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Influence of agricultural practices and seasons on the abundance and community structure of culturable pseudomonads in soils under no-till management in Argentina

--Manuscript Draft--

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Abstract:	Background and aims Pseudomonas are common inhabitants of rhizospheres and soils, and it is known that soil types and crops species influence their population density and structure. 20x106 ha are cultivated under no-tillage in Argentina and there is a need to find new biologically-based soil quality indexes to distinguish between sustainable and non-sustainable agricultural practices. Pseudomonads abundance and community structure were analyzed in no-till soils with different agricultural practices, in productive fields along 400 km of Argentinean Pampas. Methods We sampled soils and root systems from agricultural plots in which sustainable or non-sustainable agricultural practices have been applied. Samples were collected in summer and winter during 2010 and 2011. Culturable fluorescent and total pseudomonads were enumerated by plating on Gould's selective medium S1. Colonies from these plates served as DNA source to carry out PCR-RFLP community structure analysis of the pseudomonads-specific marker genes oprF and gacA. Results Abundance of total and fluorescent culturable pseudomonads in bulk soils was influenced by seasonal changes and agricultural practices. Rhizospheric counts from the same crop were affected by agricultural treatments. Also, crop species influenced pseudomonads density in the rhizosphere. Combined PCR-RFLP profile of both genes showed a seasonal grouping of samples. Conclusions Sustainable soil management seems to favor pseudomonads development in soils, favoring root colonization of crops from those plots. Crop species influence total pseudomonads load of rhizospheres and its community structure. Total or relative pseudomonads load could function as soil quality indicator of good agricultural practices.
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reconsidered for publication should you be prepared to incorporate major revisions. Both reviewers find the topic of your manuscript interesting and fitting for Plant and Soil. However, the aim of the work is not clearly described and the manuscript needs to be drastically rewritten. Some conclusions are not justified based on the results presented. The reviewers have made numerous valuable suggestions to improve the study".

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*Covering Letter

Universidad Nacional de Quilmes

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Bernal, October 11th, 2013

Dear Editors of Plant and Soil:

My colleagues and I wish to submit the manuscript entitled: "Influence of agricultural practices and seasons on the abundance and community structure of culturable pseudomonads in soils under no-till management in Argentina", for publication in the journal Plant and Soil.

In this manuscript, we report the application of a previously published work, in which we developed a combination of selective count and PCR-RFLP assays to study pseudomonads in bulk soil and rhizospheric samples from agricultural plots under no-till management in Argentina. We sampled those plots during two consecutive years, in summer and winter. The results highlight the effects of agricultural treatments and seasons on pseudomonads density. We confirmed the rhizosphere effect on this bacterial genus, and the effects exerted by crop species and geographical location on the community structure.

All authors agree with the submission of the work as it stands and declare that do not have any conflict of interest. We have followed the instructions for authors available in your web site for its preparation. References have been compiled and formatted using Mendeley with the corresponding output style for Plant and Soil. Figures have been prepared in CorelDraw and exported as TIFF files. We are providing supplementary material with information that we think it helps to the review process (two Tables and four Figures).

We hope you and the referees will deem the manuscript suitable for publication as an article in Plant and Soil.

Sincerely yours,

Lic. Betina Agaras

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Revised version including track changes

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Influence of agricultural practices and seasons on the abundance and community structure of culturable pseudomonads in soils under no-till management in Argentina

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Abbreviations

NT: no-tillage; GAP: Good Agricultural Practices; BAP: Bad Agricultural Practices; NE: natural environment; TP: total pseudomonas abundance; FP: fluorescent pseudomonas abundance; TH: total heterotrophic bacteria; RFLP: Restriction Fragment Length Polymorphism

Abstract

Background and aims *Pseudomonas* are common inhabitants of rhizospheres and soils, and it is known that soil types and crops species influence their population density and structure. $20x10^6$ ha are cultivated under no-tillage in Argentina and there is a need to find new biologically-based soil quality indexes to distinguish between sustainable and non-sustainable agricultural practices. Pseudomonads abundance and community structure were analyzed in no-till soils with different agricultural practices, in productive fields along 400 km of Argentinean Pampas.

Methods We sampled soils and root systems from agricultural plots in which sustainable or non-sustainable agricultural practices have been applied. Samples were collected in summer and winter during 2010 and 2011. Culturable fluorescent and total pseudomonads were enumerated by plating on Gould's selective medium S1. Colonies from these plates served as DNA source to carry out PCR-RFLP community structure analysis of the pseudomonads-specific marker genes *oprF* and *gacA*.

Results Abundance of total and fluorescent culturable pseudomonads in bulk soils was influenced by seasonal changes and agricultural practices. Rhizospheric counts from the same crop were affected by agricultural treatments. Also, crop species influenced pseudomonads density in the rhizosphere. Combined PCR-RFLP profile of both genes showed a seasonal grouping of samples.

Conclusions Sustainable soil management seems to favor pseudomonads development in soils, favoring root colonization of crops from those plots. Crop species influence total pseudomonads load of rhizospheres and its community structure. Total or relative pseudomonads load could function as soil quality indicator of good agricultural practices.

Keywords

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1. Introduction

Soil microbiology is a continuously growing discipline that aims to understand the assembly of microbial communities dwelling in soil, and their contribution to plant health, nutrient cycles and biochemistry of soil (Cho and Tiedje 2000). In recent years, increasing efforts were made to characterize the bacterial community of agricultural soil, and particularly, to study the effect that agricultural practices have on microbial community structures (Costa et al. 2006b; Costa et al. 2006c; Picard et al. 2008; Cycoń and Piotrowska-Seget 2009; Figuerola et al. 2012; Ding et al. 2013). In Argentina, almost $20x10^6$ ha (78.5% of the arable land) are under no-till management (AAPRESID; www.aapresid.org.ar/superficie/). Moreover, this dominant practice in our country is expanding towards marginal soils with more challenging climatic and nutritional conditions. When no-tillage is accompanied by crop rotation and rational use of chemical fertilizers/pesticides, soil quality increases over time (Derpsch et al 2010); such kind of soil management is referred to as Good Agricultural Practices (GAP) (FAO 2003; AAPRESID 2013). GAP tend to preserve soil quality and high productivity (Leoni Velazco 2013). By contrast, no-tillage associated with mono-cropping and misuse of fertilizers/pesticides induce soil quality decline (low crop yields, accumulation of chemical products in soil, higher soil erosion and higher incidence of plant diseases) (FAO 2003; Raaijmakers et al. 2009; AAPRESID 2013; Leoni Velazco 2013); these are considered Bad Agricultural Practices (BAP), i.e., the absence of GAP (AAPRESID 2013b). Traditionally, the assessment of soil quality, and therefore,

84 the distinction between plots managed under GAP or BAP, has been
85 determined on the basis of a set of chemical and physical indicators (Doran and
86 Zeiss 2000; Schloter et al. 2003; Aparicio and Costa 2007). However, after
87 more than four decades of no-till farming in Argentina, soil productivity could not
88 always be directly linked to chemical and physical indicators, and prompted
89 farmers to seek for biological predictors of soil sustainability and productivity.
90 Provided the complexity of the soil ecosystem, identifying biological indicators of
91 soil quality and sustainable agricultural practices may require multidisciplinary
92 approaches and cooperation between public and private initiatives (Wall 2011).
93 Among soil bacteria, *Pseudomonas* is one of the most diverse and
94 widespread genus in ecosystems, due to its physiological and genetic
95 adaptability (Stanier et al. 1966; Palleroni and Moore 2004; Silby et al. 2011).
96 Many soil pseudomonads strongly colonize plant roots and promote plant
97 growth by means of direct or indirect mechanisms, thus being regarded as plant
98 growth promoting rhizobacteria (PGPR). In particular, a number of siderophore-
99 producing fluorescent pseudomonads (Budzikiewicz 1993) indirectly contribute
100 to plant health by antagonizing diverse plant pathogens (Haas and Défago
101 2005). Evidence gathered from various cropping systems at diverse
102 geographical sites and by different authors, strongly suggest that the
103 assemblage of pseudomonads communities in soil is influenced by plant
104 species, soil conditions, crop management system, and interactions with other
105 biological agents like predators and competitors (for a review, see Picard and
106 Bosco, 2008). We thus hypothesized that: 1) the amount and composition of
107 pseudomonads communities in agricultural soils under no-till management in
108 Argentina are influenced by the agricultural practice (GAP or BAP); 2)

pseudomonads abundance and community structure are not static and have a seasonal dynamics; 3) pseudomonads communities are geographically distinct. Therefore, the aim of this work was twofold: i) to analyze the influence of agricultural practices season and soil location, on the abundance and community structure of culturable pseudomonads in soil and rhizosphere of Argentinean crop fields under no-till management; ii) to identify quantitative and/or qualitative potential indicators of Good Agricultural Practices under no-till management.

2. Materials and methods

2.1. Sampling sites and sample collection

Bulk soil and plant root samples were collected from three different soil managements: Good Agricultural Practices (GAP), Bad Agricultural Practices (BAP), and natural pastures where no agriculture was practiced for at least 30 years (Natural environment, NE) as reference. As we wanted to compare agricultural management regardless soil type, the block of treatments was replicated in productive fields at four different geographical sites, distributed across a 400 km west-east transect in the most productive region in the Argentinean Pampas, near the villages of Bengolea and Monte Buey (Córdoba province), Pergamino (Buenos Aires province), and Viale (Entre Ríos province). The geographical coordinates, physicochemical properties and the records of

agricultural management and crop yields of the sampled fields are available elsewhere (Figuerola et al. 2012; Agaras et al. 2012).

In every plot treatment-site, we obtained three replicate samples of soil and root systems (when indicated). Each replicate sample was collected at a distance of ca. 50 m and corresponded to a composite of 16–20 top soil cylinders (0-10 cm) taken within a 5-m^2 spot. The whole sampling scheme was carried out in two consecutive years (2010 and 2011), both in mid-summer (February) and in late winter (September). Root samples were obtained only in summer, when crop plants were present in all GAP and BAP plots (Online Resource 1). Their root systems were separated to analyze the rhizosphere community (Agaras et al. 2012). Soil and root samples were stored at 4°C for no longer than 30 days (Sheppard and Addison 2006) before being processed for bacterial counts and community structure analysis.

2.2. Processing of soil and rhizosphere samples for bacterial enumeration

Soil and rhizosphere samples were processed as described previously (Raaijmakers and Weller 2001; Agaras et al. 2012). Briefly, 1g of soil or 2g of root material (roots with tightly adhered soil) were thoroughly agitated in 10-volumes of saline solution (0.85% (w/w) NaCl) and sonicated in a water bath (40 Khz, 160 W, Testlab TB04, Argentina). Thereafter, tubes with soil or root samples were centrifuged at 50×*g* at room temperature for 1 min, and the supernatant was recovered in new clean tubes. Serial dilutions of each soil or rhizosphere suspensions were plated in triplicate on Gould's S1 plates (Gould

et al., 1985) to count culturable pseudomonads (TP), and on 10% TSA (tryptone-soy agar, Biokar) to count total heterotrophic mesophilic bacteria (TH). Both media were supplemented with cycloheximide (100 μg/ml) to inhibit growth of fungi and yeasts. Colony counts were done after 48 h of incubation at 28 °C. . The abundance of fluorescent pseudomonads (FP) was estimated as the number of colony forming units (CFU) that showed surrounding fluorescent halos upon exposure of S1 plates to UV light (290nm). Moisture content of soil samples was estimated as weight loss upon drying at 62 °C for 7 days, and then used to express the soil bacterial abundance as CFU per g of dry soil.

2.3. PCR-RFLP analyses of culturable pseudomonads

To study the structure of the culturable community of pseudomonads, S1 plates were washed to recover bacterial cells from colonies as described previously (Agaras et al. 2012). Templates for PCR reactions were prepared by incubating the suspensions for 15 min at 100ºC, centrifuging at 14000rpm for 2 min and recovering supernatants in new clean 1.5-ml tubes. Thermal lysates were stored at -20ºC. For PCR analysis, equal volumes of DNA templates from each plot replicate were pooled into a single representative sample per treatment at each geographical site. We have already shown that RFLP patterns from pooled samples are equal to the sum of the three patterns from every biological replicate (Agaras et al. 2012). PCR-RFLP assays of *oprF and gacA* genes were performed as described previously (Agaras et al. 2012). Briefly, primers *oprF*-FW2 (5'-ATCGGYTACTTCHTBACHGA-3') and *oprF*-Rev2 (5'-CCNACGGAGTCRGTRTGRCC-3') served to amplify an internal *oprF*

fragment of 602-683 bp, and primers *gacA*-Fw (5'-TGATTARGGKSYTRGTDGTCG-3') and *gacA*-Rev (5'-ATCATCARSGCRATCTGGAT-3') to amplify an internal 480 bp-*gacA* fragment. The *oprF* amplicons were digested separately with *Hae*III (Promega) and *Taq*I (Fermentas), whereas *gacA* amplicons were treated with *Hae*III and *Mbo*I (Fermentas). Incubations were performed for 3 h at the corresponding temperatures (37 °C for *Hae*III and *Mbo*I; 62°C for *Taq*I). Restriction products were separated by electrophoresis in 2% agarose gels (Invitrogen, USA) in 0.5× TBE at 6 V/cm for 2 h. Gels were stained with ethidium bromide and DNA banding patterns were visualized under UV light. A 50 bp DNA marker (PB-L, Argentina) was included to normalize gels before multiple RFLP pattern comparison.

2.4. Statistics

For every treatment (three) within each geographical site (four), the number of replicate samples was three (n = 3). Plant roots were sampled in parallel to have 3 replicates per treatment and per site. So, the sampling at every season consisted in a total of 4 sites × 3 treatments × 3 replicates = 36 soil samples, and an equivalent number of rhizosphere samples (only for summer samplings). Every sample was processed and plated in triplicate for CFU counts. Thus, the data from plate counts correspond to averages of 3 replicate plates x 3 replicate samples = 9 replicates per treatment and per site. CFU values were log10(x+50)-transformed prior to statistical analyses to ensure normalization. Generalized Linear Mixed Models (GLMM, Di Rienzo et al., 2012) were applied

to analyze TH, TP and FP plate count results, and it was followed, when appropriate, with “Least Significant Difference” tests (Fisher LSD) using Infostat software (Di Rienzo et al. 2013), to evaluate significant differences between values. TP/TH and FP/TP ratios were transformed prior to statistical analysis, using the angular transformation recommended to normalize percentage and proportion values (Knudsen and Curtis 1947). Then, values were analyzed with GLMM (Di Rienzo et al. 2012). In all cases, statistics were done at $p < 0.05$.

DNA banding patterns were analyzed with the GelCompar II V. 4.602 software (Applied Maths, Kortrijk, Belgium) by Pearson correlation based clustering (Rademaker and de Bruijn 1999), using the densitometric curves obtained from each pattern. Clusters were performed with Ward algorithm. Cophenetic correlation values higher than 80 indicate that the resulting dendograms correspond to the original matrix (van Verseveld and Röling 2008). Analysis was performed using the Composite data set tool of GelCompar. This analysis allows performing comparisons of the combined RFLP patterns generated by both enzymes for each gene, or even of the PCR-RFLP patterns obtained from the four treatments (2 enzymes x 2 genes), such that the possibilities to find differences in the composition of pseudomonads community structure among samples, were maximized.

3. Results

3.1. The abundance of pseudomonads in bulk soil differs among no-till agricultural practices and seasons

For each geographical site, the abundance of total heterotrophic mesophilic bacteria (TH) in bulk soil was not significantly influenced by season or treatment. Overall, TH counts ranged from 7.31 ± 0.26 log CFU/g (Pergamino, NE, September 2011) to 6.15 ± 0.15 log CFU/g (Monte Buey, GAP plots, February 2010) (Online resource 2). In contrast, we found that the abundance of total pseudomonads (TP) in bulk soil showed a wider variation among all samples, ranging from 2.56 ± 0.97 log CFU/g (Bengolea, BAP, February 2011) to 6.39 ± 0.02 log CFU/g (Viale, NE, September 2011) (Figure 1). Similarly, the abundance of fluorescent pseudomonads (FP) in bulk soil ranged from 1.94 ± 0.23 log CFU/g (Bengolea, BAP, February 2011) to 6.01 ± 0.31 log CFU/g (Viale, NE, September 2010), thus representing between 6 and 95% of TP (Figure 1). In terms of the land use history of no-till plots, TP and FP counts were, in most cases, higher in GAP plots than in BAP ones (13 out 16 cases; Figure 1).

Considering the geographical site as a random factor effect there was no statistical interaction between treatment and season, thus we could evaluate those effects independently. The analysis of soil management effect showed that both TP and FP counts were significantly higher in bulk soil samples from GAP plots than in those from BAP plots (LSD, $p < 0.05$) (Figure 2a). However, the pseudomonads load of natural pastures (NE) was significantly higher than in cropped soils (Figure 2a).

As expected from the observed relative variations in TH and TP counts, the proportion of pseudomonads to total heterotrophic bacteria (TP/TH) was also higher in most GAP samples than the TP/TH ratio of BAP samples (Figure 2b). In fact, when the geographical location was considered as a random effect,

TP/TH values were also significantly higher in GAP than in BAP plots ($p < 0.05$) (Figure 2c). Instead, the proportion of fluorescent pseudomonads in relation to total pseudomonads (FP/TP) did not show any significant difference between managements; only agricultural plots (whether GAP or BAP) had in general higher FP/TP ratios than the corresponding NE reference plots (Online Resource 3).

In addition to crop management, the sampling season influenced pseudomonads abundance in bulk soil. TP and FP counts samples were higher in soils samples taken in late winter than in summer for three out of the four sampling locations (Figure 1). As for the cropping practice effect, when the geographic location was considered as a random factor, winter samples had a significantly higher load of TP and FP than those from summer (Figure 3a). This effect was reflected in the TP/TH as well, being soil samples taken in summer those with the lower TP/TH ratios (Figure 3b). The sampling season had not any clear influence on soil FP/TP ratios (Online Resource 3).

3.2. Total load of pseudomonads community in rhizospheric soils differs between agricultural practices and crop species

The abundance of total pseudomonads and of fluorescent pseudomonads in rhizosphere samples could only be quantified from crops that were present in the agricultural plots in summer samplings (Online resource 1). The so-called “rhizospheric effect” (Hiltner 1904; Lynch and Whipps 1990; Warembourg 1997) was verified, as in all cases, the TP load of sampled roots was on average 3-4 orders of magnitude higher than the load of the

283 corresponding sampled bulk soil (Figure 4a), and also, TP/TH ratios were much
284 higher in rhizospheres (Online resource 4) than in the corresponding bulk soils
285 (Figure 2c), reflecting the selective enrichment of pseudomonads in the root
286 zone.

287 The nature of rhizodepositions depends on plant species, thus impacting
288 on the community structure of the rhizosphere (Lynch and Whipps 1990; Bais et
289 al. 2006; Costa et al. 2006b; Garbeva et al. 2008). In this regard, we observed
290 that the rhizosphere of crop species (maize and soybean) supported
291 significantly higher TP and FP loads than the rhizosphere of natural pastures
292 (Figure 4a), and consequently, higher TP/TH values (Online Resource 4).
293 However, the rhizosphere of soybean showed significantly higher TH, TP and
294 FP loads than the maize rhizosphere ($p < 0.05$, Figure 4b). This comparison
295 was only possible for GAP plots in which both maize and soybean are included
296 in the crop rotation (Online Resource 1), even among different years
297 considering that the sampling time effect was not statistically sound ($p > 0.05$).

298 As soybean was the crop species present in most sampled plots in this
299 study (Online Resource 1), we could evaluate the impact of agricultural
300 practices on soybean rhizosphere colonization by pseudomonads, provided
301 there was no interaction among sampling time and treatment during the
302 statistical analysis. We observed that the rhizosphere of soybean from GAP
303 plots supported significantly higher TH and pseudomonads densities than those
304 from BAP plots ($p < 0.05$, Figure 4c). Although this effect was numerically
305 reflected into higher soybean rhizospheric TP/TH values for GAP plots (Online
306 resource 4), this difference was not statistically significant ($p = 0.1318$).
307

3.3. Seasonal and geographical effects on community structure

PCR analysis targeting pseudomonads-specific genes *oprF* and *gacA*, allowed monitoring changes in pseudomonads communities in complex ecosystems, as is the case of soil and rhizosphere (Costa et al. 2006a; Bodilis et al. 2006; Agaras et al. 2012). The PCR-RFLP method used in this study provided an image of the genetic composition of the most abundant pseudomonads of every sampled soil or rhizosphere (Agaras et al 2012).

The comparative analysis of composite RFLP patterns from all sampled soils revealed that the pseudomonads community structure was mainly shaped by the sampling season (Figure 5). Notably, samples from the same geographical site appeared clustered within every seasonal group with more than 50% of similarity, especially in summer samples (Figure 5). Rhizosphere pseudomonads fingerprints could be compared to those from their corresponding soils, only for summer samples. In this case, we observed that only *gacA* profiles could distinguish soil and rhizosphere communities by sampling time (Online Resource 5), whereas *oprF* profiles failed to group samples in clear-cut clusters (data not shown). When comparing all summer soil and rhizosphere samples, the major clustering force was the sampling time (Figure 6). However, as observed for bulk soil, samples from the same geographical location were clustered into groups with more than 50% of similarity within major annual clusters (Figure 6). Finally, we evaluated the effect of crop species on the community structure. For this analysis, we compared rhizospheres of maize and soybean, both from GAP plots of summer sampling

times. We found that samples were clearly grouped by crop species (>85% of similarity), independently of the geographical site and sampling date (Online Resource 6).

4. Discussion

Several studies reported on the effects that agricultural practices and crop species have on the structure of the microbial community in soil and rhizosphere in European farms (Gomes et al. 2001; Smalla et al. 2001; Garbeva et al. 2004a; Berg et al. 2005; Costa et al. 2006b; Govaerts et al. 2007; Costa et al. 2007; Mittal and Johri 2008; Cycoń and Piotrowska-Seget 2009; Meyer et al. 2010; Ding et al. 2013). In Argentina, where agriculture is one of the main economic activities, these studies are incipient (Montecchia et al. 2011; Figuerola et al. 2012). Given the massive adoption of no-till management in Argentinean agricultural soils and the need to develop reliable biological indicators of soil quality and crop productivity to help distinguish among sustainable (GAP) and non-sustainable (BAP) agricultural practices, a multidisciplinary consortium was launched to study different biological components of the soil ecosystem in no-till plots (Wall 2011). Here, we have characterized the abundance and community structure of culturable pseudomonads, a bacterial genus intimately related to plant-growth promotion (Lugtenberg and Kamilova 2009).

4.1. Agricultural treatments and seasons influence pseudomonads abundance in bulk soils

Culture-dependent methods are still very useful when working with bacterial genera for which selective media are available to quantify them in complex samples, as is the case of pseudomonads (De Leij et al. 1993; Edwards et al. 2001; Landa et al. 2002; Dell'Amico et al. 2005). Here, we have detected total culturable pseudomonads in the 10^2-10^6 CFU/g range in agricultural bulk soils from the central pampas by plating in Gould's medium (Figure 1). The maximal records roughly correspond to <0.01% of the total bacterial cells estimated to be present in soils (Bach et al. 2002; Lloyd-Jones et al. 2005), and are consistent with 16S DNA pyrosequencing analyses of the same samples, which detected pseudomonads sequences with a relative abundance of <0.1% of the total bacterial OTUs (Figuerola et al. 2012). Thus, the simplicity and reproducibility of CFU counts turn this traditional method into a suitable tool to monitor quantitative changes in pseudomonads community as affected by cropping practices, and fulfills the requirement of simple and reliable methods for the identification of suitable biological indicators of soil quality (Nielsen and Winding 2002). Culture-independent approaches are required to monitor the dynamics of the whole *Pseudomonas* population.

Soil type is a determinant factor for structuring its microbial community (Latour et al. 1996; Cho and Tiedje 2000; Garbeva et al. 2004a; Berg and Smalla 2009). In this work, we considered soil type as a random factor for the statistical analysis of data as we aimed to evaluate the influence of the agricultural practices on the *Pseudomonas* community. In fact, we found an overall similar pattern of variation in the abundance of pseudomonads in soils from different locations (Figure 1). In agreement with previous observations

(Garbeva et al. 2004b; Garbeva et al. 2008), we found that agricultural practices have a neat effect on the total load of pseudomonads (TP) in soil, whereas they do not significantly change the abundance of heterotrophic bacteria (Figure 2a and Online resource 2). Among the tested cropping practices, we observed that no-till plots under GAP had higher loads of TP and FP than BAP plots (Figure 2). This suggests that no-tillage accompanied by crop rotation and rational use of chemical fertilizers/pesticides (e.g., GAP) promote highest populations of culturable pseudomonads, most probably because these plots offer a more favorable environment for this bacterial genus and its interactions with other biological components. In the particular case of the fluorescent pseudomonads, which is a sub-group related to plant-growth promotion, its high abundance may increase their chance to access crop roots and to colonize them, cooperating with their development and/or health. These results seem to be in conflict with the reported build up of antifungal-producer pseudomonads associated with take-all decline of wheat after long-term monocropping (Weller et al. 2002; Weller et al. 2007). However, such well-documented beneficial effect of monocropping is tightly linked to a particular geography with soil and climate conditions that restrict crop alternatives (Raaijmakers et al. 2009). In Argentina in a context of widespread implementation of no-till management and the observed benefits that sustainable practices (GAP) have on crop yields, monocultures are clearly discouraged (Asociación Argentina de Productores en Siembra Directa 2013). Moreover, it is well known that crop rotation is one of the oldest management strategies for favoring plant nutrition and for controlling plant diseases (Leoni Velazco 2013). Thus, no-tillage associated with crop rotation both enhance agricultural yields and soil health (Acosta-Martínez et al.

2007; Govaerts et al. 2007; Meriles et al. 2009; Derpsch et al. 2010), whereas monocropping tends to increase fungal disease predominance and leads to higher fertilization doses to supply the plant requirements (Leoni Velazco 2013; Pérez-Brandán et al. 2014).

In addition to the influence of the cropping practice, we found a strong seasonal effect on the abundance of total and fluorescent pseudomonads, with a significant drop in CFU counts in summer (Figure 3), as reported for other bacterial groups (Cookson et al. 2006; Prevost-boure et al. 2011; Rasche et al. 2011). Such seasonal shifts occurred irrespective of the presence or absence of winter cover crops in both GAP and BAP plots (Figuerola et al. 2012). Most likely, changes in weather conditions would be the critical factor that explains seasonality of soil pseudomonads abundance, as our statistical analysis did not show any interaction between treatments and seasons. The summer air temperature in the sampled region tends to be 10 ºC higher than in winter periods (INDEC 2013), thus increasing soil water evaporation. In fact, soil sampled in late winter (September) showed ca. 1.5% more moisture than those sampled in summer (data not shown). It has been already demonstrated that pseudomonads survival is negatively affected by soil dryness and higher temperatures (Moffett et al. 1983; O'Callaghan et al. 2001). In line with this explanation, we observed a positive correlation among pseudomonads counts and soil moisture (Figure 7), thus providing further support to the notion that the lower abundance of pseudomonads in no-till plots is due to their relative low moisture and higher temperatures to which they are exposed in summer.

To summarize, we found that pseudomonads load in bulk soils under no-till management is positively influenced by sustainable cropping practices (GAP)

433 and that shows seasonality achieving higher densities in late winter compared
434 to summer.
435
436 ### *4.2. Impact of crop species and agricultural treatments on*
437 ### *pseudomonads abundance in the rhizosphere*
438
439 As reported elsewhere (Lugtenberg et al. 2001), pseudomonads were
440 strongly enriched in all sampled rhizospheres in comparison to the
441 corresponding bulk soils, irrespective of the location, season and plant species
442 (Figure 4a). The rhizosphere colonization level of both crops were comparable
443 to those reported elsewhere (Kuklinsky-Sobral et al. 2004; McSpadden
444 Gardener et al. 2005; Picard et al. 2008; Von Felten et al. 2010). However,
445 soybean rhizospheres showed significantly higher microbial counts (TH, TP and
446 FP) than those of maize, independently of the plot location (Figure 4b).
447 Soybean root systems bear thinner roots, which results in a higher exposed
448 surface area on a fresh weight basis that may explain the higher counts for
449 soybean root samples. Further support to this explanation comes from the fact
450 that TP/TH ratios showed similar values for soybean and maize from GAP plots
451 (Online Resource 4), suggesting that the relative abundance of pseudomonads
452 in the maize and soybean rhizospheres was similar. Nevertheless, we observed
453 that the proportion of fluorescent pseudomonads (FP/TP) were higher for maize
454 than for soybean (data not shown), suggesting that maize rhizodepositions
455 result relatively more attractive for fluorescent pseudomonads than soybean
456 rhizodepositions. In this regard, McSpadden Gardener and collaborators have
457 shown that corn rhizosphere were significantly more enriched in DAPG-

458 producing fluorescent pseudomonads than the rhizosphere of soybean in the
459 same sampled soils (McSpadden Gardener et al. 2005).
460 In terms of cropping practice, we found that the rhizosphere of soybean
461 plants supported significantly higher densities of pseudomonads in GAP plots
462 (Figure 4c) suggesting that crop rotation under no-till management promotes
463 higher root colonization by pseudomonads than a putative positive selective
464 force by monocropping of soybean present in BAP plots. This could be
465 explained by the higher pseudomonads abundance found in GAP bulk soils
466 (Figure 2a). Thus, our results suggest that the positive effect that GAP have on
467 the build up of pseudomonads in bulk soil, may also be reflected into the
468 rhizosphere of the plants cropped under sustainable practices.
469
470 ***4.3. Seasonality as the main shaping factor of pseudomonads***
471 ***communities in bulk soil and rhizosphere***
472
473 We have previously shown that PCR-RFLP fingerprinting of *oprF* and
474 *gacA* genes is a useful, specific and relatively simple tool to characterize the
475 community structure of culturable pseudomonads in bulk soil and rhizosphere
476 samples (Agaras et al. 2012). Here, we have systematically applied the same
477 method to study the effects of agricultural practices, seasons and soil
478 geography on the community structure of the culturable pseudomonads.
479 Notably, both multiple comparisons of PCR-RFLP patterns from pseudomonads
480 recovered from all bulk soil samples at the four sampling times (Figure 5), as
481 well as from bulk soils and their corresponding rhizospheres in summer
482 samplings (Figure 6) revealed a seasonal clustering of the most abundant

pseudomonads. Within each seasonal cluster, those samples with highest similarity corresponded to the same geographical site. Finally, no significant grouping by agricultural practices (GAP or BAP) was found according to the community structure profiles (Figures 5 and 6). Thus, our results suggest that the season of the year seems to be the main factor that influences community structure. Such seasonal dynamics depends, however, on the local composition of pseudomonads species inhabiting each bulk and rhizospheric soil at every sampling location (Figures 5 and 6). Such geographic effect was particularly evident for the *gacA* gene profiling (Online Resource 5). As the *gacA* gene product is part of the global regulatory two-component system GacS/GacA that coordinates phenotypes related to stress resistance, ecological fitness and interactions with eukaryotes (Heeb and Haas 2001), its local sequence conservation and lower sequence evolution rate may reflect its relevance for cell physiology and adaptation. By contrast, the *oprF* gene seems to be less affected by minimal mutations that reduce its degree of endemicity.

Our fingerprinting data also showed that the *Pseudomonas* community structure was strongly influenced by the crop species (soybean or maize), independently of the geographical site (Online Resource 6). Thus, the nature of crop species not only may determine the abundance of total rhizosphere pseudomonads (Figure 4), but also selectively recruits specific pseudomonads from the microbial repertoire of the surrounding bulk soil within each location at a particular season (Lynch and Whipps 1990, McSpadden Gardener et al. 2005; Mittal and Johri 2008).

5. Conclusions

To summarize, we found that: 1) Good Agricultural Practices (GAP), based on no-till management with crop rotation and rational use of chemical fertilizers/pesticides, performed a positive effect on the culturable TP and FP loads in bulk soils when comparing with plots in which these practices are absent and monocropping prevails (BAP); 2) total (TP) or relative (TP/TH) pseudomonads abundance could contribute as a biological indicator of good agricultural practices; 3) the quantity of soil pseudomonads shows seasonality with higher densities in late winter compared to summer; 4) whereas pseudomonads (TP and FP) are more abundant in bulk soils from natural pastures (NE), crop rhizospheres achieve higher pseudomonads loads than the rhizosphere of natural pastures; 5) at least for soybean, GAP promote rhizospheric enrichment of pseudomonads (TP and FP); 6) the differences recorded in pseudomonads abundance in soils and rhizospheres were not translated into differences in the community structure of the most abundant culturable population, as the genetic structure was rather preserved at each geographical site and it was mainly influenced by the sampling season and the crop species, with a dynamic pattern that could be related with climate changes.

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Fig. 1 Seasonal dynamics of culturable pseudomonads abundance in bulk soils from no-till agricultural plots at different geographical locations in Argentina. Black bars: total pseudomonads (TP); grey bars: fluorescent pseudomonads (FP). NE, natural pastures; GAP, good agricultural practices; BAP, bad agricultural practices. Samples were taken in summer (Feb) or late winter (Sept) in 2010 and 2011. Different letters indicate significant statistical difference (LSD, $p < 0.05$) between average log CFUs for each bacterial group (TP, uppercase; FP, lowercase), within each geographical site: Bengolea (a), Monte Buey (b), Pergamino (c), and Viale (d). Error bars indicate SE. Arrows highlight those samples within one period for which the TP load was NE>GAP>BAP (13 out of 16 cases)

Fig. 2 Influence of agricultural practices on the abundance of pseudomonads in bulk soil from no-till plots. a) black bars: total pseudomonads (TP); grey bars: fluorescent pseudomonads (FP). NE, natural pastures; GAP, good agricultural practices; BAP, bad agricultural practices.. Different letters indicate significant statistical difference (LSD, $p < 0.05$) between average log CFUs for each bacterial group (TP, uppercase; FP, lowercase), considering soils as random factor effect. Error bars indicate SE. b) Point density plot showing the relative abundance of pseudomonads to heterotrophic mesophilic bacteria (TP/TH) for each geographical site under the same treatment (NE, GAP or BAP). c) point density plot showing the TP/TH ratios as a function of the treatment, independently of the geographical site. Shown are average values and different letters indicate significant statistical differences (LSD, $p < 0.05$).

Fig. 3 Seasonality of pseudomonads abundance in bulk soil from no-till plots. a) black bars: total pseudomonads (TP); grey bars: fluorescent pseudomonads (FP). Shown are average values and different letters indicate significant statistical differences (LSD, $p < 0.05$). b) point density plot showing the TP/TH ratios as a function of the sampling period, independently of the geographical site and treatment. Shown are average values and different letters indicate significant statistical differences (LSD, $p < 0.05$).

Fig. 4 Influence of agricultural practices and crop species on the abundance of pseudomonads in the rhizosphere. a) Rhizospheric effect. Solid bars: rhizosphere abundance; dashed bars: bulk soil abundance; black bars: total pseudomonads (TP); grey bars: fluorescent pseudomonads (FP). Shown are average values and different letters indicate significant statistical differences among rhizosphere (normal) or soil samples (italic) (LSD, $p < 0.05$), for each group (TP, uppercase; FP, lowercase). b) Rhizosphere abundance in different crop species cultivated in GAP plots. c) Abundance of rhizospheric pseudomonads as influenced by cropping practice. White bars: total heterotrophic bacteria (TH); black bars: TP; grey bars: FP. Shown are average values + SE. Different letters indicate significant statistical difference (LSD, $p < 0.05$) between average values for each bacterial group (TH, TP or FP) under every condition, considering geographic sites as random factor effect..

Fig. 5 Seasonal and geographical clustering of culturable pseudomonads community in bulk soil from no-till plots. The figure shows a dendogram generated by the Pearson correlation method upon comparison of the *gacA*-

836 *oprF* composite RFLP patterns from all sampled soils. Each sample is denoted
837 with an acronym composed of a first letter for the origin of the sample (S for
838 soil), a second for the geographical location (B for Bengolea, M for Monte Buey,
839 P for Pergamino and V for Viale), followed by the treatment code (NE, GAP or
840 BAP) and the sampling period (1, February 2010; 2, September 2010; 3,
841 February 2011; 4, September 2011). Clustering was performed with the Ward
842 algorithm. Cophenetic correlation values are indicated at each node.
843
844 **Fig. 6** Seasonal and geographical clustering of culturable pseudomonads
845 community in rhizosphere and bulk soil from no-till plots sampled in summer.
846 See legend to Fig. 5 for references. In every sample acronym, the first letter
847 denotes the origin of the sample: S, bulk soil; R, rhizosphere. Groups of
848 samples with > 50% of similarity are enclosed within dashed boxes.
849
850 **Figure 7**. Pseudomonads abundance in bulk soil positively correlates with
851 soil moisture. The correlation coefficient (r = 0.36) was calculated with the
852 Pearson method. Moisture content was determined by the laboratory group of
853 Ing. Iglesias, from the BIOSPAS consortium (Wall 2011). Dispersion graphic
854 shows a moderate positive correlation between parameters.
855

Electronic Supplementary material

Online Resource 1. Crop species present in each plot in summer samplings.

Online Resource 2. Total heterotrophic (TH) plate counts at each geographical site, at every sampling time and under every treatment. Mean values are expressed as log (CFU/g dry soil + 50) ± standard deviation (SD). Parenthetically, different letters indicate statistically significant differences (LSD Fisher, $p < 0.05$), when values were analyzed by GLMM.

Online Resource 3. Point density plots of FP/TP ratios of soils samples. Ratios are expressed as percentage values. We show: all ratios analyzed by agricultural treatment (a); and all ratios analyzed by sampling time (b). Mean values are presented, and asterisk indicates a significant statistical difference (LSD, $p < 0.05$). Whilst FP/TP ratios seem to have a trend GAP>BAP>NE, no seasonal effect was clearly evident.

Online Resource 4. Point density plots of TP/TH ratios of rhizosphere samples, grouped by crop species and agricultural treatment, at each geographical site. Ratios are expressed as percentage values. Mean values of all samples, taking location as a random factor, are presented.

Online Resource 5. Seasonal and geographical clustering of culturable

881 pseudomonads community in bulk soil from no-till plots based on *gacA* gene.
882 The figure shows a dendogram generated by the Pearson correlation of RFLP
883 patterns obtained from *gacA* treatment with both enzymes. February 2010 is
884 shown as 1 (■), and February 2011, as 3 (■). The names of samples are
885 acronyms, as explained in Figures 5 and 6. Clustering was performed with the
886 Ward algorithm. Cophenetic correlation values are indicated at each node.
887 Samples are divided by their sampling time in two big clusters, independently of
888 their origin (soil or rhizosphere).
889

Online Resource 6. Crop species effect on community structure of pseudomonads. Soybean (■) and maize (■)rhizospheres cultivated in GAP plots were compared. February 2010 is shown as 1, and February 2011, as 3. The names of samples are acronyms as in Figures 5 and 6. Dendogram was obtained with the Pearson correlation of RFLP patterns, and clustering was performed with the Ward algorithm. Cophenetic correlation values are indicated at each node. Samples are divided mainly by crop species.

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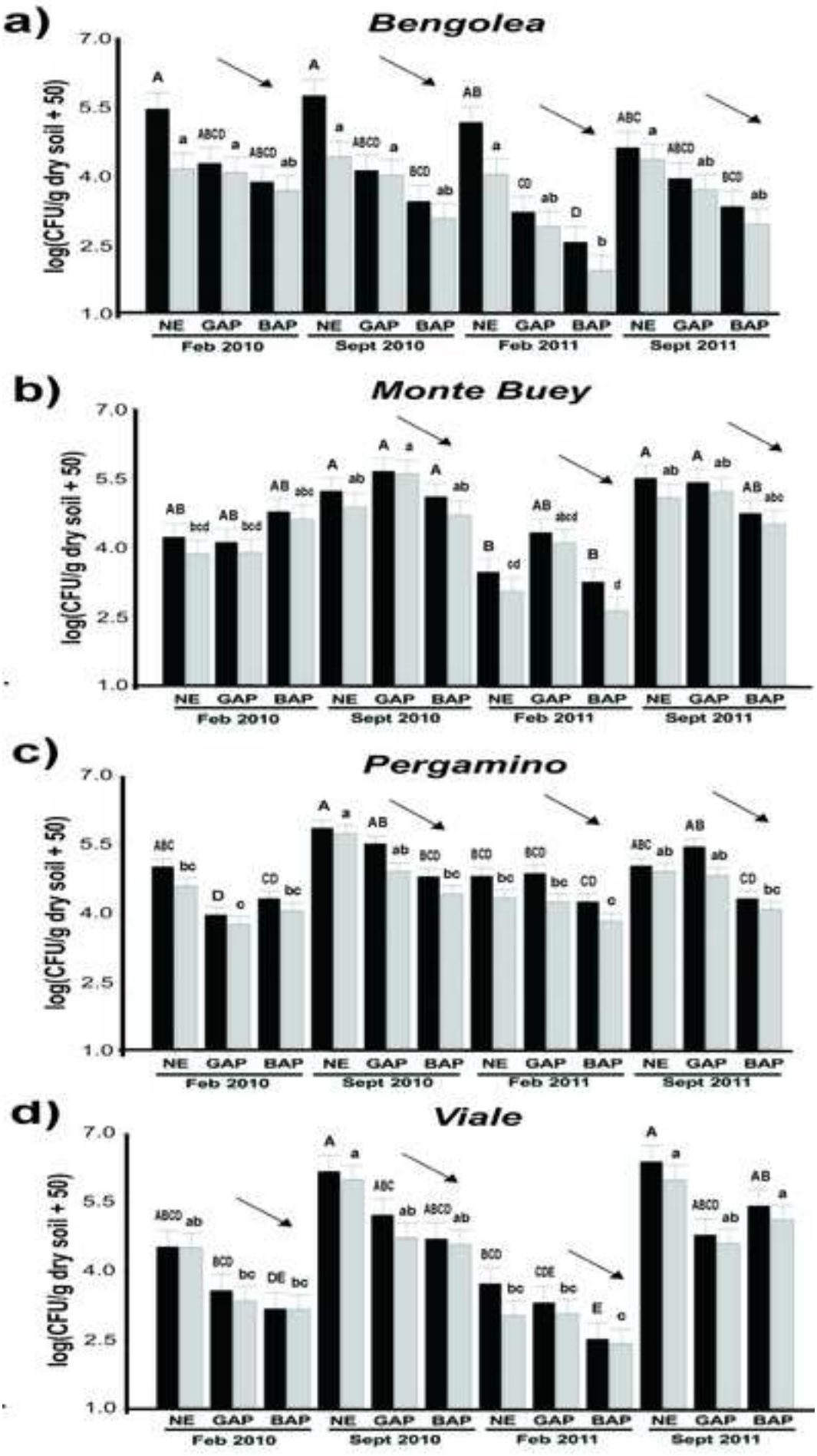

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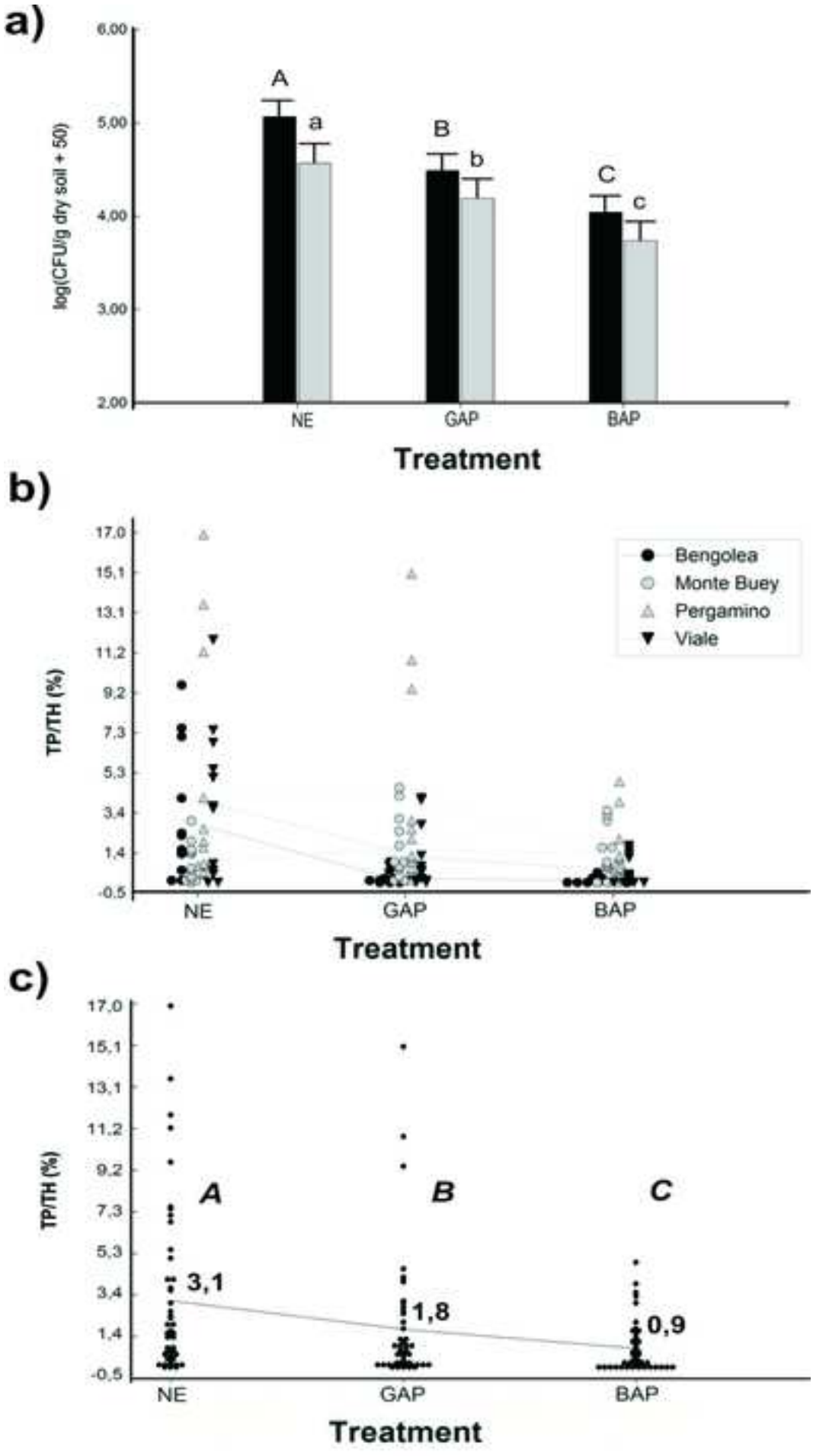

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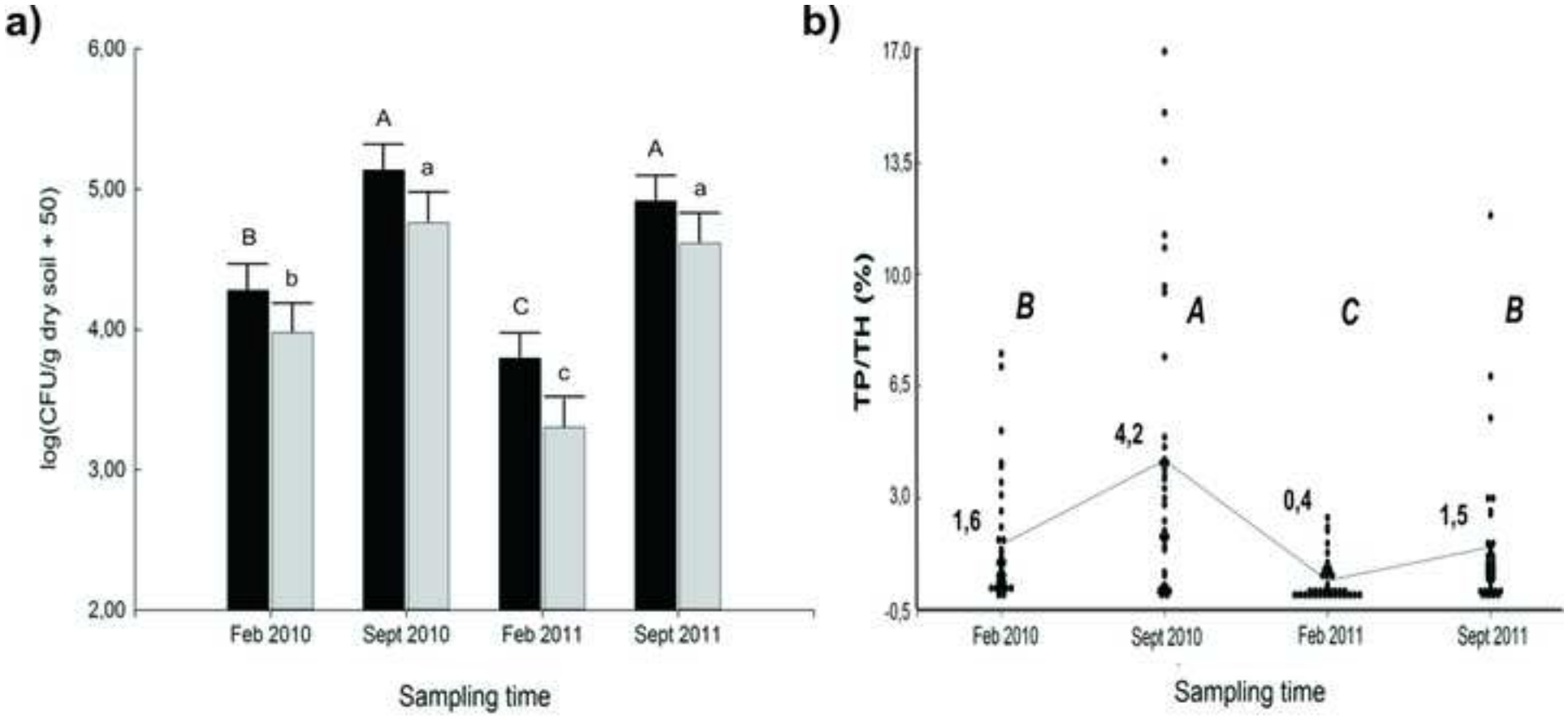

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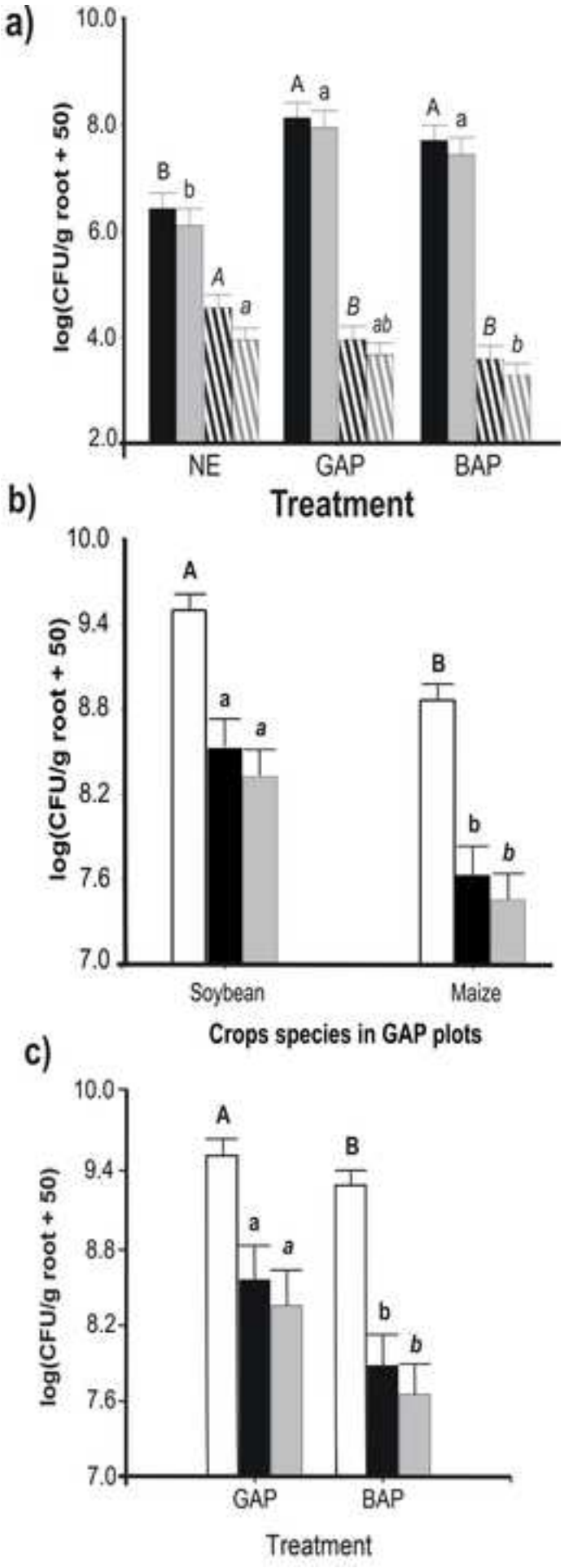

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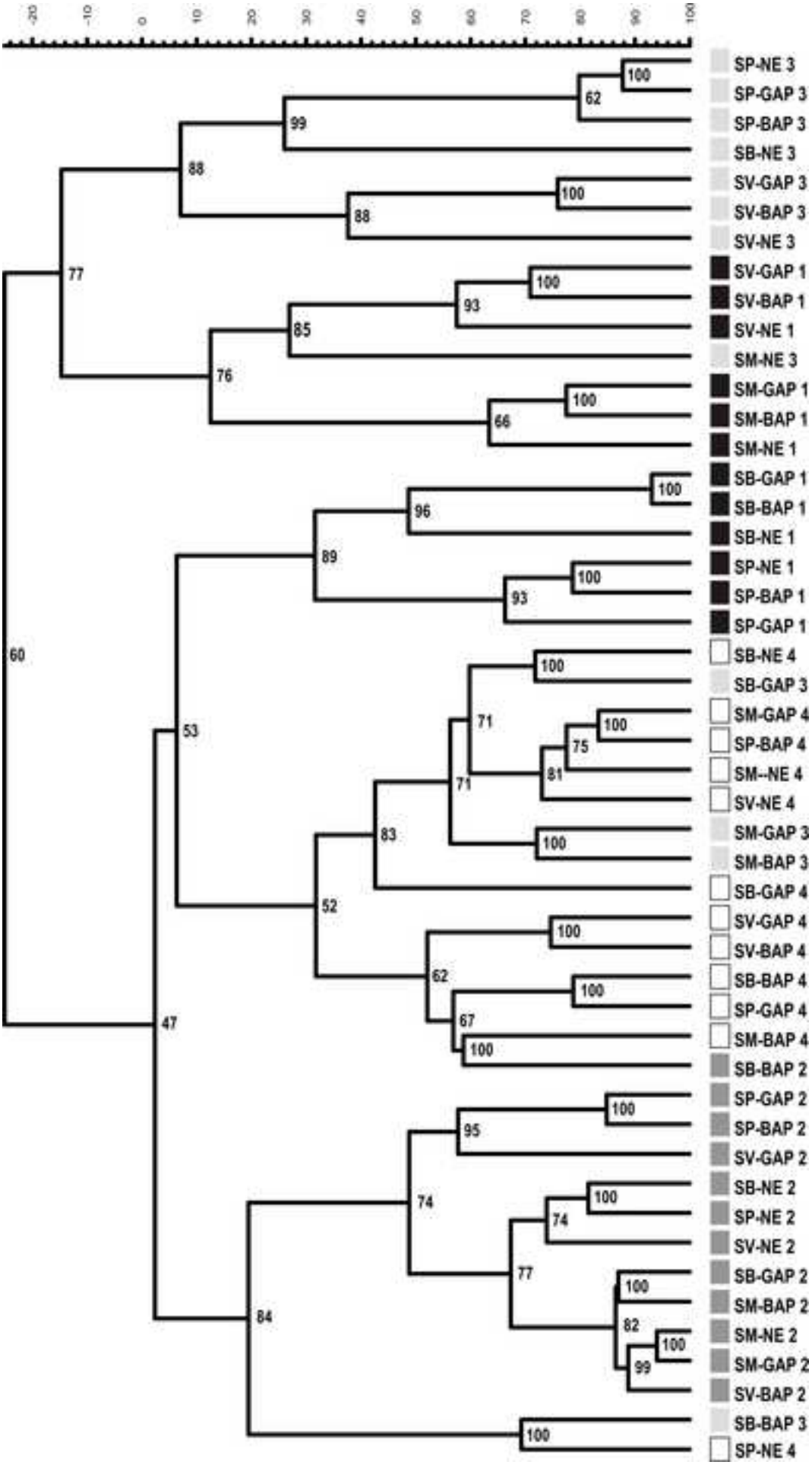

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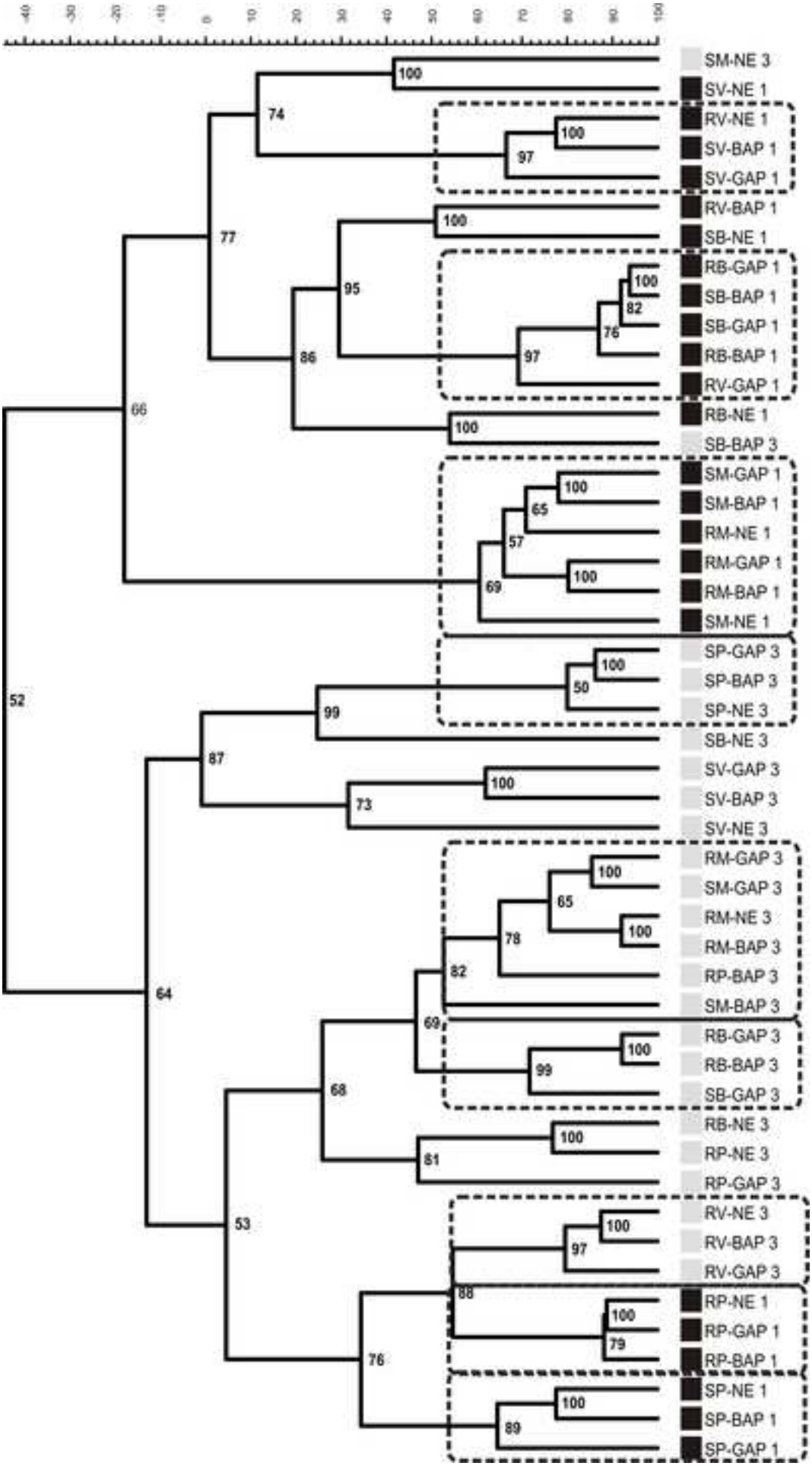

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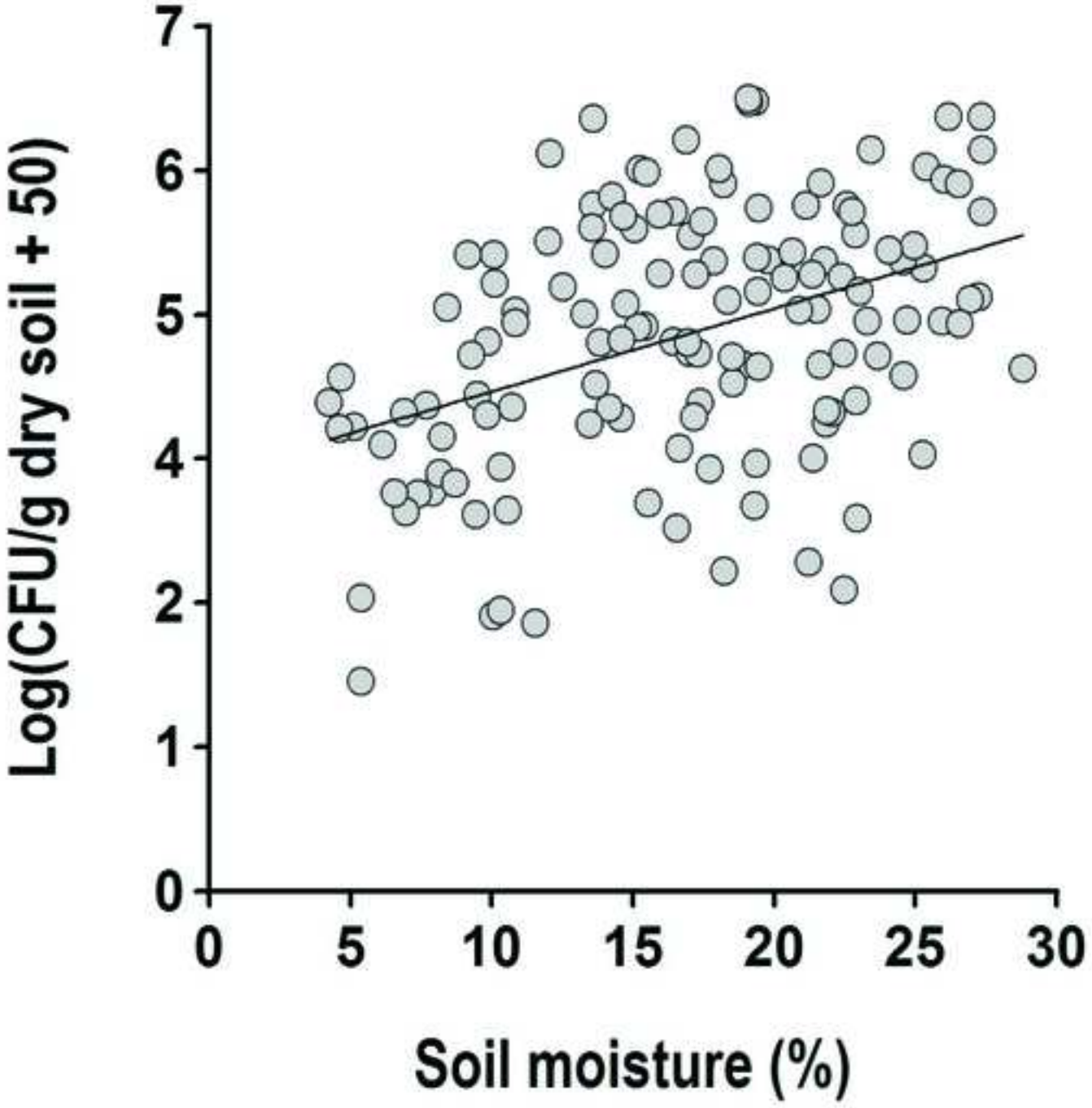

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