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Production of Plant Growth-Regulating Substances by the Vesicular-Arbuscular Mycorrhizal Fungus *Glomus mosseae*

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Glomus mosseae, a representative species of *Endogonaceae* (*Phycomycetes*) able to form vesicular-arbuscular mycorrhiza, was investigated for phytohormone production. Spores of *G. mosseae* were axenically germinated in water, and the resultant mycelial growth was assayed by standard procedures for extracting plant hormones from microbial cultures. Paper partition chromatography and specific bioassays were used to separate and identify plant growth-regulating substances. The microorganism synthesized at least two gibberellin-like substances, one with R_f corresponding in position to authentic gibberellic acid, and four substances with the properties of cytokinins.

There is much evidence that soil microorganisms can affect plant growth, morphology, and physiology, and that some of these effects can be explained by their production of growth-regulating substances (6). Microbial production of hormones in the root zone could provide an additional supply of these biologically active substances, since they are taken up and used by the plant (6, 16).

It has been suggested (8) that some of the effects produced by vesicular-arbuscular (VA) mycorrhiza could be hormonal, and Allen et al. (1) demonstrated that VA mycorrhizal infection increases the level of cytokinins in the host plant. However, VA endomycorrhizal fungi have not been proved to synthesize these substances because of difficulties in axenic culture. We recently described a method that uses a liquid medium (water) by which spores of *Glomus mosseae* (Nicol. & Gerd.) can be readily germinated axenically (5). After spores germinated, this liquid medium was found to contain substances that behaved as auxins in specific bioassays (J. M. Barea and C. Azcón-Aguilar, Abstr. 2nd Int. Symp. Microb. Ecol. 1980. 18–8, p. 130).

This paper reports experiments to verify the presence of gibberellin and cytokinin-like substances in such cultures of *G. mosseae*.

MATERIALS AND METHODS

Axenic culture of *G. mosseae*. Resting spores of the endomycorrhizal fungus *G. mosseae* were excised from sporocarps, and the surface was sterilized with streptomycin and chloramine (11). The culture system consisted of glass test tubes (20 cm by 20 mm) with 10 ml of distilled water (pH 7.0) in which a strip of filter paper was partially immersed (5). The tubes were autoclaved at 120°C for 20 min. Batches of about 100 surface-sterilized spores were transferred with sterile capillary pipettes to each of 10 replicate tubes and placed onto the filter paper above the level of the water. Series of control tubes containing uninoculated strips were also prepared. Incubation was at 22 to 24°C in the dark. After 20 days of growth, the cultures were investigated for the presence of hormone-like substances. Sterility was first checked on nutrient broth, and only uncontaminated tubes were used. Spore germination was assessed under a dissecting microscope, and cultures with nearly 100 germinated spores showing well-developed mycelia and bearing a number of secondary spores were selected. Six of these selected cultures were extracted separately.

Extraction and chromatography of plant growth regulators. The pieces of filter paper were eluted with 80% ethanol overnight and then washed twice with fresh ethanol (17). After evaporation of the ethanol at room temperature, the water from the corresponding tube was added to the aqueous residue. Then the combined solution was acidified with 0.1 N HCl to pH 2.8 to 3.0 and shaken three times with ethyl acetate (10 ml). In the organic fraction, the auxins, gibberellins, and part of the cytokinins are recovered (3). However, since there was no interference from nutrients in the medium, the acidified solutions were also directly evaporated under vacuum at 40°C. In this residue, all of the possible hormonal activity must be present. The residues, whatever the extraction procedure used, were dissolved in methanol (0.3 ml) for chromatography. Replicate 100-μl drops of each extract were run for 16 to 18 h by descending paper partition chromatography (Whatman no. 1), using as the solvent freshly mixed isopropanol-ammonia-water (10:1:1, by volume). Co-chromatography with standard of authentic substances was used to help identification. Chromatograms were dried for 7 days to remove solvents, and 10 equal strips representing the sequence of R_f values 0.1 to 1.0 were used for specific bioassays.

Bioassays. Gibberellin-like substances were assayed by two tests: extension of lettuce hypocotyls (7) and

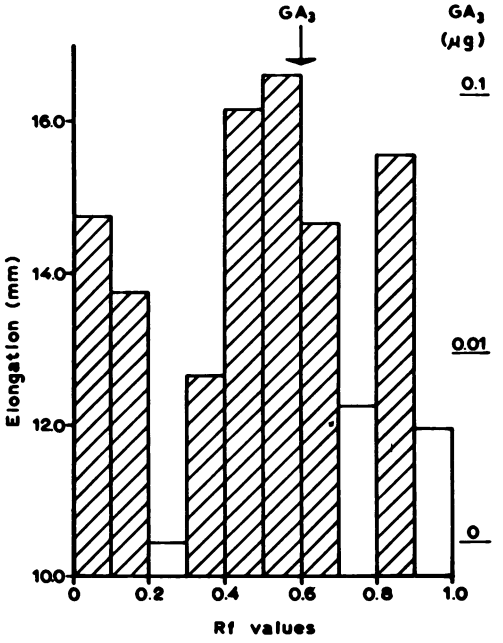

FIG. 1. Gibberellin bioassay by measuring the extension of lettuce (*Lactuca sativa*) hycocotyls. Effects of eluates of each R_f value from chromatograms of *G. mosseae* cultures and of different amounts of authentic gibberellic acid (GA_3). Arrow indicates position of authentic GA_3 cochromatographed. In all figures, shaded portions represent significant activity at the 5% level.

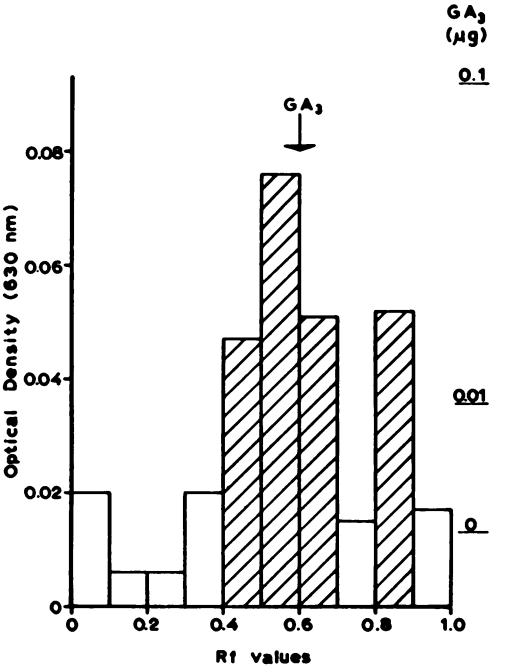

FIG. 2. Barley (*Hordeum vulgare*) endosperm bioassay for gibberellins. Portions of 0.2 ml sampled from the corresponding test solutions (2.0 ml, containing three embryoless pieces of endosperm, eluates of each R_f value from chromatograms of *G. mosseae* cultures, or different amounts of authentic gibberellic acid) were assayed for reducing sugars by the *o*-toluidine test, and the optical density of the resultant solutions was measured. Arrow indicates position of authentic gibberellic acid (GA_3) cochromatographed.

the barley endosperm bioassay (12). In the last case, the gibberellin-induced reducing sugars released from embryoless pieces of barley endosperm were assessed by the *o*-toluidine test (9). Cytokinins were detected by the bioassay using excised radish cotyledons (10) and also by the method based on chlorophyll retention in oat leaves (17). In all bioassays, a dose-response curve with authentic substances was prepared.

RESULTS

Different batches of *Glomus* cultures were extracted and separated by paper chromatography and bioassayed. Figures 1 through 4 show representative histograms of the effects produced in each bioassay.

Uninoculated culture medium gave neither gibberellin nor cytokinin activity in the bioassays. Nevertheless, any slight effect of the blanks was substracted from the effect achieved by the fungal culture at the corresponding R_f values.

Gibberellin-like substances. Substances promoting lettuce hypocotyl extension occurred in three zones of the chromatograms with peaks of activity at R_f 0.1, 0.6, and 0.9 (Fig. 1). The substances with a peak at R_f 0.6 corresponded in position to authentic GA_3 (gibberellic acid) and behaved similarly in the barley endosperm bioassay (Fig. 2). The substance at R_f 0.9 also significantly stimulated the release of reducing sugars in the barley endosperm test, but that at R_f 0.1 did not. This phenomenon is usually due to the differing sensitivities of the test materials used in the various bioassays for the nearly 40 gibberellins already known.

Cytokinin-like substances. Figures 3 and 4 show the effects of *G. mosseae* culture extracts on chlorophyll retention and on the radish cotyledon expansion test, respectively. These two biological assays indicated that cytokinin-like substances occurred in four regions on the chromatograms with peaks of activity at R_f 0.1, 0.4, 0.7, and 1.0.

DISCUSSION

The specific bioassays for plant hormones demonstrated that extracts of *G. mosseae* cultures contained substances with the properties of gibberellins and cytokinins, as we previously found for auxins (Barea and Azcón-Aguilar,

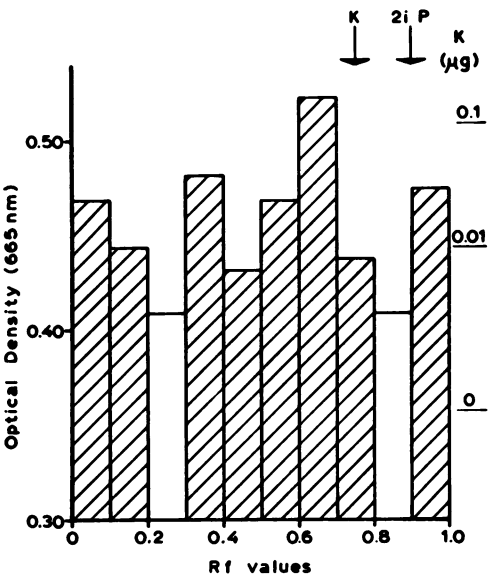

FIG. 3. Cytokinin bioassay by measuring optical density of chlorophyll retained by three excised first leaves of oat (*Avena sativa*). Effect of eluates of each R_f value from chromatograms of *G. mosseae* cultures and of different amounts of authentic kinetin. Arrows indicate positions of authentic substances cochromatographed: K, kinetin; 2iP, *N*-6(Δ-isopentenyl)adenine.

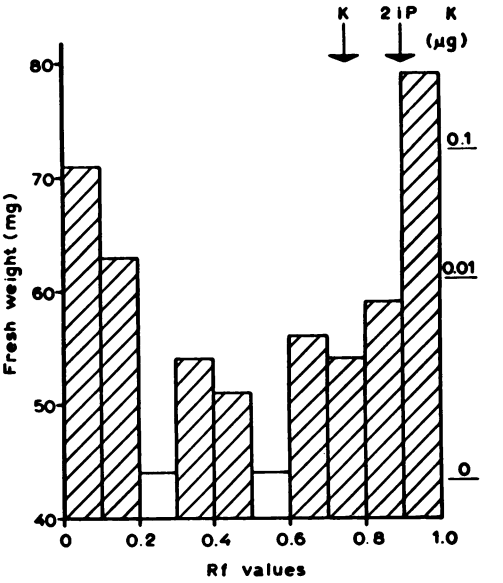

FIG. 4. Cytokinin bioassay using excised radish (*Raphanus sativus*) cotyledons (seven small cotyledons). Effect of eluates of each R_f value from chromatograms of *G. mosseae* cultures and of different amounts of authentic kinetin. Arrows indicate positions of authentic substances cochromatographed: K, kinetin; 2iP, *N*-6(Δ-isopentenyl)adenine.

Abstr. 2nd Int. Symp. Microb. Ecol. 1980, p. 130). Although the results presented here speak for themselves on this biosynthetic ability of *G. mosseae*, a representative species of the VA mycorrhizal fungi, we are currently attempting a more accurate characterization of the biological activity of the growth substances produced by *G. mosseae* and also to extend this research to other species.

The enhancement of uptake of phosphorus and other nutrients by fungal hyphae is the primary mechanism responsible for plant growth stimulation by VA mycorrhiza, as is well accepted (8). However, mycorrhizal benefits may not be limited to improved nutrient uptake, and the production of plant hormones by these mutualistic fungi could also be involved with their effects on plant metabolic process. The morphological and physiological changes that microbial plant hormones can induce in the host plant may favor the establishment of VA symbiosis and its activity, thus leading to a greater rate of nutrient absorption by the plant (2). In fact, it is known that gibberellins increase leaf area and the development of lateral roots, that cytokinins are involved in many basic processes of plant growth, including improvement of photosynthetic rate, and that auxins control root formation and increase elasticity of the cell wall (14, 15). All of these activities can affect the formation or effectivity of VA mycorrhiza. Furthermore, we have demonstrated previously that auxins, gibberellins, and cytokinins applied to the rooting medium enhance plant growth and mycorrhiza formation (2), and that a direct effect of the plant hormones on the establishment of VA symbiosis cannot be excluded.

The reported increases in the production of cytokinins in VA mycorrhizal plants (1) raise the question of whether these substances are produced by the fungus or by the plant in response to the fungal infection. Our results clearly show that at least part of the contribution is from the endosymbiont itself.

Assuming that hormones play a role in the infection mechanism of legume roots by *Rhizobium* (13), the formation of these substances by endomycorrhizal fungi could help to explain some interactions between these microorganisms in establishing dual symbioses with legumes (4).

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