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Plant-microbe interactions endorse growth by uplifting microbial community structure of *Bacopa monnieri* rhizosphere under nematode stress

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ABSTRACT

The modification of rhizosphere microbial diversity and ecological processes are of rising interest as shifting in microbial community structure impacts the mutual role of host–microbe interactions. Nevertheless, the connection between host-microbial community diversity, their function under biotic stress in addition to their impact on plant performances is poorly understood. The study was designed with the aim to analyze the tripartite interactions among *Chitiniphilus* sp., *Streptomyces* sp. and their combination with indigenous rhizospheric microbial population of *Bacopa monnieri* for enhancing the plant growth and bacoside A content under *Meloidogyne incognita* stress. Overall, plants treated with the microbial combination recorded enhanced growth as illustrated by significantly higher biomass (2.0 fold), nitrogen uptake (1.8 fold) and bacoside A content (1.3 fold) along with biocontrol efficacy (58.5%) under nematode infected field. The denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S-rDNA revealed that microbial inoculations are major initiators of bacterial community structure in the plant rhizosphere. Additionally, the plants treated with microbial combination showed maximum diversity viz., Shannon's (3.29), Margalef's (4.21), and Simpson's (0.96) indices. Likewise the metabolic profiling data also showed a significant variation among the diversity and evenness indices upon microbial application on the native microflora. We surmise that the application of beneficial microbes in combinational mode not only helped in improving the microbial community structure but also successfully enhanced plant and soil health under biotic stress.

1. Introduction

Plant rhizospheric zone is the superlative example of microbial activity based on the defense of the root system against phytopathogens which indirectly and directly bestow multifarious benefits to the plant health (Mendes et al., 2014). Rhizospheric soils have reemerged as a recent area of research because of the growing emphasis on the sustainable and organic agriculture. In addition, microbial alteration is being looked upon as a strategy to reduce the exploitation of chemical fertilizers or pesticides for soil-borne disease management (Cha et al., 2016). Plant diseases cause considerable agricultural yield losses annually, and management of phyto-nematodes is one of the prime agricultural management concerns faced by the farmers worldwide. Recently, the application of antagonistic microorganisms such as chitinolytic microbes to the rhizospheric soil has been shown to have suppressive property against plant diseases caused by phyto-nematode along with the additional benefit of providing ecological fitness to their host plant (Bhattacharya et al., 2007; Gupta et al., 2017a). Importantly, the combination of microbes meets the requirements for sustainable agriculture by minimizing the use of chemical fertilizers and nematicides in crop production. The microbes with beneficial activities are well known for their usefulness as biocontrol since they reside in the rhizospheric region of host plants, facilitating the vanguard resistance to the plant roots (Daayf et al., 2012; Banuelos et al., 2014). Thus, since last decade in order to achieve the said target; exploitation of the beneficial antagonistic microbes has been vigorously validated in the natural conditions (Kavino et al., 2010; Gupta and Pandey, 2015).

Various biotic stresses affect many fundamental processes that collectively lead to the truncated plant growth. In addition to the harmful impact on the host, pathogenicity of nematodes has also been widely known to shift the microbial dynamics by manipulating rhizodeposits released by the host plant (Briar et al., 2011). The rhizospheric microbial communities are manipulated by the plant roots that are known to secrete numerous forms of organic substrates (Studer et al., 2016).

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The quantity and composition of these organic substances are distinct in diverse plant species and cultivars which also alter during the plant growth development including old and young parts of the root system (Brink, 2016). Consequently the diversity of rhizospheric bacterial communities which frequently utilize the organic substances that are secreted by plant root also changes (Hirsch et al., 2013). Utilization of antagonistic strains has been frequently reported in context with the plant disease management; a part of which also plays a crucial role in amelioration of the indigenous rhizospheric microflora (Felici et al., 2008; Singh et al., 2015). An important factor in the development of antagonistic microbes as an efficient biocontrol for commercial application includes their communication with the indigenous dynamics of rhizospheric microflora. However, the possible environmental impact related to the microbial inoculation has not yet been explored much. To date, no comprehensive relationship has been established between microbes mediated changes in microbial community structure and function effect on plant performance along with concomitant suppression of diseases caused by phyto-nematodes.

Bacopa monnieri (L.) Pennell (Scrophulariaceae), an economically important medicinal plant is commonly grown in Asia, Australia, and America. Recent studies have proved that its active constituent used in the treatment of insomnia, insanity, depression, psychosis, epilepsy, asthma and to improve memory related functions (Russo and Borrelli, 2005). According to the National Medicinal Plant Board (NMPB, India), this medicinal plant was placed second in a priority list of the most important medicinal plants and the annual cultivation of *Bacopa* is growing day by day owing to the application of its active constituent bacosides. The plant is commonly invaded by the root knot nematode, *Meliodogyne incognita* (Gupta et al., 2017a). The *M. incognita* is an avid pest which enters in roots and forms root galls thereby decreasing the worth of the plant.

Therefore, the present investigation was aimed to determine the influence of chitinolytic microbe's viz., *Chitiniphilus* sp. MTN22 and *Streptomyces* sp. MTN14 single as well as in combination on the stability of native rhizospheric bacterial communities under *M. incognita* stress in *B. monnieri.* Further, the molecular abundance of bacterial 16S-rRNA, *chiA* gene (chitinase) and PCR-DGGE (denaturing gradient gel electrophoresis) of phylogenetic gene (16S-rRNA) was performed to analyze the bacterial community diversity shift during plant-microbe-nematode interactions in rhizospheric soil samples. Simultaneously, the microbial carbon utilization profiling (Biolog) of rhizospheric soil provides a functional parameter to analyze the metabolic potential of rhizospheric microflora. This is the first report that has used gene-based methodologies and biolog data for elucidating the shifting of the rhizosphere microbial community function under biotic stress.

2. Materials and methods

2.1. Bioinoculants and nematode inoculum preparation

Chitiniphilus sp. MTN22 (KF699070) and *Streptomyces* sp. MTN14 (KF699062) were isolated from *B. monnieri* and chosen on the basis of their nematicidal and chitinolytic activities explored previously (Gupta et al., 2017b). For mass production, the culture was inoculated and the cell density of the microbes was maintained at 10^8 colony forming units (CFU in 0.85% saline) ml^{-1}. The pathogen, *M. incognita* culture was regularly maintained on *Solanum melongena* L. pusa purple long at greenhouse level. The nematode inoculum was prepared using a modified extraction technique of Hussey and Boerma (1981) followed by Baermann funnel method (Hooper et al., 2005).

2.2. Field experimentation

Field trials were performed at experimental farm, CSIR-CIMAP, Lucknow (26.85 °N, 80.92 °E). The soil of experimental plot was sick with pathogen; *M. incognita* so that the treatment (without pathogen) was eliminated. Initially, the average population of phyto-nematodes *M. incognita*, *Tylenchorhynchus vulgaris* and *Pratylenchus thornei* population present in the soil were 652/200 cm^3, followed by *T. vulgaris* (183/200) cm^3 and *P. thornei* (91/200 cm^3) in ratio of 7:2:1. The *M. incognita* density in all the plots was the highest compared to *T. vulgaris* and *P. thornei* population, therefore a detailed investigation was performed only for *M. incognita*. The infected field soil was sandy loam in nature and alkaline in nature (pH 8.2, EC 0.42 $dS\,m^{-1}$) containing 158:115:11.42 $kg\,ha^{-1}$of N: P: K along with ∼55% water holding capacity. The *B. monnieri* runners were transplanted in microplots (12 m^2) using randomized complete block design at a spacing of 30 x 45 cm between each plot of treatments. Each plot contained six rows consisting of six plants in each. Proper moisture was given at 3–4 days intervals. On maturation of the plant (90 days) the result for various growth paramerters such as fresh and dry herb yield were recorded. Extraction and estimation of bacoside A content was performed via high-performance liquid chromatography (HPLC) analysis according to Gupta et al., (1998). Root knot index (RKI) were examined according to Oka et al., (2007) on 0–10 scale (based on nematode population per root) to determine the level of pathogenicity.

Nutritional value (N, P, K^+ and Na^+) of dry plant samples were determined by Jackson et al. (1973). Kjeldahl method was used to analyze the total available N content in plant sample whereas, the amount of K and P was measured with HNO_3 + H_2O_2using flame photometer and FIA (Flow Inject Analyzer Foss FIASTAR 5000), respectively. The concentration of Na^+(with 2:1 nitric (HNO_3)-perchloric acid ($HClO_4$) mixture) was calculated via a flame photometer.

2.3. Enzymatic studies

The amount of chlorophyll and carotenoid was analyzed according to Wojtyla et al., (2016). The powdered leaves of *Bacopa* plant samples (100 mg) were extracted with CH_3OH:H_2O (9:1, v/v) solution. The absorbance of the samples was recorded at 663, 645, 480 and 510 nm. Lipid peroxidation (LPO) of membrane lipids was calculated by measuring the increase in absorbance at A_{535} due to the formation of an insoluble pink coloured thiobarituric acid (TBA) - malondialdehyde complex (Hodges et al., 1999). Total phenolic content (TPC) was quantified by using Folin–Ciocalteu method (Rover and Brown, 2013) and the results were expressed as mg gallic acid equivalent per g of dried sample. Total flavonoid content (TFC) was measured by recording the absorbance of samples at 510 nm according to Bajalan et al., (2016) and concentration was calculated by using the standard curve of rutin ($mg\,g^{-1}$). Free radical scavenging activity (FRSA) was analyzed using the 1, 1-diphenyl-2-picryl-hydrazil (DPPH) reagent method by taking the absorbance of *Bacopa* samples at 517 nm as described by Hussain et al., (2008). Total antioxidant capacity (TAC) was measured using ascorbic acid as a standard by phosphomolybdate method (Fraga et al., 2014).

2.4. Soil DNA extraction and chitinase activity measurement

Total soil DNA was extracted from rhizospheric soil samples of (250 mg) of *B. monnieri* treated with selected microbes using the Power Soil DNA Isolation kit (MoBio Laboratories, USA) and eluted into 50 μl sterilized RNAse-DNAse free water according to the manufacturer's protocol. Extracted DNA was further quantified using a NanoDrop apparatus (ThermoFisher Scientific, Germany) and confirmed by 0.8% gel electrophoresis. For Polymerase chain reaction (PCR) amplification; the extracted DNA was diluted 10 times and stored at −20 °C. The soil chitinase activity from each treatment pot^{-1} was analyzed using 4-methylumbelliferyl-$(GlcNAc)^2$ [4MU- $(GlcNAc)^2$] method. A standard curve was prepared by using 4MU (Sigma-Aldrich, USA) and the result was expressed in units to millimoles of 4MU hydrolyzed per $ml^{-1}\,g^{-1}$ soil (Johnson-Rollings et al., 2014).

2.5. Quantitative PCR based on 16S rRNA and chiA genes

Quantitative Real time PCR of the 16S rRNA and *chiA* genes was carried out with Real-Time PCR System (Applied Biosystems, the Netherlands) by using a Maxima SYBR Green system (ThermoFisher Scientific, Germany). PCR involving two different primer sets, 357 F (5′ CCTACGGGAGGCAGCAG-3′) and 907R (5′ − CCGTCAATTCFTTTRAG-TTT-3′) for 16S rRNA gene (V3-V5 region) and GA1F (5′- CGTCGACA TCGACTGGGA-3′) and GA1R (5′-ACGCCGGTCCAGCC-3′) was used for the *chiA* gene. The conditions of PCR were established according to the manufacturer instructions by the fluorochrome detection system. The number of copies of gene in particular soil sample was analyzed by the standard curve (eight pointed) of 10^1 to 10^8 copies of V3-V5 region of 16S rRNA (approximately 586 bp) and *chiA* (approximately 450 bp) gene products amplified from the pure template DNA of chitinase producing strain (*Streptomyces griseus*).

2.6. PCR- Denaturing gradient gel electrophoresis (DGGE) profiling of bacterial V3-V5 region (16S rRNA)

The V3-V5 region of 16S rRNA gene was amplified by nested PCR of 16S rRNA gene using the above primers set 357 F (with GC clamp) and 907R. All the PCR reactions were carried out by using 25 ng (per µl) of template DNA and the PCR cycle consisted 95 °C for 5 min (initial denaturation), followed by further denaturation at 94 °C for 1 min, annealing at the 55 °C for 1 min and elongation at 72 °C for 1 min for 40 cycle followed by a one cycle of final elongation step (72 °C for 10 min). DGGE was carried out using a DCode universal mutation detection system (Bio-Rad Laboratories) as described in the manufacturer's instructions. The amplified products of V3-V5 region (with GC clamp; 100 ng) were loaded onto 6% (w/v) polyacrylamide gels (40% acrylamide/bis 37.5:1; BioRad) with gene-specific denaturing gradient (7 M urea and 40%, v/v, formamide) (Chen et al., 2012). The separation of fragments in gel was done in running buffer (TAE buffer; 40 mM Tri-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) at 60 °C, 15 min at 200 V, followed by 75 V for an additional 16 h. Further, the gel was stained with EtBr (10 mg mL^{-1}) in 1X TAE buffer for half an hour before being photographed. The soil microbial community diversity indexes such as Shannon_Wiener, [$H = \Sigma^S$ Pi ln pi], Margalef's [$M = (S-1) / \ln(n)$] and Simpson [$C = \Sigma Pi^2$] index were also measured, where (Pi) is the ratio of the activity on a specific substrate to the sum of activities on all present substrates (S) is the total band number in one sample, and (n) is the number of bands present in the test sample.

2.7. Microbial diversity analysis using carbon source utilization pattern

Eco and MT plates of Biolog (Biolog, Inc., USA) were used to analyze the carbon utilization profiling of four different microflora rich rhizospheric soil samples (Nemergut et al., 2013). Different carbon substrates (2%) were dispensed in to each well of MT plates in triplicate and allowed for drying at room temperature in a sterile air laminar flow cabinet. Ten gram of each soil sample was shaken in sterile 0.85% saline (90 ml) for 60 min and diluted up to 10^{-3}. Subsequently, 150 µL of dilution was transferred in each well of Eco and MT plates of Biolog and incubated at 28 ± 2 °C for 7days. Utilization of carbon sources was indicated by Tetrazolium dye (redox indicator dye), which changed the color of well from colorless to purple. The rate of utilization was calculated regularly after 24 h by recording absorbance at 590. Carbon utilization pattern of each microplate was denoted by average well color development (AWCD) which was based on the absorbance during incubation (Nannipieri et al., 2003). The results were measured from day 1–7 at 590 nm. The samples absorbance values for the wells with C sources were blanked against the control well in the plate. The negative values were considered as 0 in subsequent data analyses. Microbial activity in each microplate was expressed as average well color development (AWCD) and was calculated as the mean of the control absorbance values for all the 95 response wells per reading time diversity, evenness indexes calculations and Principal component analysis (PCA) according to Button et al., (2016).

2.8. Statistical analysis

All the experiments were done in three technical replicates and three biological replicates. Analysis of variance (ANOVA) was applied using LSD (least significant difference) at significance level $P \leq 0.05$ to analyze significant difference between control and treatments by SPSS package (V21, SPSS Inc., Chicago, IL). The cluster analysis and dendrogram preparation was done using the NTSYSpc (2.2, Exeter Software, USA). The clustering was carried out on the basis of band patterns observed in DGGE gels.

3. Results

3.1. Plant growth, nematode management, nutrient uptake and bacoside A content

Significant ($P \leq 0.05$) increment in plant growth parameters was observed following the application of chitinolytic microbes treatments under field conditions (Fig. 1). The maximum increase in fresh and dry biomass was achieved when plants were treated with the microbial combination (2.0 and 1.9 fold, respectively) followed by alone *Streptomyces* sp. (1.7 and 1.6 fold, respectively), and *Chitiniphilus* sp. (1.5 and 1.4 fold, respectively) as compared to the control plants (Table S1). The maximum biocontrol efficacy was observed in the consortium of microbes (58.8%) followed by single microbial inoculation *Chitiniphilus* sp. (41.2%), and *Streptomyces* sp. (38.8%) treated plants (Table S1). Application of the microbes also play a significant role in the synthesis of bacoside A as its content was enhanced by 1.3 fold and 1.2 fold in dual combination and alone *Streptomyces* sp. treatments respectively followed by *Chitiniphilus* sp. as compared to the control set (Fig. S1). Content of nitrogen, potassium, and phosphorus was negatively affected under nematode-stressed condition which was further significantly improved by the application of microbes in the plants (Figs. S2a, b and c). Interestingly, the plants without any microbial application revealed a drastic elevation in the sodium levels as compared to the treated plants (Fig. S2d).

3.2. Effect of bioinoculants on the phytochemical activities

The content of total chlorophyll and carotenoid varied in different treatments depicted in Figs. S3a, b and c. Non-significant increment in chlorophyll b content was found in all the treatments (Fig. S3b). Whereas untreated plants under nematode-stress showed an increase in LPO content followed by marked reduction in chlorophyll content (Fig. S3d). The chlorophyll and carotenoid content of the plants was observed to be negatively correlated with the level of LPO under pathogenic stress condition.

In the present study, the applications of the microbes showed significantly higher activity of FRSA (1.2–1.3 fold) and TAC (1.2–1.5 fold) with respect to infected control plants (Figs. S3e and f). Similarly, in comparison to the control plants the application of microbes in plants under pathogenic stress condition was found to increase the level of total TPC (72.3–81.4 mg g^{-1}) and TFC (11.8–19.8 mg g^{-1}) (Figs. S3g and h).

3.3. Chitinase activity

Crude cell suspensions of rhizospheric soil were used to analyze the efficacy of potent microbes in terms of chitinase activity (Fig. 2a). The highest chitinase activity was found in dual microbial combination treated soil (2.7 fold) followed by *Streptomyces* sp. (2.2 fold) and *Chitiniphilus* sp. (1.8 fold). Compared to the control treatments, the

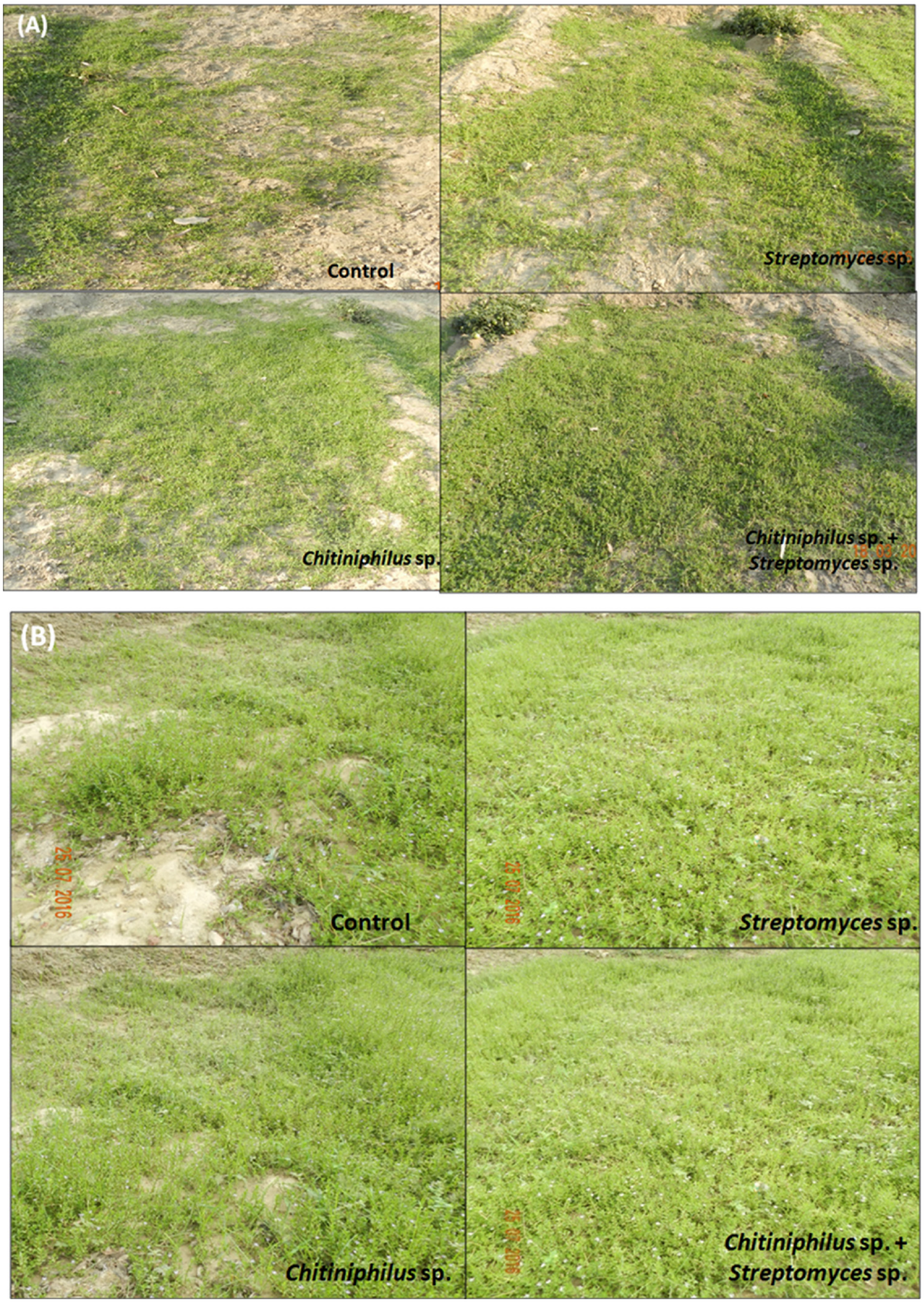

Fig. 1. Effect of *Chitiniphilus* sp. (CHI) and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations on plant growth (A) after 45 and (B) 90 days of *B. monnieri* under *M. incognita* infected field conditions. Control: without any microbial treatment.

consortium treated rhizospheric sample demonstrated significantly higher level of chitinase activity ($P \leq 0.05$).

3.4. Abundance of 16S-rRNA and chiA genes

RT-PCR based observations subjected to copy number of 16S-rRNA gene was used to determine the bacterial load in the rhizospheric soil (Fig. 2b). The dynamics of copy number (represents sizes of the bacterial communities per unit mass) showed distinctness across the treatments, maximum presence of 16S-rRNA gene copies g^{-1} soil in the microbial combination as compared to control. In fact, the plants treated with consortium showed highest abundance of 16S-rRNA gene rather than other treatments (Fig. 2b). Whereas, least intensity of copy number of gene was found in the control plants differing significantly from other treatments. Similarly, in case of *chiA*, the copy number of the gene also followed same pattern in the treatments as 16S-rRNA gene ranging from 2.2 to 4.9 gene copy numbers g^{-1} of soil samples. The least copy numbers of the genes were found in the single treatments *Chitiniphilus* sp. and *Streptomyces* sp. samples, while the microbial combination samples showed a tendency to have higher abundance (Fig. 2b).

3.5. PCR- DGGE analysis of bacterial 16S rRNA

Denaturing gradient gel electrophoresis based analysis of 16S-rRNA gene was performed to magnify the shift in microbial community after

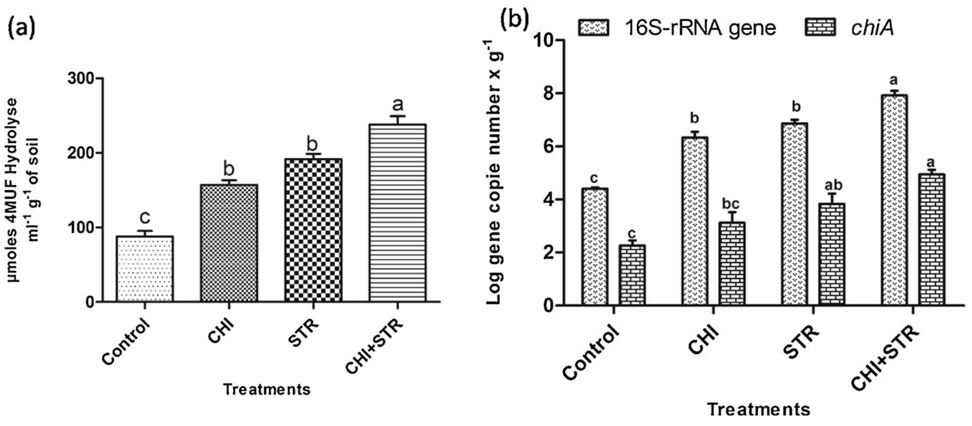

Fig. 2. Effect of *Chitiniphilus* sp. (CHI) and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations (CHI + STR) on chitinase activity measurements assayed with 4MU-(GlcNAc)^2 and real-time quantification of 16S rRNA and *chiA* genes across four different treatments (per gram of fresh material). Error bars represent standard error for triplicate assays on the same pooled soil sample. Different letters show significant differences among the control and treatments ($P \le 0.05$; LSD test).

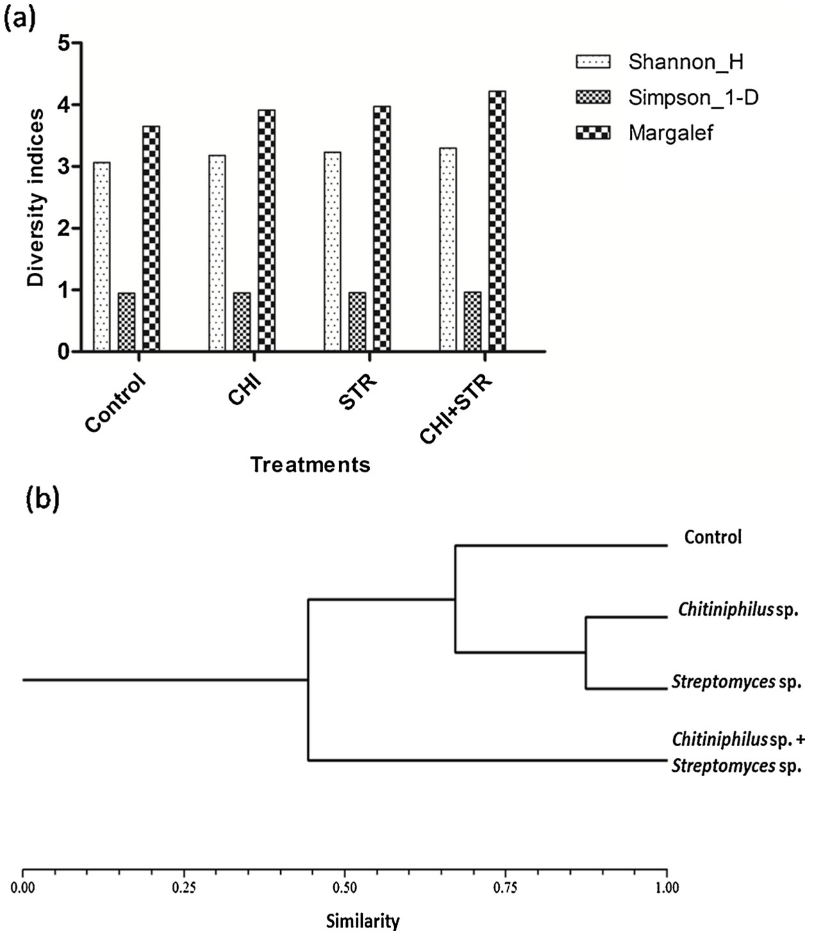

Fig. 3. (a) Diversity indices of bacterial microbial population and (b) dendrogram representing genetic similarity of bacterial-community profiles as estimated using DGGE bands generated from soil samples treated with *Chitiniphilus* sp. (CHI) and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations (CHI + STR) under field conditions.

the treatment of chitinolytic microbes under pathogenic stress condition. The treatment of different chitinolytic microbes positively manipulated the dynamics of bacterial community of host plants rhizosphere. DGGE based profiling of microbial diversity showed highest abundance of 16S-rRNA gene in consortium based application followed by the single treatments. Maximum number of prominent bands of 16S-rRNA gene with respect to bacterial population was observed in the rhizospheric soil supplemented with consortium as compared to other treatments and control (Fig. S4). Application of alone microbial treatment in the rhizospheric soil also showed significant pattern, but its frequency was lesser than the dual microbial treatment. In the case of the control soil (untreated) set least banding pattern was localized on gel as compared to the treated ones. Application of the different microbes positively regulated the bacterial diversity associated with the rhizospheric soil as revealed by the results of diversity (Shannon's), richness (Margalef's), and dominance (Simpson's) indices (Fig. 3a). Shannon's index and Margalef's index showed maximum diversity and richness in the microbial combination soil samples. Maximum value of Shannon's and Margalef's indices was observed in the application of consortium, whereas least was recorded in the rhizospheric soil of control treatment over other treatments pointing out the negative impacts of the nematode pathogencity on the diversity of soil microflora. The different diversity indices have been denoted in Fig. 3a. Sample based analysis of the bacterial community present in the rhizospheric soil showed two distinct major clusters; one of which comprised with microbial combination only whereas, the second group contained other treatments including untreated control and single microbial treatments (Fig. 3b).

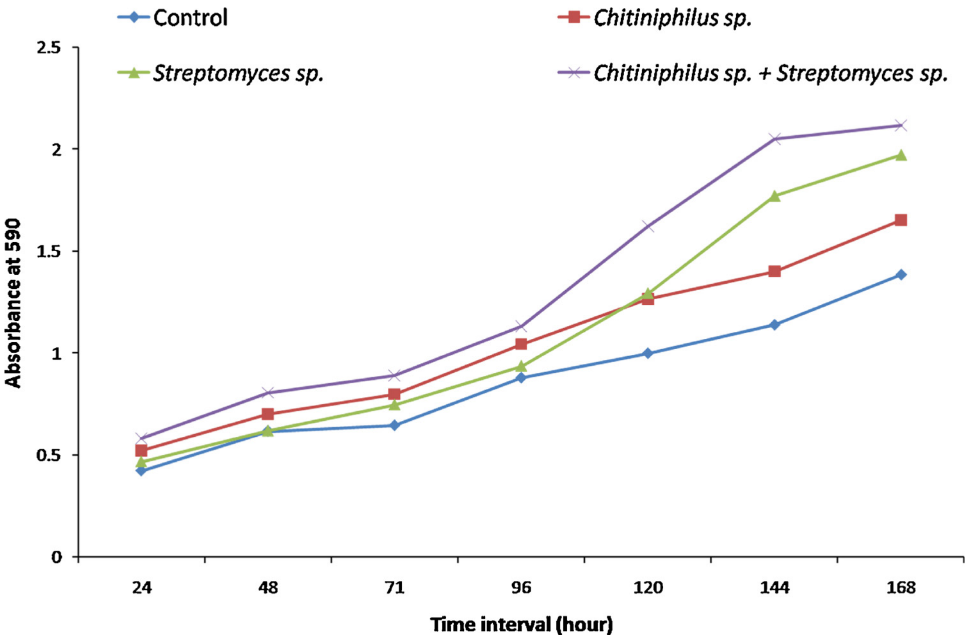

Fig. 4. Average well color development (AWCD) based on substrate utilization pattern on Biolog Eco plates in control (untreated), *Chitiniphilus* sp. (CHI), and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations (CHI + STR) in the *B. monnieri* rhizosphere microflora, respectively. Results were recorded for day 1–7 at 590 nm. Microbial activity in each microplate, expressed as AWCD.

3.6. Microbial diversity using carbon source utilization pattern

Average well color development (AWCD) was calculated to observe the impact of potent chitinolytic microbes (singly as well as in combination) on the carbon substrates utilization profiling of rhizospheric microflora on Biolog Eco plates under the pathogenic stress condition (Fig. 4). *Bacopa* rhizospheric soil treated with consortium showed highest carbon utilization pattern whereas the lowest utilization was observed in the control plants under pathogenic stress conditions (Fig. 4). We further analyzed the microbial community of rhizospheric soil using complex natural substrates generally found in plant root exudates by using Biolog MT plates.

Variation between all the samples was documented and analyzed by using different diversity indices (McIntosh, Shannon, and Simpson; Table 1). The plants treated with microbial combination revealed considerable ($P \leq 0.05$) variations in the diversity and evenness indices under nematode infection condition and recorded maximum enhancement followed by other treatments (Table 1). Principle component analysis was performed to exhibit the huge data of Biolog in a confined way. Furthermore, the result also describes the variation of the treatments and helps in understanding their diversity and distinctness among the soil samples. Out of the 31 carbon substrates included in the Biolog Eco plates, 20 different substrates such as carbohydrates, amino acids, cellulose and chitin derivatives were utilized significantly higher ($P \leq 0.05$) (Figs. S5 and S6). Substrate utilization pattern based metabolic profiling of four different rhizospheric soil samples confirmed the variation as they were distinctly different from each other (Fig. S5). Although in the present investigation, the metabolic abilities of microflora inhabiting in the microbial combination treated sample were displayed considerably higher utilization activities than those of the other treatments, the utilization pattern of different carbon substrates and the intensity of utilization were different among all the samples. Most of substrates were dominantly utilized by the microbial combination treated soil followed by the single and control samples signifying a specific response towards the nutritional conditions. Since the Biolog datasets are too large, we further subjected the data sets for principal component analysis, a mathematical algorithm that lessens the dimensionality of the data while retaining most of the variation in the data set. The percentage of variance explained by the first component is 44.36% whereas it was 23.48% for the second (Fig. 5a and b). PCA analysis using Biolog Eco plates of the four different rhizospheric soils showed separate distribution of microflora. i.e., control, *Chitiniphilus* sp., *Streptomyces* sp. and their combinations in the *B. monnieri* rhizosphere microflora (Fig. 5b). Thus, the observation revealed that there was different dynamics of soil microflora under pathogenic stress condition (Fig. 5a and b).

The MT MicroPlate offers a standardized micro-method for performing the sole carbon source utilization patterns and we opted for different carbon sources belonging to amino acids (serine, phenylalanine, glutamine, L-proline, methionine, and isoleucine), carbohydrates (glucose, arabinose, fructose, and lactose), polymers (chitin and dextrin), carboxylic acids (citric acid, lactic acid, and succinic acid) and miscellaneous products (gelatin, casein, carboxymethyl cellulose (CMC), glycerol and urea) (Figs. S5 and S6). Maximum substrate utilization was recorded for glucose followed by proline, casein, and chitin by microbial communities in the microbial treated soil. The second highest consumption of complex substrate was recorded for proline in microbial combination treatment whereas minimum utilization was found in the control soil (Supplementary Fig. S5). These carbon sources were selected on the basis of previous reports of compounds released as

Table 1

Diversity and Evenness index of *B. monnieri* rhizosphere treated with *Chitiniphilus* sp. (CHI) and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations, based on substrate utilization pattern Biolog Eco plates containing different carbon sources (Plants were harvested after 90 days). Data are means of six replicates ± standard error. Different letters show significant differences among the control and treatments ($P \leq 0.05$; LSD test).

Treatments	Shannon diversity	Shannon evenness	McIntosh diversity	McIntosh evenness	Simpson diversity
Control	3.394 ± 0.0008^{bc}	0.989 ± 0.0002^{bc}	0.978 ± 0.0003^{a}	0.992 ± 0.00015^{b}	0.993 ± 0.0011^{a}
Chitiniphilus sp.	3.401 ± 0.013^{b}	0.990 ± 0.0038^{bc}	0.961 ± 0.0005^{ab}	0.993 ± 0.001^{b}	0.988 ± 0.0002^{ab}
Streptomyces sp.	3.409 ± 0.009^{b}	0.993 ± 0.0002^{b}	0.944 ± 0.001^{a}	0.995 ± 0.0006^{ab}	0.984 ± 0.001^{a}
Chitiniphilus sp. + *Streptomyces* sp.	3.427 ± 0.001^{a}	0.998 ± 0.002^{a}	0.937 ± 0.002^{a}	0.998 ± 0.0013^{a}	0.983 ± 0.001^{a}

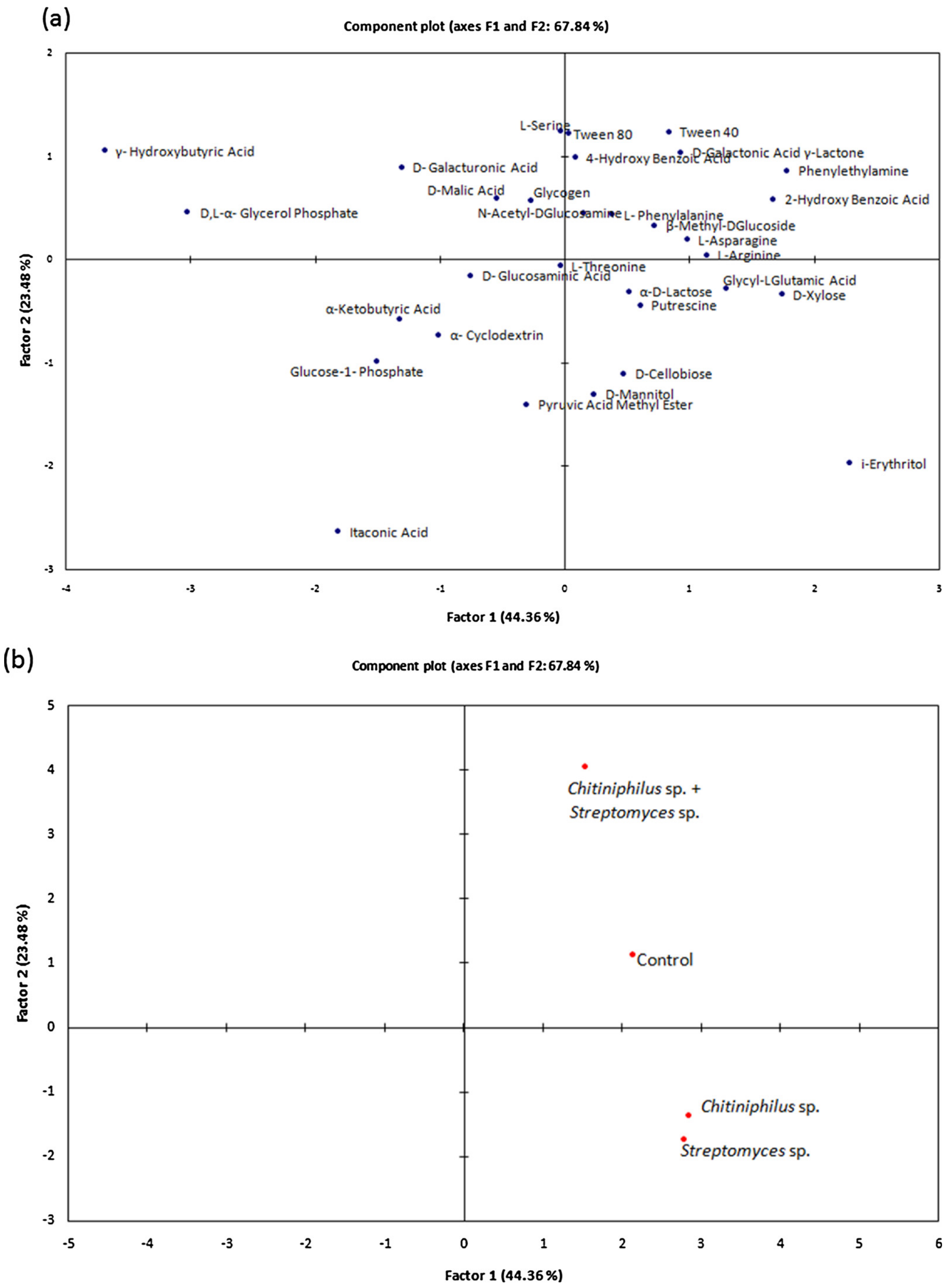

Fig. 5. (a) Analysis of the metabolic patterns based on the Biolog Ecoplate results and (b) Principal component analysis (PCA) of carbon source utilization pattern on Biolog Eco plates (Biolog, Inc., Hayward, CA, USA) of in control (untreated), *Chitiniphilus* sp. (CHI), and *Streptomyces* sp. (STR) microbes applied singly as well as in combinations (CHI + STR) in the rhizosphere of *B. monnieri* grown under nematode stress (field conditions). In this analysis, principal component axis 1 and 2 explain most of the variance in the data cumulatively (F1 = 44.4% and F2 = 23.5%). Data on Biolog Eco plates were recorded every 24 h at 590 nm with an automated microplate reader (BioTek Instruments Inc., USA). At 4th day PCA was performed on blank subtracted data divided by the average well color development (AWCD). The plotted data are averages of three independent experiments.

plant root exudates, providing biotic and abiotic stress resistance in-order to make the experiment ecologically more relevant for testing the rhizospheric soil microbial communities and soil microbes. Of the different carbon sources tested most of them L-proline, methionine, isoleucine, glucose, fructose, lactose chitin, dextrin, gelatin, CMC, glycerol and urea were maximally utilized in the rhizosphere of microbial combination treatments while serine, phenylalanine and arabinose were maximally utilized by the microflora of the *Chitiniphilus* sp. treatment and glutamine. Similarly, the various carboxylic acids were strongly utilized by the *Streptomyces* sp. treated soil rhizosphere (Figs. S6c and d).

4. Discussion

Plant-microbe interactions in natural ecosystems shifted microbial community structure and function along with improved *B. monnieri* growth under nematode stress. By and large, microbes had significant effect on plant performance in terms of fresh and dry herb yield and disease suppression, which increased with microbes in combinational mode. Application of microbe in combination could act as an elicitor for higher induction of plant defence machinery such as antioxidant and phenolic (Singh et al., 2016). The antagonistic potential of microbes in natural conditions showed disease suppression. Thus, it could be hypothesized that the diminution of the root-knot index might be possibly by the production of chitinase enzymes and plant systemic resistance which in totality restrained nematodes incursion and subsequent infestation (Gupta et al., 2017c). The microbes under nematode stress also ameliorated the effects of photosynthetic pigments like chlorophyll and carotenoids to a certain point. The enhanced content of photosynthetic pigments in the microbial combination treated plants could be probably because of the increased growth (Gupta et al., 2016a). Also, the microbe-treated plants have been shown to modify the abundance and composition of plant defense compounds such as phenolic, flavonoid and antioxidants. Previously, the reduction in LPO content was found to be associated with reduced membrane damage during enhanced stress resistance (Nath et al., 2016; Singh and Gaur, 2017). The maximum level of scavengers including total antioxidant capacity and free radical scavenging activity of microbes-treated plants positively interrelated with bacoside A and negatively with disease development in *B. monnieri*. Bacoside A is considered as a major active component of *B. monnieri* known to have defensive activities against chemical-induced liver toxicity, morphine-induced cerebral toxicity and various wound healing activities (Gupta et al., 2017a; Gupta et al., 2017b).

In previous findings, it was well defined that beneficial microbial inoculation not only ameliorated the secondary metabolites production in the host plants but also provided clear evidence for the improved nutrient uptake by the bioinoculants (Gupta et al., 2016b; Calvo et al., 2017; Gupta et al., 2017c). The beneficial microbes are known for their capacity to uptake and mobilize soil nutrients and endorse growth (Miransari, 2013; Gupta et al., 2015; Pii et al., 2015). Thus, it could be suggested that enhancement in plant growth parameters could be possibly by the effect of microbial application in soil which played a role in nutrient uptake.

The gene-based methodologies were applied to investigate chitinase and 16S-rRNA for relative abundances in contrast with the total bacterial cells present. By evaluating chitinase activity in the different microbial treatments using soil crude extract expectedly chitinase activity was observed at diverse levels under the microbial inoculation applied probably because of the presence of nematode decomposition material (Poulsen et al., 2008). Enhanced activity of chitinase in the microbial combination treatment may further be possibly be owed to the presence of easier nutrient sources in the soil. Also, as it is well defined that the *chiA* gene is present among diverse radiations of the bacterial cells. The abundance of 16S-rRNA and *chiA* gene in the microbial combination treated plant rhizosphere may again be majorly speculated due to the increase in chitinolytic bacterial community size.

To illustrate the bacterial community's size, PCR-DGGE profiling suggested that the inoculation of chitinolytic microbes in synergistic manner enhanced bacterial diversity and abundance along with increased bacterial community structure substantially as indicated by Margalef's and Shannon index. Enhancement in the bacterial abundance and diversity as a result of the inoculation of microbes has been well established (Bakker et al., 2013; Lareen et al., 2016). The phylogenetic analysis carried out with the aim to determine the similarity of the bacterial communities as affected by various microbial treatments showed that the degree of microbial inoculation positively influenced the rhizospheric bacterial community. The diversity indices in addition to phylogenetic analysis suggest a noticeable additive result of microbial treatment on the bacterial community structure as correlated with the relative abundance profiling of 16S-rRNA on the rhizosphere bacterial diversity. Previously many workers have revealed that the elevated colonization of microbes might affect the species composition of the rhizospheric bacterial community by raising the useful microbial populations (Chowdhury et al., 2013; Kröber et al., 2014). The above information suggest that the effective colonization of inoculated microbes in combination mode could have resulted in an extreme amplification in microbial abundance and diversity that would have probably supported the plants to accomplish improved strength and tolerance via better nutrient utilization.

Community-level physiological profiling further indicated significant variation between diversity and evenness indices of Shannon, Simpson, McIntosh, and related evenness indices between treated and control ones. AWCD provided the information about the diversity of microbial communities in *B. monnieri* rhizosphere microflora treated with the chitinolytic microbes under nematode stressed conditions. The overall results were statistically authenticated by PCA analysis which showed highest alteration in microbial community structure in case of microbial combination treated *Bacopa* rhizosphere soil than the control and single microbes treated treatments which revealed slight modifications. The soil surrounding the plant roots signify a rich source of dissimilar, plentiful, and reliable sugar substrates, phenolics, organic acids, amino acids, proteins and mucilage that drive underground plant-microbe interactions (Rasmann and Turlings, 2016). The substrates present in the root exudates play an important function in stimulation of microbes which may further enhance plant defense network via antimicrobial agents, triggering or interruption of biofilm development, or by acting as an easier source of nutrients for energy. The noteworthy discrimination ability of methionine, isoleucine, L-proline, glucose, fructose, lactose chitin, dextrin, gelatin, CMC, glycerol and urea along with of the differential carbon substrate utilization patterns in the rhizosphere of microbial combination plants have imperative advantages. The leverage provides an indirect vision of microbial communities representing a possible means for escalating existing information of temporal and spatial dynamics of the microbial communities associated with the plant root system. Taken together, the data (biolog eco and micro plates) recommend that the indigenous microbial communities in the microbial combination treated soil samples are specialized to utilize it's relatively complex and rich substrates with respect to the other treatments.

Thus the comprehensively designed study delineates a strong link between microbial-induced changes in microbial community structure using PCR-DGGE (stable community structure of the total bacterial population), carbon utilization tests (metabolic properties of the culturable bacteria) with the resultant nematode disease suppression and enhanced plant performance. These results put the rhizosphere bacterial population structure in the center of the broad, multi-mechanism model that envisions the impact of microbes on plant performance and health to be a function of complex interactions between many biological components of the plant- microbe-pathogen system (Fig. 6). Overall the study is in conformity with the emerging view of encouraging microbial inoculants application in agricultural crops including medicinal and aromatic crops for enhanced ecosystem functioning, plant productivity and plant resistance to phyto-nematode diseases.

5. Conclusions

The present findings suggest the significance of synergistic effect of microbes in imparting rhizospheric soil bacterial community resilience under biotic stress. The synergistic role of chitinolytic microbes viz., *Chitiniphilus* sp. and *Streptomyces* sp. in enhancing microbial diversity, abundance along with improvisation in plant health by managing nematode infestation may pave innovative ways to develop strategies for the fabrication of advance agricultural productivity in an ecologically favorable approach. However, further analysis are required to better understand the mechanisms by which the plant-microbes-pathogen interactions affects the rhizospheric microbial community, whereby the study of the function of rhizo-deposition in orchestrating microbial communities is of prime importance.

Conflict of interest

The authors declare no potential conflict of interest.

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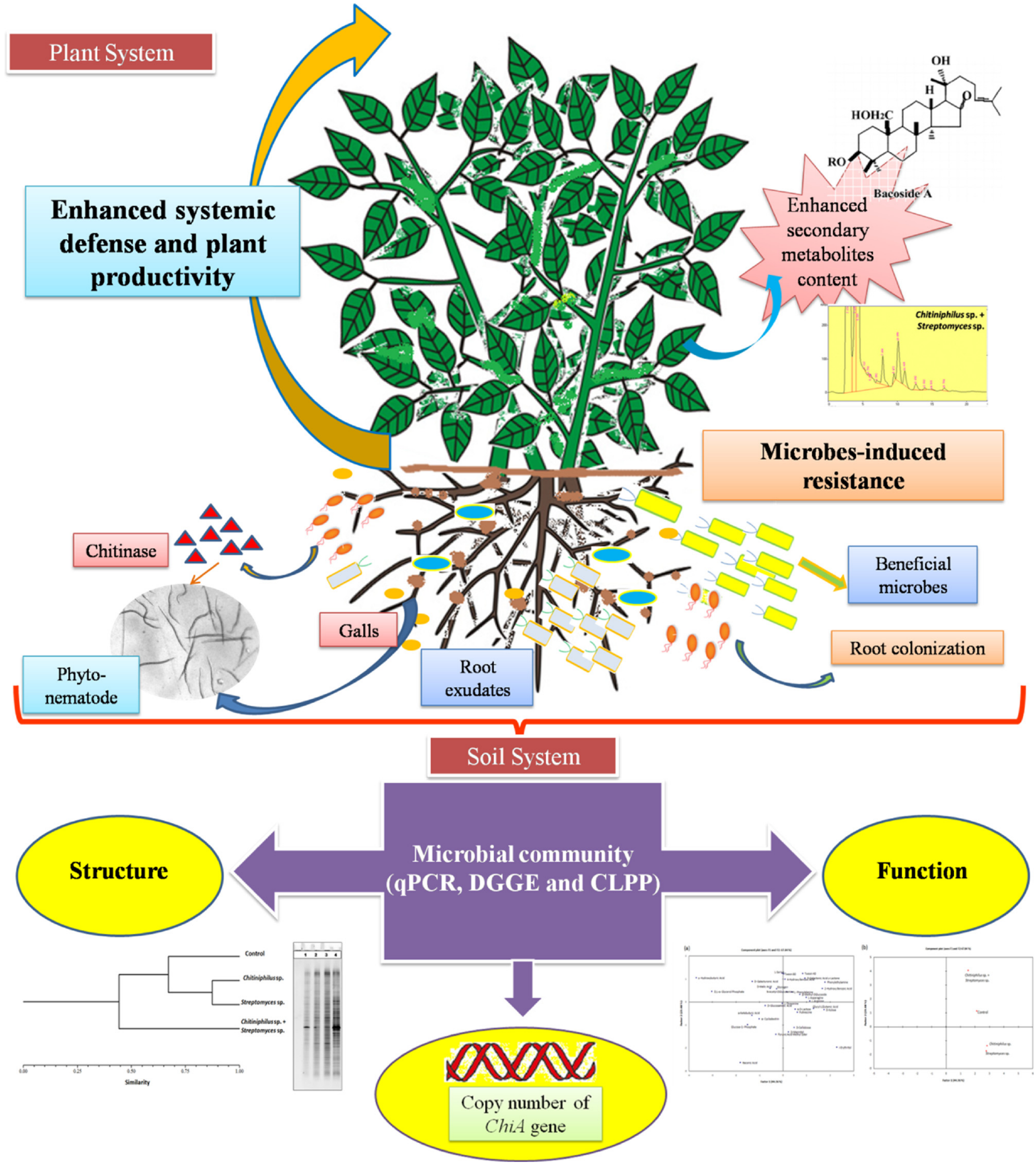

Fig. 6. Mechanistic figure representing microbes mediated resistance and secondary metabolite enhancement by uplifting microbial community structure and function of *B. monnieri* rhizosphere against nematode stress.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.10.006.

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