

# The effect of trap plants on the population diversity of *Bradyrhizobium japonicum*

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## Abstract

One hundred and four isolates of *Bradyrhizobium japonicum* were isolated from nodules of two different trap plants, viz. Soya bean cultivars, Maple Glen and Orford which were inoculated with two different soil samples (Ottawa and St-Hugus soils). All isolates were clustered based on PCR/RFLP of 16S-23S rRNA genes. RFLP analysis was performed to characterize all the isolates using six different endonuclease enzymes. The data was analyzed by using Jamp software. Using dendrogram data, all the isolates were grouped into six different clusters. There were four and five clusters of *Bradyrhizobium japonicum* in Ottawa and St-Hugus soils, respectively. Three clusters were common between two cultivars of Soya bean when inoculated with Ottawa soil and four common clusters were recognized when trap plants inoculated with St-Hugus soil. In Ottawa soil, cluster I was not detected by Orford cultivar, likewise in St-Hugus soil, cluster VI was not detected by Maple Glen cultivar of Soya bean. Isolates of cluster III were dominantly trapped when Maple Glen and Orford cultivars inoculated with Ottawa soil but isolates from clusters I, IV and III were trapped when they were inoculated with St-Hugus soil. Since different cultivars trapped different isolate types it can be concluded that for population studies of rhizobial bacteria different trap plants can provide a better composition of native population of bacteria.

**Keywords:** *Bradyrhizobium japonicum*, 16S-23SrRNA, PCR/RFLP, Trap Plant.

## INTRODUCTION

There is a little information about population structure of indigenous rhizobia in the soil. However, knowl-

edge of changes in population structure of rhizobia is very important from ecological point of view. By having a feasible technique to get this kind of information, it would be possible to study the ecological effects of agricultural practices on the population structure of rhizobia in soil samples. Furthermore any changes that occur to introduced rhizobia into the soils can be monitored. Availability of a representative indigenous rhizobial population isolated from soil samples is the most important part of this kind of studies. For this reason many scientists have developed several selective media in order to have a representative rhizobial population (Bromfield *et al.*, 1994 and Kinkle *et al.*, 1994). Selective media is generally good for just one genus of rhizobia and it is not recommended for a wide range of rhizobia species. In addition, selective media is used for direct isolation of bacteria from soil and still isolate confirmation of identity of isolate is necessary after isolation process, which is very time consuming (Laguerre *et al.*, 1993). Isolation of rhizobia from host plant nodules has an advantage because competitive strains will be selected by plant and less abundant strains still have a chance to be presented in the selected population. It is well known that symbiotic relationships between plant and bacteria mediated by plant compounds such as flavonoides. Each plant species leaks a unique signature of these kinds of compounds from their roots. The quantity and quality of root exudates depend to a certain extent on the soil chemical and physical properties, but in all cases determines the microbial community of the rhizosphere. Symbionts like *Rhizobium* are well tuned to the composition and quantity of root exudates. Constanza *et al.* (2000) showed that rhizobial bacteria isolated from plants grown under elevated CO<sub>2</sub> were genetical-

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ly different from those isolates obtained from plants grown under ambient conditions. On the other hand, bacteria from genera *Rhizobium* and *Bradyrhizobium* produce several kinds of surface polysaccharides such as exopolysaccharides and lipopolysaccharides (Kannenberg *et al.*, 1998). These compounds are recognized by plants and lead to the induction of specific biochemical and physiological reactions (Hohn *et al.*, 1997). The amount of these compounds that produced by bacteria is very closely related with the environmental factors such as acidity and calcium concentrations (Maccio *et al.*, 2002). Different legume plant excretes different compounds, which makes this symbiotic relationship quite specific. It also seems that different cultivars of each host plant species can preferably pick up different rhizobial isolates from soil samples (Orgambide *et al.*, 1994). Probably by using more cultivars of host plant a better composition of native population structure can be detected from soil samples for ecological studies.

During the last decade, biologists have employed a variety of molecular techniques to address questions regarding population diversity. Analysis of 16S (small subunit) rRNA gene and more recently, the 16S–23S internal transcribed spacer (ITS) has figured heavily in these studies, particularly those involving prokaryotic microorganisms. (Ligon *et al.*, 1991). Restriction enzyme digestion of the 16S–23S ITS region has been used for phylogenetic analyses of strains of nonphotosynthetic eubacterial genera (Navarro *et al.*, 1992). Analysis of 16S–23S internal transcribed spacer also has been used for studying the population structure of rhizobia bacteria. Diversity of *Phaseolus*-nodulating rhizobial populations in acid soil amended with lime was studied by using PCR/RFLP technique (Andrade *et al.*, 2002).

The objective of this study was to assess the changes in the population structure of *Bradyrhizobium japonicum*, which has been isolated from two different cultivars of Soya bean. In addition, the discriminative ability of PCR/RFLP technique of 16S–23S rRNA genes within species of rhizobial bacteria was examined.

## MATERIALS AND METHODS

**Isolation procedure:** Two cultivars of Soya bean viz., Maple Gelen and Orford were grown in 104 Leonardo Jars (52 for each cultivar) and then inoculated with Ottawa and St-Hugus soil samples (26 for each soil).

After 8 weeks, root nodules were collected (one from each jar) and sterilized by immersing in 70% alcohol for 3 min, rinsed with sterile deionized water and then placed in 0.1% acidified mercuric chloride solution for 3 min, followed by repeated rinses in sterile water. Nodules were then crushed in sterile water and nodule content was streaked onto yeast extract-Mannitol agar plates (Somasegaran and Hoben, 1994), and incubated at 28°C for 10 days. Single colonies were selected from isolates that appeared and re-inoculated onto Soya bean plants (specific cultivar) for authentication before being used for further studies. A total of 104 isolates of *Bradyrhizobium japonicum* were isolated from the nodules of two different cultivars from two soils.

**DNA extraction:** Small-scale preparations of total bacterial DNA were obtained by growing each strain in 5 ml of Tryptone yeast (TY) broth medium for seven days. Bacterial cells of individual isolates were pelleted by spinning 2 ml overnight culture in eppendorf tubes and resuspended in 250- $\mu$ l resuspension buffer (50 mM Tris-Cl; 10 mM EDTA and pH 8). Resuspended cells were treated with 250  $\mu$ l of the lysis buffer (200 mM NaOH; 1% SDS) and mixed by inverting eppendorf tubes until the solution became viscous and slightly clear. The resulting solution was treated with 350- $\mu$ l neutralization buffer (3.0 M potassium acetate, pH 5.5) and centrifuged at 14000 rpm for 10 min. The supernatant from each isolate was collected in a fresh eppendorf tube and the extracted DNA was precipitated by adding isopropanol, followed by washing with 70% (v/v) ethanol. The DNA of the individual isolates was dried under vacuum and re-dissolved in 80  $\mu$ l TE buffer (10 mM Tris adjusted to pH 8 with HCl, 1 mM EDTA) (Cullen and Hirsch, 1992).

**PCR condition:** For this reaction the following primers are used; Forward 5'-tgcggetggatcacctcctt-3' and Reverse 5'-ccgggttccccattcgg-3'. PCR reaction was performed as described by Williams *et al.*, (1990) with some modifications. DNA amplification was performed in a total volume of 50  $\mu$ l containing; 5  $\mu$ l 10 $\times$ PCR reaction buffer, 1  $\mu$ l dNTP (10 mM), 1  $\mu$ l of each primers (5  $\mu$ M), 41  $\mu$ l distilled water, 0.5 unit Tag DNA polymerase and 1  $\mu$ l of DNA sample.

The following PCR reaction was performed with the following amplification cycles; initial denaturation at 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 54°C for 1 min and 72°C for 2 min and the final extension at 72°C for 3 min. The PCR products were ana-

lyzed in 1.4 % agarose gel and visualized under UV light.

**RFLP of the intergenic space (IGS) region:** Ten  $\mu$ l of PCR products were digested with different restriction enzymes viz., *MspI*, *AluI*, *HinfI*, *Sau3A*, *DdeI* and *CelI* at 37°C for 3h. Digested PCR products were separated on 2% agarose gel after running in gel for 6h at 70 volts. Scoring of +/- bands was done and cluster analysis was performed using Jamp software.

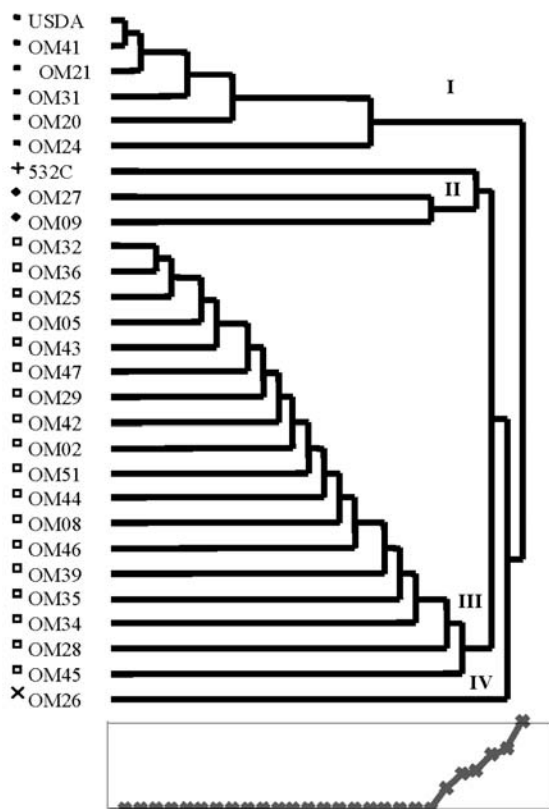
## RESULTS

The amplification of intergenic space region (IGS) of 16S-23S rRNA gene gave PCR product of about 900 kb for all the isolates. Some isolates also had two different operons of rRNA gene. Ten different restriction enzymes were tried finally six enzymes viz. *MspI*, *AluI*, *HinfI*, *Sau3A*, *DdeI* and *CelI* were selected for RFLP of amplified IGS region of all isolates. Isolates

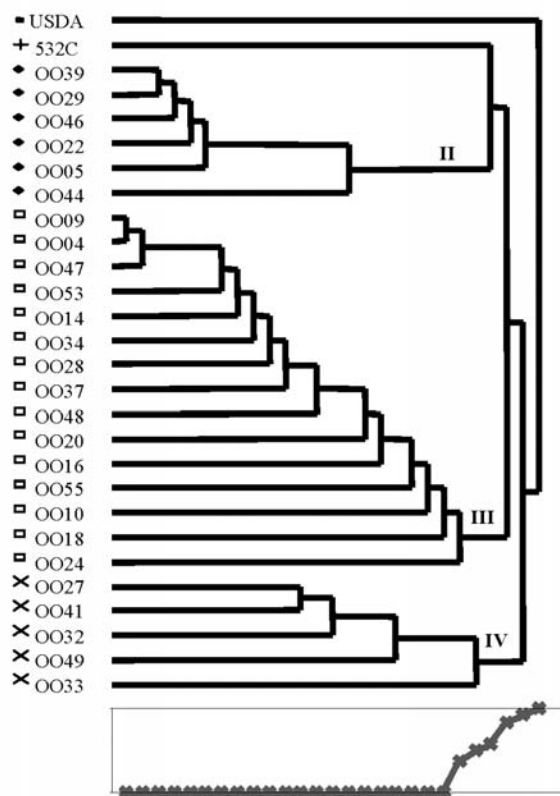
were clustered based on the number and the size of produced fragments using Jamp software.

Twenty six isolates of *Bradyrhizobium japonicum* isolated from nodules of Maple Glen, and inoculated with Ottawa soil were grouped based on RFLP of PCR products. They were clustered in four different clusters (I, II, III and IV) were obtained (Fig.1). Cluster I, II, III and IV included 19, 7, 70 and 4 percent of the population in the Ottawa soil respectively when Maple Glen was the trap plant. Clusters of I and III were dominant in the population of *Bradyrhizobium japonicum* in Ottawa soils. Isolate 532C (this strain is used as inoculant in Canadian soils) which was not detected in Ottawa soils when Maple Glen cultivar was used as a trap plant was distinct from other isolates as evident by dendrogram. The isolate, 532C (the strain used as inoculant in Canadian soils) was detected in Ottawa soils when Maple Glen cultivar used as a trap plant was distinct from other isolates in clusters as evident by dendrogram.

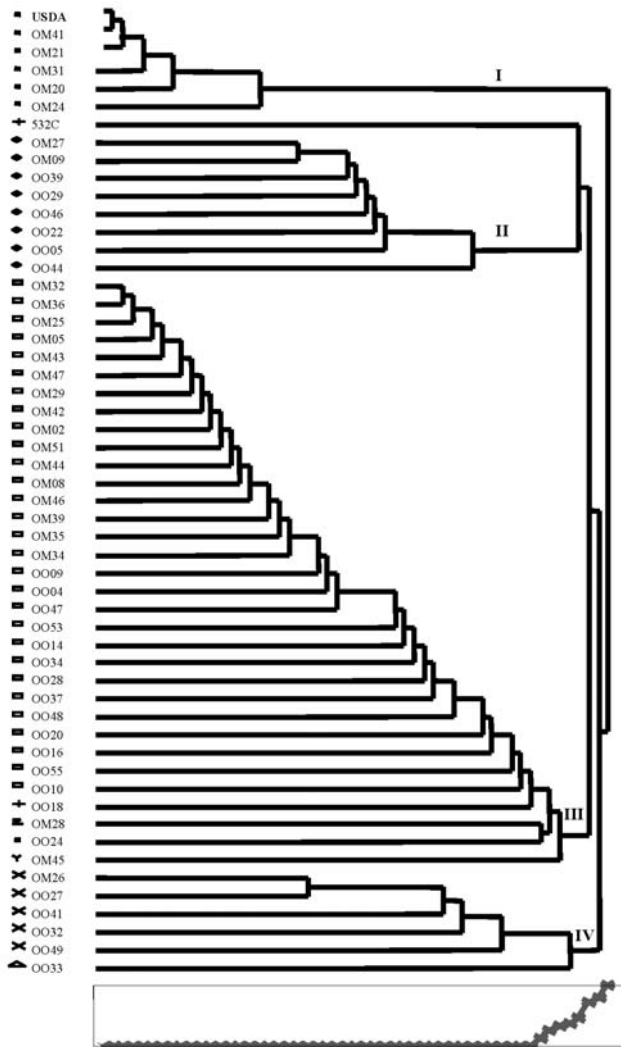
Isolates of *Bradyrhizobium japonicum* isolated from



**Figure 1.** Dendrogram of 26 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Maple Glen cultivar) inoculated with Ottawa soil. Clustering was done based on PCR/RFLP of IGS region of 16S-23S rRNA gene using six different restriction enzymes. (O stands for Ottawa soil and M stands for Maple Glen cultivar).



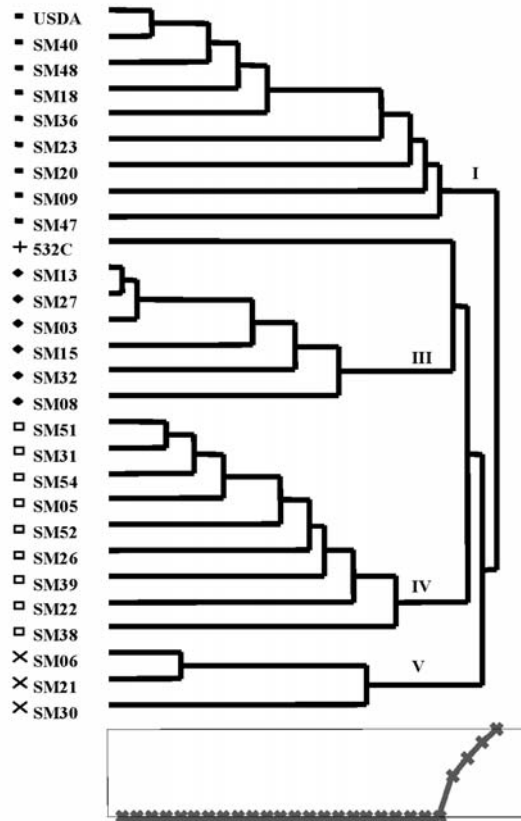
**Figure 2.** Dendrogram of 26 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Orford cultivar) inoculated with Ottawa soil. Clustering was done based on PCR/RFLP of IGS region of 16S-23S rRNA gene using six different restriction enzymes. (The first O stands for Ottawa soil and the second O stands for Orford cultivar).



**Figure 3.** Dendrogram of 52 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Maple and Orford cultivars) inoculated with Ottawa soil. Clustering was done based on PCR/RFLP of IGS region 16S-23S rRNA gene by using six different endonuclease enzymes. (The first O stands for Ottawa soil. The second O and M stand for Maple Glen cultivars).

root nodules of Orford cultivar that was inoculated with Ottawa soil were grouped into three different clusters II, III and IV based on PCR/RFLP pattern (Fig. 2). Each cluster formed 23, 58 and 19 percentage of the population of *Bradyrhizobium japonicum* in Ottawa soils when Orford cultivar was used as trap plant. The isolates, USDA110 and 532C were totally distinct from other isolates from Orford cultivar. A high percentage of root nodules of Orford cultivar have been occupied by isolates from cluster III.

In figure 3 all 52 isolates of *Bradyrhizobium japonicum* (isolated from both trap plant) when inoculated with Ottawa soil were clustered based on PCR /RFLP



**Figure 4.** Dendrogram of 26 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Maple Glen cultivar) inoculated with St-Hugus soil. Clustering was done based on PCR/RFLP of IGS region 16S-23S rRNA gene using six different restriction enzymes. (S stands for St-Hugus soil and M stands for Maple Glen cultivar).

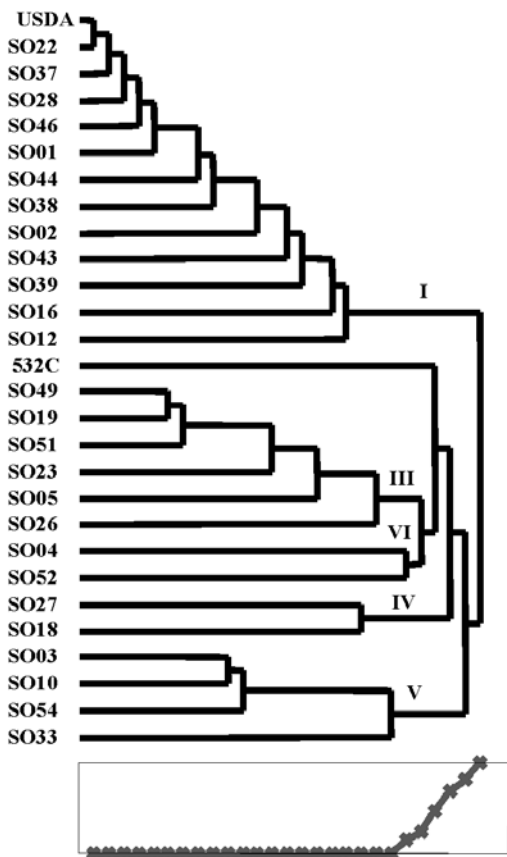
analysis. Clusters I, II, III and IV were formed 10, 15, 63 and 12 percentage of the population of *Bradyrhizobium japonicum* in the Ottawa soils respectively. It is evident that using two trap plants provides a better representative population of indigenous rhizobia bacteria in soil samples.

The results of PCR /RFLP analysis of 26 isolates of *Bradyrhizobium japonicum* isolated from root nodules of Maple Glen cultivar inoculated with St-Hugus soil showed that all isolates could be grouped in four different clusters (Fig. 4). Three clusters viz. I, III and IV were quite similar to those obtained from Ottawa soil and whereas unique cluster V was just detected in St-Hugus soil. Bacteria from cluster V formed a minority of the population (12%) of *Bradyrhizobium japonicum* and 30, 23 and 35% percentage of nodules of Maple Glen cultivar were occupied by isolates from clusters I, II and IV, respectively.

All isolates *Bradyrhizobium japonicum* isolated

from root nodules Orford cultivar inoculated by St-Hugus soil were grouped in 5 different clusters (Fig. 5). Clusters I, III, IV and V were similar to those which were isolated from root nodules of Maple Glen cultivar. Most nodules were occupied by cluster I (46%) whereas, clusters III, IV and V occupied 23, 8 and 15 percentage of nodules, respectively. One new cluster (VI) was detected by Orford cultivar in St-Hugus soil. This new cluster has been found neither among the isolates from other soils nor among isolates from Maple Glen cultivar of Soya bean plants. It seems that Orford cultivar just able to pick up some isolates from cluster VI.

Fifty two isolates of *Bradyrhizobium japonicum* (isolated from both the host plants) inoculated with St-Hugus soil clustered based on PCR/RFLP. Clusters I, III, IV, V and VI were formed (which occupied must be deleted) 38, 23, 22, 13 and 4 percentage of the population respectively (Fig. 6).

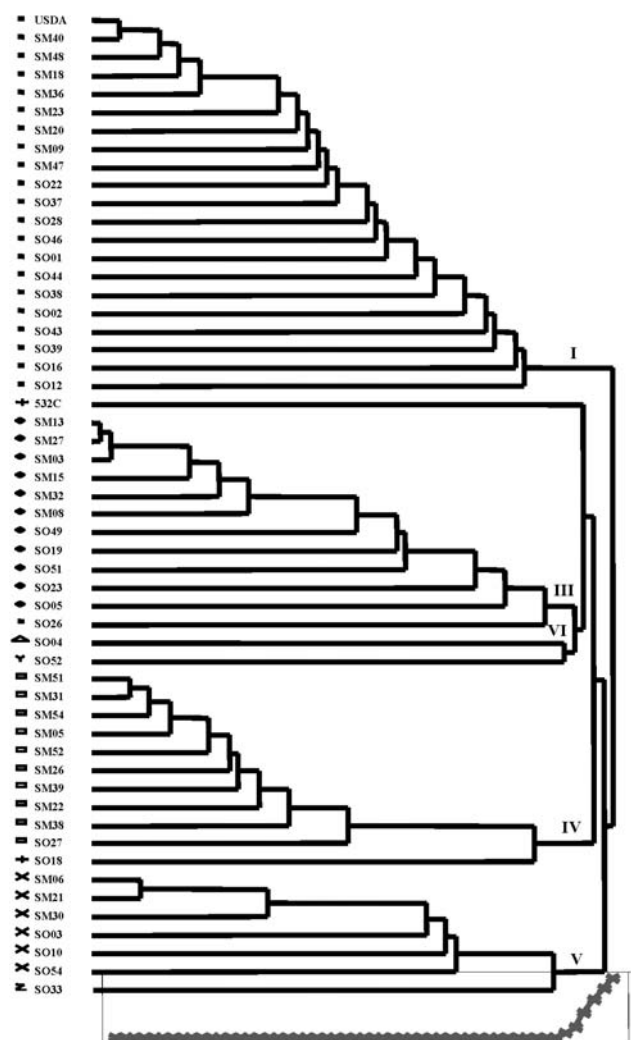


**Figure 5.** Dendrogram of 26 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Orford cultivar) inoculated with St-Hugus soil. Clustering was done based on PCR/RFLP of IGS region 16S-23S rRNA gene by using six different restriction enzymes. (S stands for St-Hugus soil and O stands for Orford cultivar).

## DISCUSSION

The results of RFLP analysis showed that endonuclease enzymes *MspI*, *AluI*, *HinfI* and *Sau3A* were more discriminative comparing with *DdeI* and *CelI*. It means that cutting sites of these two enzymes in the space region of 16S-23S rRNA of *Bradyrhizobium japonicum* isolates are not enough for RFLP technique.

In Ottawa soils 90% of isolates of *Bradyrhizobium japonicum* belonged to clusters I and III (Fig. 1). Cluster I formed 19% of the population and they were similar to USDA110 strain (for many years, USDA110 strain was used as a commercial inoculant in the United State of America for Soya bean inoculation),



**Figure 6.** Dendrogram of 52 isolates of *Bradyrhizobium japonicum* isolated from Soya bean nodules (Maple Glen and Orford cultivars) inoculated with St-Hugus soil. Clustering was done based on PCR/RFLP of IGS region 16S-23S rRNA gene using six different restriction enzymes. (S stands for St-Hugus soil, M and O stand for Maple Glen and Orford cultivars respectively).

which means Ottawa soil has also been inoculated with this strain or isolates similar to USDA110 are present in Ottawa soil. No isolates was similar to 532C strain in Ottawa soil. The results also showed that Maple Glen cultivar preferably nodulated by isolates from cluster III when inoculated with Ottawa soil. It is also possible that isolates in clusters III have a high frequency in the indigenous population of bradyrhizobial bacteria in Ottawa soil. Having a high competitive ability could be another explanation for a large number of isolates in cluster III.

The results of PCR/RFLP analysis of 26 isolates *Bradyrhizobium japonicum* isolated from Soya bean nodules of Orford cultivar inoculated with Ottawa soil showed that when the trap plant changed the isolate types and their frequency also changed. *Bradyrhizobium japonicum* bacteria that were isolated from Orford cultivar were grouped based on the RFLP patterns into three different clusters II, III and IV (Fig. 2). These clusters were similar to those, which were isolated from Maple Glen cultivar but their frequency was different. The percentage of isolates within clusters II, III and IV were 23, 58 and 19, respectively. The number of isolates in clusters II and IV increased comparing to those isolates, which were trapped by Maple Glen cultivar.

There was no isolate similar to either USDA110 or 532C when Orford cultivar was used as a trapped plant in Ottawa soil. This means that Orford cultivar did not trap any isolates of cluster I when inoculated by Ottawa soil. In order to confirm the data, 26 more isolates were isolated from Soya bean (Orford cultivar), which inoculated with Ottawa soil. Again isolates from cluster I were not detected. Obviously these isolates were present in Ottawa soil as they were present in the Maple Glen nodules, but many biotic and abiotic factors are involved in nodulation procedure. The results showed that both cultivars of Soya bean (Orford and Maple Glen) nodulated dominantly by isolates from cluster III in Ottawa soils.

When both host plants were used for trapping the rhizobial bacteria, all isolates from clusters I, II, III and IV were detected (Fig. 3). RFLP data also showed that by using two different host plants a better representative sample could be obtained from the rhizobial population.

The results were repeated in St-Hugus soil. Maple Glen trapped four Cluster I, III, IV and V when inoculated by St-Hugus soil. Orford cultivar trapped five clusters I, III, IV, V and VI when inoculated by St-Hugus soil. These results also showed that just a single trap plant is not able to pick up all isolates present in

the soil sample and using two trap plants would be better for having a good representative sample of indigenous rhizobia population. For example, Maple Glen cultivar was not nodulated by isolates from cluster VI in St-Hugus soil or isolates from cluster IV were picked up much better by Maple Glen as compared to Orford cultivar.

It can be concluded that the composition of population structure of *Bradyrhizobium japonicum* was affected by type of host plant. Two different clusters (I and VI) were not detected by Orford cultivar in Ottawa soil and by Maple Gelen cultivar in St-Hugus soils.

Similar results were obtained by Constanza *et al.* (2000). They showed that rhizobial bacteria isolated from plants grown under elevated CO<sub>2</sub> were genetically different from those isolates obtained from plants grown under ambient conditions. They also showed that a shift in the community composition of *R. leguminosarum* bv. *trifolii* occurred as a result of an increased atmospheric CO<sub>2</sub> concentration.

Bacteria from genera *Rhizobium* and *Bradyrhizobium* produce several kinds of surface polysaccharides such as exopolysaccharides and lipopolysacchides (Kannenberg *et al.*, 1998). These compounds are recognized by plants and lead to the induction of specific biochemical and physiological reactions (Hohn *et al.*, 1997). The amount of these compounds, which is produced by bacteria, is closely related with the environmental factors such as acidity and calcium concentrations (Maccio *et al.*, 2002). It also seems that different cultivars of each host plant species preferably picks up rhizobial bacteria from soil samples. Therefore, using just one host plant for isolating rhizobial bacteria population from soil samples is not sufficient to be a good representative of native population of rhizobial bacteria especially for ecological studies.

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