PHYSIOLOGICAL CHANGES AGAINST MELOIDOGYNE INCognITA IN RHIZOBACTERIAL TREATED EGGPLANT UNDER ORGANIC CONDITIONS

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ABSTRACT

Field trials and laboratory experiments were conducted to evaluate the efficacy of rhizobacterial treatments for controlling *Meloidogyne incognita* (Root Knot Nematodes, RKN) on brinjal crop. Biochemical and histological analyses of treated plants were performed to check the extent and mechanism of activity of bacterial treatments. Disease percentage, Shoot length, Shoot weight, Root length and Root weight were deliberated and significant differences were recorded in these aspects. Findings also illustrated significant difference in the quantity of total phenolic contents of control (0.3g/kg) and RB5 (3.4g/kg) plants. Quantification of peroxidase (PO) revealed the significant distinction in control (0.6g/kg) and treated plants (2.54g/kg), whereas peroxidase contents were also variable among different rhizobacterial treatments. In case of terpenoids control (0.29g/kg) and RB1 (0.54g/kg) were notably varied from RB2 (0.83g/kg), RB3 (1g/kg) and RB4 (1.02g/kg). Here, RB5 again showed maximum amount of terpenoids (1.23g/kg). Findings declared all the treatments significantly effective than the control treatment with reference to Ascorbic acid and Polyphenol oxidase (PPO) contents. Collectively, analyses determined the ability of brinjal plant to activate its defenses more rapidly against *M. incognita* under the influence of rhizobacterial treatment RB5.

**Key words:** Biochemical defenses; Disease incidence; Induced systemic resistance; Root galls.

INTRODUCTION

Eggplant (*Solanum melongena* L.) commonly known as brinjal is generally a vegetable of summer season. It is cultivated both in open agricultural fields and under greenhouse conditions. It is very important dietary item containing water (92.7%), protein (1.4%) and vitamin A (Murslain et al., 2013). In Pakistan, the annual production of brinjal is 84707 tonnes and it is cultivated on an area of 8490 ha (FAO, 2011). The harvested average yield is very low due to the attack of various pests such as insects, fungi, bacteria, viruses and nematodes. These pathogenic nematodes are one of the most ancient and diverse type of organisms on earth (unsegmented round worms) that belongs to phylum Nematoda, existing for an estimated period of one billion years (Wang et al. 1999).

These pathogens of brinjal crop, including root knot nematodes are the serious pathogen of brinjal crop and cause significant annual losses of the yield in Pakistan (Anwar et al., 2009). These losses range 10% to 100% in Pakistan (Shahid et al., 2007). The severe attacks of *Meloidogyne incognita* have been reported by a number of scientific studies. (Dhawan and Sethi, 1976; Netscher and Sikora, 1990). *M. incognita* is categorized as a major plant parasitic nematode in tropical and subtropical areas of the world including Pakistan (Anwar et al., 2007). Root knot nematodes are classified as not only an important pest of brinjal in Pakistan, but its host range is also very diverse.

Plant roots are the most targeted source of food for nematodes. Cultivated plants are affected by the genus *Meloidogyne*, which belongs to Root Knot Nematodes (RKN) and is represented by over 90 species (Moens et al., 2009). However, plant resistance possesses major advantage of being self-protection system that is more effective and cost-effective method to manage nematode diseases (Starr and Roberts, 2004). It also considered more convenient than other disease controlling strategies (e.g. chemical and cultural control methods) due to its significant disease control potential and environment friendly nature. Whereas the most damaging species of nematodes for agricultural crops are *M. incognita*, *M. javanica* and *M. arenarrian* (Sasser et al., 1982). Microbes with the highest suppressive potential include pathogenic rhizobacteria, fungi infecting nematode eggs, fungi with general antagonizing effects and obligate parasitic bacteria (Whipps and Davies, 2000).

*Trichoderma* and *Purpure ocillium* genera are the most promising biocontrol fungi for *Meloidogyne* spp. (Dababat et al., 2006; Affokpon et al., 2011; Wilson and Jackson, 2013). Endospores of *Pasteuria penetrans* and
rhizobacteria (e.g. Bacillus firmus) have also been well investigated for nematode management (Wilson and Jackson, 2013). It has been recorded that female nematodes treated with Pasteuria harvested low number of eggs.

Several studies have identified a number of plant growth promoting rhizobacteria (PGPR) strains hampering nematode attacks through a mechanism called induced systemic resistance (Van Loon et al., 1998; Ahmad et al., 2014a). All these studies declare ISR as an efficient, ecofriendly and cost-effective strategy to manage plant diseases. Moreover, management of RKN through ISR would yield better vegetable crops and would be advancement in plant protection program. Therefore, this study has been performed to control attack of root knot nematode on brinjal plants using rhizobacterial treatments. This behavior may have the involvement of elevation of plant defenses under the activity of bacterial treatment, hence termed as induced systemic resistance (ISR). This study will provide an efficient control strategy for the nematode disease, and will also help researchers to understand the basis of resistance in brinjal plants.

**MATERIALS AND METHODS**

**Procurement of Host plant and RKN:** Different eggplant cultivars cultivated in district Lahore were procured from various agricultural farmhouses and screened for the susceptible cultivar for RKN attack. The most virulent strain of RKN was procured from Plant Nematology Lab, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.

**Experimental design:** The susceptible cultivar was subjected to pathogen RKN (host pathogen system) under greenhouse conditions (22±2 °C) to minimize the influences of environmental factors. Rhizobacterial species which have been already assessed for successful induction of systemic resistance in vegetable crop plants (other than brinjal) were procured from Fungal Biotechnology Lab, Fermentation Lab, and First Fungal Culture Bank of Pakistan (FCBP). Inducer species include Bacillus sp IAGS-571 (RB1), B. fortis (RB2), B. farraginis (RB3), B. thuringiensis IAGS 174 (RB4) and B. subtilis IAGS 572 (RB5). One set of pots without bacterial treatments were injected with M. incognita to serve as control treatment.

**Experimental setup:** Two weeks old susceptible brinjal seedlings grown in transplant trays were transferred to the pots of 14” diameter (@ 3plants/pot). Pots were filled with organic brinjal cultivation media (formulated by mixing 9 quarts compost, 3 quarts garden soil, 1/2 cup blood meal, 3 quarts sawdust and 1/2 cup bone meal). Organic growth media was obtained from Vegetable Cultivation Farms of University of the Punjab, Lahore. Two weeks after transplantation; plants were inoculated with freshly processed inducer bacteria according to RCBD (experimental design). Bacterial inoculum was prepared according to Markey J. (1996) and the prepared suspension was constantly stirred to keep it well mixed. Each Rhizobacterial strain suspension (RB1, RB2, RB3, RB4 and RB5) was applied as a treatment in a separate set of pots leaving one set without bacterial treatment to serve as ‘control’. Each treatment contained 3 replicates ensuring the reliability of results.

**RKN inoculum preparation and application:** Pure culture of RKN was maintained in-vivo on eggplant roots. Inoculum of RKN was prepared from mother culture; egg masses were stained, separated, processed and hatched to obtain second stage juveniles (J2s). J2s from 3 month old infected eggplant soil were extracted using modified Baermann method (Luc et al., 2005) and nematode suspension was obtained after 48 hours. Brinjal plants having bacterial inoculations were treated with second stage juveniles of M. incognita @ 250-300 per plant after 8 days of bacterial inoculations.

**Determination of bacterial activity:** Plants were incubated for 30 days post RKN application. Randomly, three plants were uprooted from each treatment to interpret deviations among different treatments. The bacterial efficacy was assessed by counting egg masses per root and number of eggs per egg mass and by biochemical analysis.

**Histological analysis:** Evaluation of host resistance boosted by rhizobacteria against nematode was accomplished by determining the disease incidence, shoot length, root length, shoot weight and root weight. Average lengths and weights from three replicates of shoot and root were taken under consideration.

**Determination of Disease incidence:** Each root system was weighed and finely chopped with scissors to 2 cm for assessment of egg masses. Determination of egg masses of M. incognita on the root system of the brinjal plants was carried out by staining roots with Phloxine B (Thies et al., 2002). This method was optimized for root knot nematode estimation. Stained egg masses were disassemble out from root and were given dynamic stirring on magnetic stirrer by immersing in Sodium hypochlorite (NaOCl, 1.0%) solution for 10 min to unshackle nematode eggs from egg mass (Stanton and O'Donnell, 1994). Number of eggs per egg mass was determined by selecting 10 egg masses from each plant; eggs were dispersed with 0.26% Sodium hypochlorite and three 0.5mL aliquots of the resulting egg suspension were counted under a dissecting microscope and the number of eggs per egg mass was calculated.

**Biochemical analysis:** Biochemical investigations were carried out in extracts using standard procedures for the
precise quantification of chemical ingredients in the
brinjal plants triggered by rhizobacteria against RKN. Biochemical assays for each treatment were
independently repeated thrice to sustain reliability of
results.

**Phenol Assay:** Pre-weighed leaves (1 g) of uprooted
plants from all treatments were plucked and crushed in a
mortar in 10 mL of 80% methanol; homogenate was
centrifuged at 10,000 rpm for 10 min. After evaporation
of supernatant, the residue was liquefied in few drops of
distilled water. Folin-ciocalteau reagent (0.25 mL) was
added following the dilution formation of 0.2 mL of
solution with 3 mL distilled water. It was assorbed for 3
min and then 1 mL of 20% (w/v) sodium carbonate was
added to it. Extract tubes were placed in boiling water for 1
min and cooled. The spectrophotometric absorbance
was recorded at 650 nm. The phenol activity was
-calculated using the following formula and was expressed
in mg catechol g⁻¹ of plant tissue (Zieslin and Ben-Zaken,
1993).

\[
Y = 0.067 \times E + 0.01
\]

\( Y \) = Phenolic contents
\( E \) = Absorbance of spectrophotometer

**Determination of Terpenoids:** Unit weight (1 g) of
leaves of uprooted plants from all treatments was
separately added to petroleum ether (10 mL) for 15 min.
The solution was filtered and its absorbance was checked
at 420 nm (Alqasoumi and Abdel-Kader, 2012).

**Estimation of Carotenoid:** Leaves of brinjal plants were
extracted with 80% acetone. The originated extracts were
centrifuged for 5 min, and then the supernatant was
accumulated. For carotenoid contents the absorbance of
the supernatant was recorded at 480 and 510 nm, on a
spectrophotometer in accordance with Maclachlan and
Zalik (1963). The quantity of carotenoid was uttered as
mg g⁻¹ fresh weight.

**Determination of Ascorbic acid:** To estimate ascorbic
acid contents, 5 mL of leaves extracts were collected into
100 mL flask and 10 mL of 4% oxalic acid was adjoined
and titrated against the dye solution. The product
manifested the pink color, and amount of ascorbic acid in
brinjal leaves was equivalent to the dye consumed
(Ibitoye, 2005).

**Assessment of Peroxidase (PO):** Leaves of uprooted
plants from all treatments were plucked, washed and
-crushed separately in a mortar in 1 mL of 0.1 M
 phosphate buffer (pH 7.0). The homogenate was
centrifuged at 14,000 rpm at 48 °C for 15 min and the
supernatant was used as enzyme source. The reaction
mixture (0.5 mL of enzyme extract, 1.5 mL of 0.05 M
pyrogallol and 0.5 mL of 1% H₂O₂) was nurtured at
(28±2 °C) temperature. Changes in absorbance were
recorded after consecutive 30 second intervals for 3 min
at 420 nm, and the boiled enzyme preparation assisted as
a blank. Change in absorbance of the reaction mixture
min⁻¹ mg⁻¹ of protein was articulated as enzyme activity
(Hammerschmidt et al., 1982).

**Polyphenol oxidase (PPO):** PPO activity in brinjal
leaves was determined by adding 50 µL leaf extracts to 3
mL of a solution comprising 100 mM Potassium
Phosphate buffer in specified circumstances (pH 6.5 and
25 mM pyrocatechol). Change in light absorbance was
recorded at 410 nm during 10 min at 30 °C (Gauillard et
al., 1993). One PPO unit was expressed as the distinction
of absorbance at 410 nm per mg soluble protein per min.

**Ribotyping and phylogenetic analysis:** Ribotyping of B.
subtilis-RB5 was carried using the method and primer set
(F: GACTGAGACCGGCCAG; R: AAAAAACATGCTACCAGCAG) of Yasin and Ahmed
(2015). The reaction mixture of PCR contained each
primer concentration 0.5 mM, deoxyxynucleoside
triphasphate mixture 0.8 mM, MgCl₂ 1.5 mM, Taq DNA
polymerase 0.6 U and genomic DNA of the bacterial
strain 20 ng to maintain the total volume of 25 µL. The
resulting amplification was sequenced and subjected to
BLAST analysis. The phylogenetic tree was developed
through software CLUSTAL-W (FS Foundation, Boston,
USA) available at NCBI genomic database. While the
sequence analysis data were provided to get more reliable
phylogenetic tree.

**Statistical Analysis:** Results were statistically analyzed
using DSAASTAT (Onofri, Italy) for their significance
through ANOVA and Duncan’s Multiple Range Test
(DMRT) at p=0.05. Data were analyzed for significant
changes in disease incidence, shoot length, shoot weight,
root length and root weight due to the application of
different treatments. Each change in alphabetic letter
represented the significant difference among recorded
data of different treatments.

RESULTS

**Histological Analysis:** Pre-invasion application of
bacterial formulations suppressed the development of
nematodes resulting in reduced number of females and
egg masses. Delayed development of juveniles into adult
nematode (♀ and ♂) due to nematocidal effects of
rhizobacterial treatments was purposeful and it was
perceived that root galling was reduced in treated brinjal
plants than control treatment (Figure 1). Plant growth was
variable according to treatments. Growth was severely
affected by nematode treatments in terms of shoot length,
shoot weight and root length. However, the root weight
was increased probably due to the pattern of giant cells in
root galling.
Figure 1: Root galls on the infected brinjal plant viz healthy plant

![Figure 1: Root galls on the infected brinjal plant viz healthy plant](image)

Figure 2. Plant growth analysis of control and bacterial treated brinjal plants. Plant growth parameters i.e. disease incidence (A), shoot length (B), shoot weight (C), root length (D) and root weight (E) were analyzed for different bacterial treatments (i.e. RB1, RB2, RB3, RB4 and RB5) and compared with negative control. Data were statistically analyzed through Analysis of Variance (ANOVA) and Duncan’s Multiple Range Test (DMRT), using MS-Excel statistical package DSAASTAT (Onofri, Italy) at p=0.05. Error bars represent the standard error among experimental treatments, and each change in alphabet represents the significant difference among data.
Figure 3: Quantification of defense related biochemicals i.e. phenolic contents (A), terpenoids (B), carotenoid (C), ascorbic acid (D), peroxidase (E) and polyphenol oxidase (F) in brinjal plants treated with different rhizobacterial treatments (i.e. RB1, RB2, RB3, RB4 and RB5). Data were statistically analyzed through Analysis of Variance (ANOVA) and Duncan’s Multiple Range Test (DMRT), using MS-Excel statistical package DSASTAT (Onofri, Italy) at p=0.05. Error bars represent the standard error among experimental treatments, and each change in alphabet represents the significant difference among data.
**DISCUSSION**

Brinjal plants when treated with different bacterial strains corresponded with provoked resistance and induced plant defense mechanisms against *Meloidogyne incognita*. Brinjal plants were treated with five different rhizobacterial species (*Bacillus subtilis, B. thuringiensis, B. fortis, B. megaterium* and *Bacillus* species) that were already checked against foliar diseases caused by fungus. An unchecked treatment was also observed for comparison in which only nematodes were applied.

Findings described the histological changes in plants grown under treated and controlled conditions. Significance difference was observed in the shoot length, shoot weight, root weight, root length and disease percentage. Shoot length of plant significant difference in terms of plant length when treatments were applied. This is mainly because of the formation of giant cells in roots caused by nematode feeding. These giant cells endow with a nutrient sink on which nematode nourish. Consequently, the plant roots were no longer able to supply nutrients to whole plant. Therefore, the growth of plant parts above the soil surface is severely affected and restricted (Mohri et al., 2005).

It was observed that plants of control treatment exhibited reduced root length; however, they showed increased root weight. The major reason behind this phenomenon is the formation of galls on roots. Those root galls multiplied the weight of the roots; however, the length of the roots remained shortened. It proved that galls formation hindered the enlargement process of the roots, while reducing the absorption area of the roots. Moreover, it also decreased the soil area from which plants could procure nutrients (Huang et al., 2015).

Findings demonstrated that treatment RB1 was significantly superior to control treatment with respect to plant height. Moreover, RB3 and RB4 significantly increased plant height than RB1 and RB2. Shoot of the Brinjal plant under different rhizobacterial treatments provided unusual outcomes and no pattern could be developed between bacterial strains and their potential to...
induce plant height. Similar results were also recorded by Yasin and Ahmed (2015) after treating rose plants with biocounter agents. This indicates that microbe-plant interactions are very complex based relations upon complex cascades of genes and proteins (Ahmad et al., 2014b). Root length of the brinjal was affected by application of treatments. In this case, RB5 was very supportive to root length, and showed the highest value than all other treatments. Roots of the Brinjal plants demonstrated variations in terms of root weight. Higher weight of roots in control plants can be due to nematodes gall formation. Increased root weight of control treatment can never be useful for plants with respect to nutrients uptake because roots had decreased length and lessened soil area to procure nutrients. Therefore, gall formation not only hinders the translocation of nutrients, but also decreases the nutrients absorption (Melakeberhan et al., 1987).

Likewise the apparent dissimilarity, significant differences were observed during the biochemical analyses of the plant leaf extracts. In bacterial treated plants biochemicals produced work as defensive agents against pathogen in brinjal and seized the nematodes attacks (Mohri et al., 2005). However, the bacteria used in this study played their part as systemic resistance inducer. The main evidence of their resistance induction activity is the increased quantities of defense related biochemicals. Those elevated defenses retarded nematode attack and reduced the development of root galls in brinjal plants, resulting into improved growth of above soil surface plant parts. This study proved that biological inducers of systemic resistance are equally effective against foliar as well as root pathogens.

In plants a large variety of secondary products formed that includes a chemically heterogeneous group called Phenol. These compounds could be significant part of the plants protection system against pests including root parasitic nematodes (Wuyts et al., 2006). Present study provided the exact quantity of phenolic contents and their change within brinjal cultivars by rhizobacterial activity. Change in phenolics also strengthens the conclusions of previous investigations of plant antinematode resistance. High concentrations of phenolics in the leaves and roots of tomato determined the resistance against M. incognita (Bajaj and Mahajan, 1977). In this study, RB5 recorded the highest quantities of phenolic compounds produced, hence ensured the elevated plant resistance against pathogens.

One of the most significant groups of plant pigments is carotenoid that plays a crucial role in determining the quality factor of fruit and vegetables (Van den Berg et al., 2000). In case of terpenoids all the treatments showed noticeably deviating result from others. RB5 produced maximum carotenoid contents than all other treatments. Same results were obtained in case of terpenoids proving RB5 as the best inducer treatment.

Various investigations proposed capacity for ascorbic acid in the defense mechanism of plants (Arrigoni et al., 1979; Melillo et al., 1983). Quantity of ascorbic acid in susceptible tomato (Solanum lycopersicum L.) cultivars was described to be lesser than in resistant cultivars (Arrigoni, 1979). Resistance of tomato plants to nematode infection diminished by reduced ascorbic acid contents in plant tissues (Arrigoni et al., 1976; Arrigoni et al., 1979 and Melillo et al., 1983). Here in case of ascorbic acid a great difference was observed between RB5 and control treatment, while RB3 and RB4 had non-significant difference to each other.

PO and PPO have been corresponding with provoked resistance and are concerned in numerous plant defense mechanisms like oxidative cross-linking of plant cell walls, lignin biosynthesis and production of vigorous oxygen species (Faize et al., 2004). Peroxidase makes cellular environment toxic and extremely unfavorable for pathogen by producing reactive species of oxygen and nitrogen (Passardi et al., 2005; Gill and Tuteja, 2010; Schaffer and Bronnikova, 2012). The most interesting feature of peroxidase is that it shows its activity only under the attack of a pathogen or any other stress conditions. Hence, do not exert extra pressure on plant defense machinery and makes wise use of energy resources (Mika et al., 2004; Liu et al., 2010). Peroxidase activity in nematodes infected roots of tomato were considered, there total peroxidase activity was twice in resistance plants as compared to susceptible (Zacheno et al., 1993). All above described studies provide reasoning upon importance of peroxidase elevation in enhanced plant resistance and present study is the reminiscent of all the above described investigations. Peroxidase and polyphenoloxidase showed variation gradually, RB5 is dominant in case of PO and PPO production as compared to all other treatment. There was significance difference in all the treatment as compared to controlled one (Figure: 3E &3F).

Recorded data from experiment showed nematieidal efficacy of bacteria hindered the progress of invading nematodes. In response to applications occurrence of phytochemicals in plants, for example presence of peroxidase and phenol in plants leaves were used to check resistance or susceptibility of host plant against M. incognita. Comparison of host-parasite relationships of M. incognita and Pratylenchus penetrans have been carried out on three cultivars of tomato (Hung and Rohde, 1973). It was reported that large number of larvae of M. incognita and P. penetrans on no account pierce the resistant cultivar of tomato due to some sort of inhibition that was provided by phenol compounds.

The study also describes phylogenetic relation among the bacterial inducer species and already reported other species. There are many studies showing that closely placed species in phylogenetic map also exhibit
similar characters (Krimitzas et al., 2013; Igea et al., 2010). Therefore, the bacterial species found in the phylogenetic map are potentially resistance inducer species. However, more studies are required to explore the extent of their resistance induction potential. Phylogenetic tree also represents the evolutionary history and relationships among different organisms and strains. Phylogenetic diagram in this study reveals that the most promising bacterial strain has evolved into only eleven bacterial strains. It proves the stability of the bacterial genome and reliability of its resistance induction potential as well (Igea et al., 2010). It can be concluded that the bacterial species can be applied safely applied in brinjal fields for disease control without evolutionary risks.

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