

Impact of *Trichoderma harzianum* Biocontrol Agent on Functional Diversity of Soil Microbial Community in Tobacco Monoculture in Argentina

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Abstract: The aim of this study was to assess the effect of *Trichoderma harzianum* R3P2 biocontrol agent on soil microbial community in tobacco monoculture. To this end, we studied the changes in soil metabolic diversity with cell suspensions methodology using Biolog[®] (Inc., Hayward, CA.) GN2 Microplates[™]. Principal component analysis showed that functional abilities of the soil microbial community in tobacco monoculture were altered by the application of the biocontrol agent. A novel taxon-independent index, Yokoyama CLPP Diversity Index, indicated that control plots had higher bioactivity than treated ones. Even though the introduced biocontrol agent resulted in a lower number of metabolized compounds, absorbance values were much higher for a set of specific compounds in control. Results suggest that the addition of biocontrol agent contributes to stimulate growth of a specific bacterial population shifting the microbial community.

Key words: Microbial diversity % Biological Control % Community level physiological profiles

INTRODUCTION

Microorganisms play a number of important roles in nature, stabilizing the ecosystem and consequently, preventing explosive growth of soil borne pathogens. Ecological studies of microbial communities have considered mainly diversity and function of species. Garland and Mills [1] introduced the use of community-level carbon source utilization patterns for estimating diversity of microbial community. Since then, this approach which has been used for comparison of microbial communities from different habitats [2, 3] has gained importance from an ecological perspective. Yokoyama [4] developed the Yokoyama CLPP Diversity Index for quantitatively describing diversity independently of taxon. It is well established that soil management practices affect the soil ecosystem and increases in soil-borne pathogens are expected in soils under monoculture system [5-7]. In Northwestern Argentina, a huge area is cultivated with tobacco, whose yield is severely curtailed by wilt caused by *Fusarium oxysporum* f.sp. *nicotianae*. As application of chemical fungicides proved to be ineffective to reduce disease, biological control appears an alternative method to control diseases. Even though, is not fully understood the

impact of biocontrol agents on functional diversity of native microbial communities [8-10).

Antagonists that produce anti-fungal metabolites alter the microbial community structure when they are applied to microhabitats [8] and this might affect crop yield and soil microbial process [11-3).

In previous studies, we investigated the ability of *Pseudomonas fluorescens*, *T. harzianum* and other naturally occurring microorganisms to reduce the population of soil borne plant pathogens in cultivated soils [12].

The purpose of this study was to assess the impact of a strain of *T. harzianum* on the soil bacterial community when it is introduced into soils monocultured with tobacco.

MATERIALS AND METHODS

Site Description and Field Sampling: The experimental plot was established in a commercial field of a private company in Villa Alberdi, Tucumán, Argentina (27°35' S; 65°36' W) during tobacco cropping season in 2004. The experiment was carried out in preparation of risk assessment studies accompanying the application of the biocontrol agent.

The field site was located in southern region of Tucumán Province characterized by the subtropical climate with a dry winter season (June to September) and a humid summer season (December to March). Mean annual rainfall ranges from 600 to 1000 mm and the average temperature is 26°C. The soil in the experimental plot was characterized as typical Hapludol, pH 6.8, with a history of 8 years of tobacco monoculture.

The experiment consisted of four blocks and was arranged in a randomized complete block design named with a number and a letter. Five sub samples were taken from each experimental plot at harvest time sites, which referred to the treatment from a depth of 0-10 cm, at five random places from each experimental plot. The soil was bulked and kept in plastic bags at 4°C until use. Disease severity was evaluated 84 days after planting and calculated according to Camporota [13] with a modified scale ranging from zero to five.

Treatments: *Trichoderma harzianum* strain R3P2 isolated from the rhizosphere of tobacco was found to be an antagonist of *Fusarium oxysporum f.sp. nicotianae*. Field experiment consisted of a non-treated control and a treatment with the biocontrol agent. *Trichoderma harzianum* was transferred from a conservation medium [14] to a liquid medium, BASE S broth [15] and incubated for seven days in an orbital shaker (250 rev min⁻¹) at 25°C. The mycelial mat was blended immediately after collection and kept at 4°C until it was used. The roots of tobacco seedlings were immersed in a 50% diluted formulation containing 5x 10⁶ *T. harzianum* propagules for 60 minutes and then planted in the soil.

Community-Level Response to a Range of Carbon Sources: Metabolic diversity of soil communities from control and treated plots was analyzed by using BIOLOG[®] Microtiter[™] GN2 plates by means of cell suspension [16, 17].

Cell Suspension Method: A 5 g sub-sample of each soil sample was added to 95 ml of sterile phosphate buffer 15 mM pH 7.0 and was shaken at 250 rev min⁻¹ for one hour. An aliquot of 100 µl of standard dilutions in phosphate buffer (10⁻⁴ to 10⁻⁶) was spread onto plates of PTYG (peptone, tryptose, yeast, glucose) agar medium [18]. Plates were incubated at 25°C for four days under darkness.

In order to choose colonies at random, plates were divided in sectors and all colonies within a chosen sector

were picked. Once colonies were purified, biomass of them was increased on the same medium and incubated for 48 hours at 28°C. Based on a modification of Tonso *et al.* [18], 40 colonies were randomly chosen to inoculate individual plates.

Inoculum from the bacterial colonies was picked up with a long cotton swab, suspended in Biolog[®] GN fluid (0.40% NaCl, 0.03% Pluronic F-68, 0.01% Gellant Gum dissolved in deionized water). Transmittance was adjusted at 75%. Aliquots of 150 µl of each bacterial suspensions were inoculated on three 96-well GN microtiter plates [1] and incubated at 28°C.

The degree of utilization of a particular substrate was quantified by measuring the intensity of color (optical density), which indicated the respiratory activity of each bacterial isolate or soil community, representing each metabolic pattern the functional attributes of the microorganism or community. Absorbance at 590 and 750 nm of GN2 plates were measured at 24, 48 and 72 hours by means of an Emax[™] plate reader (Molecular Devices[®], Inc., Sunnyvale, CA, USA).

The optical density (O.D.) values of Biolog[®] microplate wells were corrected considering the background color developed in the control well A1.

Negative scores were set to zero. In order to reduce the variable-to-sample ratio [19] for processing data from cell suspension samples, the 95-carbon sources were grouped in 11 chemical groups. The average absorbance for the wells corresponding to each group was calculated. We considered only those metabolic C sources with scores >0.35 contributed to principal components.

Functional Diversity: The influence of *T. harzianum* on functional diversity of soils was analyzed by means of the average well color development (AWCD) of the microplate [20].

The CLPP Yokoyama Diversity Index describes the relative complexity of systems which might even have different number of components. It is in proportion to square distances between bacterial components and in inverse proportion to the number of components. The DI was calculated as follows,

$$DI = (E d_i)^2 / n$$

where:

d_i = cluster separation,

n = total number of colonies of the cell suspension sample.

The AWCD was used to compare the individual color data and to discriminate the carbon sources most intensively used by microbial communities in each microplate.

By a modification of the method of Vahjen *et al.* [21], using color absorbancies instead of binary data, we identified the substrates that contributed mainly to the activity of the microbial community which were those that showed higher absorbancies compared with the average plate absorbance.

RESULTS

The analysis of disease severity showed that there was no significant difference among treatments. The community-level-physiological profiling (CLPP) of both untreated and treated soils with *T. harzianum* had similar profiles.

Multivariate results for the control and *T. harzianum* treated plots at 48 h of incubation, are presented in Figure 1. Functional characteristics of some bacterial members of control soil were different from those of *T. harzianum* treated soils.

The first component accounted for 65% of variance while the second component accounted for 20%. This indicates that a total of 85% of variance was represented in the two dimensional graph (Figure 1). The eigenvector scores in Table 1 shows the contribution of each carbon source to the CLPP groups.

Bacterial metabolic profile of unaltered soil sites utilized mainly carbohydrates and carboxylic acids, while in soils treated with *T. harzianum*, used mainly phosphorous compounds.

Table 1: Carbon compounds utilized by microbial communities from soil collected under tobacco field.

Samples were collected at harvest time and processed according to cells suspension methodology

Chemical Group	PC1	PC2
Var (%) ^a	60	25
Carbohydrates	0.18	-0.52
Esters	0.04	-0.15
Polymers	0.07	-0.25
Carboxylic Acids	0.14	-0.51
Alcohols	0.35	-0.09
Amides	0.08	-0.10
Phosphorilated Compounds	0.55	0.57
Amino Acids	0.38	0.03
Aromatic Compounds	0.56	-0.09
Brominated Compounds	0.05	-0.10
Amines	0.23	-0.16

^aPercentage of variance explained by each principal component.

The sign of the scores are indicative of a positive or negative association of the different chemical groups consumption.

Carbon sources like alcohols, aromatic compounds and amino acids correlated to PC1 had negative response in the tightly clustered samples from control and treated soils. In turn, the same substrates and phosphorus compounds shown high correlation with *T. harzianum* treated samples while carbohydrates and carboxylic acids correlated to PC2 and exhibited mainly utilization by control samples (Figure 1 and Table 1).

The analysis of functional diversity among bacterial components showed that diversity as Yokoyama CLPP Diversity Index was lower for treated plots (DI, 3.68) compared with the control soils (DI, 4.41).

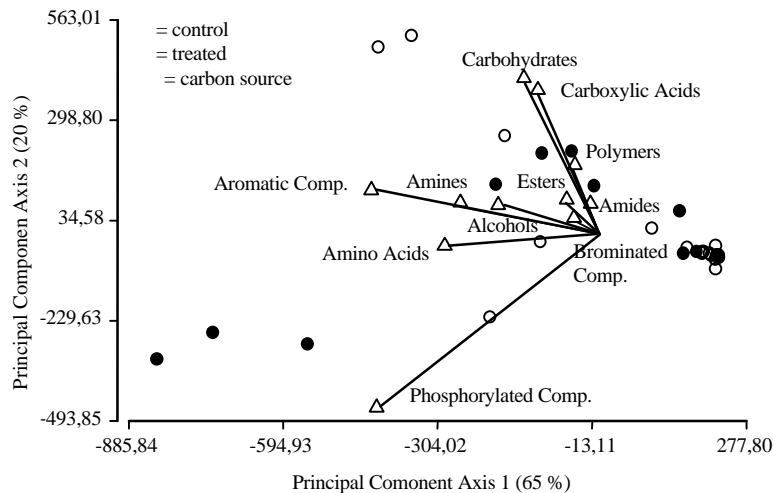


Fig. 1: Biplot of community-level-carbon source utilization by soil microorganisms through cell suspension methodology

Among the most intensely utilized substrates, after 48 h of incubation, microplates from control and treated plots shown different metabolic patterns. Substrates like bromo succinic acid, m-Inositol, sucrose, citric acid, d-galacturonic acid, d-glucosaminic acid d, L-"-Glycerol Phosphate, hydroxi-L-proline were highly utilized in treated samples. On the contrary, substrates like 2-Aminoethanol, "-D-Glucose, D-Cellobiose, D-Galactose, D-Maninitol, D-Mannose, D-Psicose, D-Trehalose, Gentobiose, i-Erithriol, l-Fucose, Maltose, N-acetyl-D-Galactosamine, L-alaninamide, Methyl pyruvate, "-cyclodextrine, Glycogen, Tween-80, "-ketobutyric-acid, "-Ketoglutaric Acid, "-Ketovaleric Acid, D-Gluconic Acid, D-Glucuronic Acid, D-L-Lactic, Acid, Malonic Acid, Propionic Acid, Sebacic Acid, Glycerol Glucuronamide, D,L-Carnitine, D-Alanine, Glycyl-L-Glutamic Acid, L-Alanine, L-Asparagine, L-Aspartic Acid, L-Histidine, L-Leucine and L-Proline were oxidized at highly rates by isolates of control plots.

DISCUSSION

From our results it is important to point out that inoculation with *T. harzianum* did not significantly modify disease severity in treated plots. However, changes in microbial metabolic profiles were detected.

The use of CLPP to assess microbial diversity is an ecologically reliable method for measuring diversity, because both sources of nutrients and the environment are the most important influencing microbial growth in the soil. CLPP approaches are therefore an important tool to show the ecological effect of the introduction of the biocontrol organisms on the ecosystem [1].

Differences due to the introduction of biocontrol agent were explainable by the functional richness. Our data suggested that substrate utilization can be applied to detect changes in the soil microflora as a response to the addition of the biocontrol agents. At Tucumán in Northwestern Argentina, the metabolic diversity of the soil bacterial community in plots with high inoculum density of *Fusarium oxysporum f.sp. nicotianae*, was altered by *T. harzianum* treatment. Similar diversity alterations of indigenous soil bacteria were observed by White *et al.* [22] in soils inoculated with *Pseudomonas fluorescens*.

Our results from the most intensely utilized substrates indicated that samples from control soils metabolized most carbon sources at higher rates than those treated with *Trichoderma*. The results of cell analysis may indicate that cell lines isolated from the soil

community evolve in response to the appropriate substrate. In agreement with Caldwell *et al.* [23], we suggest that microbial community of treated soils may be a mixture of related organisms and the members could be associated through specific functional behavior. Data reflected that metabolic diversity in soils treated with the biocontrol agent decreased. Presumably, the addition of *T. harzianum* in the soils stimulated growth of a specific bacterial population, formed by dominant functions in the community.

The differentiation of the most intensely utilized substrates in their chemical nature led to differences in terms of response of soil treated with *T. harzianum*. According to Vahjen *et al.* [21], the analysis of specific responses is important to interpret the results, facilitating the detection of differences among treatments that could be covered in overall analyses of substrates. We distinguished the intensely used substrates from those that were not or only weakly utilized in order to obtain a different indicator of soil biological perturbations induced by the biocontrol agent introduction.

The metabolic richness of the soil microflora was reduced in treated soils. Our data, in agreement with Bosio and Scow [24], suggest that the most affected carbon sources were carbohydrates followed by amino acids and carboxylic acids, which were consumed at lower level (76%) than in control plots. A set of carbon sources were consistently highly utilized by the bacterial community of *T. harzianum* treated soils, which includes bromide and phosphor compounds, whereas polymers, amines, amides and esters were used at higher rates by the soil community of control plots. Zak *et al.* [25] and Garland [20] stated that carbohydrates contributed mostly to the overall activity and appeared to respond mainly to perturbations in the soil community.

Scores of absorbance showed that microbial community of treated plots exhibited much higher degrees of oxidation of m-inositol, citric acid, d-galacturonic acid, D-glucosamine acid, hydroxy-L-proline and L-pyroglutamic and relative low value in control plots. Haack *et al* [26] found that communities with high members of antagonistic *Pseudomonas* reduced m-inositol and hidroxy-proline at higher rates than other carbon sources. Furthermore, they reported that m-inositol contributed significantly to differentiate microbial communities.

Microorganisms should be introduced to soils at high levels since a rapid decline of the population occurs because of soil resistance to introduced biocontrol agents

due to abiotic or biotic factors [10]. Lower levels of carbohydrates and carboxylic acids consumption have been associated with specialization in the succession of microbial communities [1]. Results [23] to date indicate significant increases in niche specialization during succession in the soil.

Microorganisms in the soil might be in a dormant state or inhibited by the effects of the general soil biota. However, the active soil biota can affect the introduced biocontrol agents and the introduced agent may be important in shifting the resident biocontrol microflora to an active state [27].

Positive response to certain substrates in community profiles generally reflects the presence of dominant functions. There seems to be specific bacterial populations of functional guilds selected by the condition of treated plots. Several reports [28, 29] have emphasized that the biotic components of the soil might play an important role in growth and control efficiency of *T. harzianum* when it is introduced into soil ecosystems. Contrary to our results, Bae and Knudsen [10] have reported that the addition of *T. harzianum* into soils resulted in a higher bacterial population. On the basis of functional diversity response, the addition of *T. harzianum* altered tobacco monocultured soil functional diversity. The biocontrol agent contributes to shift functional profiles of dominant members resulting in a reduction of substrate utilization by the copiotrophic bacterial communities of treated soil samples.

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