Gibberellin A_4 Production by *Sphaceloma manihoticola*, Causal Agent of Cassava Superelongation Disease

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ABSTRACT

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Symptoms of superelongation disease of cassava (caused by *Sphaceloma manihoticola*), include striking internode elongation, suggesting a hormonal role in the disease. Gibberellin A_4 was purified from culture filtrate of pathogenic isolates by base-acid partitioning with ethyl acetate followed by silica gel column chromatography. Biologically active fractions were identified by lettuce seedling hypocotyl elongation bioassay. The fungal product was identified as gibberellin A_4 by combined gas-liquid chromatography and mass spectrometry. Treatment of plants with known gibberellin A_4 and that purified from pathogen filtrate reproduced secondary symptoms.

Cassava (*Manihot esculenta* Crantz, also known as manioc, tapioca, or yuca) is a tropical root crop in the Euphorbiaceae. Its starchy, swollen roots are the staple food for hundreds of millions of people in the tropics. In world production it ranks seventh in tonnage and is the third most important tropical crop behind rice and sweet potatoes (7). In many parts of the world the leaves, which are high in protein, also are consumed as a green vegetable. Because of its high potential productivity (up to 54 tonnes/ha) in experimental yield trials without fertilizer, insecticides, or fungicides (2), tolerance of poor soil (6), potential as a livestock feed (12), and presently relatively "unimproved" genetic state, cassava offers considerable promise to calorie-short developing nations in the tropics. Cassava also is being seriously considered as a carbohydrate source for ethanol production for fuel (19). Given the present and potential importance of this crop, basic information on pathogens and pests is of considerable importance.

Until recently cassava was thought to be relatively disease-free (16); however, this probably was due to lack of attention by agricultural scientists. In 1972, a previously unreported anthracnose-like disease of cassava, called "superelongation disease," was brought to the attention of plant pathologists (3). First reported in Colombia, the disease since has been reported in several Latin American countries, but it is not known to occur in Africa or Asia. Precise losses are not known, although entire fields often are devastated (10).

Superelongation disease, which affects leaves, petioles, and stems, is caused by a fungus that has been identified as *Sphaceloma manihoticola* Bitancourt and Jenkins (10). Primary symptoms of the disease are necrotic leaf spots and stem and petiole cankers. Secondary symptoms include a striking elongation of the internodes (brittle, spindly stems, where elongated) and leaves misshapen by asymmetrical growth around leaf spots. Acervuli are formed in necrotic areas on leaves and stems.

In some of the early studies of this disease Krausz (10) attempted, without success, to isolate a fungus-produced plant growth regulator responsible for the internode elongation. Plant hormones are known to be involved in many plant diseases. The classic case is that of "bakanae" disease of rice caused by *Gibberella fujikuroi* (Saw.) Wr. from which the plant hormone gibberellin (GA) was first isolated. The most obvious symptom of that disease is internode elongation of juvenile rice plants, not unlike that observed in superelongation disease of cassava. Given this similarity in symptoms, J. Carlos Lozano suggested that a gibberellin or a gibberellinlike compound produced by *S. manihoticola* may be responsible for the observed hypertrophy. The study described below was undertaken to determine if the pathogen is capable of producing a GA in vitro that causes secondary symptoms in the absence of the pathogen.

It is of interest that no fungi other than *G. fujikuroi* have been shown unequivocally to produce GA's. In the late 1950's Curtis (5) superficially tested a number of fungi for GA production and obtained negative results. Pegg (13,14) has demonstrated biological activity resembling that of GA's in extracts from several species of fungi. These substances were never identified, and it is not clear that they were of fungal origin. Helminthosporol, which is produced by *Cochliobolus sativus*, was shown to have GA-like biological activity, but was subsequently shown not to be a GA (9,18).

MATERIALS AND METHODS

Maintenance of the pathogen. Isolates 27, 35, 39, 40, and 45, were obtained from J. Carlos Lozano of the International Center for Tropical Agriculture (CIAT), Colombia. Isolates were maintained on potato dextrose agar plus yeast extract (PDAY) at 24 C (10). Cultures for hormone extraction and pathogenicity trials were grown in Czapek solution (Difco) at 24–27 C in a Metabolyte Water Bath Shaker at 150 rpm under laboratory lighting. In preliminary experiments, levels of hormone production and colony growth were compared under conditions of continuous agitation and no agitation. Because growth was very limited under no agitation and yielded little biologically active compound, the fungus was grown under continuous agitation.

Proof of pathogenicity. Conditions for inoculations were based on those described by Krausz (10). Stem cuttings from unlignified stems of cassava plants (cultivar Llanera) were rooted in 15.2-cm-diameter (6-inch) pots containing Cornell Greenhouse Mix potting medium in a mist chamber under ambient temperature (18–30 C) and lighting. Mist was applied for 15 sec every 12 min. After 1 wk to 10 days in the mist chamber, plants were placed in the greenhouse for 1 wk.

Vigorously growing plants were selected and inoculated with spores obtained from the broth cultures described above. Ten to 20 ml of broth containing abundantly sporulating colonies and 1–10

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$\times 10^5$ spores per milliliter were sprayed onto the leaves, petioles and stems of young cassava plants. Inoculated plants were placed in a moist chamber at 100% RH and 18–24 C. After 48–72 hr the plants were removed from the moist chamber and placed in the greenhouse under a 24-hr photoperiod. Illumination was provided by two Sylvania cool-white fluorescent lamps placed 30–50 cm above each row of plants. Plants were observed daily.

The pathogen was reisolated from the suscepts by macerating lesions from surface sterilized leaves in sterile distilled water and pouring the resultant tissue suspension on solidified water agar (2%) containing 100 ppm streptomycin. After 48–72 hr, isolates from the *Sphaceloma*-like mycelia that grew from tissue pieces and that resembled the pathogen were transferred to PDAY and compared with the pathogen at a later date. Reisolations attempted on PDAY proved to be unsatisfactory because vigorously growing contaminants prohibited successful reisolation of the pathogen.

Purification of fungal hormone-like products. Mycelium and spores of colonies grown in 200 ml of liquid medium for 11–24 days as described above were removed from broth by centrifugation at 16,300 *g* for 10–20 min. Culture supernatant was adjusted to pH 8.3 with NaOH and partitioned three times with ethyl acetate (1:1, v/v); the aqueous fraction was retained. In preliminary experiments, the ethyl acetate fraction was bioassayed before being discarded and found to lack hormonal activity. The combined aqueous fractions were adjusted to pH 3.0 with HCl and partitioned three times with ethyl acetate (1:1, v/v). During the partitioning a viscous substance produced by the fungus tended to form a persistent emulsion which was broken by centrifugation at 2,000 *g* for 15 min. The ethyl acetate fractions were retained, combined, and evaporated to dryness under reduced pressure at 30 C. The residue was taken up in a small amount of methanol and transferred to glass fiber disks for silica gel column chromatography.

The silica gel column was prepared as described by Powell and Tautvydas (15) except that the column was not packed with air pressure from a hand-held squeeze bulb. The glass fiber disks containing residue were placed on the silica gel column and eluted stepwise with 25-ml quantities of increasing percentages of ethyl acetate in hexane saturated with 0.5 M HCOOH. The following solutions were used: 0, 1, 3, 6, 9, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, and 100% ethyl acetate in hexane. After it was established that the only fractions which consistently showed major biological activity in the lettuce hypocotyl assay were those eluted with 20 and 25% ethyl acetate in hexane, the following solutions were used: 0%, 15%, 20% (3×), and 25%. Fractions were collected separately, reduced to dryness, and redissolved in 1.0 ml of methanol.

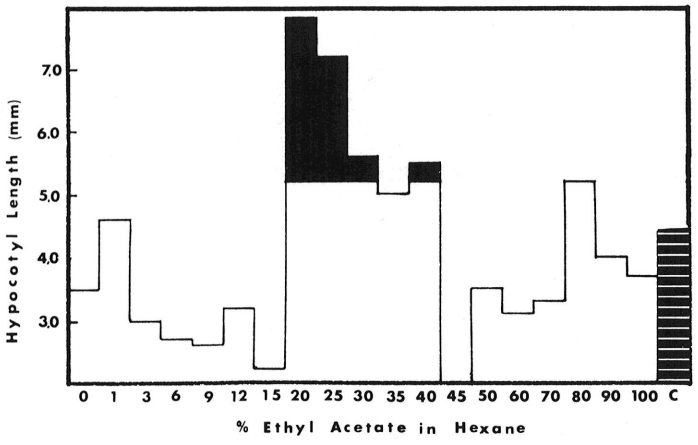

Fig. 1. Results of a typical lettuce seedling bioassay for gibberellinlike activity in all fractions from silica gel column chromatography. Shaded areas show hypocotyl length significantly greater than control. Hatched bar signifies water control. Elongation in the 25% fraction is equivalent to that caused by 0.015 μg GA_3 and is 10% of material obtained from isolate 27 of *Sphaceloma manihoticola* grown for 28 days in 250 ml of culture broth.

Sterile Czapek's solution was taken through the entire purification procedure as a medium and solvent check. All solvents were redistilled prior to use and all glassware was passed through a self-cleaning oven for 4–5 hr to ash all organic matter.

Bioassay. The lettuce seedling hypocotyl elongation bioassay was selected because of its specificity for GA and relatively high sensitivity to most of the known GA's (17). Five commercially available lettuce cultivars (Black Seeded Simpson, Salad Bowl, Butter Crunch, Ithaca, and Dark Green Boston) were tested for relative sensitivity to known concentrations of GA_3. Ithaca proved to be most sensitive and was selected for all subsequent bioassays.

Bioassays of 10, 1.0, and 0.1% of the column fractions were conducted in a manner similar to that described by Stoddart et al (17).

Portions of the biologically active fractions from the silica gel column were dissolved in methanol and methylated with ethereal diazomethane (4). Dry samples of the methylated fractions were taken up in dry pyridine and treated with a mixture of 0.1 ml trimethyl chlorosilane and 0.2 ml hexamethyldisilazane to produce the trimethylsilyl ether of the methyl ester (TMSiMe ester) (4).

Gas-liquid chromatography (GC) of the methyl (Me) ester and TMSiMe ester were conducted on a Perkin-Elmer 900 gas chromatograph on 3% SP-2100 (100/120-mesh) packed in a 1.828-m (6-ft), 4 mm ID glass column. Nitrogen carrier gas flow was about 40 ml/min and the temperature was programmed at 4°/min from 175 C. Hydrogen flame ionization detection was used.

Combined gas liquid chromatography-mass spectrometry (GC-MS) was conducted on a Finnigan 3300 Gas Chromatograph/Mass Spectrometer. Helium flow was 15–20 ml/min through 2% OV-1 packed in a 1.219-m (4-ft), 2 mm ID glass column. Temperature was programmed at 4°/min from 170 C. Ionization was by electron impact at 70 ev.

The amount of biologically active compound produced was not calculated in terms of dry weight of mycelium. However, comparing GC peak area with known quantities of the methyl esters of GA_7 and GA_4 ($MeGA_7$ and $MeGA_4$) permitted an estimate of the quantity of material subjected to GC-MS.

Treatment of cassava with gibberellin. Cassava plants of the cultivar Fowel Fat, rooted as described above, were treated with 10 μg of known GA_4 in 20% ethanol and with an estimated similar concentration of *S. manihoticola*-produced GA_4 by placing a 10 μl drop of 1 μg/μl solution with 0.2% Tween-20 on the growing tip. This treatment was repeated every 2 days during 6 days for a total of 30 μg per plant. The youngest discernible internode was marked at the time of treatment and measured 10 days later. Preliminary experiments with GA_3 established this concentration as adequate and that this method of treatment was superior to injection.

RESULTS

Growth of *S. manihoticola* on solid and liquid media generally was slow and highly variable. Morphology of young colonies of all isolates varied from a yeast-like growth of budding, unicellular propagules and short hyphal strands to a distinctly mycelial form. Regardless of young colony morphology, all colonies on solid media eventually became raised, convoluted, and bound in a gelatinous matrix as described by Krausz (10). Colony color was equally variable within and among isolates. Colors generally ranged from nearly pure white through yellow to deep reddish purple and black. Such variability also was observed within colonies and sectoring occasionally was observed.

Proof of pathogenicity. Some difficulty was encountered in proving pathogenicity of the isolates. In some attempts, too few lesions developed to permit successful reisolation of the pathogen. In others, pure cultures were not obtained because the slow-growing pathogen was overwhelmed by contaminating organisms. Eventually, however, pathogenicity was demonstrated for the two isolates for which data are presented below.

The development of symptoms following inoculation closely resembled those described by Krausz (10). The principle difference was that a very striking yellow halo about 1–2 mm wide appeared surrounding leaf spots. In addition, infection only occurred on one

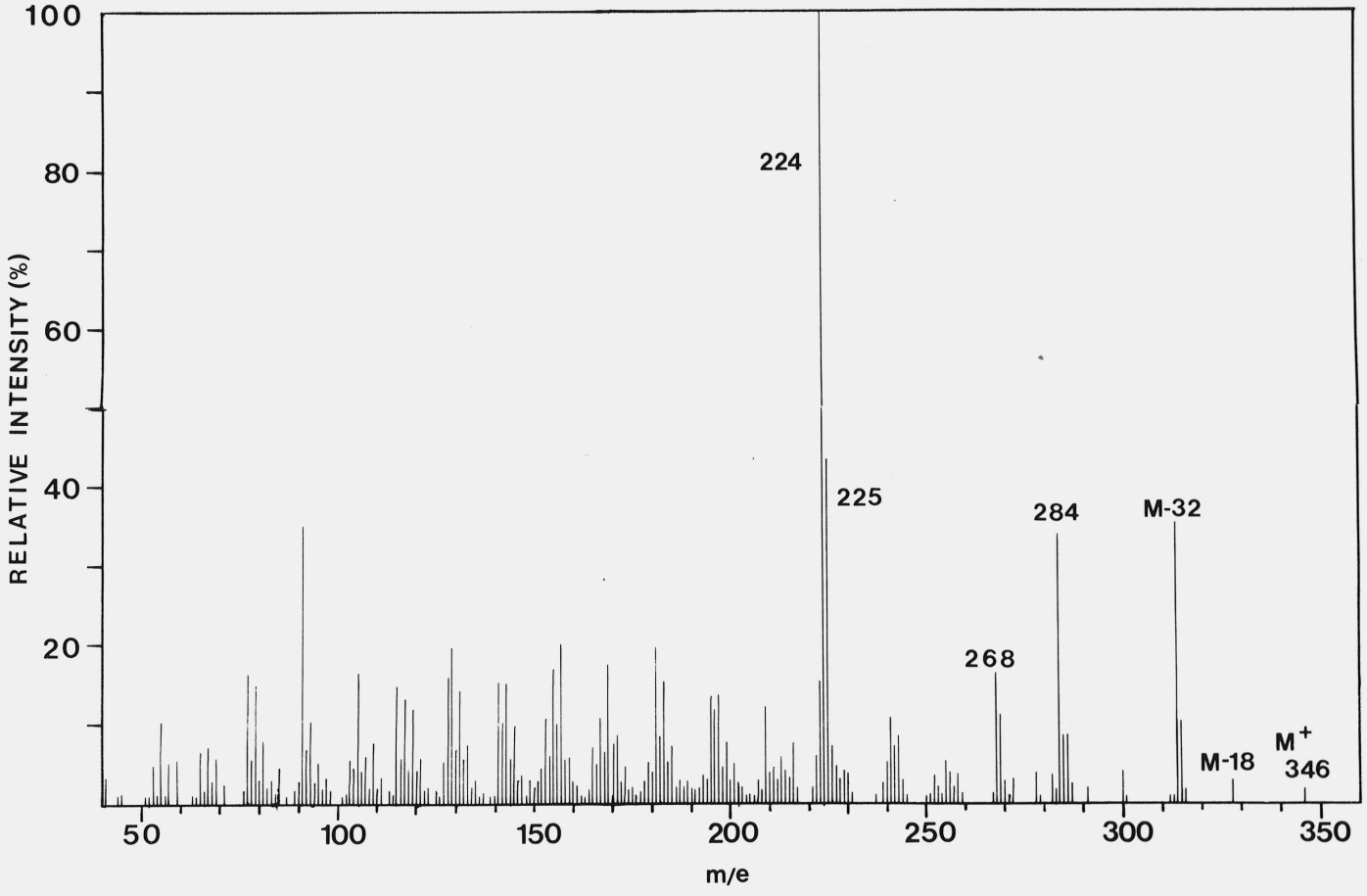

Fig. 2. Mass spectrum of the methyl ester of material from biologically active fraction 20% from silica gel column chromatography. Sample was obtained from culture filtrate of isolate 35 of *Sphaceloma manihoticola*. M^+ = parent ion. M−32 = parent ion minus 32 mass units, m/e = mass-to-charge ratio.

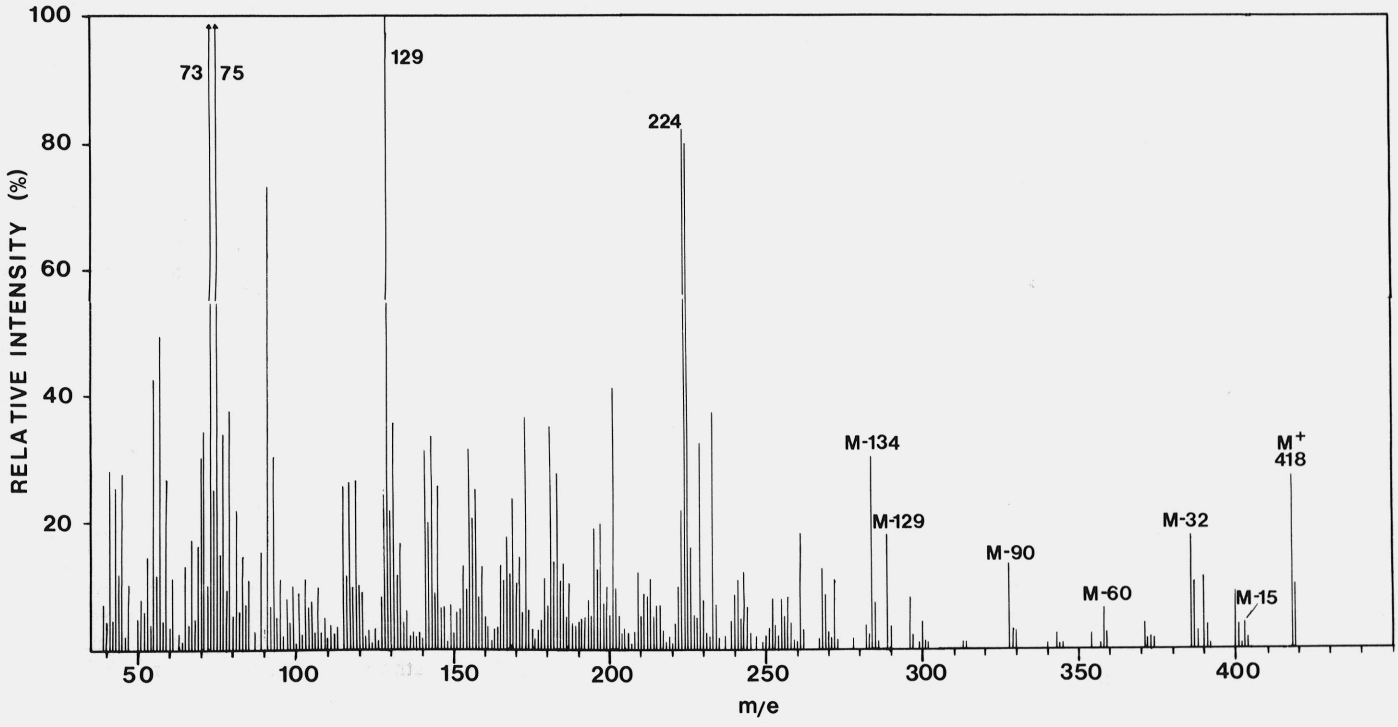

Fig. 3. Mass spectrum of the trimethylsilyl ether of the methyl ester of material from biologically active fraction 20% from silica gel column chromatography. Sample was obtained from culture filtrate of isolate 35 of *Sphaceloma manihoticola*. M^+ = parent ion. M−32 = parent ion minus 32 mass units. m/e = mass-to-charge ratio.

or two immature, expanding leaves, even though entire plants were covered with inoculum.

Purification of fungal hormone-like products. Ten purifications from all isolates yielded biological activity. Results of a typical bioassay following acid-base partitioning with ethyl acetate and silica gel column chromatography are presented in Fig. 1. Principal active fractions were always eluted by 20% and 25% ethyl acetate in hexane. Occasionally, activity was observed in other fractions such as 40% (Fig. 1); however, these results were not routinely reproducible and did not yield an adequate amount of material for analysis. Zones of apparent inhibition of seedling hypocotyl elongation were also observed on occasion, as in the 45% fraction (Fig. 1). This is a common occurrence in bioassays of partially purified extracts containing gibberellins (4). Biological activity from 1 L of culture filtrate from isolate 35 after 12 days of incubation was approximately equivalent to that caused by 700 μg of GA_3.

Bioassay of the culture filtrate and acidic ethyl acetate fractions from partitioning prior to column chromatography did not reliably indicate the presence of biological activity in the preparation. Bioassay did not indicate the presence of active material in the ethyl acetate fraction extracted from basic solution. Bioassays of sterile Czapek's solution carried through the entire purification process were likewise negative.

Preliminary GC on the Perkin-Elmer 900 Gas Chromatograph suggested that the methylated fractions with biological activity were quite pure. There were usually only two or three well-separated peaks. The predominant peak always had a retention time of about 23.4 min. This was slightly longer than that of known $MeGA_7$ and essentially identical to that of standard $MeGA_4$ under the same conditions. Other peaks had shorter retention time and were not always present in biologically active fractions.

The MS of the Me ester of the predominant GC peak of isolate 35 is characterized by a base peak at m/e 224 and parent ion (M+) at m/e 346 (Fig. 2). Other prominent peaks are at m/e 225, 204/205, 268, 285/286, 314/315, and 328. The MS of the TMSiMe ester of the fungal product is characterized by a base peak at m/e 129 and parent ion (M+) at m/e 418 (Fig. 3). The m/e 129 is taken as base peak instead of m/e 73 which is associated with the TMSi ether group and is common to all TMSi ethers of GA's (1). Other prominant peaks are m/e 224/225, 233, 261, 284, 289, and 328.

Sensitivity of cassava to gibberellin A_4. The lengths of internodes following treatment by GA_4 and the fungal product are presented in Table 1. The observed elongation is equivalent to that induced by treatment with 0.1 μg/μl GA_3. In addition to elongation, the stems were observed to be spindly, thinner, and more brittle than normal stems. Leaf size and the number of lobes per leaf appeared to be reduced. These observations compare favorably with Krausz's (10) description of secondary symptoms of the disease in the field.

DISCUSSION

That *Sphaceloma manihoticola* produces a plant growth regulator in vitro is clearly demonstrated by the results summarized in Fig. 1. That the substance in question is a GA and is GA_4 is supported by several observations. First, the extraction and purification method is one of those used to isolate GA's from higher plants (4,15). The GA in question elutes from the silica gel column in fractions 20 and 25% which is the known position of elution of monohydroxylated GA's such as GA_4 (4,15). Third, lettuce seedling hypocotyl elongation is specific in response to the GA's among known plant growth regulators (17). Fourth, the fragmentation patterns of Me esters (Fig. 2) and TMSiMe esters (Fig. 3) of the compounds contained in the biologically active fractions are characteristic of GA's. Mass spectra of TMSiMe esters of GA's all include fragment ions at M−15, M−31/32, M−59/60, M−89/90, m/e 73, and m/e 75, and MS of Me esters show characteristic fragment ions M−18 and M−32 (1).

Given a parent ion of 346 for a methylated monohydroxylated GA, the predicted parent ion of the TMSi derivative will be 418, as observed. Inspection of the structures of the 52 known GA's (8) reveals that only GA_4, GA_5, GA_7, GA_{10}, GA_{20}, GA_{31}, GA_{37}, GA_{40}, GA_{44}, GA_{45}, and GA_{51} are monohydroxylated (with only one carboxyl group). Binks et al (1) have stated that 3-hydroxylated GA's have characteristic very strong peaks at m/e 129 in mass spectra of the MeTMSi derivatives. This is the case for the compound under consideration (Fig. 3). The only monohydroxylated GA's with the hydroxyl group on the 3-carbon are GA_4 and GA_7. The TMSi ethers of $MeGA_4$ and $MeGA_7$ also are distinguished from those of other GA's by strong peaks at M−134/135 and at M−193/194 (1), as observed.

GA_4 and GA_7 readily may be distinguished by the mass spectra of their Me esters and TMSiMe esters. $MeGA_4$ has a parent ion (M+) of m/e 346 whereas $MeGA_7$ has M+ of m/e 348. Furthermore, a base peak at m/e 224 is characteristic of $MeGA_4$, whereas for $MeGA_7$ m/e 281 is the base peak and there is no major peak at m/e 225/224. The mass spectrum of $MeGA_7$ also contains a single major peak at m/e 155, whereas that of $MeGA_4$ has three peaks of comparable intensity at m/e 157−155, (20,21). The mass spectrum of $MeGA_4$TMSi has a major peak at M−129, whereas this peak is absent from the spectrum of $MeGA_7$TMSi (1). Confirmation that the fungal product was GA_4 was obtained by demonstrating that the MS of standard $MeGA_4$ and $MeGA_4$TSMi run under the same conditions produced spectra identical to the respective esters of the fungal product.

It would appear that, given the accumulation of GA_4 in *S. manihoticola* liquid culture, GA_4 is an end product of a fungal biosynthetic pathway. This is of interest in that for the known gibberellin synthesis pathways in *G. fujikuroi*, GA_4 is an intermediate compound that does not accumulate in appreciable quantities (8). Thus, GA synthesis by *S. manihoticola* may proceed differently from that of *G. fujikuroi* and, therefore, be a useful tool for studying GA biosynthesis. That the fungus produces GA_4 relatively abundantly also may have immediate practical value as a source for commercial growth promotion products which contain GA_4.

Since most organisms shown to produce GA's produce more than one kind (8), it seems unlikely that GA_4 is the only GA produced by this organism. Biological activity occasionally observed in fractions other than 20 and 25% may have resulted from small amounts of other GA's produced as intermediate products in GA_4 synthesis.

The role of the pathogen-produced hormone in pathogenesis is unclear. GA_3 and GA_4 applied to cassava plants grossly affect only juvenile tissue and infection apparently only occurs on juvenile tissues. That application of the hormone in the absence of the pathogen duplicates some of the symptoms, and that all isolates tested are capable of producing the hormone, further supports a hormonal role in the disease, but in no way establishes that the fungal product is essential for the successful development of a pathogenic relationship. It is not unusual for a cassava plant suffering from superelongation disease to exhibit no hypertrophy of the internodes, especially during the dry season (10). This could simply indicate that the juvenile tissue is not sensitive to the hormone under certain conditions, rather than that none is produced by the pathogen.

Muretsev and Globus (11) suggested that GA production by *G. fujikuroi* may play an essential role in pathogen nutrition. They

TABLE 1. Comparison of internode lengths of cassava plants treated with known gibberellin A_4 (GA_4) and a purified extract (GA_s) from *Sphaceloma manihoticola* grown in a liquid medium

Internode[a]	Mean internode length (mm)[b]		
	GA_4	GA_s	Control
1st	37.3	38.7	7.7
2nd	49.0	56.7	2.7
3rd	45.0	60.1	2.3

[a] Internodes are numbered in order of development after treatment.
[b] Three replicate plants each received 10 μl of test solution every 2 days for 6 days. Known GA_4 concentration was 1 μg/μl. Purified product from *Sphaceloma manihoticola* (GA_s) was approximately 1 μg/μl. All solutions were in 20% ethanol with 0.2% Tween-20.

found a negative correlation between the ability of isolates of the fungus to produce high levels of GA and hydrolytic enzymes, such as amylases. They concluded that production of GA's by the fungus permits isolates with low hydrolytic enzyme production to obtain sugars from the plant by hormonally stimulating hydrolysis of large carbohydrates. Similar studies of *S. manihoticola* could contribute substantially to our understanding of the role of pathogen-produced higher plant hormones in pathogenesis.

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