and boron enriched *T. viride* (mixture) (10g/5kg of pot soil) talc formulation in black gram than other treatments. The defense enzymes *viz*., superoxide dismutase and phenolic content also increased proving the positive effect of micronutrient enriched *T. viride.*

***Key words :*** Defense enzymes, *Macrophomina phaseolina*, micronutrients, *Trichoderma viride*.

## Introduction

Black gram, also known as urdbean, mash, black maple contains 26% protein (three-fold higher than cereals), 55% carbohydrates, 32% starch and about 10% dietary fibers (both insoluble and soluble) and form an important source of vegetarian diet. This pathogen is both seed and soil inhabitant in nature (Dhingra and Khare, 1973). The seed and soil-borne nature of the pathogen is a major hurdle in the successful management of the disease. Hence, chemical control of the pathogen has been recommended by many workers (Bhimsen *et al*., 1995 and Prameela Devi and Singh, 1977). But the fungus is reported to have developed resistance to some of the fungicides (Kumar and Shastry, 1979 and Anitha *et al*., 1989). The high cost of chemicals required for soil application, development of fungicide resistance by target pathogen and the hazardous effects of chemicals on the ecosystem have forced the scientists to develop alternate methods to control this disease. Hence, the use of biocontrol agent *viz., T. viride* and mineral nutrients which are devoid of any of the above adverse effects by soil applicationwas studied against *M. phaseolina*under pot culture conditions.

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## Materials and Methods

### Isolation of pathogens and bio control agents

The root rot pathogen was isolated from infected dry root rot plants using potato dextrose agar (PDA) as culture medium. The biocontrol agent *Trichoderma* is isolated from rhizosphere soils of black gram using Trichoderma selective medium (TSM) (Elad and Chet, 1983). The individual colonies of *Trichoderma* were identified based on the morphological characters (Webster and Lomas, 1964).

### Effect of nutrients on the mycelial growth of *M. phaseolina*

The efficacy of micronutrients *viz*., zinc sulphate, boron, ammonium sulphate, ferrous sulphate, calcium

sulphate and magnesium sulphate at 3 levels *viz*., 250, 500 and 750 ppm (w/v) concentration was tested on the mycelial growth of *M. phaseolina*by poisoned food technique (Schmitz, 1930) in three replications. The difference in colony diameter between poisoned medium and control was used to calculate the per cent inhibition (Paul and Mishra, 1993).

**Effect of nutrients on the biomass production and sporulation by *Trichoderma viride* (liquid broth)**

To study the effect of zinc sulphate on the growth and sporulation of *T. viride*, yeast molasses medium was used as the basal medium at three different ppm concentration 250, 500 and 750. Inoculated flasks were incubated at room temperature for fifteen days. Mycelial mat was harvested on pre-weighed filter paper (Whatman No. 42) and oven dried at $60^0$C for 48 hours (Singh and Malhotra, 1994). The dry weight of the mycelial mat was recorded. The same procedure was followed for boron and sporulation was studied demonstrated by Jayaraj and Ramabadran (1998).

**Effect of nutrient and nutrient enriched talc formulation against *M. phaseolina*under pot culture condition**

The virulent isolate of *M. phaseolina*was mass multiplied in the sand-maize medium and was mixed with the potting mixture. Surface sterilized black gram seeds (Co-6) were sown in pots containing potting mixture @ 3 seeds per pot. The treatments of pot culture experiments were as follows.

**Treatments**

$T_1$ - Soil application of *T. viride*a lone (20 g/5 kg of pot soil) – talc formulation.

$T_2$ - Soil application of zinc sulphate (12.5 mg/5 kg of pot soil).

$T_3$ - Soil application of boron (5.0 mg/5 kg of pot soil).

$T_4$ - Soil application of zinc suphate enriched *T. viride* (15g/5kg of pot soil) - talc formulation.

$T_5$ - Soil application of boron enriched *T. viride* (20g/5kg of pot soil) - talc formulation.

$T_6$ - Soil application of zinc sulphate and boron enriched *T. viride* (mixture) (10g/5kg of pot soil) - talc formulation.

$T_7$ - Inoculated control.

$T_8$ - Uninoculated control.

The experiment was conducted in completely randomized block design replicated thrice. The observations on wilt incidence were done at 15, 30, 45, 60 and 75 days after sowing (DAS). The per cent disease incidence was assessed using the following formula.

**Per cent Disease Incidence (PDI)**

$$= \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

**Nutrient analysis in plant and soil sample**

The soil and plant samples were collected at 30 days after sowing. The plants uprooted at each stage were washed with water to remove soil particles and separated into shoot and root portions then washed with dilute HCl and shade dried.

**Plant nutrient (zinc) analysis**

After recording dry weight, plant samples (shoot, root and seed) were ground to a fine powder in a Willey mill and used for the estimation of Zn content. The Zn content in various plant parts (shoot and root) was determined by using the procedure outlined by Jackson (1973).

**Boron estimation in plants**

Take five mlof the triple acid extract which is diluted up to 50 ml was taken in a 25 ml volumetric flask, to which four ml of the buffer and four ml Azomethine - H reagent were added and allowed for half an hour for colour development. The volume was made up and the color intensity was measured at 420 nm using UV - Visible Spectrophotometer (Cary 50 Scan - varion) (Page *et al*., 1982).

**Soil nutrient (zinc) analysis**

**DTPA extractable Zn**

Ten gram soil sample was weighed into polythene shaking bottle, added with 20 ml of 0.005 M DTPA extractant and kept in mechanical shaker for two hours. The 0.005 M DTPA extractant was prepared by mixing 0.005 M DiethyleneTriaminePenta Acetic Acid, 0.1 M Triethanolamine and 0.01 M $CaC1_2$ which was finally adjusted to a pH of 7.3 using 1:1 diluted HCl. The extract was filtered using Whatman No.42 filter paper and the DTPA Extractable Zn content was estimated using Atomic Absorption Spectrometer (GBC Avanta model).

**Boron estimation in soil**

Azomethine-H solution was prepared by dissolving 0.45 g of Azomethine-H in 100 ml of 1 per cent ascorbic acid. The buffer solution was prepared by dissolving 250 g of ammonium acetate and 15 g of Na salt of EDTA (Disodium dihydrogen Ethylene Diamine Tetra Acetic acid) in 400 ml of double distilled water. All the reagents were dissolved and 125 ml of acetic acid and was added to the solution and mixed thoroughly and remaining procedure is followed by Berger and Troug (1940).

**Assay of defense-related enzymes and compounds**

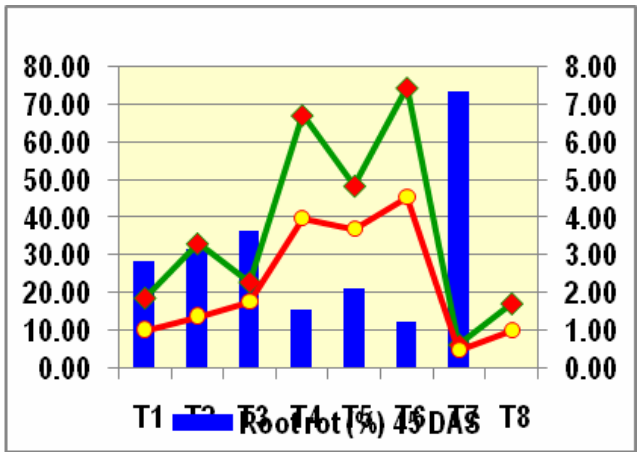


**Fig. 1 :** Effect of nutrients and nutrient enriched *Trichoderma viride* (talc formulation) on the root rot incidence of blackgram.

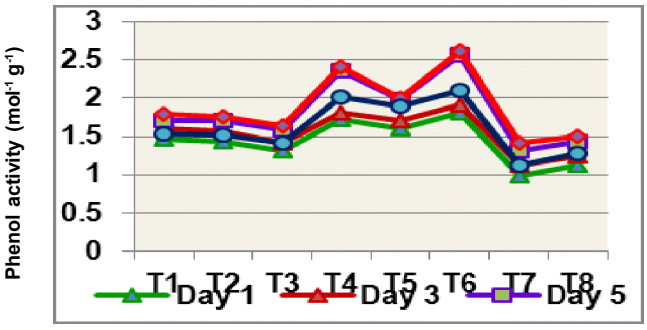


**Fig. 2 :** Induction of phenol activity *Macrophomina phaseolina* challenged inoculated black gram treated with nutrients and nutrient enriched *Trichoderma viride*.

**against root rot of black gram**

**Collection of plant samples**

Samples were collected from different treatments to study the Induced Systemic Resistance (ISR) in response to *M. phaseolina*and *T. viride* under glasshouse conditions. Leaves from bioagents and nutrients treated plants with or without root rot and wilt were collected at 1st day, 3rd day, 5th day, 7th day and 9th day, which were maintained at same conditions.

The enzyme activity wasexpressed as µM of trans-cinnamic acid/ min/g fresh weight of tissue.

**Phenol content**

Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of fresh tissue was homogenized in 10 ml of 80 per cent methanol and agitated for 15 minutes at 70°C. One ml of the methanol extract was added to 5 ml of distilled water and 250 µl of FolinCiocalteau reagent (1N) and the solution was kept at 25°C. After three min. one ml of saturated solution of $Na_2CO_3$ and one ml of distilled water

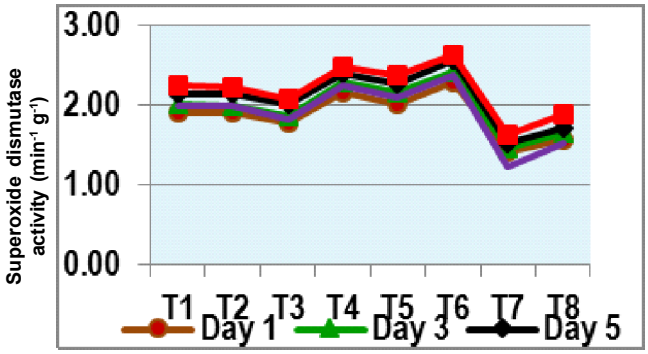


**Fig. 3 :** Induction of superoxide dismutase activity in *Macrophomina phaseolina* challenged inoculated black gram treated with nutrients and nutrient enriched *Trichoderma viride*.

was added and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue colour was measured using a GS 5703 AT spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reagent with a phenol solution ($C_6H_5OH$) and expressed as catechol equivalents $g^{-1}$ of fresh tissue.

**Assay of superoxide dismutase (SOD)**

The enzyme extract was prepared by homogenizing 1 g root tissue in 2 ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 10000 rpm at 4°C for 30 min. The supernatant served as enzyme source and SOD activity was determined as its ability to inhibit the photochemical reduction of NBT (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin. 0.1 mm EDTA and 100 µl of the enzyme extract and the riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent lamp at 25°C. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non-illuminated in parallel to the sample tubes for blank. Each extract was subtracted from the blank and mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of NBT photo-reduction. The SOD activity was expressed in SOD units $g^{-1}$ tissue (50% NBT inhibition = 1 unit) (Belid El-Moshaty *et al*., 1993).

## Results and Discussion

**Effect of nutrients on the mycelial growth of *M. phaseolina***

The efficacy of micronutrients *viz*., zinc sulphate, boron, ammonium sulphate, ferrous sulphate, calcium

**Table 1 :** Effect of nutrients on mycelial growth of *Macrophomina phaseolina* incitant of root rot of black gram on solid and liquid medium.

| Nutrients | Mycelial growth (mm) | | | Mycelial dry weight (mg) | | |
|---|---|---|---|---|---|---|
| | 250 ppm | 500 ppm | 750 ppm | 250 ppm | 500 ppm | 750 ppm |
| Zinc sulphate | 76.49$^{fg}$ | 62.36$^{c}$ | 32.70$^{a}$ | 675.51$^{d}$ | 577.22$^{c}$ | 406.15$^{a}$ |
| Boron | 81.70$^{hi}$ | 69.20$^{d}$ | 42.36$^{b}$ | 720.76$^{e}$ | 724.85$^{e}$ | 524.52$^{b}$ |
| Ammonium sulphate | 86.89$^{j}$ | 76.50$^{g}$ | 71.26d$^{e}$ | 786.85$^{ghi}$ | 767.10$^{fgj}$ | 741.32$^{ef}$ |
| Ferrous sulphate | 87.48$^{j}$ | 80.20$^{ghi}$ | 73.90$^{ef}$ | 855.12$^{kl}$ | 830.32$^{k}$ | 784.03$^{h}$ |
| Calcium sulphate | 88.00$^{j}$ | 82.72$^{gi}$ | 79.00$^{ghi}$ | 871.52$^{l}$ | 867.55$^{jkl}$ | 827.85$^{ijk}$ |
| Magnesium sulphate | 87.29$^{j}$ | 78.60$^{h}$ | 76.66$^{g}$ | 885.71$^{l}$ | 826.65$^{ik}$ | 808.91$^{hij}$ |
| Control | 90.00$^{j}$ | 90.00$^{j}$ | 90.00$^{j}$ | 998.61$^{m}$ | 998.73$^{m}$ | 999.00$^{m}$ |

Values are mean of three replications. Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P = 0.05.

**Table 2:** Effect of nutrients and nutrient enriched *Trichodermaviride*(talc formulation) on the root rot incidence of black gram

| Treatments Soil application | Germination (%) | Root rot (%) 45 DAS | Shoot length (cm) | Root length (cm) | DMP ($g^3$ seedlings$^{-1}$) | Zinc uptake (mg/pot) | Boron ptake (mg/pot) | Vigour index | Yield (gm/pot) |
|---|---|---|---|---|---|---|---|---|---|
| $T_1$ -*T. viride*(20 g/5 kg of pot soil) – talc formulation | 94 (75.82) | 28.66$^{e}$ (97.10) | 18.8$^{d}$ | 11.5$^{d}$ | 0.0632$^{d}$ | 1.88$^{f}$ | 1.02$^{f}$ | 2230.4$^{d}$ | 8.79$^{d}$ |
| $T_2$ -$Znso_4$ (12.5 mg/5 kg of pot soil) | 92 (73.57) | 31.66$^{f}$ (102.72) | 18.3$^{d}$ | 11.3$^{d}$ | 0.0638$^{d}$ | 3.32$^{d}$ | 1.38$^{e}$ | 2254.4$^{d}$ | 8.58$^{d}$ |
| $T_3$ –Boron (5.0 mg/5 kg of pot soil) | 91 (72.54) | 36.42$^{g}$ (111.35) | 16.6$^{e}$ | 08.2$^{e}$ | 0.0533$^{e}$ | 2.30$^{e}$ | 1.76$^{d}$ | 1832.8$^{e}$ | 7.52$^{e}$ |
| $T_4$ -*T.viride*+$ZnSo_4$ (15g/5kg of pot soil) – talc formulation | 95 (77.07) | 15.50$^{c}$ (69.55) | 24.5$^{b}$ | 18.1$^{b}$ | 0.0768$^{b}$ | 6.72$^{b}$ | 3.99$^{b}$ | 2629.6$^{b}$ | 13.47$^{b}$ |
| $T_5$ -*T.viride*+Boron (20g/5kg of pot soil)- talc formulation | 92 (73.57) | 21.33$^{d}$ (82.51) | 21.0$^{c}$ | 14.5$^{c}$ | 0.0682$^{c}$ | 4.85$^{c}$ | 3.70$^{c}$ | 2479.0$^{c}$ | 11.25$^{c}$ |
| $T_6$ -*T.viride*+$ZnSo_4$+Boron (10g/ 5kg of pot soil)- talc formulation | 95 (77.07) | 12.50$^{b}$ (62.12) | 27.0$^{a}$ | 22.8$^{a}$ | 0.0883$^{a}$ | 7.45$^{a}$ | 4.54$^{a}$ | 2920.0$^{a}$ | 16.38$^{a}$ |
| $T_7$ -Inoculated control | 91 (72.54) | 73.54$^{h}$ (177.15) | 11.3$^{g}$ | 05.1$^{g}$ | 0.0314$^{g}$ | 0.64$^{h}$ | 0.49$^{h}$ | 1389.2$^{g}$ | 4.21$^{g}$ |
| $T_8$ -Un inoculated control | 100 (90.00) | 0.00$^{a}$ (0.86) | 15.0$^{f}$ | 10.8$^{f}$ | 0.0428$^{f}$ | 1.73$^{g}$ | 1.00$^{g}$ | 1600.0$^{f}$ | 6.98$^{f}$ |

Values are mean of three replications. Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at P = 0.05.Figures in parentheses are arcsine transformed value.

sulphate and magnesium sulphate at 3 levels *viz*., 250, 500 and 750 ppm (w/v) concentration was tested on the mycelial growth of *M. phaseolina*. Among all zinc sulphate and boron inhibited the mycelial growth of the pathogen both in the solid and liquid medium.

### Effect of nutrients and nutrient enriched *Trichodermaviride*fungal antagonist on the root rot incidence of black gram

Pot culture experiments were conducted with the effective bioagent *viz*., *T. viride*along with nutrients like Zinc sulphate and boron to find out its efficacy against root rot disease (*M. phaseolina*). Incidence of root rot was recorded at regular intervals and the final recorded reading was on 45 DAS (table 2).

### Nutrient analysis in plant and soil sample

The *T. viride* enriched zinc sulphate and boron had recorded minimum incidence of root rot under pot culture studies. The *T. viride* enriched zinc sulphate and boron had reduced the root rot (12.50%) incidence of black gram and increased the shoot length (27.00 cm), root length (22.80 cm), DMP (0.0883 g/3 seedlings), zinc (7.45 mg/pot) and boron uptake (4.54 mg/pot), vigour index (2920) and ultimately an increased yield (16.38 g/pot)

(table 2). The nutrient enriched *T. viride* was able to reduce the soil-borne disease significantly and increased the nutrient uptake. This pot culture experiment proved that the nutrients enriched *T. viride*had reduced the root rot incidence and increased the shoot length, root length, DMP, zinc and boron uptake, vigour index and ultimately reflected in an increased yield and it is depicted in the fig. 1.

**Assay of defense-related enzymes**

**Phenol content**

The increase in phenol activity was noticed upto seven days after inoculation and there after decreased in all the treatments. The phenol activity was maximum in the combined application of zinc sulphate and boron enriched *T. viride* (2.628) challenged with *M. phaseolina.* The next best was zinc sulphate enriched *T. viride*followed by boron enriched *T. viride* 2.414, 2.003 (*M. phaseolina*). Inoculated control and uninoculated control showed lowest Phenol activity as compared to other treatments at 9 days after inoculation. The Phenol activity was maximum in the combined application of zinc sulphate and boron enriched *T. viride* was depicted in fig. 2.

**Assay of superoxide dismutase (SOD)**

The increase in SOD activity was noticed upto seven days after inoculation and there after decreased in all the treatments. The SOD activity was maximum in the combined application of zinc sulphate and boron enriched *T. viride* (2.64) challenged with *M. phaseolina*. The next best was zinc sulphate enriched *T. viride*followed by boron enriched *T. viride* 2.49, 2.37 (*M. phaseolina*). Inoculated control and uninoculated control showed lowest SOD activity as compared to other treatments at 9 days after inoculation. The SOD activity was maximum in the combined application of zinc sulphate and boron enriched *T. viride* was depicted in fig. 3.

The effect of mineral nutrients on plant disease control has received considerable attention over the year, but little of this attention has been directed towards the trace elements *viz*., zinc, silicon, boron, calicium etc. Zinc sulphate at 500 ppm concentration retarded the mycelia growth and mycelia dry weight of *Rhizoctoniasolani* (Lakpale *et al*., 1997). The nutrient enriched *T. viride* was formulated in the talc powder and was used in pot culture studies to find out its efficacy against the soil borne diseases of pulses black gram. Soil application of talc based formulation of *T. harzianum*, *T. polysporum* and *T. viride* effectively controlled the root rot (*M. phaseolina*) of eggplant under field condition (Ramezani, 2008). Arora *et al*. (2012) suggested that B and Zn application under both normal and salinity stress provides better chance of survival of seeds and seedlings of green gram (*Vigna radiata*), which lead to better growth and yield.Plant-microbe associations enhance the defense capacity of the plant and effectively ward off a broad spectrum of pathogens (Pozo *et al.*, 2005). Phenolic compounds enhance the mechanical strength of host cell wall and also inhibit the invading pathogenic organisms. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi.* Benhamou *et al.* (2000) reported that an endophytic bacterium, *Serratiaply muthica* induced the accumulation of phenolics in cucumber roots against *P. ultimum*. Manganese and zinc are co-factors of Super Oxide Dismutase (SOD), which considered enzymatic antioxidant, hence alleviate the harmful effect of Reactive Oxygen Species (ROS free radicals) caused by fungal stress. These findings are in agreement with Kostas and Christos (2006).

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