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CONTENTS

Effects of *Brassica napus* seed meal amendment on soil populations of resident bacteria and *Naegleria americana*, and the unsuitability of arachidonic acid as a protozoan-specific marker

Michael F. Cohen[1,2*] and Mark Mazzola[1]

[1]Tree Fruit Research Laboratory

USDA-Agricultural Research Service

1104 N. Western Ave.

Wenatchee, WA 98801, USA

[2]Department of Biology

Sonoma State University

1801 East Cotati Ave.

Rohnert Park, CA 94928, USA

[*]Corresponding author:

Email: cohenm@sonoma.edu

Tel: 1-707-664-3413

Fax: 1-707-664-3012

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Abstract

Bacterial and protozoan populations were monitored in orchard soil amended with *Brassica napus* seed meal. Numbers of fluorescent pseudomonads peaked one week following incorporation of the amendment and declined thereafter whereas populations of *Streptomyces* spp. increased to stable levels nearly 20-fold higher than in non-treated soil. The amoeba-flagellate *Naegleria americana*, isolated from the amended soil, showed a marked feeding preference in culture for fluorescent pseudomonads relative to *Streptomyces* spp. Amended soils showed rapid declines in RSM-derived *cis*-oleic acid that paralleled increases in the numbers of bacteria and proportion of bacterial fatty acid markers in the soil. Arachidonic acid, which reached peak levels one week after seed meal incorporation, was found in *Pythium* spp. and *Mortierella* spp. but not in the two most abundant protozoa isolated from soil. These data indicate that arachidonic acid is not a suitable marker for protozoan quantification.

Introduction

Organic amendments, such as the seed meal of *Brassica napus*, are routinely applied to orchard soils to improve soil physical properties, provide for plant nutrition and to suppress pathogens that incite root rotting diseases. Increases in the abundance of protozoa have been documented in organically amended soils (Forge et al. 2003) with the particle size of the amendment influencing the ultimate size of this population (Vestergaard et al. 2001). Selective feeding by protozoa can alter soil bacterial community structures (Jurgens et al. 1999; Ronn et al. 2002). With a filamentous morphology that prevents internalization, *Streptomyces* spp. are generally less edible than smaller fast-growing bacteria such as *Pseudomonas* spp. (Pussard et al. 1994). Although a differential trend in the populations of these two bacterial genera has been observed in response to *B. napus* seed meal amendment (Cohen et al., 2005),

the role, if any, of protozoan grazing in determining this outcome has yet to be examined. For the present study, we first sought to monitor by cultural and fatty acid analysis changes in bacteria and protozoan populations in seed meal-amended orchard soil. Secondly, we determined in vitro the relative susceptibility of *Streptomyces* spp., *Pseudomonas* spp. and other bacteria to feeding by *Naegleria americana* trophozoites.

Materials and Methods

Plant residues. *Brassica napus* cv. Athena seed meal (5.8% N, 1.2% P, 1.3% K, 0.6% S) was kindly provided by Jack Brown of the University of Idaho, Moscow. The "flake" preparation used in experiments consisted of material that was retained following sieving on a 2 mm metal mesh. *B. napus* powder was prepared by grinding flakes in a blender and utilizing the material that passed through a 1 mm metal mesh. Unless otherwise noted the powder seed meal was utilized. Ground soybean flour (6.25% N) was purchased from Bob's Red Mill Natural Foods (Milwaukie, OR). Percent mineral dry weights of plant tissues were determined by Soiltest Farm Consultants, Inc. (Moses Lake, WA).

Soil and soil treatments. Soil from the Columbia View Experimental (CV) orchard had a water content of 17.0% at the time of collection. Orchard history, soil properties, and soil collection procedures have been published previously (Mazzola 1998, 1999). Upon the initiation of an experiment and at bi-weekly intervals 2 L of CV soil in 4-L plastic containers was misted to achieve water content of 19.0 ± 1.0% and kept covered on a greenhouse bench at 23 ± 5 ˚C. Immediately prior to the initial watering, organic amendments were mixed into soils at 0.5% (vol/vol). For some soil treatments, to inhibit oomycetes, Ridomil Gold ® (48% wt/vol mefenoxam) was added at 15 mg a.i. kg soil^{-1} simultaneous, if applicable, with seed meal incorporation.

For pasteurization, 2 L of soil contained in a heat-resistant plastic bag was steamed at 102 ˚C for 3 h, cooled overnight, and the cycle repeated once before utilizing the soil. Pasteurized *B. napus* seed meal was obtained by similarly treating with steam but was additionally dried overnight at 80 ˚C prior to incorporation into soil.

Isolation and quantification of soil microorganisms. All microbial population density values are reported relative to soil dry weight. From each treatment the top 2-cm of soil in the container was sampled from five random sites for dry weight determination. Soil sub-samples collected from these same sampling locations were resuspended in Page's modified Neff's amoebae saline (PAS: 120 mg NaCl, 4 mg $MgSO_4 \cdot 7H_2O$, and 4 mg $CaCl_2 \cdot 2H_2O$ per 1 L of 1 mM pH 7 phosphate buffer). Serial dilutions were plated in triplicate onto 1.5% agar containing 1/50th-strength Tripticase Soy broth (TSA), KB+ (Simon and Ridge 1974), PSSM (Mazzola et al. 2001), and 1/10th-strength Potato Dextrose broth (PDA) supplemented with 100 µg ml^{-1} streptomycin sulfate and 75 µg ml^{-1} kanamycin and incubated at 24 ˚C to estimate populations of *Streptomyces* spp., fluorescent pseudomonads, *Pythium* spp. and total fungi, respectively. *Bacillus* spp. endospores were quantified by boiling the dilution tubes for 15 min prior to plating in triplicate onto 1/10th-strength TSA and incubating as above. Plates were monitored for appearance of colonies over the course of six days. For *Streptomyces* spp., colonies exhibiting a growth characteristic representative of

members belonging to this genus, were subjected to microscopic examination (100 ×) for confirmation of identity.

Common fungal colony types from the dilution plates were subcultured into 1/5th-strength Potato Dextrose broth and incubated for one week at room temperature with 100 rpm shaking and the DNA isolated with the MoBio (Carlsbad, CA) Microbial DNA Isolation Kit. The primers ITS4 and ITS5 (White et al. 1990) were used to prepare sequencing template by amplifying the internal transcribed spacer regions 1 and 2, the entire 5.8S gene and a portion of the 28S gene of nuclear ribosomal DNA. The amplified gene fragments were directly sequenced by use of a Dye Terminator Cycle Sequencing Quick Start Kit and a CEQ 8000, Genetic Analysis System capillary-based DNA sequencer (Beckman Coulter, Fullerton, CA) with ITS1 (White et al. 1990) as the sequencing primer.

Assays were conducted to determine relative sensitivity of fungi and oomycetes to mefenoxam. A 5 mm agar plug was excised from the edge of an actively growing colony on 1/5 PDA and placed in the center of a fresh 1/5 PDA plate or the same medium amended with RidomilGold® at 15 µg ml^{-1}. After a period of time sufficient to allow mycelial growth onto the new medium, linear growth was determined by measuring the distances covered over various time points up to 72 h after inoculation.

To estimate numbers of protozoa in the soil, five 1 ml aliquots of each serial dilution prepared from the soil suspension described above were combined with 1 ml 1/50th-strength tripticase soy broth in 24-well microtiter plates. After 7 d of incubation at 14 ˚C the contents at the bottom of each well was mixed and 15 µl removed for microscopic examination at 100X. Numbers of protozoa in the original sample were estimated according to the most probable number (mpn) method (Alexander 1982). Soil population size estimates for any given protozoan are only approximate due to competition pressures in the wells. For instance, a common *Bodo* sp. was rarely found to co-occur in wells with small flagellates.

An amoeba, often appearing to be xenic (i.e. without other protozoa) in wells containing the most diluted samples of *B. napus*-amended soils, was isolated by transfer of 15 µl from a well to the surface of an 8-cm diameter petri dish containing 1.5% agar supplemented with 100 µg ml^{-1} ampicillin that had been spread evenly with ~10^{10} heat-killed *E. coli* cells as a food source. The agar surface was overlaid with PAS liquid supplemented with 200 µg ml^{-1} each of penicillin G and streptomycin sulfate. The trophozoites of the amoeba, termed strain C1, had a maximal length of approximately 20 µm and could be induced to form a transient bi-flagellated stage by incubation in 2 mM Tris buffer (pH 7.2) (De Jonckheere et al. 2001). The maximum temperature allowing for growth of the amoeba was 35 ˚C. Genomic DNA was prepared from harvested trophozoites using the method of Charette and Cosson (2004). The ITS4 and ITS5 primers were used as above to prepare a PCR-amplified template for direct sequencing. The 465-bp sequence (GenBank accession no. DQ015967) resulting from use of ITS4 as the sequencing primer was found by blastN analysis to overlap without mismatches to a published 393-bp sequence from the genome of *Naegleria americana* type strain CCAP1518/1G (De Jonckheere 2004). An identical rDNA product was obtained from two other amoeba cultures that were isolated from *B. napus*-amended CV soil and prepared in a similar manner.

Feeding assay. Bacteria were assayed for susceptibility to feeding by *N. americana* C1 following a modified procedure of Upadhyay (1968). Bacteria chosen for feeding susceptibility analysis were classified according to their gram morphology. Gram negative bacteria included 66 strains of fluorescent pseudomonads (64 isolates from orchard soils, *P. fluorescens* Q2-87 and *P. putida* 2C8), *Agrobacterium rhizogenes* R1000, *Azosprillum* sp. LOD4, *Erwinia amylovara* 87-70, *Escherichia coli* DH5α, *Pantoea agglomerans* E325, *Roseomonas gilardii* ATCC 49956 and six isolates from CV soil of undetermined taxonomic position. Gram positive bacteria were twelve strains of streptomycetes (11 isolates from orchard soil and *S. griseovirdis*), *Arthrobacter ilicis*, *Bacillus megaterium*, *Bacillus mycoides* PVL2*, Corynebacterium mediolanum*, *Micrococcus varians*, *Rhodococcus* sp. APG1 and eleven isolates from CV soil of undetermined taxonomic position. A bacterial colony was suspended in water and 20 µl lined across the surface of a 1/50th-strength TSA plate. Up to five separate strains of bacteria were assayed per plate. After overnight incubation at 14 ˚C, 5 µl of a harvested amoeba suspension was spotted to one end of the linear bacterial growth and the plates were returned to the incubator. After 5 d incubation, zones of clearing were measured.

Phospholipid fatty acid analysis. Fatty acids were extracted from triplicate samples of soils or cultures of specific microorganisms, and methylated to form fatty acid methyl esters (FAME) by use of the Ester-Linked method described by Schutter and Dick (2000). The effect of soil treatment on the relative contribution of bacterial communities to total fatty acid content in soils was estimated by summing the following bacterial fatty acid markers (Fierer et al. 2003; Vestal and White 1989): 15:0, *a*15:0, *i*15:0, *i*16:0, 16:1ω9*cis*, *a*17:0, cy17:0, *i*17:0, 18:1ω5*cis* and cy19:0. Fatty acids are designated as X:YωZ, where X is the number of carbon atoms, Y the number of double bonds and Z the position of the ultimate double bond from the terminal methyl group. Notations are "cy" for cyclopropane group and *"i"* and "*a*" for *iso*- and *anteiso*-branched fatty acids.

Data analysis. Statistical analyses were conducted using Sigma-Stat, version 3.1 (Systat Software, Point Richmond, CA). Microbial soil population data were transformed to $\log_{10}$ values and subjected to analysis of variance and means separation using the Tukey test. Unless otherwise noted, data are reported as means ± standard error.

Results

***Effect of amendments on soil bacterial populations*.** The physical characteristics of the *B. napus* seed meal had a demonstrable impact on transformation of the resident soil microbial community. We have reported that increased tolerance of apple to *R. solani* in RSM-amended soils consistently correlates with a higher density of *Streptomyces* spp. in the soil (Cohen et al. 2005; Mazzola et al. 2001). In this experiment, *Streptomyces* spp. in the soil reached densities about 20-fold higher in the *B. napus* powder-amended soil and 5 to 10-fold higher in *B. napus* flake-amended soil compared to that in non-treated soil (Fig. 1B). Also, both amended soils exhibited an approximate 10-fold increase in the numbers of heat-resistant endospores, an indicator of *Bacillus* spp. density (Fig. 1C). After an initial stimulation, populations of total culturable bacteria on 1/50th-strength TSA (Fig. 1A) and fluorescent pseudomonands

(Fig. 1D), unlike both *Streptomyces* spp. and *Bacillus* spp., decreased in CV soil amended with either *B. napus* powder or flakes. Many *Streptomyces* spp. produce antibacterial compounds but fluorescent pseudomonads are known to be resistant to a wide spectrum of antibiotics and, accordingly, among 100 *Streptomyces* isolates tested none were found to inhibit growth of a *Pseudomonas fluorescens* strain (data not shown).

Numbers of endospore cfu in CV soil that had been amended 32 d prior with *B. napus* powder and then pasteurized reached 2.0 ± 0.1 x 10^7 cfu g^{-1} soil, over 100-fold higher than in *B. napus*-amended non-pasteurized soil (~10^5 cfu g^{-1}, see Fig. 1C), while *Streptomyces* spp. populations were below the limit of detection (< 10^5 cfu g^{-1}).

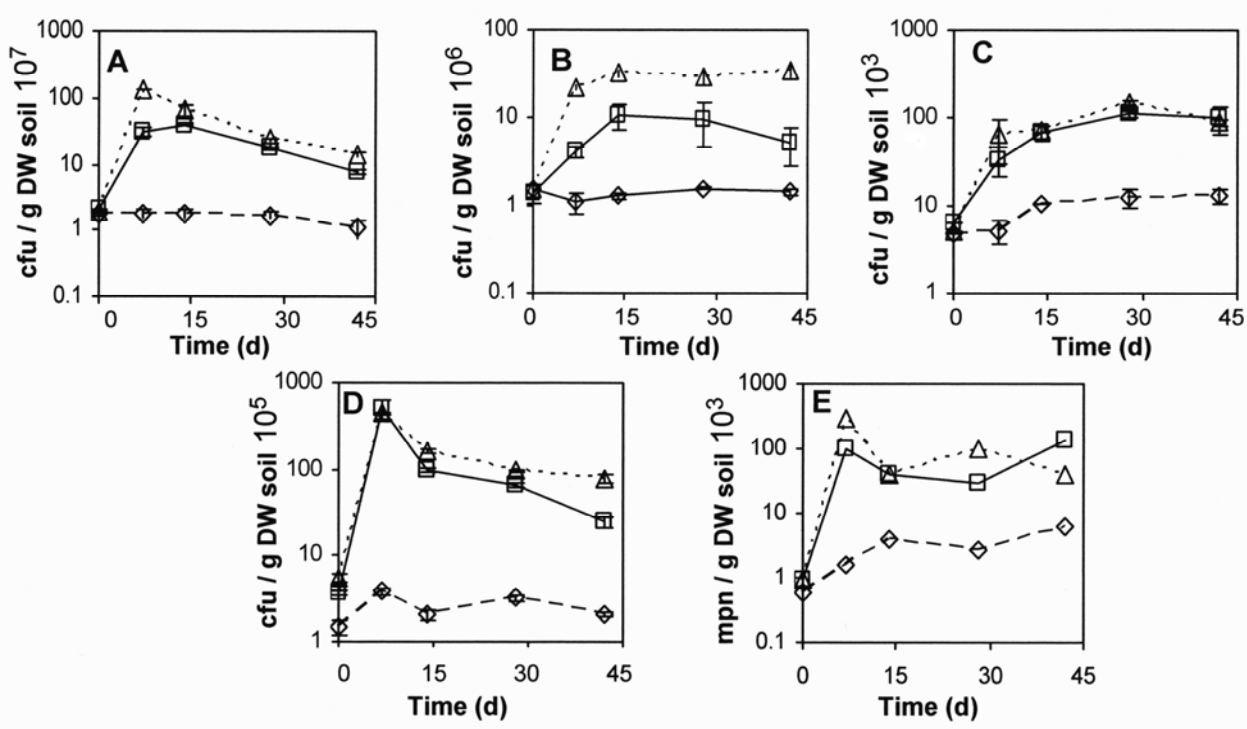

Fig. 1. Effect of soil amendment with *B. napus* cv. Athena seed meal (◊, control; □, flaked; Δ, powdered) on populations of total culturable bacteria (A), *Streptomyces* spp. (B), *Bacillus* spp. endospores that survived boiling (C), fluorescent pseudomonads (D), and protozoa that were detectable after 7 d of culturing (E).

Mortierella spp. in amended soils. One week after treatment, the numbers of fungal cfu were 1 x 10^5 g^{-1} in *B. napus* powder and flake-amended soil, and 2 x 10^5 g^{-1} in soil amended with soy flour. The predominant culturable fungi, constituting ~80% of all colonies, in all of the above amended soils had morphological characteristics of *Mortierella* spp. The sequence of a PCR amplified region of rDNA from an isolate of the predominant fungal type taken from four isolates were identical over their aligned spans and were found by Blast analysis to be closely related to *Mortierella* spp. sequences found in the GenBank database. A 577-bp sequence from one isolate was deposited in GenBank (accession no. DQ092495).

Effect of amendments on soil protozoan populations. *B. napus* powder and flake-amended soils induced increases in protozoan numbers within a week after incorporating either amendment (Fig. 1E). A modest increase in protozoan numbers

occurred in the control soil over the course of the experiment (Fig. 1E), probably in response to the watering regime since increases in soil moisture tension alone can stimulate reproduction of soil protozoa (Darbyshire 1994). In measurements taken between one and six weeks following amendment of CV soil with *B. napus* flake or powder, approximately one-half of all culturable protozoa populations were comprised of *Naegleria americana* amoeba and *Bodo* sp. flagellates. In the non-amended CV soil, *N. americana* never exceeded 10% of total protozoan numbers and *Bodo* sp. were not detected. Protozoan communities of similar size and composition developed in CV soil 7 d after amendment with soy flour or *B. napus* powder that had been pasteurized and heat-dried (data not shown).

Trophozoite feeding upon bacteria. *N. americana* exhibited significant ($P < 0.05$) preferential feeding of fluorescent pseudomonads and other bacteria relative to *Streptomyces* spp. On an agar surface feeding assay, after 5 days at 14˚C, *N. americana* trophozoites did not migrate across any of 12 *Streptomyces* strains tested but established an average 13.5 ± 1.2 mm (mean ± SE) zone of clearing on confluent growth of 62 out of 66 fluorescent pseudomonad isolates tested; trophozoites showed no migration onto the cells of the other four isolates. Among the 12 non-pseudomonad gram negative bacteria examined in the feeding assay, all were fed upon with an average 10.0 ± 2.8 mm zone of clearing. Among the 17 non-streptomycete gram positive bacteria examined, 14 were fed upon with a 13.4 ± 2.4 mm average zone of clearing; the species not fed upon were *A. ilicis, B. mycoides* and an isolate of unknown identity from CV soil.

As expected, the density of recoverable bacteria was substantially lower within zones of clearing than areas of bacterial growth that were not grazed upon by *N. americana*. Feeding on the rifampicin-resistant *P. putida* strain 2C8-28C (Gu and Mazzola 2001) was examined in more detail. The 13.0 ± 0.6 mm ($n = 3$) zone of clearing observed on this strain was typical of the pseudomonads. Recovery of *P. putida* cells rinsed from 5 mm^2 agar plugs and plated to selective medium was 6.0 ± 2.7 x 10^3 cfu/mm^2 ($n = 3$) from within the zones of clearing and was 8.4 ± 0.9 x 10^6 cfu/mm^2 ($n = 3$) from within the portion of confluent growth that had not been fed upon. *N. americana* cells were present in the zone of clearing at 2.6 ± 1.1 x 10^3 cells/mm^2 ($n = 3$) as determined by microscopic count of the plug rinse.

Amendment-induced changes in soil fatty acid composition. Analysis of extracts from *B. napus* powder and flake-amended CV soils revealed rapid logarithmic declines in the major fatty acid in the seed meal, *cis*-oleic acid (18:1ω9*cis*) (Fig. 2A), a suitable energy source for many microorganisms. Consistent with this and the observed increase in the numbers of total culturable bacteria (Fig. 1A), we found an increase in the proportion of bacterial fatty acid markers in the soils, indicating both catabolism and transformation of seed meal fatty acids into bacterial biomass (Fig. 2B).

It has been suggested that levels of arachidonic acid (AA, 20:4 ω6,9,12,15*cis*) can serve as a marker of protozoan density (Vestal and White 1989). Therefore, we monitored levels of AA as well as eicosapentaenoic acid (EPA, 20:5 ω6,9,12,15,18*cis*) in the seed meal-amended soils. Levels of both fatty acids peaked in the first week following amendment with *B. napus* powder or flakes and slowly declined thereafter (Fig. 2C & D).

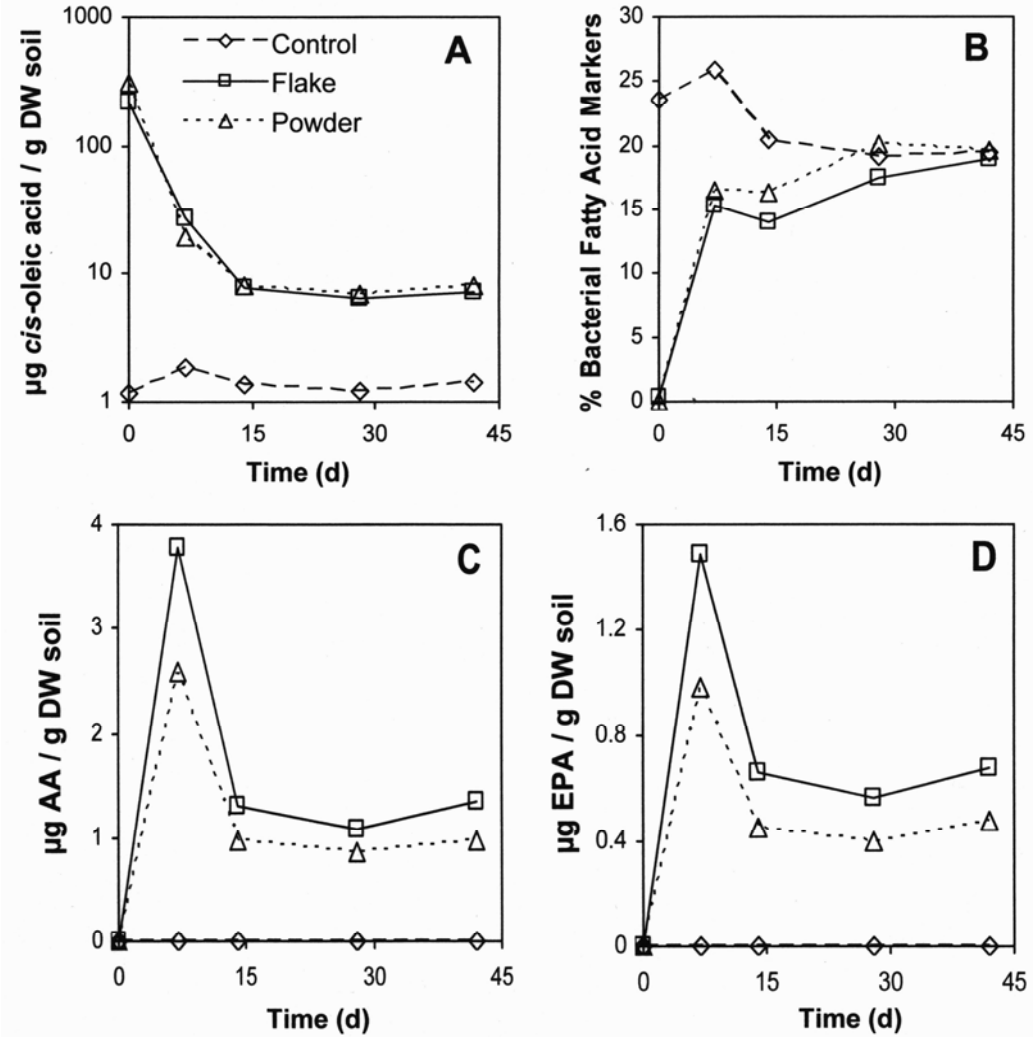

Fig. 2. Selected fatty acids monitored in CV soil amended with flaked or powdered *B. napus* cv. Athena seed meal. Amounts of *cis*-oleic acid (A); proportions of bacterial fatty acid markers (listed in the Materials and Methods) (B); amounts of arachidonic acid (AA) (C); and amounts of eicosapentaenoic acid (EPA) (D).

AA and EPA were not found in the two predominant culturable protozoa, *N. americana* and the *Bodo* sp. (Fig. 3). However, these fatty acids were found to occur in similar proportions in cultured mycelia of *P. heterothallicum, P. intermedium, P. sylvaticum* and *P. ultimum* (Fig. 3). A previous report found similar fatty acid compositions for *P. sylvaticum*, *P. spinosum* and *P. ultimum* (Müller et al. 1994). Typical of most *Mortierella* species (Amano et al. 1992), AA represented a substantial proportion of fatty acids in the *Mortierella* sp. isolated from CV soil (Fig. 3) and our cultures displayed near doublings in AA as a proportion of total fatty acids from 7 d to 21 d of incubation (data not shown).

Treatment of *B. napus* powder-amended soil with the anti-oomycete agent mefenoxam prevented the increase in AA and EPA that was otherwise observed in CV

soil that did not receive mefenoxam application. In mefenoxam-treated CV soil measured 7 d after incorporating *B. napus* powder, AA was detected in only one of four

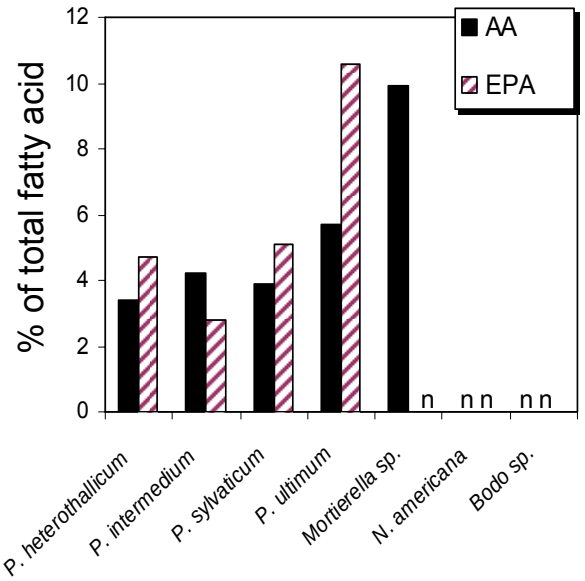

Fig. 3. Percent AA and EPA in fatty acids extracted from *Pythium* spp. and *Mortierella* spp isolated from CV soil. Strains were cultured 7 d at room temperature (21 to 23 °C) on full-strength PDA for *Pythium* spp. and in 1/5-strength PD broth with 100 rpm shaking for *Mortierella* spp. Values are averaged duplicate measurements for the *Pythium* strains and quadruplet measurements for the *Mortierella* sp. n, fatty acid not detected.

samples and EPA was not detected. For in vitro growth assays, as expected, *Pythium* spp. isolates from orchard soils were completely inhibited by 15 µg mefenoxam ml^{-1} in agar medium whereas *Mortierella* spp. isolates showed no inhibition of growth (data not shown).

Discussion

The preferential survival of *Streptomyces* spp. relative to fluorescent pseuodomonads in *B. napus* seed meal amended soils may bear some relation to the differential susceptibility of these organisms to feeding by protozoa. The most common protozoan found in the amended soils, an amoeba-flagellate identified as *Naegleria americana*, showed no capacity to utilize *Streptomyces* spp. as a substrate in culture but readily consumed most strains of fluorescent pseudomonas at a significantly higher rate. However, soy-amended soils, which developed protozoan communities of similar size and composition, do not display a dramatic drop in fluorescent pseudomonad populations (Cohen et al. 2005), indicating that factors other than or in addition to protozoan grazing are responsible for the differential survival seen in *B. napus* seed meal amended soil. Preferential utilization of this seed meal by *Streptomyces* spp. in the soil may be such a factor; the extensive repertoire of extracellular enzymes made by *Streptomyces* spp. permits *B. napus* seed meal to serve as high-yield feedstock for commercial production of *Streptomyces* spp. (Brabban and Edwards 1996).

A widely cited reference holds AA as an ecological marker for protozoan density (Vestal and White 1989). In the current study, AA was not detected in extracts from xenic cultures of *N. americana* nor a *Bodo* sp., both common culturable protozoa found in *B. napus* seed meal amended CV soil. Based on our data it is more likely that the post-amendment amplification of *Pythium* spp., which reach near 10^4 cfu g^{-1} in soil amended with *B. napus* seed meal (Cohen et al. 2005), is responsible for the abrupt and transient increase in AA and EPA levels. The near prevention of AA accumulation in amended soil treated with mefenoxam, to which zygomycetes are not sensitive, implies that *Mortierella* spp. are relatively minor contributors to soil AA. Other known sources of AA and EPA that can be isolated from orchard soils include the oomycetes *Phytophthora* spp. (Müller et al. 1994), and nematodes (Chen et al. 2001). We did not examine the possibility that AA synthesis by protozoa may occur in the soil environment and is perhaps dependent on precursors present in seed meal. Regardless, based on the range of organisms that can accumulate this fatty acid, it is clear that AA is not a specific indicator of protozoan densities in soils.

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