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Antagonistic interactions among bacteria inhabiting pineapple

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ABSTRACT

Pineapple is the second most important tropical fruit in international trade and Mexico ranks ninth in world production. Pink disease is asymptomatic in the field and is characterized by the production of dark discoloration (amber-reddish brown) and its initial effects are not recognized until the fruits are cored and canned. Hence pink disease is considered a major problem in the pineapple canning industry.

Four hundred and eighty isolates of pineapple were tested for antagonistic activity vs. *Tatumella*, causal agent of pink disease, and strain UAPS07070 was selected for further assays.

Population dynamics were explored in co-inoculation *in vitro* and in *planta* with strain UAPS07070 and *T. ptyseos* UAPS07007. The population of UAPS07007, the producer strain of the disease severely declined in comparison with the controls.

This work contributes to the knowledge of the ecology of pink disease in pineapple, as well as to the comprehension of the interactions between microorganisms colonizing the habitat of the plant.

1. Introduction

In the entire world, pineapple [*Ananas comosus* (L.) Merr] is considered as the second most economically important tropical fruit. Its trade mainly consists of canned slices (1,065,000 t), and frozen juice concentrate (215,000 t) (Bartholomew et al., 2003). There are few reports on the diversity of microbial inhabitants of pineapple plants. Several bacterial phytopathogens are known to cause diseases in this plant, such as *Dickeya chrysanthemi* (Lim and Lowings, 1978; Melo et al., 1974), *Tatumella morbirosei*, previously misidentified as *Pantoea citrea* (Brady et al., 2010; Cha et al., 1997), *Tatumella ptyseos* (Marín-Cevada et al., 2010), *Pantoea ananas* (Dye, 1969), and possibly some microorganisms belonging to the family Acetobacteraceae (Kontaxis and Hayward, 1978) like *Acetobacter peroxydans* (Serrano, 1928). This plant is also colonized by endophytes like *Gluconacetobacter diazotrophicus* (Tapia-Hernández et al., 2000), *Burkholderia* sp. (Magalhães Cruz et al., 2001; Weber et al., 1999), bacteria related to *Azospirillum* (Weber et al., 1999), and *Asaia bogorensis*, a microorganism that showed beneficial effects on its growth (Weber et al., 2010).

Pink disease of pineapple has been reported in different places around the world (Cha et al., 1997; Marín-Cevada et al., 2006; Rohrbach, 1983). This disease is asymptomatic in fresh fruits but generates considerable economic loses to the canning industry. Bacterial gluconate metabolites provoke an orange-brown pigmentation in affected fruits at pasteurization temperatures (Pujol and Kado, 1999, 2000). The enterobacteria *T. morbirosei* and *T. ptyseos* have been demonstrated as causal agents of pink disease (Brady et al., 2010; Marín-Cevada et al., 2010).

Burkholderia species are widely distributed in soil, water and plant rhizosphere (Coenye and Vandamme, 2003). A significant number of species are pathogenic to plants, animals or humans, although some of them have been used in biocontrol (De Costa and Erabadupitiya, 2005; Fridlender et al., 1993; Hebbar et al., 1998; Parke et al., 1991). Furthermore, some species are plant growth promoters and others have the potential to remediate polluted environments (Caballero-Mellado et al., 2004, 2007; Estrada-de los Santos et al., 2001; Folsom et al., 1990; Hebbar et al., 1998; McLoughlin et al., 1992; Parke et al., 1991; Vandamme et al., 2002). Different plants, including pineapple, are inhabited by isolates of this genus (Weber et al., 1999).

In this study, the antagonistic activity of a pineapple bacterial isolate against a causative agent of pink disease was evaluated using *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Bacterial strains

Bacteria isolated from pinapple plants grown in the Papaloapan Basin, Mexico were streaked on TESMA medium (Marín-Cevada

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et al., 2010). *Burkholderia gladioli* ATCC 10248^T, *B. unamae* MTI-641^T, *B. plantarii* LMG 9035^T, *B. silvatlantica* SRMrh-20^T, *B. saccharii* DSM1716^T and *B. tropica* Ppe8^T were grown in culture medium BAc (Estrada-de los Santos et al., 2001).

2.2. Detection of antagonist activity in solid medium and strain selection

The antagonist activity of pineapple isolates versus *T. ptyseos* UAPS07007, a pink disease producer strain (Marín-Cevada et al., 2010), was tested using the double layer agar test (Muñoz-Rojas et al., 2005). The isolates were incubated in TESMA broth for 24 h at 30 °C (150 rpm). The cultures were adjusted to an optical density of 0.05 at 620 nm and 20 μl was inoculated on glass Petri dishes containing TESMA agar. After incubation for 24 h at 30 °C, the bacterial growth was removed using a sterile glass slide. The plates were exposed to chloroform vapors for 1 h to ensure bacterial death. The glass plates were left opened in the laminar flow cabinet for 1.5 h in order to allow complete evaporation of the chloroform. *T. ptyseos* UAPS07007 was grown in TESMA broth under the same conditions and adjusted to an optical density of 0.05 at 620 nm. Three hundred microlitres of bacterial culture was mixed in 5.7 ml of molten TESMA agar at 37 °C, homogenized and was immediately poured evenly on the layer of agar where presumably antagonistic strains had been previously grown. Halos formed in the upper layer of the plates after incubation for 24 h were considered as indicators of inhibitory activity (Parke et al., 1991). Strains that consistently showed antagonistic activity and that showed larger halos were selected.

2.3. Bacterial identification and molecular analysis

Phenotypic characterization of UAPS07070 included biochemical tests, SDS-PAGE of whole proteins and siderophore production. Both the biochemical and the antimicrobial susceptibility tests were performed with a MicroScan Automated System (Dade Behring Co.).

The SDS-PAGE assays and the extraction of cellular proteins were done as described previously (Estrada-de los Santos et al., 2001), except that the bacteria were cultured in MN broth (Muñoz-Rojas et al., 2005). Siderophore detection was performed by a modified assay on Chromeazurol-S (CAS) agar plates (Caballero-Mellado et al., 2004). Initially, UAPS07070 was grown in liquid TESMA medium and then transferred to Cas-amino acid solid media (CAA), adding succinic acid instead of MM9 minimal medium. Bacterial growth was then inoculated (20 μl) on modified CAS medium (CAS-CAA agar). Since siderophores are produced under iron limiting conditions, detection of antibacterial substances was examined on media TESMA with and without $FeCl_3$ (20 ppm) and incubated at 30 °C for 24 h.

Genotypic identification was performed by analysis of the sequences of 16S rRNA and *recA*. Briefly, bacterial cells were grown overnight in TESMA medium at 30 °C, centrifuged and washed with 10 mM $MgSO_4$. DNA was extracted and purified with a commercial kit (Wizard Genomic DNA Purification kit, Promega). A 16S rRNA amplicon obtained with primers fD1 and rD1 (Weisburg et al., 1991), and a *rec*A PCR product amplified with primers BUR1 and BUR2 (Payne et al., 2005) were sequenced with the same primers at the Instituto de Biotecnología (UNAM, Mexico). The 16S rRNA PCR conditions were as follows: initial denaturation (3 min at 94 °C); followed by 27 amplification cycles (30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C); followed by a final elongation (10 min at 72 °C). The PCR mixture contained 150 μM dNTPs, 3 mM $MgCl_2$, 3 μM of each primer (fD1 and rD1), 1× Taq buffer and Taq polymerase 1.2 U (Altaenzymes). The *recA* amplification and electrophoresis conditions were according to Estrada-de los Santos et al. (2001).

2.4. Antagonistic activity in liquid medium

The pink disease causative agent *T. ptyseos* UAPS07007 (resistant to 0.5 M NaCl), and the isolate UAPS07070 (resistant to gentamycin 30 μg/ml) were grown in TESMA medium for 24 h and their populations adjusted to OD 0.05 at 620 nm. One hundred microlitres of each strain was inoculated both in co-cultures and individually in 50 ml of liquid medium in 250 ml Erlenmeyer flasks and incubated at 150 rpm for 96 h at 30 °C. At 24, 48, 72 and 96 h after inoculation, 1 ml aliquots were sampled from each culture. Bacterial cultures were serially diluted with 10 mM $MgSO_4{\cdot}7H_2O$. Serial dilutions were plated on TESMA agar with or without gentamycin (30 μg/ml) or NaCl (0.5 M) in six replicates, and bacterial counts were determined after incubating for 24 h.

2.5. Antagonistic activity in plants

Cells were inoculated in TESMA broth at 30 °C and incubated at 150 rpm for 24 h. The cultures were washed with 10 mM $MgSO_4{\cdot}7H_2O$ and the cultures were adjusted as previously described in section 2.4. Suspensions of strain *T. ptyseos* UAPS07007 and strain UAPS07070 (1.0×10^8 CFU/ml) were prepared both individually for the controls and in a 1:1 mixture for the treatments. Micropropagated sterile pineapple plantlets (var. Smooth Cayenne) were obtained by crown meristem tissue-culture. Seven month plantlets were inoculated under sterile conditions by immersing the roots in the corresponding bacterial suspension for 1 h (1.0×10^8 CFU/ml). Inoculated plantlets were transplanted in pots containing sterile silica sand and maintained in a controlled environment for 60 days (28 °C and 30% humidity, day/night cycle of 14/10 h). The plantlets were watered every three days with 30 ml of modified Hoagland's solution, basal salt mixture with the macro- and micronutrients as reported by Hoagland and Arnon (1950). The bacterial numbers in the rhizosphere and roots were determined at 10, 30 and 60 days post-inoculation. Eight replicate plants were analyzed independently each time. The plantlets were removed from the pots, and the roots were separated from the rest of the plant and vortexed for 15 s in sterile 10 mM $MgSO_4{\cdot}7H_2O$ in a 1:10 (w/v) proportion. The resulting suspension, considered to contain the rhizospheric bacteria, was serially diluted with sterile 10 mM $MgSO_4{\cdot}7H_2O$ and plated on TESMA agar containing gentamycin (30 μg/ml) or NaCl (0.5 M) for bacterial counting.

For determining endophytic colonization, the roots were surface sterilized with 1% Chloramine T for 10 min followed by seven washes in sterile distilled water and then macerated in sterile 10 mM $MgSO_4{\cdot}7H_2O$ in a 1:10 (w/v) proportion. The macerates were serially diluted and plated on TESMA agar containing gentamycin (30 μg/ml) or NaCl (0.5 M) and bacterial numbers determinated as described above.

2.6. Statistics

Antagonism data were tested statistically each time point with ANOVA and statistically significant differences were determined by the Tukey test using Statgraphics Plus software (v. 4.1).

2.7. Evaluation of antagonistic spectrum

The spectrum of inhibitory activity by UAPS07070 against the following bacteria and fungi was assessed by measuring the inhibition halo formed in double layer assays: *Pseudomonas aeruginosa* PAO1^T, *Staphylococcus aureus* subsp. *aureus* ATCC 33807, *Raoultella planticola* ATCC 33531^T, *Ustilago maydis* 521, *Candida albicans* ATCC

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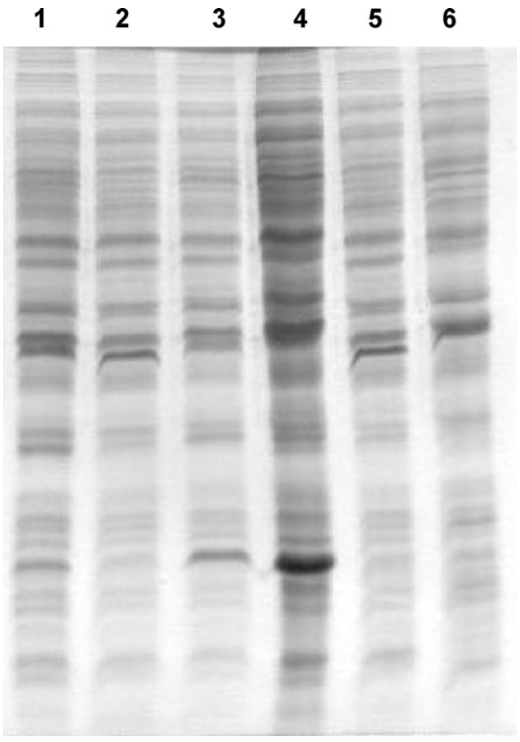

Fig. 1. Total protein profile (SDS-PAGE) of selected antagonistic isolates from pineapple. (1) UAPS07070; (2) *B. gladioli* ATCC10248^T; (3) *B. gladioli* CBe821; (4) *B. gladioli* ASC744; (5) *B. plantarii* LMG 9035^T; (6) *B. caryophylli* ATCC 25418^T.

10231^T, *Pantoea ananatis* LMG 2665^T, *Escherichia coli* 62348-69, *B. cepacia* ATCC 25416^T, *B. cenocepacia* J2315, *B. vietnamiensis* LMG 10929^T, *B. latens* LMG 24064^T, *B. multivorans* LMG 13010^T, *B. dolosa* LMG 18943^T, *B. stabilis* LMG 14294^T, *Azospirillum brasilense* DSM 1843 and *Gluconacetobacter diazotrophicus* PAl5^T.

2.8. Activity of the isolate UAPS07070 in in vitro pigmentation test

Sterile canned pineapple juice was inoculated with *T. ptyseos* UAPS07007 or with UAPS07070 alone and in co-culture. Cells were incubated at 30 °C, 150 rpm.

Two 1 ml aliquots were collected after 0, 5, 8, 12, 24, 48 and 72 h of incubation. One aliquot was heated at 110 °C for 5 min. After cooling to room temperature, the cells were centrifuged (10 min, 15,000 rpm) and the absorbance (420 nm) of the supernatants was determined.

3. Results

3.1. Selection and identification of antagonist isolate

Among 480 bacterial isolates tested, 17 strains consistently showed inhibition against *T. ptyseos* UAPS07007, and among them UAPS07070 was selected for further assays. This strain was selected since apparently showed larger halos than other strains (Table S1, Supplementary Material). BlastN with the NCBI database of sequences JN107997 and JN104387 showed 99% identity of both with *Burkholderia gladioli* 16S rRNA and *recA* sequences, *E* values = 0.0, and 4×10^{-155}, respectively. Clustal comparison of those sequences also showed identical results. The results of biochemical tests (data not shown) also suggested that isolate UAPS07070 belonged to the genus *Burkholderia*.

UAPS07070 showed similar whole-cell proteins pattern with *B. gladioli* ATCC 10248^T by visual analysis (Fig. 1). Hence, strain

Table 1
Antagonistic activity by *B. gladioli* UAPS07070 in liquid co-culture.[a]

Strain	Time (h)				
	0	24	48	72	96
T. ptyseos UAPS07007	3.50×10^{7A} (5.80×10^6)	1.63×10^{9A} (2.30×10^8)	2.00×10^{8A} (8.00×10^7)	2.40×10^{8A} (6.90×10^7)	3.03×10^{9A} (2.30×10^8)
T. ptyseos UAPS07007 in co-culture	2.93×10^{7A} (2.90×10^6)	1.18×10^{8B} (4.00×10^7)	2.00×10^{8A} (2.00×10^7)	5.67×10^{7B} (3.00×10^6)	6.67×10^{6B} (7.00×10^5)
B. gladioli UAPS07070	2.50×10^{7A} (2.80×10^6)	3.00×10^{8A} (3.40×10^7)	3.50×10^{9A} (7.00×10^8)	4.20×10^{9A} (7.00×10^8)	4.20×10^{9A} (1.07×10^9)
B. gladioli UAPS07070 in co-culture	1.33×10^{7B} (1.60×10^6)	3.80×10^{8A} (8.10×10^7)	1.50×10^{9A} (9.00×10^7)	3.27×10^{9A} (2.30×10^8)	2.10×10^{9B} (4.00×10^8)

Distinct superscript letters reflect statistically significant difference in an individual species density between mono- and co-culture at an individual time point.
[a] CFU/ml in liquid TESMA medium, mean followed by standard error of the mean in parenthesis. All the assays were performed with six replicates after the indicated incubation time. The strains were incubated either alone or together (co-culture).

Table 2
Relationship among bacterial inoculation and pigmentation decrease.

Time (h)	*T. ptyseos* UAPS07007[a]	*T. ptyseos* UAPS07007 and *B. gladioli* UAPS07070[a]
0	0.000 (0.000) ^{A}	0.000 (0.000) ^{A}
5	0.463 (0.020) ^{A}	0.140 (0.017) ^{B}
8	0.526 (0.032) ^{A}	0.470 (0.017) ^{A}
12	0.750 (0.080) ^{A}	0.583 (0.026) ^{A}
24	1.390 (0.034) ^{A}	0.290 (0.028) ^{B}
48	1.520 (0.040) ^{A}	0.050 (0.017) ^{B}
72	1.796 (0.037) ^{A}	0.000 (0.000) ^{B}

[a] Absorbance (629 nm) of inoculated pineapple juice, media followed by the standard error of the mean. All tests were performed in triplicate and comparisons were done each time point, ($P < 0.05$).

UAPS07070 was identified as belonging to the species *Burkholderia gladioli*.

3.2. Antagonistic activity and pigmentation decrease in liquid medium

Bacterial growth inhibition of *T. ptyseos* UAPS07007 by *B. gladioli* UAPS07070 could be detected after 24 h in liquid medium. The antagonistic activity was more evident in the late exponential phase of growth (Table 1). The pigmentation produced by *T. ptyseos* UAPS07007 diminished after 12 h when growing in co-inoculation with *B. gladioli* UAPS07070 (Table 2). *B. gladioli* UAPS07070 was not able to produce any pigmentation.

3.3. Antagonistic activity in pineapple plants

At 10 and 30 days after inoculation of pineapple plantlets, there was no significant difference in *T. ptyseos* UAPS07007 cell number,

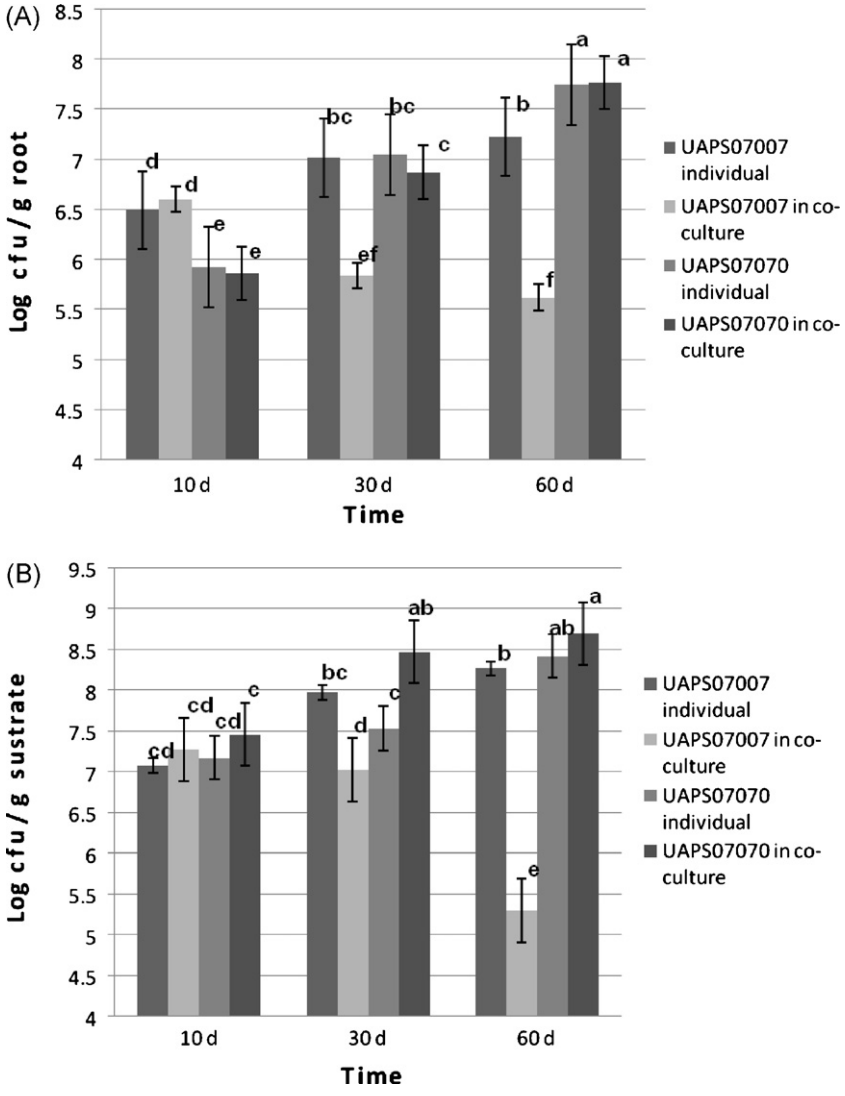

Fig. 2. *In planta* antagonism of UAPS07070 strain against Pink disease producing strain in root (A) and rizosphere (B). There are no differences statistical among treatments with same letter (Tukey test based on the standard error). In each treatment eight replicates were tested each time point.

either alone or in co-inoculation with *B. gladioli* UAPS07070, both in the rhizosphere or within the root. However, 60 days after inoculation, the population of the pink disease producing strain showed a noticeable decrease in both environments when co-inoculated with *B. gladioli* UAPS07070 (Fig. 2).

3.4. Evaluation of antagonistic spectrum

Strain *B. gladioli* UAPS07070 displayed inhibitory activity against all the bacteria and fungi strains tested with inhibition halos ranging from 10 to 45 mm (Table S2, Supplementary Material), similar size to the halo against *T. ptyseos* (45 mm).

4. Discussion

Plant diseases cause major yields decrease so it would be desirable to find alternatives for their control. Attempts for controlling Pink disease in pineapple include the production of resistant varieties and the use of biological control methods; for example, the antagonistic activity of *Paenibacillus validus* (formerly *Bacillus gordonae*) against *T. morbirosei* has been very promising in the laboratory (Kado, 2003). However, its effectiveness and economic benefits have not been determined. This work aimed to detect bacterial populations that could inhibit the causative agent of Pink disease. Antagonistic effects observed in *in vitro* experiments are not necessarily observed *in vivo* because of the intervention of uncontrolled variables such as the production of exudates, activity of native flora, and the different physical and structural environments (Ownley and Windham, 2003). Notably, the isolate UAPS07070 obtained from pineapple fruit and identified as *Burkholderia gladioli,* showed inhibitory activity *in vitro* and *in planta* against *T. ptyseos*, a pink disease causative agent. In other experiments some *B. gladioli* strains have been found that are able to inhibit the growth of fungi on fruits like lemon and apple (Cirvilleri et al., 2006; Whitby et al., 2000). Some isolates identified as *B. gladioli* promote plant growth (J. Caballero, unpublished results; Mamta et al., 2010), while other isolates have the ability to solubilize inorganic phosphate (Mamta et al., 2010; Sashidhar and Podile, 2010). Most of the antagonistic strains in this work were isolated from apparently healthy pineapple fruits, suggesting a possible natural inhibition of Pink disease in pineapple fields. The plants inoculated with *B. gladioli* UAPS07070 did not show apparent damage (data not shown). Additionally, co-inoculation experiments in liquid medium with *B. gladioli* UAPS07070 against *T. ptyseos* UAPS07007 showed a decrease in pigmentation by the Pink disease-producing strain (Table 2). These results suggest that *B. gladioli* UAPS07070 could degrade the chromogenic molecule or its precursors, or also could inhibit its synthesis. A density-dependent production of the pigment could also partially explain its decline. *B. gladioli* UAPS07070 possesses a wide range inhibitory mechanism since it inhibited not only *T. ptyseos* but other Gram negative and Gram positive bacteria, and even fungi (Supplementary Material). The antagonistic activity was not inhibited in the presence of Fe^{3+} (Results not shown), consequently a siderophore nature of the inhibitor is ruled out.

Nevertheless, field application should not be recommended until ecological knowledge becomes extensive, since *B. gladioli* has been described as a pathogen of some flowers (Cirvilleri et al., 2006; Coenye and Vandamme, 2003) and crops (Kato et al., 1992; Lincoln et al., 1991; Maeda et al., 2006; Sashidhar and Podile, 2010; Whitby et al., 2000), and it has even been found as an opportunistic pathogen in immune-compromised patients with respiratory infections (Whitby et al., 2000).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apsoil.2011.11.014.

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