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PHYSIOLOGICAL CHANGES AGAINST *MELOIDOGYNE INCOGNITA* IN RHIZOBACTERIAL TREATED EGGPLANT UNDER ORGANIC CONDITIONS

[*]A. Ibrahim[1], A. A. Shahid[2], S. Noreen[3] and A. Ahmad[1,4]

[1]Institute of Agricultural sciences, University of the Punjab, Lahore-54590, Pakistan
[2]Center for Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore-54590, Pakistan
[3]Institute of Pure and Applied Biology, Bahauddin Zakariya University. Multan-60800, Pakistan.
[4]Institute for Medicinal Plants, College of Plant Science and Technology, Huazhong (Central China) Agricultural University
[*]Corresponding Author Email: asmaibrahim12@gmail.com

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 (un-segmented 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. arenarian* (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 assorted 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 l0 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% H_2O_2) 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: GACTGAGACACGGCCCAG; R: AAACCACATGCTCCACCGCT) of Yasin and Ahmed (2015). The reaction mixture of PCR contained each primer concentration 0.5 mM, deoxynucleoside triphosphate mixture 0.8 mM, $MgCl_2$ 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 nematicidal 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

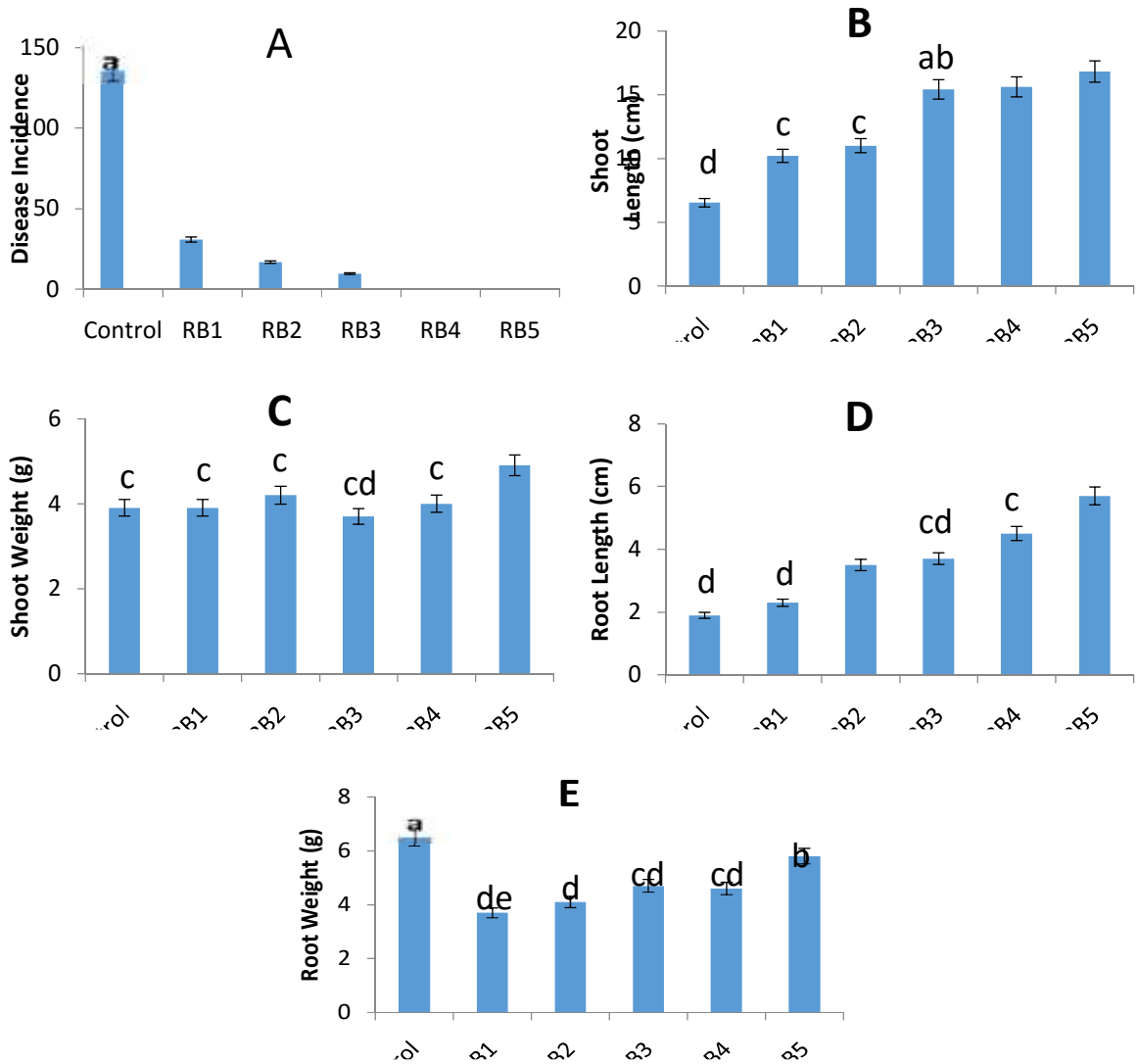

Figure 2. Plant growth analysis of control and bacterial treated brinjal plants. Plant growth parameters i.e. disease incidence (A), shoot length (B), shoot weight (C), root length (D) and root weight (E) were analyzed for different bacterial treatments (i.e. RB1, RB2, RB3, RB4 and RB5) and compared 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.

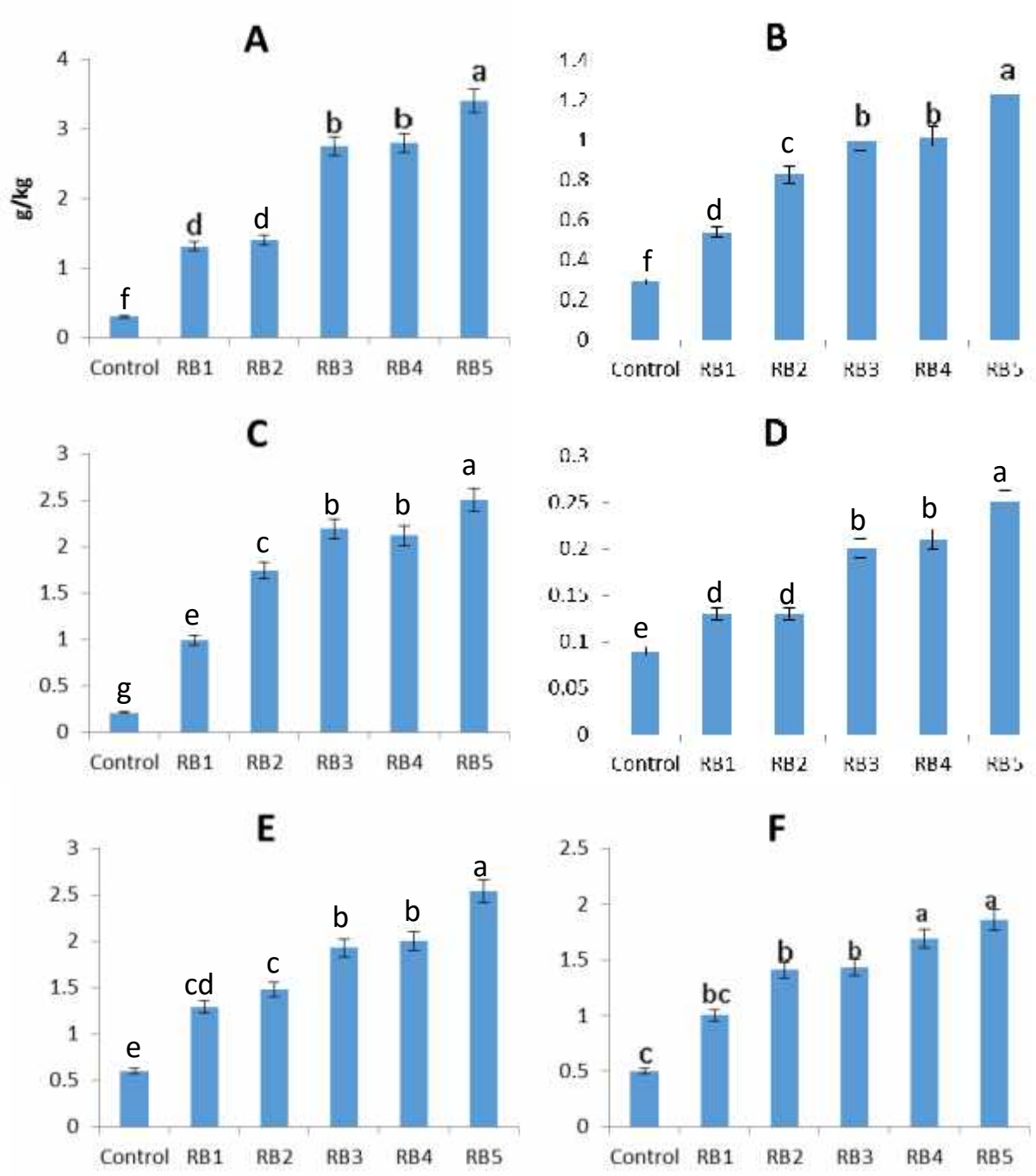

Figure 3: Quantification of defense related biochemicals i.e. phenolic contents (A), terpenoids (B), carotenoid (C), ascorbic acid (D), peroxidase (E) and polyphenol oxidase (F) in brinjal plants treated with different rhizobacterial treatments (i.e. RB1, RB2, RB3, RB4 and RB5). Data were statistically analyzed through Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT), using MS-Excel statistical package 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.

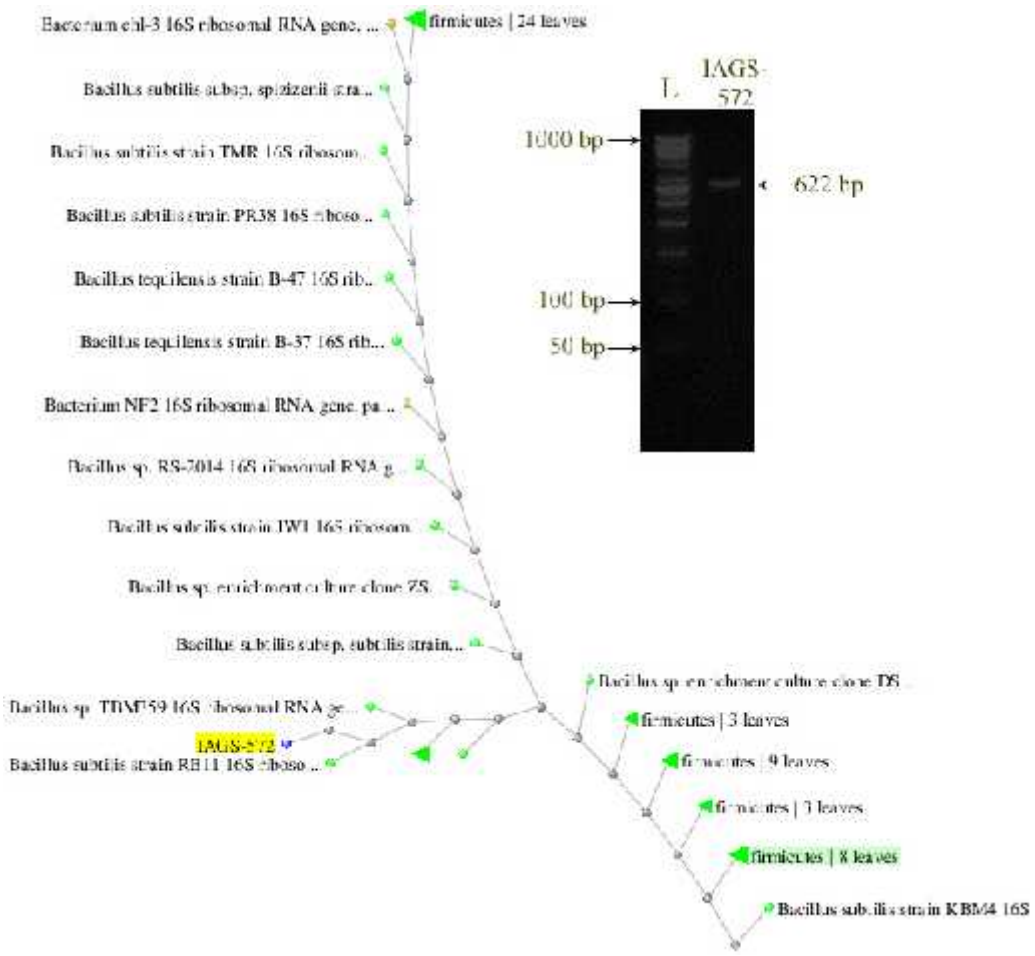

Figure 4: Electrophoresis gel image of ribotyping of *Bacillus subtilis* (IAGS-572), and dendrogram showing phylogenetic distances among *B. subtilis* and different other microbial strains. Bacterial strain IAGS-572 has been highlighted to focus its position among other microbial strains.

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 biocontrol agents. This indicates that microbe-plant interactions are very complex relations based 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 anti-nematode 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 plat 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 (Zacheo *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 polyphenyloxidase 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 nematicidal 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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