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ASSESSMENT OF IMPACT OF *PARTHENIUM HYSTEROPHORUS* (L.) ON SOIL BACTERIAL POPULATION

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Parthenium hysterophorus

Amendment

Genomic analysis

Strain

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ABSTRACT

The paper deals with the effect of *Parthenium hysterophorus* L. on soil bacterial population. Different proportion (5, 10, 15 and 20%) of shed dried powdered *Parthenium* (whole plant) was mixed with the soil collected from agroecosystem from the depth of 10-15cm. Bacterial population was analyzed over a period of 45 days using dilution plate method and results obtained were compared with control. On 15th day in control culture, the bacterial population was $44.6 \pm 1.9798 \times 10^9$ which significantly decreased to $33.4 \pm 1.5556 \times 10^9$, $28.0 \pm 0.8485 \times 10^9$, $21.2 \pm 0.4242 \times 10^9$ and $9.85 \pm 0.4949 \times 10^9$ respectively in 5, 10, 15 and 20% of *Parthenium* amended soil. On 30th day, bacterial population observed was $39.6 \pm 3.6769 \times 10^9$, $32.4 \pm 3.5355 \times 10^9$, $25.6 \pm 1.6971 \times 10^9$ and $17.5 \pm 0.9899 \times 10^9$ which was also less than control bacterial population ($49.0 \pm 3.2526 \times 10^9$). A similar trend of decrease in bacterial population was obtained on 45th day. A two way ANOVA revealed the variation of bacterial population with respect to concentration of *Parthenium* powder and duration of amendment statistically significant ($F = 175.2671$, df 4, 2; $p < 0.001$ and $F = 30.55931$, df 4, 2; $p < 0.001$). Morphological details of different bacterial colonies showed increase in population of irregular – undulate colony while other showed decrease in population with increase of concentration of *Parthenium* in soil. Genomic analysis revealed that this *Parthenium* resistant colony was *Bacillus cereus* strain MBL13.

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INTRODUCTION

In a living soil, around 100 elements and billions of microorganisms work together in synergy. Presence of microorganisms differentiates a living soil from a dead soil. Soil microorganisms help in decomposition of residual agrochemicals in soil, more efficient release of nutrients from organic matter, increase yield over a period of time and to increase the resistance of plants to pests and other diseases etc. (Higa, 1993; Higa, 1994; Sangakkara and Higa, 2000; Sangakkara and Weerasekera, 2001; Bruggenwert *et al*., 2001). They are also responsible for cohering soil particles and for providing hormones, nutrients and minerals in a useable form to the plants via the root ecology. Soil microbes are also responsible for sequestration of green house gases. Methane is an important greenhouse gas which contributes about 15% to global warming (Rodhe, 1990). Soil microorganisms, which oxidize atmospheric methane, are responsible for an estimated 5-10% of the total removal of atmospheric methane (Cicerone and Oremland, 1988).

Effective microorganisms are reported to assert a powerful regenerative effect on soil (Higa, 1994), that helps to re-establish a balanced soil ecology and to combat oxidative corrosion in plants and humans (Deiana *et al*., 2002). Their population ranges from 100 million to 3 billion in a gram of soil and there are about 60,000 different bacteria species, which live in the top 10cm of soil where organic matter is present. Congress grass (world's 7 most notorious weed), after decomposition acts as a sink of organic matter in soil but besides this it also affects the soil microorganisms most.

Parthenium hysterophorus L. (congress grass, family – Asteraceae) is an exotic species said to be originated in Mexico and has entered India from USA through imported food grains or cereals got for experimental purpose (Vertak, 1968; Lonkar *et al*., 1974). *Parthenium* is an aggressive colonizer of degraded areas with poor ground cover and exposed soil such as fallow wastelands, roadsides and overgrazed pastures. It is estimated that about 5 million hectares area in India has been invaded by this weed (Vertak, 1968). The main toxin of this plant is sesquiterpene lactones (Oudhia and Tripathi, 1998). After completing its life cycle, the plant dies and whatever biomass they contain accumulates in the soil along with their toxins. These toxins affect the beneficial organisms of soil. Reports on effect of insecticides on soil bacteria, and biology of *Parthenium hysterophorus* are available (Navie *et al.,* 1996; Ahmed and Ahmad, 2006), but no report on toxic impact of *Parthenium hysterophorus* on bacterial population of soil is available. The present study therefore, has been undertaken to assess its impact on soil bacteria.

MATERIALS AND METHODS

Soil sample

Soil was collected with the help of sterilized equipments from a depth of 10-15cm from the agro ecosystem around Deptt. of Zoology, Ranchi University, Ranchi. The characteristics of soil analyzed are given in Table 1.

Table 1: Edaphic profile of experimental soil

Characteristics	Value (M ±SD)
pH	6.37±0.21
Organic Carbon(mg / g soil)	8.31±1.92
Nitrogen(mg / g soil)	0.58±0.11
Phosphorous(Kg / hec.)	31.93±2.97
Potassium(Kg / hec.)	158.4±8.57

Plant material

Whole plant of *Parthenium* collected from densely infested area was chopped, shade dried and powdered to be used in experiments.

Experimental setup

In the experiment, 5, 10, 15 and 20% powder of *Parthenium* was mixed in soil collected from agro ecosystem and kept in moist condition in plastic container. One container without *Parthenium* was kept as control.

Soil bacteria culture

Bacterial culture was done from *Parthenium* amended and control soil, over a period of 45 days using dilution plate method (Thornton, 1922; Thom and Raper, 1945). 1mL inoculums of the primary suspension

were taken and Czapek Dox agar media (peptone -10g /L, NaCl- 5g/L, beef extract- 10g/L, agar- 15g/L, pH-7) was used for culture. The petriplates (diameter 100mm) were incubated at 37°C for 48h. Colonies so cultured were isolated and retained for subsequent screening like pure culture, gram staining and genomic analysis, on the basis of which their phylogenetic tree was constructed.

Genomic Analysis

For genomic analysis DNA was isolated from the pure culture of *Parthenium* resistant colony. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1420 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database (Marchler-Bauer *et al.*, 2002; Pruitt *et al.*, 2005). Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program and the phylogenetic tree was constructed using Molecular evolutionary genetics analysis software version 4.0 (MEGA 4) (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Morphological details pertaining to different bacterial colonies on nutrient agar plates are presented in Table 2. The developed bacterial colonies on the nutrient agar plates with respect to their shape and margin were of 4 types i.e. circular-entire, irregular-undulate, punctiform and filamentous. In control, 50% of the colonies were circular-entire, 46% were punctiform and 4% of the colonies were irregular-undulate. Colony with filamentous shape and margin was absent in control. The elevation of circular-entire colonies were either flat (20%), raised (75%) or convex (5%) and with white (40%) or green (60%) colour. The elevation of punctiform colonies were only flat, with green (50%) and white (50%) colour. While the elevation and colour of irregular-undulate colonies was only flat and white respectively. In 5% *Parthenium* amended soil, the developed colonies were circular-entire (26%), punctiform (64%), irregular-undulate (8%) and filamentous (2%). The elevation and colour of all the four colonies were same as control except filamentous colony. The filamentous colony had only flat elevation and white colour. Morphological details of bacterial colonies obtained in 5, 10, 15 and 20% *Parthenium* amended soil (Table 2) showed that the population of different colonies obtained in these samples was either less than the control or they showed little variation. Only colony with irregular shape and undulate margin showed a significant increase from 4% (control) to 20% as concentration of *Parthenium* in soil increased. The bacteria constituting irregular-undulate colonies were observed to be bacilli and their response to gram's stain was positive (Fig. 1).

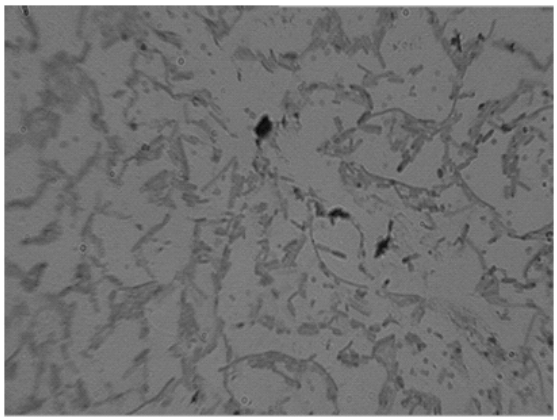

Figure 1: Gram staining of *Parthenium* resistant colony

Bacterial population of *Parthenium* amended and control soil is presented in Fig 2. In control, the bacterial population was $44.6 \pm 1.9798 \times 10^9$, which significantly decreased to $33.4 \pm 1.5556 \times 10^9$, 28.0 0.8485×10^9, $21.2 \pm 0.4242 \times 10^9$ and $9.85 \pm 0.4949 \times 10^9$ respectively in 5, 10, 15 and 20% of *Parthenium* amended soil on 15th day of experiment. On 30th day, the bacterial population observed was $49.0 \pm 3.2526 \times 10^9$, $39.6 \pm 3.6769 \times 10^9$ and $32.40 \pm 3.5355 \times 10^9$, $25.60 \pm 1.6971 \times 10^9$ and $17.50 \pm 0.9899 \times 10^9$ in control, 5, 10, 15 and 20% of *Parthenium* amended soil respectively. On 45th day, a similar trend of decrease in bacterial population was observed viz. $53.10 \pm 1.2727 \times 10^9$ (control), $37.3 \pm 1.8384 \times 10^9$ (5%), $35.30 \pm 0.9899 \times 10^9$ (10%), $29.5 \pm 1.4142 \times 10^9$ (15%), and

Table 2: Morphological details of bacterial colonies in culture condition

% of *Parthenium*	Shape	Margin	Elevation	Colour
Control	46% punctiform	46% entire	100% flat	50% green 50% white
	50% circular	50% entire	20% flat 75% raised 5% convex	40% white 60% green
	4% irregular	4% undiluted	100% flat	100% white
5% *Parthenium*	64% punctiform	64% entire	100% flat	50% green 50% white
	26% circular	26% entire	20% flat 75% raised 5% convex	40% white 60% green
	8% irregular	8% undiluted	100% flat	100% white
	2% filamentous		100% flat	100% white
10 % *Parthenium*	45% punctiform	45% entire	100% flat	50% green 50% white
	41% circular	41% entire	20% flat 75% raised 5% convex	40% white 60% green
	12% irregular	12% undiluted	100% flat	100% white
	2% filamentous		100% flat	100% white
15% *Parthenium*	36% punctiform	36% entire	100% flat	50% green 50% white
	47% circular	47% entire	20% flat 75% raised 5% convex	40% white 60% green
	15% irregular	15% undiluted	100% flat	100% white
	2% filamentous		100% flat	100% white
20% *Parthenium*	25% punctiform	25% entire	100% flat	50% green 50% white
	52% circular	52% entire	20% flat 75% raised 5% convex	40% white 60% green
	20% irregular	20% undiluted	100% flat	100% white
	3% filamentous		100% flat	100% white

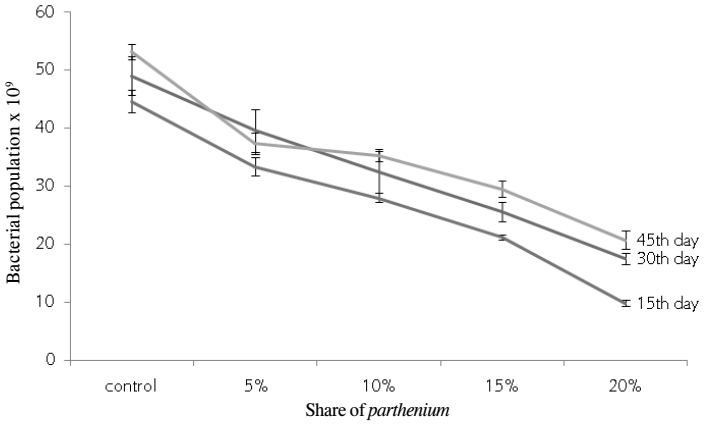

Figure 2: Bacterial population (values are per g of soil sample)

Table 3: Analysis of variance (Two way)

Variation	SS	Df	MS	F	Significance
Between Conc.	1820.20733	4	455.05183	175.2671	$p < 0.001$
Between days	158.684333	2	79.342167	30.55931	$p < 0.001$
Error	20.7706667	8	2.5963333		

$20.70 \pm 1.5556 \times 10^9$ (20% *Parthenium* amended soil). Ahmed and Ahmad (2006) reported that insecticide, chlorpyrifos causes significant reduction in number of soil bacteria. The bacterial population data were subjected to two way analysis of variance. The analysis revealed the variation of bacterial population statistically significant with respect to different concentration of *Parthenium* in soil and different amendment days (Table 3).

Genomic analysis of the *Parthenium* resistant irregular - undulate colony (PH-1) which gradually increased in number during the experiment, was done and the phylogenetic tree was constructed. The culture, which was labeled as PH-1 was found to be *Bacillus cereus* strain MBL13 (Genbank Accession Number: GQ148914.1) based on nucleotide homology and phylogenetic analysis.

Bacillus cereus which showed resistance to *Parthenium* is an aerobic, endospore-forming and mobile Gram-positive rod shaped bacteria. It is an opportunistic pathogen cause food poisoning manifested by diarroheal or emetic syndromes (Drobniewski, 1993; Helgason *et al.*, 2000; Ivanova *et al.*, 2003). *Bacillus cereus* MBL13 is a collagenase-producing bacterium and is able to degrade animal bones (Liu *et al.,* 2010).

By PCR of fragment of 16S rDNA gene from the isolated DNA of bacterium, a single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel (Fig. 3). A similar band of 1500 bp has been observed for some more soil isolated bacteria such as *Aeromonas punctata* strain JM 10 (GenBank Accession Number: GU205197.1), *Bacillus* sp. BFF-3 (GenBank Accession Number: EF031071.1) and *Bacillus* sp. HBUM 84231 (GenBank Accession Number: EU158325.1). Table 4 provides information about other close homologs for *Bacillus cereus* strain MBL13 based on BLAST data and phylogenetic tree of *Bacillus cereus* MBL13 is shown in Fig. 4. It was observed that *Bacillus cereus* strain MBL13 is closely related to *Bacillus* sp. Ts -116 and *Bacillus thuringiensis* strain 61436 and their maximum score is 2614, 2612 respectively which is equal to total score. They show 99% and 100% sequence similarity in query coverage of amino acids respectively (Table 4). Other close homologs of *Bacillus cereus* strain MBL13 also scored maximum score equals to total score. Query coverage of all other homologs is 100% except for *Bacillus cereus* isolate HKS2-1 whose query coverage is 99% (Table 4). The expectation value (E) of all these *Bacillus* strain was 0.0, which determines that all the 10 close homologs of *Bacillus* strain are homogenous in comparison to *Bacillus cereus* strain MBL13.

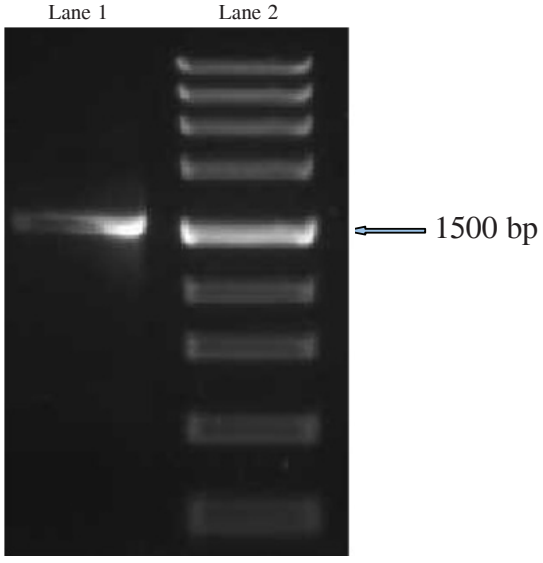

Figure 3: Gel image of 16S rDNA amplicon
Lane 1: 16S rDNA amplicon band; Lane 2: DNA marker

PH-1 which was the sample for genomic analysis shows bootstrap value or bootstrap percentage of 55 (at node) with GQ148914.1 (Fig. 4). Felsenstein (1985) proposed that bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade; alternative nodes can be rejected if they occur in less than 5% of the bootstrap estimates. Following Felsenstein (1985) description, it was concluded that PH-1 and GQ148914.1 (*Bacillus cereus* strain MBL13) are sister species and thus a clade (monophyletic group). Hillis and Bull (1993) also stated that bootstrap values of 50% or more may be overestimates of accuracy. Bootstrap value between GU190368.1 (*Bacillus* sp. Ts-116) and GQ148914.1 monophyletic group is 47%. FJ932761.1 (*Bacillus thuringiensis* strain 61436) shows bootstrap value of 52 with clade of *Bacillus cereus* strain MBL13. This value also signifies that FJ932761.1 is a close member of Bacillus cereus monophyletic group. DQ289058.1, HM047298.1, GU269268.1, GU250444.1, GU250443.1, GU120652.1 and EU622630.1 are also other descendants of ancestor of

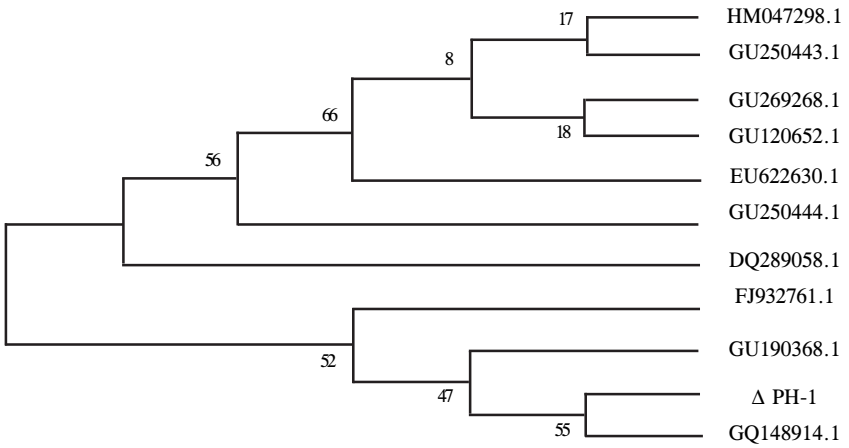

Figure 4: Phylogenetic tree showing position of Δ PH – 1 (*Bacillus cereus* strain MBL13)

Table 4: Close homologs of *Bacillus cereus* strain MBL13

Accession	Description	Max. score	Total score	Query coverage	E value	Max ident
GQ148914.1	*Bacillus cereus* strain MBL13	2623	2623	100%	0	100%
GU190368.1	*Bacillus* sp. Ts-116	2614	2614	99%	0	99%
FJ932761.1	*Bacillus thuringiensis* strain 61436	2612	2612	100%	0	99%
DQ289058.1	*Bacillus cereus isolate* HKS 2-1	2610	2610	99%	0	99%
HM047298.1	*Bacillus thuringiensis* strain ZJU03	2606	2606	100%	0	99%
GU269268.1	*Bacillus cereus* strain P-12	2606	2606	100%	0	99%
GU250444.1	*Bacillus cereus* strain BFE 5384	2606	2606	100%	0	99%
GU250443.1	*Bacillus cereus* strain BFE 5392	2606	2606	100%	0	99%
GU120652.1	*Bacillus thuringiensis* strain IWF24	2606	2606	100%	0	99%
EU622630.1	*Bacillus* sp. NS-4	2606	2606	100%	0	99%

Max. score = maximum score; E value = expected value; Max. ident = maximum identification

Bacillus cereus strain MBL13 (GQ148914.1) but they are far from GQ148914.1 in comparison to *Bacillus* sp. Ts-116 (GU190368.1) and *Bacillus thuringiensis* strain 61436 (FJ932761.1), but all belongs to same monophyletic group.

Several studies have been dedicated to a comparison of *Bacillus thuringiensis* (Bt) and *Bacillus cereus* (Bc) on the basis of characters not related to the production of Insecticidal crystal proteins (Hendriksen and Hansen, 1998). *Bacillus thuringiensis* (Bt) is a facultative anaerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore. Bt is a member of the Bc group, which also contains *Bacillus cereus* (Bc), *B. mycoides* and *B. anthracis*. Furthermore, the psychrotolerant *B. weihenstephanensis* has recently been proposed as a new member of the group (Lechner *et al.*, 1998). Bt can only be distinguished from *Bacillus cereus* by the production during the sporulation process of one or more inclusion bodies, which have been found to be toxic for invertebrates, primarily insect species in the orders *Coleoptera*, *Diptera* and *Lepidoptera* (De Barjac, 1981; Andrews *et al.*, 1987). Phenotypic differentiation of Bt and Bc is not possible on the basis of morphology or utilization of organic compounds (Baumann *et al.*, 1984; Logan and Berkeley, 1984; Priest *et al.,* 1988), characterization of cell content of fatty acids (Väisänen *et al.*, 1991) or sugars (Wunschel *et al.*, 1994) and enterotoxin production (Damgaard *et al.*, 1996(a); Hansen and Hendriksen, 1997(a)). Likewise, genotypic differentiation of Bt and Bc is not possible by DNA homology analysis (Kaneko *et al.*, 1978), 16S rDNA sequencing (Ash *et al.*, 1991); analysis of the 16S-23S internal transcribed sequence (Wunschel *et al.*, 1994; Bourque *et al.*, 1995) and PCR analysis of genes encoding Bc-like toxic products (Damgaard *et al.*, 1996b; Asano *et al.*, 1997; Hansen and Hendriksen, 1997b). Giffel *et al.*, (1997) found differences in 16S rDNA sequences between a limited number Bt and Bc. Beattie *et al.*, (1998) were able to discriminate among members of the Bc group by Fourier transform infrared spectroscopy, and Brousseau *et al.*, (1992) were able to distinguish Bt and Bc by random amplified polymorphic DNA

fingerprinting. However, the transfer of ICP encoding plasmids from Bt to Bc makes the receptor Bc indistinguishable from Bt, and vice versa (González *et al.*, 1981, 1982).

Hence it is difficult to assign any reason as why Bc is resistant to toxicity of *Parthenium hysterophorus* while others of the same group and having phylogenetic relation are not.

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