Plant and Soil **266:** 41–46, 2004.

Persistence and impact on microorganisms of *Bacillus thuringiensis* proteins in some Zimbabwean soils

P. Muchaonyerwa[1,2,4], S. Waladde[2], P. Nyamugafata[1], S. Mpepereki[1] & G.G. Ristori[3]
[1]*Department of Soil Science and Agricultural Engineering, University of Zimbabwe, Box MP 167, Mt Pleasant, Harare.* [2]*Department of Zoology, University of Fort Hare, 5700 Alice, South Africa.* [3]*Istituto Genesi Ecologia Suolo, CNR Firenze, Italy.* [4]*Corresponding author**

Received 7 April 2003. Accepted in revised form 5 November 2003

Key words: Bacillus thuringiensis, bioassays, crystal proteins, microorganisms, persistence, *Plutella xylostella*

Abstract

The persistence of the *Bacillus thuringiensis* subsp. *kurstaki* (Btk) toxin (Cry1Ab protein) from Bt maize (MON810, Yieldgard®) residues incorporated in a vertisol (739 g clay kg^{-1}) was investigated. The maize residues were incubated in the soil for 4 weeks, and activity of the toxin in the residues was bioassayed using larvae of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). Corrected mortality of *P. xylostella* in the bioassays decreased from 76% to 30% in less than a week of incubation in the soil. In addition to the above observations, the effects of Btk, Bt subsp. *israelensis* (Bti), and Bt subsp. *tenebrionis* (Btt) proteins on the soil microbiota were examined using a vertisol, an alfisol, and an oxisol. The pre-incubated soils (7 days after moisture adjustment) were treated with crystal proteins of Btk, Bti, and Btt and incubated for further a 7-day period. Microbial biomass carbon (MBC) and counts of culturable bacteria and fungi were determined. The proteins did not show effects on MBC or bacterial and fungal counts, possibly as a result of adsorption of the proteins on soil particles, which could have rendered the proteins inaccessible for microbial utilization. Microbial biomass carbon and counts arranged in decreasing order were vertisol>oxisol>alfisol, similar to the amounts of organic C and clay in the soils. However, bacteria and fungi counts were higher in the vertisol than in the alfisol and the oxisol soils. Our observations suggest that larvicidal proteins produced by different subspecies of Bt and Bt maize could persist in tropical soils as a result of adsorption on soil clays but that there were no observable effect on the soil microbiota.

Introduction

Crops that are genetically engineered to produce *Bacillus thuringiensis* (Bt) toxins release the proteins to soils as components of root exudates (Saxena et al., 1999) and crop residues (Tapp and Stotzky, 1998), and there is no general consensus on persistence of the proteins in soils. Saxena and Stotzky (2001) reported that Cry1Ab protein released in root exudates from Bt maize remained larvicidal for > 180 days and Tapp and Stotzky (1998) reported that purified Cry1Ab protein was toxic > 234 days after addition to soil, presumably as a result of binding on clay particles. Sims and Holden (1996) reported a 90% decrease in bioactivity of Cry1Ab protein in 15 days in soil. Persistence of these proteins in soils poses a potential hazard on non-target insects, particularly in tropical soils where Bt crops are being introduced. Whereas Bt crystal proteins are selectively pesticidal to larvae of different insect orders (Schnepf et al., 1998), their toxicity to other invertebrates (Acarina, Nematoda, Collembola and Hymenoptera) inhabiting the soil has been reported (Addison, 1993). Little attention has been paid to effects that Bt proteins might have on indigenous soil microorganisms and the information available is often confusing (Addison, 1993). Here we report our observations on the persistence of Bt subsp.

*FAX No: +27-40-6531669.
E-mail: muchaonyerwa@yahoo.com

kurstaki (Btk) toxin (Cry1Ab) from Bt maize residues in a tropical vertisol. In addition, we examined the effects of the proteins from Btk, Bt subsp. *israelensis* (Bti), and Bt subsp. *tenebrionis* (Btt) on the soil microbiota.

Materials and methods

Persistence of Cry1Ab protein from Bt maize residues in soil

Bt maize (MON810, Yieldgard® producing the Cry1Ab protein, and its near-isogenic line (DC-26) (supplied by Monsanto, Italiana SpA) were grown at the University of Fort Hare (South Africa) on an alfisol (pH 7.0, 150 g clay kg^{-1}). Plants were grown for 10 weeks, and residues (roots, stems, and leaves) were dried at 40 °C (to avoid denaturing the Cry1Ab protein) until constant weight was achieved, ground to < 1 mm (Retsch Mühle mill, Adolf Dietz Electromotorenfabrik motor, 1.1 kW, 220 V), and stored at 4 °C. Samples (< 2 mm) of a vertisol (pH 7.5, 739 g clay kg^{-1}, 12.5 g organic C kg^{-1}) from Chisumbanje (Zimbabwe) amended with the maize residues (10% w/w), in 250-mL flasks, were adjusted to the 33-kPa water tension (60% w/w, pressure plate method) (Anderson and Ingram, 1993). The particle-size fraction < 2 μm was referred to as clay. A high application rate (10%) was used in order to have detectable levels of the Cry1Ab toxin so that persistence of its toxic effects could be monitored using larvae of the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae), which has a low susceptibility to the toxin in feeding bioassays (Peferoen, 1997). The experiment had two treatments: soil amended with Bt maize residues and soil amended with residues of near-isogenic maize, which served as the control. Each treatment was replicated three times. The flasks were loosely capped by unscrewing the lids, incubated at 25 °C for 4 weeks, 5 g samples were taken weekly from each replicate, stored at 4 °C, and 1 g was homogenized in 4 mL of phosphate buffer (137 mM NaCl, 1 mM KH_2PO_4, 16 mM $Na_2HPO_4.2H_2O$, 3 mM KCl, 0.05% Tween 20, pH 7.6), with an electric stirrer (NORU model NR913-7204) for 30 s.

A modified leaf-painting assay, as used by MacIntosh et al. (1990) to test activity of *Bacillus thuringiensis* proteins on alfalfa weevil, was used. Ten non-sterile rape (*Brassica napus*) seedlings (at two-leaf stage) were dipped into each soil extract for 30 s, air-dried, and each seedling was then placed into 10 cm long (2 cm diam), open-ended glass tubing with the roots in contact with damp cotton wool plugged at one end. A 2nd instar larva of *P. xylostella* was introduced onto the treated rape seedlings via the open end of the glass tubing, which was then plugged with dry cotton wool.

The larva was allowed to feed on the treated leaves for at least three days. The experimental room was maintained at 25 ± 1 °C, 40–50% relative humidity, and a 16:8 day: night photoperiod (Mori, 1986). Faecal pellets, produced by each feeding larva were counted and recorded after 48 h. Mortality was recorded after 72 h, analysis of variance (ANOVA) of the treatment means (Genstat 5 Release 4.1, 1997) performed for the 4-week incubation period. The data were corrected for natural mortality in the controls (Abbott, 1925).

Table 1. Properties of the Zimbabwean soils used in this study

Soil property	Vertisol	Alfisol	Oxisol
	Origin of soil		
	Chisumbanje	Chiredzi	Harare
Clay (g kg^{-1})	739	250	500
Organic carbon (g kg^{-1})	12.5	5.2	11.7
CEC (cmol kg^{-1})	69.9	11.1	9.1
pH (1:5, soil:water)	7.5	6.5	5.8

Effect of Bt crystal proteins on the soil microbiota

Tropical soils collected from three different localities in Zimbabwe were used. Details regarding the properties and places of origin of the soils are given in Table 1. In a study by Muchaonyerwa et al. (2002), the *Bacillus thuringiensis* subsp. *tenebrionis* (Btt) toxin adsorbed and bound in large amounts on clay fractions of the vertisol and the alfisol used in this study, and it was necessary to test its effects on microorganisms in these soils. The toxin was purified from the commercial preparation (Novodor FC, Abbott Laboratories) using 0.1 M 3-N-morpholinopropane sulphonic acid (MOPS) buffer (pH 7.8) containing 1 M potassium thiocyanide (KSCN) (as used by Venkateswerlu and Stotzky (1990) and Tapp et al. (1994), for purification of the toxin from Btk) and modified by addition of 0.5 M β-mercaptoethanol instead of dithiothreitol. The crystal proteins from *Bacillus thuringiensis* subsp. *kurstaki* (Btk) and *Bacillus thuringiensis* subsp. *israelensis* (Bti) were extracted from commercial pre-

parations, Delfin (Norvatis) and Turbac CD (Abbott Laboratories). The crystals were separated by suspending the commercial preparation in distilled water, homogenizing, and centrifuging for 15 min in a swinging bucket rotor at a gradient of 70–200 $\times$ g. The crystals were obtained in the white phase and stored at 4 °C until used.

The alfisol and oxisol were adjusted to field capacity (FC) (33 kPa) and the vertisol to 60% of FC, so that both soil moisture and aeration would not limit growth of microorganisms. The vertisol contained large amounts of expanding clay minerals, which could result in low aeration (< 10% of porosity) at FC. Soil samples were pre-incubated in the dark at 28 °C for 7 days before a solution of Btt toxin and suspensions of Btk and Bti crystal proteins were added. The proteins were added at 6.3 μg protein g^{-1} soil and incubated at 28 °C for 7 days. Each treatment was replicated four times, and unamended soils were used as controls. Microbial biomass carbon (MBC) was determined by the fumigation extraction (FE) procedure (Rice et al., 1996), and the values were corrected for the proportion of microbial biomass C extractable from soil by this method (Kc = 0.35) (Howarth and Paul, 1994). Bacterial counts were determined by spread plating 0.1 mL aliquots of the soil suspensions from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions on Plate Count Agar (Wollum II, 1982), in triplicate, and incubated at 28 °C for 3 days. The spread plate method was more convenient and gave more consistent results than pour plate, in preliminary checks for enumerating soil microorganisms. Only plates with between 30 and 300 colonies were counted. To determine fungal colony-forming units (CFU), aliquots from the 10^{-2} and 10^{-3} soil dilutions were spread-plated on Martin's Rose Bengal medium (Wollum II, 1982) in triplicate and incubated at 28 °C for 5 days. Only plates with between 10 and 100 colonies were counted.

Logarithmic (base 10) transformations of microbial count data were performed. Two-way analyses of variance (ANOVA), with main factors as soil and protein treatment, were performed on both the MBC and the log-transformed microbial counts data using a Genstat statistical package (Genstat 5 Release 4.1, 1997).

Table 2. Microbial biomass carbon (MBC), expressed as mean ± standard error of the mean (SEM), in soils treated with Bt proteins

Soil	MBC (μg g^{-1})			
	Control	Btk crystals	Btt toxin	Bti crystals
Vertisol	608 ± 45.9	637 ± 65.8	618 ± 65.8	643 ± 19.7
Alfisol	168 ± 1.0	192 ± 32.5	173 ± 3.0	193 ± 7.9
Oxisol	356 ± 58.3	397 ± 19.8	393 ± 28.1	359 ± 65.8

Results

Persistence of Cry1Ab protein in soil

Mortality of *P. xylostella* depended on the type of maize extract (Bt maize or near-isogenic control) and incubation time ($p < 0.01$). Mortality was higher in the treatments with soil-Bt maize extracts than with the near-isogenic controls throughout the incubation period. Mortality of larvae fed on rape seedlings treated with extract from soil amended with Bt maize decreased rapidly from 80% to 50% within the first week of incubation. The initial mortality (80%) was higher than those for weeks 1 up to 4, which were comparable. Mortality in the controls (leaves treated with either phosphate buffer or with a homogenate of soil-near-isogenic maize) remained between 20% and 30% for the entire 4-week period and could have been caused by stresses resulting from handling and confinement of the larvae in the tubes. Corrected mortality decreased within the first week of incubation from 76% to about 30% and then remained essentially constant to week 4 (Figure 1). There was a linear relationship between frass production and larval mortality (Figure 2).

Effects of Bt proteins on microorganisms

MBC in the soils was not affected by the addition of any of the proteins (Table 2). Soil type had a significant effect, independent of the proteins, on MBC ($p < 0.05$). MBC levels for all treatments were in the order: vertisol >oxisol >alfisol. Protein treatment had no effect on bacterial counts ($p > 0.05$) and on CFU of fungi ($p > 0.05$). The three protein treatments did not increase or decrease bacterial counts (Table 3) and CFU of fungi (Table 4) in the three soil types. Bacterial counts were higher in the vertisol than in the oxisol and alfisol.

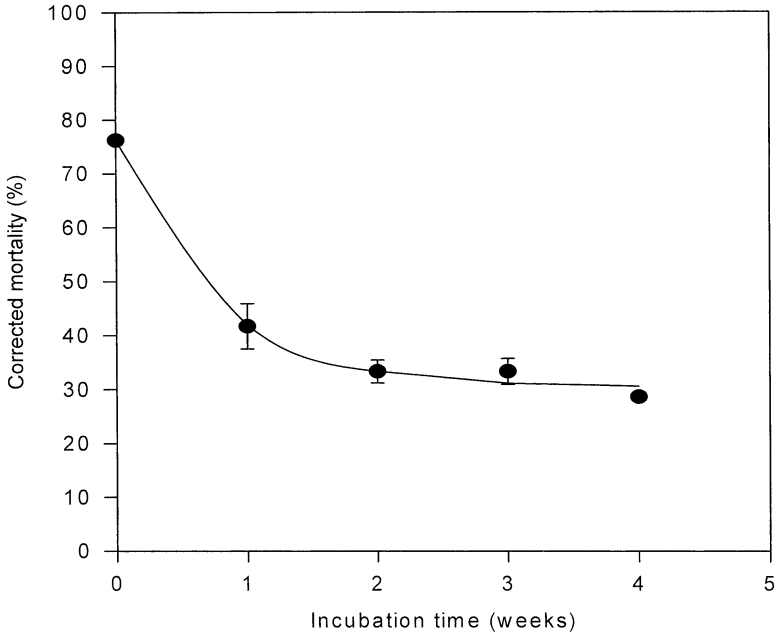

Figure 1. Corrected mortality of *Plutella xylostella* larvae fed on rape seedlings treated with extracts from soil containing plant residues of Bt maize.

Table 3. Culturable bacterial counts (mean ± SEM) in soil treated with Bt proteins

Soil	Bt proteins			
	Control	Btk crystals	Btt toxin	Bti crystals
	Bacterial counts (x 106 g^{-1} soil)			
vertisol	24.6 ± 5.10	43.7 ± 14.12	36.3 ± 4.25	33.1 ± 2.99
alfisol	7.4 ± 1.05	9.6 ± 2.03	9.1 ± 0.53	8.1 ± 1.24
oxisol	4.8 ± 1.66	6.6 ± 1.35	3.4 ± 0.60	5.0 ± 1.18

Table 4. Fungal colony-forming units (CFU) (mean ± SEM) in soils treated with Bt proteins

Soil	Bt proteins			
	Control	Btk crystals	Btt toxin	Bti crystals
	Fungal count (x 10^4 CFU g^{-1} soil)			
vertisol	6.6 ± 1.19	9.6 ± 0.97	10.2 ± 1.08	9.3 ± 0.87
alfisol	1.8 ± 0.31	2.1 ± 0.23	2.5 ± 0.28	2.7 ± 0.58
oxisol	2.0 ± 0.33	2.6 ± 0.27	2.2 ± 0.38	1.9 ± 0.57

Discussion

These results showed that Cry1Ab protein from Bt maize could persist in tropical soils for several weeks without losing insecticidal properties. Tapp and Stotzky (1998) and Saxena and Stotzky (2001) reported longer persistence with purified Btk toxin (> 234 days) and root exudates and biomass of Bt maize (> 180 days), respectively. The rapid decrease in mortality in the first week could have been a result of the utilization of the readily available Cry1Ab protein in the tissue by soil microorganisms. Binding of the protein on clay and organic matter could have rendered it unavailable for utilization, resulting in the plateau in mortality for the rest of the incubation period. Several studies have shown that Bt proteins bound on clays and humic acids resisted microbial decomposition and retained their bioactivity (e.g., Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001), probably because their structures were not modified upon binding on the solids (Tapp et al., 1994). Our observations suggest that the Cry1Ab protein has a potential to persist and accumulate in tropical soils. This scenario may arise if Bt maize is grown in soils with high clay contents thus contributing to a continuous incorporation and accumulation of Bt maize residues and the Cry1Ab protein in soil. There is need to study the effects of the Cry1Ab protein, and its persistence in soils, on non-target insects.

Bt proteins had no observable effects on MBC and bacterial and fungal counts in any of the soils evaluated. The findings were in agreement with those of

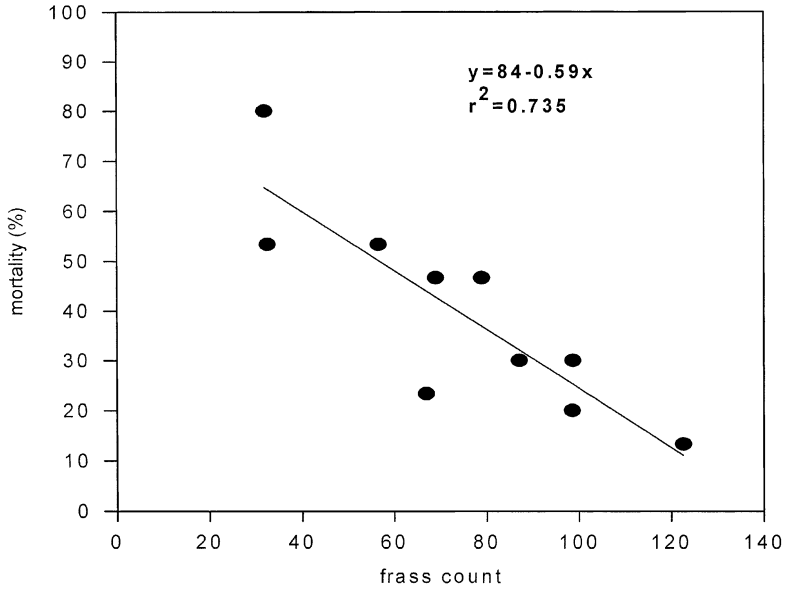

Figure 2. Relationship between frass production and mortality of *Plutella xylostella* larvae.

Saxena and Stotzky (2001), who also reported that the Cry1Ab protein in root exudates and biomass of Bt maize was not toxic to earthworms, nematodes, protozoa, bacteria, and fungi in soil. This could have been a result of adsorption of the protein on soil clays (Tapp et al., 1994; Muchaonyerwa et al., 2002), which rendered the protein molecules not readily available for microbial utilisation. However, Koskella and Stotzky (2002) observed that clay-bound and free insecticidal toxins from Btk, Btt, and Bti did not affect the growth of a variety of bacteria, fungi, and algae in mixed and pure cultures.

Petras and Casida (1985) observed increases in populations of bacteria, including actinomycetes, fungi, and nematodes after the addition of a commercial preparation of Btk to soil, which implied that the crystal proteins were used as a substrate. In our study, microbial biomass and fungal counts appeared to be related primarily to the amounts of organic C in the soils rather than to the addition of the Bt proteins.

Our observations may be useful relative to the concerns about the possible negative effects of Bt proteins from genetically engineered plants to the soil microbiota. However, more sensitive molecular methods are needed to establish any possible shifts in community composition and effects on diversity of soil microorganisms as a result of Bt proteins.

Acknowledgements

The European Community funded the work under the MICROBE Project (grant INCO-DCIC18CT97-0135). Special thanks go to Dr Luca Calamai and Dr O. L. Pantani for purifying the Bt proteins. We thank Dr A. Fereres and Dr C. Chenu for providing assistance in the course of these investigations.

References

Abbott W S 1925 A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Addison J A 1993 Persistence and non-target effects of *Bacillus thuringiensis* in soil: A review. Can. J. For. Res. 23, 2329–2342.

Anderson J M and Ingram J S I 1993 Tropical Soil Biology and Fetility; A Handbook of Methods 2nd ed. CAB International, Wallingford, Oxon, UK. 313 pp.

Crecchio C and Stotzky G 1998 Insecticidal activity and biodegradation of protein from *Bacillus thuringiensis* sub-species *kurstaki* bound to humic acids from soil. Soil Biol. Biochem. 30, 463–470.

Crecchio C and Stotzky G 2001 Biodegradation and insecticidal activity of the protein from *Bacillus thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite-humic acids-Al hydroxypolymers. Soil Biol. Biochem. 33, 573–581.

Genstat 5, Release 4.1 1997 Lawes Agricultural Trust, Rothamsted Experiment Station, UK.

Howarth W R and Paul E A 1994 Microbial biomass. *In* Methods of Soil Analysis Part 2: Microbiological and Biochemical Properties. Eds. R W Weaver, J S Angle and P S Bottomley. pp. 753–773. Soil Sci. Soc. Am. Book Series 5. SSSA, Madison, WI.

Koskella J and Stotzky G 1997 Microbial utilization of free and clay-bound insecticidal proteins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. Appl. Environ. Microbiol. 63, 3561–3568.

Koskella J and Stotzky G 2002 Larvicidal toxins from *Bacillus thuringiensis* subsp. *kurstaki*, morrisoni (strain *tenebrionis*), and *israelensis* have no microbicidal or microbiostatic activity against selected bacteria, fungi, and algae in vitro. Can. J. Microbiol. 48, 262–267.

MacIntosh S C, Stone T B, Sims S R, Hunst P L, Greenplate J T, Marrone P G, Perlak F J, Fischhoff D A and Fuchs R L 1990. Specificity and efficacy of purified Bt proteins against agronomically important insects. J. Invert. Pathol. 56, 258–266.

Mori H 1986 Effectiveness of dead-spore *Bacillus thuringiensis* formulation against diamond-back moth. *In* Diamond-back Moth Management. Ed. T D Griggs. pp. 157–164. Proceedings of the First International Workshop, 11–15 March 1985, Tainan. Shanhua, Asian Vegetable Research, Taiwan.

Muchaonyerwa P, Chenu C, Pantani O L, Calamai L, Nyamugafata P and Mpepereki S 2002 Adsorption of the insecticidal toxin from *Bacillus thuringiensis* subsp. *tenebrionis* to clay fractions of tropical soils. *In* Developments in Soil Science. Vol. 28B. Eds. A Violante, P M Huang, J M Bollag and L Gianfreda. pp. 59–68. Elsevier, Amsterdam.

Peferoen M 1997 Insect control with transgenic plants expressing *Bacillus thuringiensis* crystal proteins. *In* Advances in Insect Control: The Role of Transgenic Plants. Eds. N Carozzi and M Koziel. pp. 21–38. Taylor and Francis Publishers, London.

Petras S F and Casida L E Jr 1985 Survival of *Bacillus thuringiensis* spores in the soil. Appl. Environ. Microbiol. 50, 1496–1501.

Rice C W, Moorman T B and Beare M 1996 Role of microbial carbon and nitrogen in soil quality. *In* Methods for Assessing Soil Quality. Eds. J D Doran and A J Jones. pp. 203–215. SSSA Spec. Publ. 49. Madison, WI.

Saxena D and Stotzky G 2001 *Bacillus thuringiensis* (Bt) protein released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. Soil Biol. Biochem. 33, 1225–1230.

Saxena D, Flores S and Stotzky G 1999 Insecticidal toxin in root exudates from Bt-corn. Nature 402, 480.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler D R and Dean D H 1998 *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806.

Sims S R and Holden L R 1996 Insect bioassay for determining soil degradation of *Bacillus thuringiensis* sub-species kurstaki Cry1Ab protein in corn tissue. Environ. Entomol. 25, 659–664.

Tapp H and Stotzky G 1998 Persistence of insecticidal protein from *Bacillus thuringiensis* subsp. *kurstaki* in soil. Soil Biol. Biochem. 30, 471–476.

Tapp H, Calamai L and Stotzky G 1994 Adsorption and binding of insecticidal proteins from *Bacillus thuringiensis* subspecies *kurstaki* and subspecies *tenebrionis* on clay minerals. Soil Biol. Biochem. 26, 663–679.

Wollum II A G 1982 Cultural methods for soil microorganisms. *In* Methods of Soil Analysis 2: Chemical and Microbiological Properties. Eds. A L Page, D R Keeney, D E Baker, R H Miller, E Jr Roscoe and J D Rhoades. pp. 781–802. ASA, SSSA, Madison, WI.