CAN SOIL PHYSICOCHEMICAL CHARACTERISTICS AFFECT THE PRESENCE OF *BACILLUS THURINGIENSIS* SPORES?

¿PUEDEN LAS CARACTERÍSTICAS FISICOQUÍMICAS DEL SUELO AFECTAR LA PRESENCIA DE ESPORAS DE *BACILLUS THURINGIENSIS*?

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Abstract

One hundred and one soil samples from several departments of Colombia were collected; for each of them, 14 different physicochemical variables were evaluated, along with the presence and number of *Bacillus thuringiensis* spores, and a statistical study was performed. No simple regression between each of the variables and the presence of *B. thuringiensis* spores was found, suggesting that the relationship between *B. thuringiensis* spores and soil must be complex. By multiple regression, however, three models with several variables were found. Moreover, Logit regression analysis revealed another model that might allow to correlate the presence of *B. thuringiensis* spores with some physicochemical variables in a given soil sample. In all models, soil pH was the most important factor. Even though soil pH had no effect by itself, the combination of soil pH along with other variables, such as CEC, nutrient availability (Ca, Mg, K), clay content and soil texture, had an effect on *B. thuringiensis* biology. The models found in the present study represent novel tools that could facilitate the understanding the ecology of *B. thuringiensis*.

Key words: Bacillus thuringiensis, ecology, Colombia, mathematic models, soil physicochemical characteristics.

Resumen

A 101 muestras de suelos de varios departamentos de Colombia se les evaluaron 14 variables fisicoquímicas y la presencia y número de esporas de *Bacillus thuringiensis*. Posteriormente se realizó un estudio estadístico. No se encontró ninguna regresión simple entre las variables fisicoquímicas y la presencia de esporas de *B. thuringiensis*, lo cual sugiere que la relación entre el número de esporas de *B. thuringiensis* y el suelo debe ser compleja. Sin embargo, mediante regresión múltiple se encontraron tres modelos con distintas variables. Por otra parte, el análisis mediante regresión Logit aportó otro modelo que correlaciona la presencia de esporas de *B. thuringiensis* con diversas variables fisicoquímicas del suelo. En todos los modelos matemáticos encontrados, el pH del suelo fue una de las variables más importantes. Aunque el pH del suelo no mostró un efecto por sí solo, la combinación del pH con otras variables, como la CEC, la disponibilidad de nutrientes (Ca, Mg, K), el contenido de arcillas y la textura del suelo, tienen un efecto sobre la biología de *B. thuringiensis*. Los modelos encontrados en este estudio aportan nuevas herramientas que pueden ayudar a entender mejor la ecología de *B. thuringiensis*.

Palabras clave: Bacillus thuringiensis, ecología, características fisicoquímicas del suelo, Colombia, modelos matemáticos.

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore-forming bacterium. It has a special feature that differentiates it from other *Bacillus* species, namely, the formation of a proteic crystal during sporulation. The crystal is composed of proteins, some of which are toxic to several insect species. Due to this feature, *B. thuringiensis* has become the most studied and used bacterium in biological control of

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insect pests and vector-borne diseases (Realpe et al., 1998; Smith and Couche, 1991). Since its discovery in 1901 in Japan by Ishiwata, new *B. thuringiensis* isolates from soil have been described. Even though its ecological niche has been controversial for the last fifty years (Damgaard et al., 1997; Helgarson et al., 2000; Maeda et al., 2000; Ohba, 1996; Smith and Couche, 1991), soil remains as the main source for *B. thuringiensis* isolation (Bravo et al., 1998; Chilcott and Wigley, 1993; Iriarte et al., 1998; Kim et al., 1998; Martin and Travers, 1989).

From a microbiological point of view, soil is one of the richest and most diverse ecosystems. It is believed that one gram of soil can hold between one million and several thousands of millions of microorganisms. The genus *Bacillus* is one of the most abundant within the group formed by 20 bacteria genera most commonly found in soil (Burbano, 1989). The type and abundance of bacteria in soil depends on three factors: the availability of microorganism's demands (energetic requirements, carbon sources, oxygen presence, micronutrients, etc.), the soil physicochemical features (ventilation, moisture, pH, temperature, nutrient availability, etc.), and the relationship with other organisms (Burbano, 1989; Gammack et al., 1992; Robert and Chenu, 1992).

Several researchers have tried to establish the relationship between soil factors and the presence or survival of *B. thuringiensis*. Hossain et al. (1997) concluded that the presence of *B. thuringiensis* in soil was negatively conditioned by the percentage of sand and positively by copper concentration. In both cases, simple regressions were established. Other studies have shown soil pH as an important factor in *B. thuringiensis* survival in soil (Dias and Sagardoy, 1998; Petras and Casida, 1985; Saleh et al., 1969; West et al., 1985). According to these studies, low soil pH levels (below 5.0) affect spore survival and cellular growth. Other factors that have been reported affecting *B. thuringiensis* survival are soil moisture (Petras and Casida, 1985; West et al., 1985), competition with other microorganisms, and nutrient availability (Saleh et al., 1969; West et al., 1985).

In this work, we aimed at establishing the relationship between the presence of *B. thuringiensis* spores and some of the soil physicochemical characteristics by multivariate analysis in order to contribute to the understanding of the soil ecology of this bacterial species.

MATERIALS AND METHODS

Soil sample collection. One hundred and one soil samples were collected from 13 departments of Colombia, with the following distribution: 24 from Cundinamarca, 11 from Boyacá, 11 from Córdoba, 10 from Antioquia, 10 from San Andrés, 9 from Huila, 8 from Meta, 6 from Bolívar, 4 from Tolima, 3 from Cesar, 3 from Valle del Cauca, 1 from Caldas, and 1 from Arauca (figure 1). All samples were collected from agricultural lands, between 0 and 35 cm depth. Two hundred grams of soil were scraped with a sterile spatula and placed in sterile plastic bags. Once in the laboratory the samples were dried at 40 ºC during 24 h and they were stored at room temperature until analysis.

Physicochemical analysis of soil samples. The following physicochemical characteristics of the soil samples were analyzed: particle size distribution (sand, silt, and clay), determined by the Bouyoucos method (Silva and Olarte, 1973); soil pH was determined according to Jackson (1958); soil moisture, according to the Association of Official Analytical Chemists (AOAC) (Jackson, 1960); exchangeable sodium, magnesium, potassium, calcium, cationic exchange capability (CEC), and base saturation (BS) by the ammonium acetate method (Bower et al., 1952); soil carbon content, according to the Schollenberger method (Schollenberger, 1945); and soil available phosphorus, by the method described by Jackson (1958).

Isolation and characterization of *B. thuringiensis*. After soil physicochemical analysis, a sub-sample of each soil was used to determine the presence of B. thuringiensis spores. Half a gram of soil was suspended in 5 ml of Luria Bertani (LB) culture broth in sterile tubes. Appropriate dilutions were heated at 80 ºC for 10 minutes in a water bath in order to select only the spores and remove the vegetative forms; a 100-µl aliquot was plated on LB agar. After 48 hours, bacterial cultures were observed and colonies com-

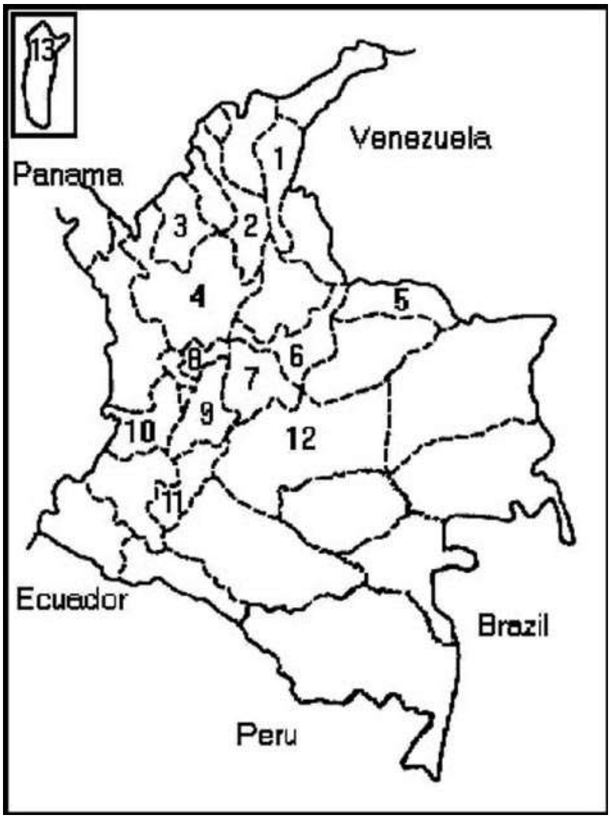

Figure 1. Distribution and number of soil samples taken in the Colombian departments. **1** = Cesar (3 soil samples); **2** = Bolívar (6); **3** = Córdoba (11); **4** = Antioquia (10); **5** = Arauca (1); **6** = Boyacá (11); **7** = Cundinamarca (24); **8** = Caldas (1); **9** = Tolima (4); **10** = Valle del Cauca (3); **11** = Huila (9); **12** = Meta (8); **13** = San Andrés isles (10)

patible with B. thuringiensis morphology were picked up (Orduz et al., 1992), and cultured in liquid M1 medium (Orduz et al., 1996). After 48 hours, isolates were observed by phase-contrast microscopy, and those with parasporal inclusion bodies were identified as B. thuringiensis. B. thuringiensis index was calculated as the number of Bacillus thuringiensis isolates obtained divided by the number of spore-forming bacterial colonies. The parasporal crystal morphology of each strain was examined. The final whole cultures (FWC), composed of spores and crystals, were kept at 4 ºC for subsequent analysis.

Bioassays. Toxicity of *B. thuringiensis* strains was determined against first larval instar of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Four hundred µl of each of the FWC were placed in 3 cm diameter plastic containers with an artificial diet (Shorey and Hale, 1965) in triplicate; they were allowed to dry and then five newly hatched *S. frugiperda* larvae were added. Larval death was recorded after 72 hours of treatment. The isolates were also tested against third larval instar of *Culex quinquefasciatus* (Diptera: Culicidae). One hundred µl of FWC were added to 100 ml of tap water in plastic containers, with 10 *C. quinquefasciatus* larvae in triplicate, and mortality was recorded after 24 hours. In addition, the toxicity range was calculated for *B. thuringiensis* strains that had shown toxicity against *C. quinquefasciatus*, by using serial FWC dilutions following the above-mentioned procedure.

Statistical analysis. Each of the soil samples were defined as the test unit, and the dependent variables were the number of *B. thuringiensis* spores per soil sample (total-Bt) and the presence or absence of *B. thuringiensis* spores in the soil samples (Bt-presence). The independent variables analyzed were (names in parenthesis when different): sand, silt and clay, pH, moisture, CEC, BS, exchangeable calcium, magnesium, potassium, sodium, soil available phosphorus (Ca, Mg, K, Na, and P, respectively), and soil carbon content (C). The variables sand, silt, and clay could not be included together in the study, since each of them can be explained from the other two and this causes severe multicolinearity problems. We decided to work with only two of them, silt and clay, because soil microorganisms interact more with small particles than with big ones. Overall, 13 independent variables were used.

The results obtained were analyzed by simple regression, principal component analysis (PCA), multiple regression, and Logit regression with the Statistica program, version 5.0 (StatSoft, Inc.). Logit regression study is useful for situations with two events, such as presence or absence of *B. thuringiensis* spores.

RESULTS

Presence of *B. thuringiensis*. Fifty-one out of 101 soil samples contained *B. thuringiensis* spores (50.5%) (figure 2). Overall, 3.241 colonies were analyzed, from which 349 were identified as *B. thuringiensis* (*B. thuringiensis* index of 0.11). The average number

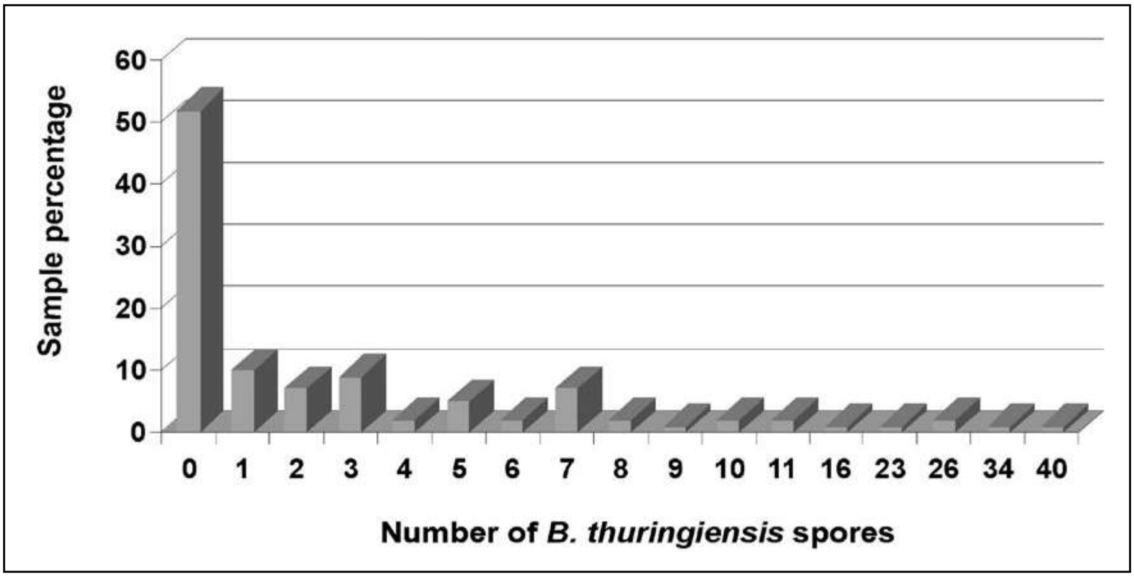

Figure 2. Percentage of positive soil samples containing different numbers of *Bacillus thuringiensis* spores per sample

of *B. thuringiensis* isolates was 7 per g of soil. The sample with the highest number of *B. thuringiensis* isolates was a soil from the town of Argentina (Huila), with a total of 40 isolates (figure 2) and a *B. thuringiensis* index of 0.65. The two soil samples with the highest index of *B. thuringiensis*, equal to 1, were found at Tausa (Cundinamarca), in which only one colony, identified as *B. thuringiensis*, was found and at Villa de Leyva (Boyacá), in which both colonies isolated belonged to the *B. thuringiensis*. No differences were found in the number of *B. thuringiensis* spores in the different departments nor at different altitudes or rain-levels (data not shown).

Bioassays showed that 255 isolates (73.06%) were toxic against third larval instar of *C. quinquefasciatus*, the best strain being 3-05410-02, which caused 80% mortality at the 10^{-6} dilution of the FWC, similar to *B. thuringiensis* serovar. *israelensis*. On the other hand, only 13 strains were found to be toxic against *S. frugiperda* (3.7%). There were 81 strains (23.21%) that did not show toxicity neither against *C. quinquefasciatus* nor against *S. frugiperda*. None of the 349 colonies of *B. thuringiensis* strains were toxic against both insect larvae. Out of the total of 349 colonies, 279 (79.94%) exhibited round crystals, 67 (19.19%) bipyramidal, and the remaining exhibited amorphous crystal.

Effect of soil physicochemical characteristics on *B. thuringiensis* presence. The correlation matrix of all variables was analyzed in order to evaluate presence of colinearity problems. Correlation coefficient values (r) between almost all variable pairs were close to zero (data not shown), even though two sets of variables were closely related, TBC and Ca (r = 0.98), and CEC and C (r = 0.85). From each pair, one variable should be eliminated, but since there was not a clear elimination criterion, it was decided to maintain them for further analysis to decide which could be the most important one for the final models. The principal component analysis indicated that the minimum number of factors that could best explain data dispersion were soil clay content, soil pH, soil moisture, CEC, and exchangeable calcium and magnesium, even though the remaining variables had enough importance to be kept on the study.

Simple regressions were analyzed between each independent variable and the two dependent variables; overall they presented little significance (table 1). The highest r^2 was 0.09, between silt content and total-Bt, and 0.073 between CEC and total-Bt. Afterwards, lineal regressions with more than one variable were investigated. By means of multiple lineal regressions, all variables and the variables transformed with different functions were studied. The best regression models obtained are shown below:

total-Bt = 4.41 pH - 1.0 CEC - 0.39 silt - 0.34 Ca

$r^2 = 0.6390$, Anova: $F = 15.49$, $p = 0.000000$

pH, CEC, silt, and Ca at 5% level of significance

$\sqrt{}$total-Bt = 1.04 pH – 0.059 silt – 0.021 BS – 0.22 C

$r^2 = 0.8578$, Anova: $F = 52.82$, $p = 0.000000$

pH, silt, BS, and C at 5% level of significance

Bt-presence = 0.41 pH - 0.02 CEC + 0.03 moisture + 0.03 Mg

$r^2 = 0.8333$, Anova: $F = 121.36$, $p = 0.000000$

pH, CEC, moisture, and Mg at 5% level of significance

In the Logit regression study, the most accurate model obtained was:

Bt-presence = -9.45 + 0.93 clay + 2.16 pH + 0.83 moisture - 0.81 CEC + 0.75 Mg + 0.53 Ca

$r^2 = 0.2943$

DISCUSSION

After a random collection of 101 soil samples from most regions of Colombia, *B. thuringiensis* was isolated from 50% of the soil samples. In other reports this percentage has been variable, between 18% and 91% (Bravo et al., 1998; Chilcott and Wigley, 1993; Iriarte et al., 1998; Ohba and Aizawa, 1986). The zones in which *B. thuringiensis* was found did not show any particular distribution.

The average number of *B. thuringiensis* isolates per g of soil (7.0) was similar to what Bravo et al. (1998) obtained in Mexico (7.8 isolates g^{-1} soil), and slightly lower to Martin and Travers (1989) data, who found 9.4 *B. thuringiensis* isolates per g of soil in samples from Central and South America. On the other hand, the *B. thuringiensis* index (0.11) was relatively low compared to those studies, where *B. thuringiensis* indexes of 0.28 and 0.24 were found, respectively. The diversity and the variability among the sampling areas in the three mentioned studies may probably explain the differences in *B. thuringiensis* indexes.

Most *B. thuringiensis* isolates (79.94%) exhibited round crystal morphology, while the remaining showed bipyramidal or amorphous crystals. Seventy-three percent of all isolates were toxic to *C. quinquefasciatus* larvae, with all these strains having round crystal morphology. It was noteworthy that the number of isolates toxic to *S. frugiperda* was low. From these lepidopteran active isolates, 10 presented bipyramidal crystals, and the remaining three contained round crystals, contrasting the results reported by Bernhard et al. (1997) from a worldwide study, where the most abundant crystal types in soil samples were irregular spherical and bipyramidal, each of them accounting for almost 25% of the total. In relation to toxicity, Bernhard et al. (1997) found that only 21% of *B. thuringiensis* isolates were toxic against *C. pipiens*, while 73% were toxic against *S. littoralis*, although these toxicity data corresponded to all the isolates and not only to those from soil samples.

In the present study, it was not possible to find a simple regression between any of the response variables (Bt-presence and total-Bt) and the soil physicochemical properties evaluated (independent variables) (table 1). These results could suggest that, in nature, the presence of *B. thuringiensis* in soil is not determined by a single factor. Moreover, Hossain et al. (1997) reported low correlation coefficients (below ± 0.62) between soil characteristics and *B. thuringiensis* index. Taking into account that this bacterium has been isolated from all over the world, it is considered to be a cosmopolitan organism (Bernhard et al., 1997; Martin and Travers, 1989), suggesting that its relationship with the soil environment must be more complex. Therefore, there is a need to study biotic factors and other soil physicochemical features that may influence *B. thuringiensis* biology, such as competition or antagonism with other soil micro and macro organisms and content of organic nutrients and toxic materials (Petras and Casida, 1985; Saleh et al., 1969; West et al., 1985).

This is the first report of *B. thuringiensis* in which methods of multivariate analysis are used to describe

Table 1. Correlation coefficient values (r^2) of simple regressions between each soil physicochemical factor and the dependent variables, number of *Bacillus thuringiensis* spores per soil sample (total-Bt) and presence or absence of *B. thuringiensis* (Bt-presence)

Factors	**total-Bt**	**Bt-presence**
Silt	0.0900	0.0025
Clay	0.0361	0.0144
PH	0.0036	0.0169
Moisture	0.0025	0.0049
Cationic exchange capability (CEC)	0.0729	0.0625
Total basic components (TBC)	0.0100	0.0004
Calcium (Ca)	0.0100	0.0001
Magnesium (Mg)	0.0064	0.0064
Potassium (K)	0.0025	0.0025
Sodium (Na)	0.0036	0.0009
Total saturation (TS)	0.0009	0.0001
Carbon percentage (C)	0.0484	0.0400
Phosphorus (P)	0.0064	0.0025

the interaction between *B. thuringiensis* and the soil physicochemical characteristics. However, the only comparable study was published by Hossain et al. (1997) analyzing 17 soil samples by simple correlation and regression analysis with 14 soil variables. The significant multiple regressions that were found in the present work confirm the complexity of the interaction of *B. thuringiensis* with the soil environment. The number of *B. thuringiensis* spores from a given sample correlated to at least four variables (79.9%), the most important being CEC and pH (figure 3). Moreover, a second analysis with the introduction of mathematical transformations of all variables showed that the square root of the spore number (total-Bt) could explain 92.6% of the variation with four variables. In both regression analyses, pH was the most important factor. The square root transformation results in high total-Bt values being more reduced than lower values, therefore the square root is grouping the data. The fact that this transformation leads to a better correlation can have two possible explanations. First, the transformation reduces data variance, giving a higher reliability. Second, by reducing the high total-Bt values, the number of *B. thuringiensis* spores have less importance, and in an extreme situation, null values remain unmodified, while values different from zero tend to 1. This equation would be assessing more accurately the presence or absence rather than the number of spores, therefore, the meaning of the dependent variable would have been changed. To confirm the second hypothesis, regressions associated to *B. thuringiensis* presence or absence (variable Bt-presence) were analyzed using multiple regression. The results obtained confirmed that the square root transforms the number of *B. thuringiensis* spores (variable total-Bt) into a variable similar to the presence or absence of *B. thuringiensis* (variable Bt-presence). In this model, the most important variable turned out to be again soil pH. Other studies have reported the effect of the combination of several biotic and abiotic factors on *B. thuringiensis* populations, but no multivariate analysis were performed (Petras and Casida, 1985; Saleh et al., 1969; West et al., 1985).

In the model inferred from the Logit regression study, comparable results were obtained concerning the biological complexity of the event analysed. Up to six independent variables were needed in order to obtain a minimum regression model ($r^2 = 0.2943$). Our findings indicate that it might be possible to correlate some soil physicochemical variables from a given soil sample (soil clay content, pH, moisture, CEC, and exchangeable Ca and Mg) with the pres-

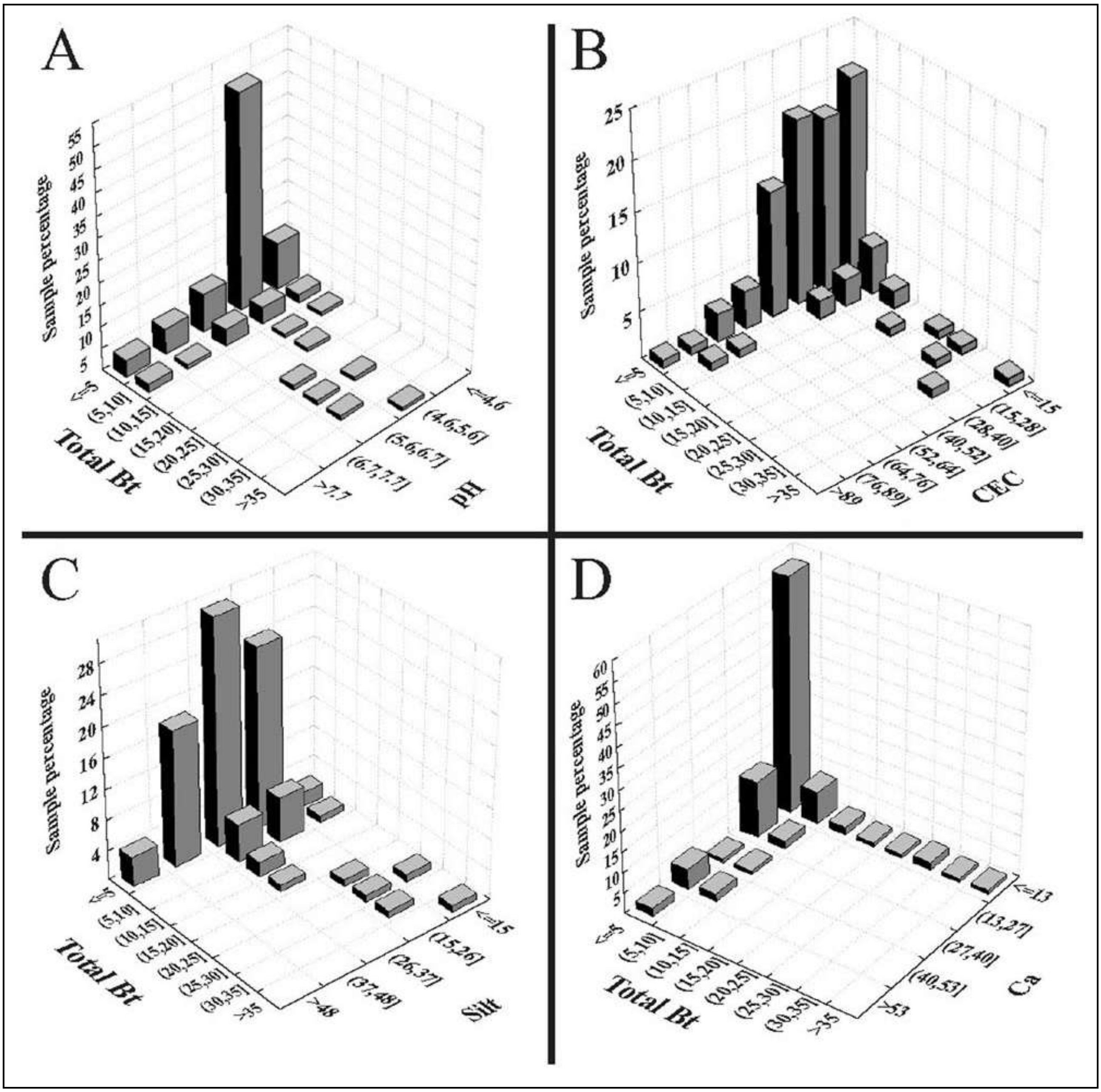

Figure 3. Bivariate histograms indicating the number of *Bacillus thuringiensis* related to the physicochemical factors of the soil samples taking part in the regression model with the following equation: total-Bt = 4.41 pH - 1.0 CEC - 0.39 silt - 0.34 Ca, (A, pH; B, CEC; C, silt; D, Ca)

ence of *B. thuringiensis* spores. Moreover, Logit regression analysis revealed another model that might allow to correlate the presence of *B. thuringiensis* with some physicochemical variables in a given soil sample. Again, it is noteworthy that among the six variables contained in the model, soil pH was the most significant. This result confirms previous *B. thuringiensis* studies, in which soil pH was evaluated (Dias and Sagardoy 1998; Petras and Casida, 1985; Saleh et al., 1969; West et al., 1985), and all of them concluded that soil pH is the single most important influential factor.

This study describes how complex the ecological niche of B. thuringiensis in soil is and shows the advantage of using statistical models in order to understand the complex relationship between microbes and their environment. The regression models reported here could become useful tools to improve the search for new B. thuringiensis isolates and to gain an insight into the possible habitat of this bacterial species in terrestrial ecosystems.

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