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Assessing insecticide and fungicide effects on the culturable soil bacterial community by analyses of variance of their DGGE fingerprinting data

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ABSTRACT

To assess the effects of three insecticides (aldicarb, chlorpyrifos, deltamethrin) and two fungicides (tebuconazole and metalaxyl + mancozeb) on the PCR-DGGE fingerprints of culturable soil bacterial communities (CSBC), a greenhouse experiment was carried out with soil samples from an Integrated System for Agroecological Production (ISAP), a Conventional Potato Production Area (CPPA) and a Secondary Forest Area (SFA) close to the CPPA. Samples were obtained at 15 day intervals starting at 32 until 77 days after sowing (DAS) to perform the PCR-DGGE analysis of the CSBC cultured on media amended with soil suspension. Analysis of variance from PCR-DGGE data indicated significant differences among treatments. Regardless the type of pesticide applied, CSBC was disturbed and similarity values varied from 5% to 90% in comparison to the control. Significant shifts on CSBC were only detected among treatments in the first two harvests, while CSBC tended to be more akin to each other at the last two harvest dates. The most significant responses observed were due to different soil sample origins, where values of 5% of similarity to the control were observed on CPPA soil. The use of analysis of variance on PCR-DGGE data was useful to a better understanding of the changes on CSBC induced by pesticides applications.

1. Introduction

A large number of pesticides is used to control potato pests in Brazil [6]. From those registered in the Ministry of Agriculture, 41 are for pest and 58 for disease control [2].

Although the use of pesticides is intended to provide satisfactory crop yields by controlling commonly occurring pests and disease in production fields, some may be toxic to the environment, as well as to humans. Reports on 320 chemicals registered for agricultural use in Brazil revealed that 18 insecticides and 5 fungicides, had potential risks to humans [10]. Adverse effects caused by pesticides are related to the central and peripheral nervous systems, in addition to elicitation of immunosuppressive or carcinogenic responses [14].

Some pesticides may accumulate throughout the food chain, affecting several trophic levels. The understanding of the impact a pesticide may cause to the environment is a complex issue, being necessary to observe the overall hierarchical chain, from a single molecule to the entire ecosystem, passing by the cell, the organism and the community [30].

The study of pesticide effects on non-target populations is an accepted strategy to evaluate its associated potential environmental risks. Among non-target populations, soil microorganisms are extremely important, since they play an essential role in nutrient turnover [3], maintaining generative capacity in agroecosystems [7]. The processes of ecological succession are, among other factors, mediated by microorganisms and depend on a fine balance of their population dynamics [23]. Under these circumstances, the impact inflicted on soil microbial populations caused by a specific pesticide is a potential indicator of the toxicity level of this product, and may represent a component of a broad study aiming to evaluate its potential impact on the environment [24].

Recently, microbial diversity has been studied through molecular methods, mainly by the analysis of the ribosomal genes, which are amplified by Polymerase Chain Reaction (PCR) and sequenced after cloning, or by the study of microbial community profiles obtained using molecular tools such as Random Amplified Polymorphic DNA – RAPD [16], Amplified Ribosomal DNA Restriction Analysis – ARDRA [42], Terminal Restriction Fragment Length Polymorphism – T-RFLP, RISA [19], Denaturing/Temperature Gradient Gel Electrophoresis – DGGE/TGGE [31] and Single-Strand Conformation Polymorphism – SSCP [38].

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DGGE fingerprints are commonly analyzed by cluster analysis (dendrogram) and/or non-parametric analysis, which pose great difficulty to establish whether clusters are significantly different. Non-parametric statistics shows some advantages to parametric approaches, such as reduction or even avoidance of bias caused by outliers. Besides, no assumptions are needed about the distribution of the analyzed values and homogeneity of variances as well as additivity of effects [21]. The disadvantage of non-parametric statistics is its complexity, power analysis, and time consuming. In contrast, parametric statistics are simple and easy to compute but rely upon the assumption of a "Gaussian" distribution. Parametric statistics are known to be generally robust even when the assumption of "Gaussian" distribution is violated [29].

The objectives of this work were to evaluate the impacts of different insecticides and fungicides on the PCR-DGGE profiles of culturable soil bacterial communities (CSBC) as compared to forest area, and to determine the potential use of the analysis of variance and mean tests on the interpretation of PCR-DGGE data.

2. Material and methods

2.1. Soil sampling and experimental design

Soil samples were collected from an Integrated System for Agroecological Production (ISAP) located in the county of Seropédica, Rio de Janeiro, Brazil. The ISAP is being subjected to agroecological management since 1993 and plant diversity is being stimulated by intercropping and the use of green manure. Soil samples were also collected from a Conventional Potato Production Area (CPPA), and in a neighboring Secondary Forest Area (SFA), both located in Brasília, DF, Brazil. Soil from SFA was used for potato cultivation because despite differences in the structure and function of heterotrophic microbial communities in forest soils have been linked primarily to the quantities and qualities of soil organic materials [40] these soils are frequently used as a reference of non disturbed environment.

In the CPPA, potato has been intensively cultivated for several years, and the use of chemical fertilizers and pesticides is the strategy to obtain high yields. The SFA is a typical area of the "Cerrado" ecosystem common in the Brazilian central plateau. In each area, soil sampling was performed at 0–20 cm depth. Soil analyses were performed according to EMBRAPA [15] and results are shown on Table 1.

The experiment was carried out in a greenhouse at Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil, from July to September 2003. This period comprises the winter season with climatic conditions relatively constant over the entire experimental period (Table 2). The experimental design was a completely randomized block with 3 replicates, in a factorial arrangement.

The factorial arrangement was composed of: 3 soils (ISAP, CPPA and SFA); 5 pesticides (3 insecticides: deltamethrin, aldicarb and chlorpyrifos; and 2 fungicides: tebuconazole and metalaxyl + mancozeb); and 4 harvest periods (32, 47, 62 and 77 days after sowing – DAS). For each soil type a control treatment without insecticide or fungicide application was assigned. This factorial arrangement resulted in a total of 216 pots, each one representing an independent experimental unit.

Table 1
Fertility analysis of the soils used to evaluate the effects of insecticides and fungicides on the culturable soil bacterial communities.

	pH	Al	Ca + Mg	Ca	Mg	P	K	C	OM	N
		Cmolc dm^{-3}				mg dm^{-3}		%		
AF	6.7	0	4–0.3	3.0	1.3	117.3	142.1	1.7	2.9	0.19
CPPA	5.4	0.3	2.7	2.1	0.6	17.7	150.3	0.4	0.6	0.13
SFA	5.5	1.5	1.2	0.9	0.3	0.7	62.1	1.5	2.6	0.15

Al, aluminum; Ca, Calcium; Mg, magnesium; P, phosphorus; K, potassium; C, carbon; OM, organic matter and N, nitrogen.

Potato cv. Achat was cultivated in 1.5 kg pots containing the same soil amount for all treatments. Pesticides were applied according to Table 3. Two spray applications of pesticides were performed at 30 and 45 DAS, to simulate field conditions, except for aldicarb, incorporated during pot preparation.

2.2. Preparation of medium for soil microorganism cultivation

A culture medium (Meio Solo – MS) was used where the nutrient source for microbial growth was soil (Zilli, 2004) [44], collected on each site described on Section 2.1. Initially, soil samples were sieved through a 2 mm wire mesh, dried at 65 °C and ground in a rolling mill [41]. Afterwards, different amounts of ground soil (10, 20, 40 and 80 g) and agar (1.5, 2 and 4 g) on a final volume of 100 mL, were tested to reach the best condition for gelling of the culturable medium. The MS medium was sterilized and poured into Petri dishes to solidify.

2.3. Soil microbial community cultivation

After removing the plants from each pot, including the root system, the soil was homogenized and a 10 g sample of bulk soil was taken and placed in Erlenmeyer flasks (250 mL) containing sterilized water (90 mL) to compose the soil suspensions used for the CSBC cultivation. The flasks were placed on a shaker at 200 rpm for 30 min. A total of 100 μL of the soil suspension from each treatment was sampled and inoculated in Petri dishes containing the corresponding MS medium. Plates were incubated until abundant microbial growth could be observed (28 °C; 5 d). After this period, sterilized water (2 mL) was added to the surface of the culturable medium, cells were mixed with a platinum loop and the suspension was collected with an automatic pipette. Approximately 1 mL of this material was transferred to a microtube (1.5 mL) and centrifuged (9300 × *g*; 15 min). The supernatant was discarded and the pellet stored at −20 °C overnight.

2.4. DNA extraction

DNA extraction was performed following the protocol described by Schwieger and Tebbe [37] and modified by Xavier et al. [43]. The stored pellets were suspended in 0.6 mL of TES buffer (0.05 M NaCl; 0.01 M EDTA; 0.05 M Tris HCl pH 8.0; 1% SDS) and vortexed. Samples were subjected to five freeze and thaw cycles, consisting of freezing in liquid nitrogen (5 min) and heating under agitation (65 °C; 180 RPM; 5 min). After each freeze and thaw cycle, samples were mixed by vortexing, 0.168 mg of proteinase K were added to each sample, followed by incubation under agitation (65 °C; 180 RPM; 1 h). At the end of the incubation period, 0.6 mL of phenol:-chloroform:isoamyl alcohol (25:24:1) was added to each sample and centrifuged (7500 × *g*; 6 min). The supernatant was transferred to a 1.5 mL microtube and 0.6 mL of chloroform:isoamyl alcohol (24:1) was added followed by centrifugation (7500 × *g*; 6 min). A 0.5 mL of the supernatant was transferred to a 1.5 mL microtube and 0.5 mL of cold isopropanol was added. Samples were incubated at −20 °C for 60 min and centrifuged (16,100 × *g*; 20 min). The supernatant was removed and the pellet was dried in a cold trap centrifuge and raised in 50 μL of TE buffer (10 mM Tris; 1 mM Na-EDTA; pH 8.0).

2.5. PCR-DGGE conditions

PCR was performed with three different dilutions of the DNA samples: 1:20, 1:40 and 1:80 in ultrapure water. After amplification each replicate was mixed together in a single microtube. For each