

SELECTIVE INFLUENCE ON POPULATIONS OF RHIZOSPHERE OR RHIZOPLANE BACTERIA AND ACTINOMYCETES BY MYCORRHIZAS FORMED BY *GLOMUS FASCICULATUM*

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(Accepted 6 September 1985)

Summary—The influence of infection by the vesicular–arbuscular (VA) mycorrhizal fungus *Glomus fasciculatum* on populations of general taxonomic and functional groups of naturally-occurring rhizosphere and rhizoplane bacteria and actinomycetes associated with roots of sweet corn (*Zea mays* var. *rugosa*) and subterranean clover (*Trifolium subterraneum* L.) was assayed on selective media. Total numbers of bacteria, but not actinomycetes, on the rhizoplane increased on plants with VA mycorrhizas (VAM) compared to plants without VAM. Bacteria and actinomycete populations were not affected quantitatively in the rhizosphere soil of VAM plants. However, VAM affected specific groups of bacteria and actinomycetes in both the rhizosphere soil and rhizoplane. Rhizosphere soil of mycorrhizal plants contained more facultative anaerobic bacteria, had fewer fluorescent pseudomonads, but had the same number of Gram-negative bacteria as non-mycorrhizal plants. Of the actinomycetes assayed, populations of both *Streptomyces* spp and chitinase-producing actinomycetes decreased in the rhizosphere, but not in the rhizoplane of mycorrhizal plants.

Leachates of VAM and non-VAM rhizosphere soil were also compared for the presence or activity of bacteria that could influence sporulation by the root pathogen *Phytophthora cinnamomi* Rands. Fewer sporangia and zoospores were produced by *P. cinnamomi* in leachates of rhizosphere soil from VAM plants than from non-VAM plants, suggesting that sporangium-inducing microorganisms had declined or sporangium-inhibitors had increased.

Since assays for specific functional groups of microorganisms revealed changes even when total numbers seemed the same, we conclude that the microbial equilibrium had been altered by formation of VA mycorrhizas.

INTRODUCTION

The plant root primarily determines the nature and extent of the saprophytic rhizosphere soil microflora, so any condition affecting root growth or metabolism will be reflected in quantitative and qualitative changes in rhizosphere microbial populations (Katznelson, 1965). The dense colonization of plant roots by vesicular–arbuscular (VA) mycorrhizal fungi and the resulting changes in root physiology and exudation (Graham *et al.*, 1981; Hayman, 1983; Schwab *et al.*, 1983) suggest that VA mycorrhizal fungi establishment of the VA mycorrhizas (VAM) association will influence the microbiology of the rhizosphere. Studies on VAM microbial interactions in the rhizosphere have focused mainly on the introduction of organisms involved in nutrient transformations such as the associative N₂-fixing bacteria *Azotobacter* (Bagyaraj and Menge, 1978; Barea *et al.*, 1973; Brown and Carr, 1979; Ho and Trappe, 1979),

Azospirillum (Barea *et al.*, 1983), and *Beijerinckia mobilis* (Manjunath *et al.*, 1981) or organisms which can solubilize inorganic phosphates (Azcon *et al.*, 1975; Azcon-Aquilar and Barea, 1978; Barea *et al.*, 1975; Raj *et al.*, 1981). Stimulation of VAM colonization by introduced bacteria (Azcon-Aquilar and Barea, 1978; Barea *et al.*, 1983; Brown and Carr, 1979), sustained bacterial populations on mycorrhizal roots (Ames *et al.*, 1984; Bagyaraj and Menge, 1978; Barea *et al.*, 1975; Raj *et al.*, 1981), synergism between bacteria and VAM on plant growth (Azcon-Aquilar and Barea, 1978; Bagyaraj and Menge, 1978; Barea *et al.*, 1975; Brown and Carr, 1979; Manjunath *et al.*, 1981) as well as the mutual antagonism observed between a VAM fungus and a *Streptomyces* isolate (Krishna *et al.*, 1982) are evidence that VA mycorrhizae and rhizosphere microorganisms can influence their mutual development and exert combined effects on plant growth. Because these studies were performed by introducing selected microorganisms, it is not yet known if VA-mycorrhizal and nonmycorrhizal roots supported a qualitatively different rhizosphere soil microflora. However,

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qualitative differences in the microbial populations associated with ectomycorrhizae compared to non-ectomycorrhizal roots have been reported, (Katznelson *et al.*, 1962; Neal *et al.*, 1964; Oswald and Ferchau, 1968).

We have investigated the possible selective influence of VAM development on the composition of naturally-occurring, rather than introduced, rhizosphere soil bacterial and actinomycete populations. General taxonomic and functional groups of microorganisms were assayed in the rhizosphere soil and rhizoplane using selective media. *Phytophthora cinnamomi*, a root fungal pathogen whose reproduction is very sensitive to the microbial activity of the soil (Marx and Bryan, 1970), was also used in a bioassay to detect microbial changes in extracts of rhizosphere soil from mycorrhizal compared to nonmycorrhizal plants.

MATERIALS AND METHODS

Preparation of plant material and treatments

Rhizosphere bacterial and actinomycete populations were compared on mycorrhizal and non-mycorrhizal sweet corn (*Zea mays* L. var. *rugosa* cv. Golden Cross Bantam) and subterranean clover (*Trifolium subterraneum* L. cv. Mt Barker) grown in nonsterile soil in the greenhouse. Nonsterile soil free from indigenous VA mycorrhizal fungi was prepared by inoculating pasteurized soil (65°C for 30 min) with a sieved garden loam suspension (200 g l⁻¹ H₂O) after which the soil was held moist for 1–2 weeks.

Sweet corn was inoculated with VAM by placing three 3-cm root pieces colonized by *Glomus fasciculatum* (Thaxter) Gerd. and Trappe, from an 8-month-old subterranean clover pot culture, beneath two surface-disinfested (3% H₂O₂ for 30 min) sweet corn seeds; controls received non-VAM root pieces. The seedlings were thinned to one plant per 15-cm dia pot and harvested when the plants began to flower (8–9 weeks).

In experiments with subterranean clover, spores of *G. fasciculatum* were used as inoculum to reduce microbial contamination. The spores were obtained from a 9-month-old subterranean clover pot culture by wet-sieving and decanting (Gerdemann and Nicholson, 1963). They were surface-disinfested by stirring in an antibiotic solution (2% w/v chloramine T + 200 µg streptomycin ml⁻¹ and 1 drop Tween 20) for 10 min, washing with 1/2 l 0.5% sodium hypochlorite solution in a sterile Büchner apparatus and rinsing with 2 l sterile distilled H₂O. The spores were mixed in soil which was used to fill hollow cylindrical spaces in the center of 15-cm dia pots created by filling the pots around an inverted 50 ml tube, firming the soil and removing the tube. This was an effective method of concentrating the spores in the vicinity of the growing root and reducing the quantity of spores needed as inoculum. The inoculum density was approx. 100 spores per 15-cm pot. Surface-disinfested (3% H₂O₂ for 30 min) clover seed were sown and thinned to one plant per pot after germination. Plants were harvested at 6 and 12 weeks after sowing. All plants were grown in the greenhouse at 24°C day, 18°C night temperatures and a 16 h photoperiod of 240 µE m⁻² s⁻¹ from high pressure sodium vapor

lamps, and were fertilized every 14 days with 20 ml of Long-Ashton nutrient solution (Hewitt, 1966) with 1/4 strength P (11 µg ml⁻¹). Nodulation occurred by native *Rhizobium* in the soil.

Assay procedure

Plants were harvested by removing the tops with a sterile scalpel and shaking loosely adhering soil from the roots. Corn roots develop yellow pigmentation when mycorrhizal and young, yellow-pigmented lateral roots were chosen for sampling. Roots of similar size were chosen from nonmycorrhizal roots from control plants. Subterranean clover roots were sampled from the middle 1/3 of the root system. Approximately 1 g (fresh wt) of roots was removed with a sterile scalpel and forceps, tapped firmly against a hard surface to remove loosely adhering soil and placed in a sterile dilution bottle containing 100 ml 0.1% water agar diluent. This diluent was chosen because of its slight viscosity which resulted in more uniform dilutions. The roots were shaken by hand 25 × in the dilution bottle, removed and serial dilutions prepared from the resulting soil suspension. Total dry wt of rhizosphere soil sampled was determined by pipetting 25 ml of the original soil suspension into tared weighing dishes, drying for 48 h at 70°C and reweighing.

Rhizoplane microorganisms were counted by quickly weighing the fresh roots from the rhizosphere soil dilution bottle, and macerating with 100 ml sterile 0.1% water agar diluent in a sterile Waring blender for 1 min at high speed. Serial dilutions were prepared in the same manner as for the rhizosphere soil and two dilutions were plated onto five replicate plates per medium. The dry weight of the root sample was determined by calculating % moisture from the fresh and dry weights of the remaining roots. Shoot weight was determined after drying for 48 h at 70°C. The number of *Rhizobium* nodules was counted on fresh roots of 6 week-old clover plants. VAM colonization was determined by clearing and staining an additional 0.5 g (fresh wt) root sample (Phillips and Hayman, 1970) and determining the proportions of 1 mm root sections containing arbuscules, vesicles, hyphae or spores of the VAM fungi.

Taxonomic or functional groups of bacteria and actinomycetes were assayed on the following media: total bacteria on 0.3% Tryptic Soy Agar (Difco), with 2 µg ml⁻¹ crystal violet solution added to assay Gram-negative bacteria (Rovira *et al.*, 1974); fluorescent *Pseudomonas* spp on Kings B + NPC (Sands and Rovira, 1970); total actinomycetes on 1.5% H₂O or chitin agar (Lingappa and Lockwood, 1962); chitinase-producing microorganisms on chitin agar (Lingappa and Lockwood, 1962); *Streptomyces* spp, identified by sporulation, on casein-glycerol medium (Kustar and Williams, 1964), and bacteria capable of anaerobic growth on a low N medium (Rennie, 1981) with 0.05% sodium thioglycolate added to the diluent as a reducing agent. The plates were held at room temperature for 5 days (bacteria) to 14 days (actinomycetes). Anaerobic incubation was carried out for 10 days in a Gas Pak (BBL Microbiology Systems, Cockeysville, Maryland) anaerobic chamber.

The microbial activity of rhizosphere soil affecting

sporangia formation and zoospore release by *Phytophthora cinnamomi* were compared from mycorrhizal and non-mycorrhizal plants. The sweet corn rhizosphere soil suspensions from the dilution platings described above and rhizosphere soil suspensions from *P. cinnamomi*-susceptible chrysanthemum plants also previously inoculated or not with *G. fasciculatum* were placed on a Lab-Line Orbit shaker overnight and vacuum filtered through Whatman No. 1 filters with a sterile Büchner apparatus. Ten ml of the suspensions were placed in each side of 100 mm 2-compartment Petri plates. Three 5-mm plugs of *P. cinnamomi* from a 48 h V-8 agar (Ribeiro, 1978) culture were placed in each compartment, and three plates per plant were used. Sporangia produced on the plugs were counted after 3 days at $50\times$. To determine viability of sporangia and numbers of zoospores released, the test solutions were chilled for 1 h at 5°C , warmed to room temperature, and two dilutions of released zoospores were prepared in sterile distilled H_2O from each of 6 test solutions per plant. One-half ml of each dilution was plated onto *Phytophthora*-selective PVRPH medium (Ribeiro, 1978). The plates were held in the dark for 2 days and the number of colonies from germinated zoospores recorded. Except for a 12-week subterranean clover harvest, all experiments were repeated once, with at least 5 plant replicates per treatment. Tissue nutrients were analyzed on 6 week-old subterranean clover

plants with an ICP Spectrophotometer by the Plant Analysis Laboratory, Department of Horticulture, Oregon State University.

The data were analyzed by one-way analysis of variance.

RESULTS

Establishment of VAM increased total number of bacteria isolated from the rhizoplane of sweet corn and clover, but did not affect numbers of actinomycetes (Fig. 1). Total numbers of bacteria and actinomycetes in rhizosphere soil were not affected by VAM (Fig. 2).

But, the presence of VAM affected specific groups of bacteria and actinomycetes isolated from the rhizosphere and rhizoplane (Figs 1 and 2). VAM increased the numbers of facultative (or spore-forming) anaerobic bacteria isolated from clover on low N medium, decreased the numbers of fluorescent *Pseudomonas* spp isolated from sweet corn, but had no effect on the total number of Gram-negative bacteria from the rhizospheres of either species. Populations of *Streptomyces* spp and chitinase-producing microorganisms, mainly actinomycetes, decreased in the rhizosphere of mycorrhizal clover. Higher numbers of fluorescent pseudomonads were isolated from the rhizoplane of mycorrhizal sweet corn (Fig. 1). Fewer sporangia were formed by *P.*

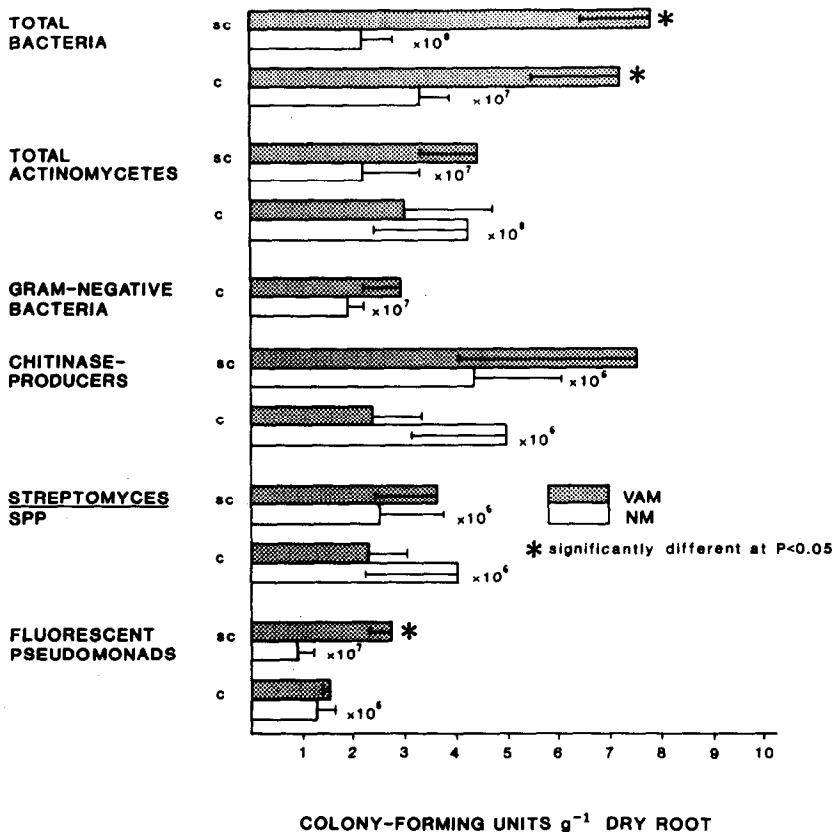


Fig. 1. Numbers of rhizoplane bacteria and actinomycetes isolated from vesicular-arbuscular mycorrhizas (VAM) or nonmycorrhizal (NM) roots of sweet corn (SC) and clover (C).

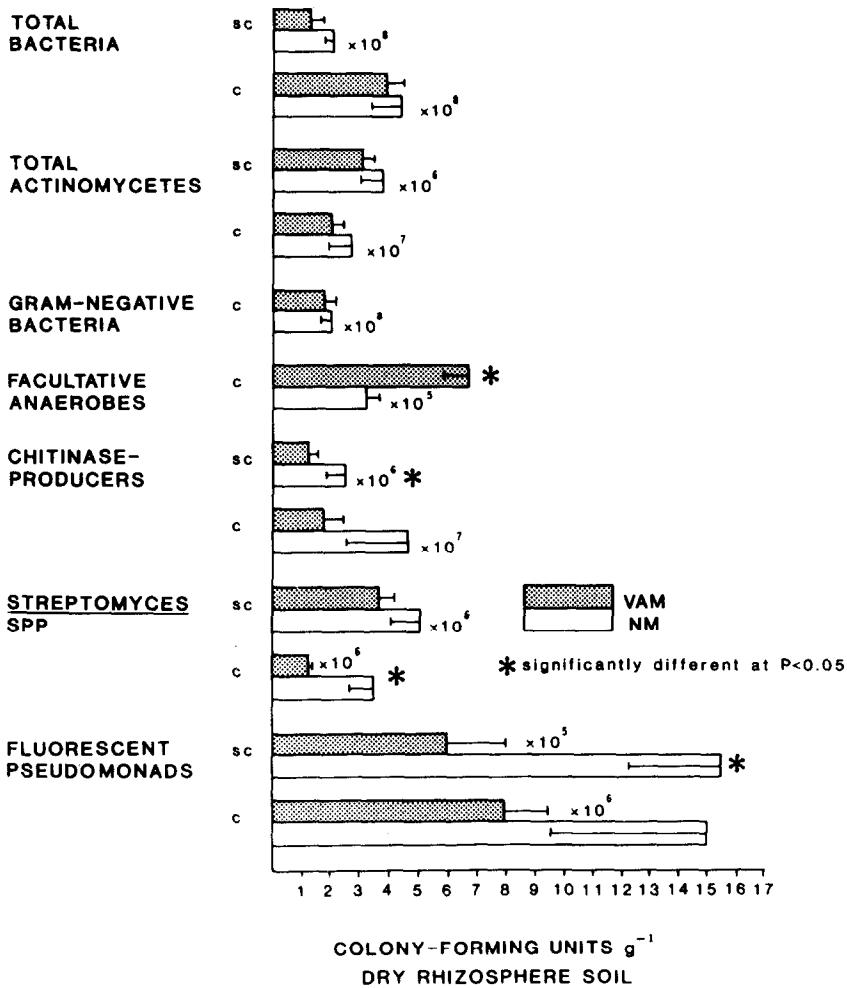


Fig. 2. Numbers of rhizosphere bacteria and actinomycetes isolated from vesicular-arbuscular mycorrhizas (VAM) or nonmycorrhizal (NM) roots of sweet corn (SC) and clover (C).

cinnamomi in leachates of VAM rhizosphere soil compared to leachates of rhizosphere soil from nonmycorrhizal plants (Fig. 3) and fewer zoospores were released.

Rhizobium nodulation was significantly enhanced

on 6 week mycorrhizal clover roots, which supported 102 total nodules or 0.73 nodules mg^{-1} root compared to 7.5 total nodules or 0.13 nodules mg^{-1} nonmycorrhizal root.

VA mycorrhizae were present in 90% of the sweet

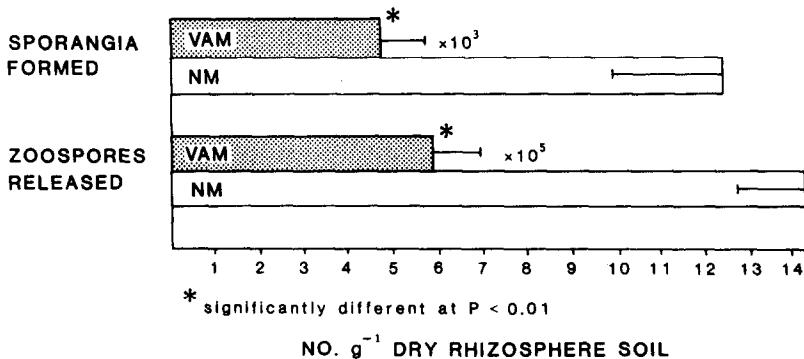


Fig. 3. Sporangia and zoospore production by *Phytophthora cinnamomi* in extracts of rhizosphere soil from vesicular-arbuscular mycorrhizas (VAM) or nonmycorrhizal (NM) roots of sweet corn and chrysanthemum.

corn root system sampled at 9 weeks; VAM infection levels in clover roots were 20% at 6 weeks and 35% at 12 weeks. VAM significantly increased root (3.33-fold at 6 weeks; 2.58-fold at 12 weeks) and shoot wt (5.70-fold at 6 weeks; 4.60-fold at 12 weeks) of clover, but tissue concentrations of N, P and K were comparable in both mycorrhizal and non-mycorrhizal plants at 6 weeks. Dry weights of mycorrhizal and nonmycorrhizal sweet corn plants were similar at 9 weeks.

DISCUSSION

Bacterial stimulation or depression of VAM formation or ectomycorrhizal fungus infection (Azcon-Aquilar and Barea, 1978; Barea *et al.*, 1983; Bowen and Theodorou, 1979; Brown and Carr, 1979) is recognized as part of the ecology of these fungi. What is generally not considered, however, is that once mycorrhizas are established, the fungal symbiont is an integral part of the rhizosphere microflora and will thus contribute to the dynamic equilibrium of the rhizosphere. Our data indicate that the rhizosphere microbial populations associated with roots of mycorrhizal plants are qualitatively different than those associated with those from nonmycorrhizal plants. This has been observed from ectomycorrhizas (Katznelson *et al.*, 1962; Neal *et al.*, 1964; Oswald and Ferchau, 1968) but has not been reported for VAM.

The ecological significance of changes in microbial populations or activities due to VAM are difficult to assess. However, the observed synergism of VAM and introduced bacteria on plant growth in greenhouse trials indicates the possible contribution of VAM in microbiological processes affecting plant productivity and plant nutrition. For example, beneficial interactive effects have been shown with VAM and a plant growth-promoting *Pseudomonas putida* isolate (Meyer and Linderman 1986), associative N₂-fixing bacteria (Bagjaraj and Menge, 1978; Barea *et al.*, 1983; Brown and Carr, 1979; Ho and Trappe, 1979; Manjunath *et al.*, 1981), P-solubilizing bacteria (Azcon *et al.*, 1975; Barea *et al.*, 1975; Raj *et al.*, 1981) and *Frankia* spp (Rose and Youngberg, 1981). The synergistic effects of VAM on nodulation and N₂-fixation by *Rhizobium* spp in greenhouse and field trials are well-documented (Barea and Azcon-Aquilar, 1983).

Establishment of VAM has been shown to reduce, have no effect, or increase plant disease (Dehne, 1982; Schenck and Kellam, 1978). But, definitive field studies are lacking and most of the available data are from greenhouse experiments using treated soil (Schenck, 1981). Under these conditions, the results mainly reflect the direct influence of VAM fungi on plant physiology and development of disease. A further aspect of plant disease development in the field is the microbial activity of the soil (Cook and Baker, 1983). Qualitative changes in microbial populations or activity in the rhizosphere by VAM fungi, therefore, may affect plant disease. For example, sporangia formation by *Phytophthora cinnamomi*, used in this study in a bioassay of microbial activity, was significantly less in the rhizosphere soil extracts from mycorrhizal roots; in addition, fewer zoospores, the primary infective propagules of the pathogen,

were released. Fewer *Streptomyces* spp and chitinase-producing microorganisms [both implicated in the biocontrol of some root-infecting fungi (Cook and Baker, 1983; Mitchell and Alexander, 1962)] were found in the rhizosphere of mycorrhizal clover and sweet corn, respectively. This indicates that biocontrol by these organisms might not be enhanced by VAM in the soil tested and secondly, that the most effective and realistic testing of potential biocontrol agents belonging to these groups would be in the presence of VA mycorrhizal fungi. Antagonism between VAM and *Streptomyces cinnamomeus* has been reported (Krishna *et al.*, 1982).

The functional and taxonomic groups of bacteria and actinomycetes assayed in this study were chosen for their ease of isolation, potential biocontrol abilities, ubiquity and differing physiology. Other qualitative assays could be devised to determine which types or activities of microorganisms involved in nutrient transformations, nutrient availability to plant and fungus, hormone production or pathogen antagonism may change as VAM established. However, we caution that extrapolation from our data to other hosts or other VAM isolates or species may not be possible without more extensive experimentation. We do suggest that data such as ours can point to ecological interactions that may otherwise be overlooked. An example of ecological significance is the isolation of a N₂-fixing bacterium from the sporocarps of ectomycorrhizal fungi by Li and Castellano (1985), confirming earlier studies by Richards and Voigt (1964), and from spores of *Glomus fasciculatum* (Ho and Trappe, 1979). In our study, more bacteria, possibly N₂-fixing, were isolated on low N medium under anaerobic incubation from the rhizosphere of VA mycorrhizal roots than nonmycorrhizal roots. Qualitative assays of rhizosphere populations or activities associated with VAM can give some insight into how the rhizosphere environment is affected by mycorrhizal establishment, and how the associated microflora contribute to the mycorrhizal effects on plants grown in various soils. Of course, we recognize that use of selective media to assay soil microorganisms is inherently limiting in that many, if not most, soil microbes do not readily grow on any artificial media.

Acknowledgements—Contribution of the Oregon State University Agricultural Experiment Station (technical paper no. 7414) in cooperation with the U.S. Department of Agriculture, Agricultural Research Service.

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