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IDENTIFICATION OF A YELLOW PIGMENT FORMED IN MAIZE ROOTS UPON MYCORRHIZAL COLONIZATION

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Key Word Index—*Zea mays*; Gramineae; roots; mycorrhizal arbuscular fungi; symbiosis; carotenoid acids.

Abstract—Roots of gramineous plants turn deep yellow upon colonization with arbuscular mycorrhizal fungi. At the same time methanolic extracts of maize roots show a polyene absorption with maxima at 375 and 395 nm that is not found in the extracts of controls. After esterification with diazomethane the compound responsible for this absorption was isolated by column chromatography and shown to be all-*E*-4,9-dimethyldodeca-2,4,6,8,10-pentaenedioic acid by NMR and mass spectrometry. The compound presumably is derived from oxidative degradation of a carotenoid.

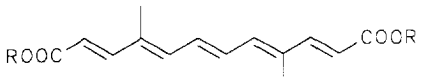
INTRODUCTION

Plant roots are often colonized by arbuscular mycorrhizal (AM) fungi of the order Glomales. These fungi form typical structures such as arbuscules, internal hyphae and often vesicles within the roots. It has been amply demonstrated that the extraradical hyphae in soils can better exploit nutrients such as phosphorus, nitrogen and cations as well as water, than the plant roots [1, 2]. Grass roots colonized by arbuscular fungi are deep yellow in colour, whereas control roots have a more brownish-white appearance. The yellow pigment of AM-colonized roots can be extracted with methanol and quantitatively determined to assess the degree of mycorrhizal colonization [3–5]. Surprisingly, the yellow pigment has not been identified chemically nor localized cytologically. A recent study [6] showed that it is deposited into the vacuole of the root parenchyma cells. It is first seen in the form of small droplets, showing Brownian molecular movement, some 3–4 weeks after mycorrhizal colonization. At the end of the vegetative period the vacuole appears to be totally filled with the yellow pigment. It is even found in endodermis cells, which are never infected by fungal structures, indicating that at least the last step of its biosynthesis must proceed within the plant cells.

RESULTS AND DISCUSSION

The formation of the substance was easily recognized by the appearance of a polyene absorption (λ_{max} = 375 nm) in the UV/Vis spectrum of root extracts. The maximal amount could be extracted 60–80 days after mycorrhizal colonization. At this time, the cut roots were frozen in liquid N_2, ground in a mortar, and the powder extracted with 90% methanol to yield a lipid extract which was prepurified by filtration through reversed phase material. However, the extract could not be separated into single components by analytical or preparative chromatography (TLC, CC, MPLC, HPLC) on silica gel or reversed phase material, respectively. Instead, a completely unresolved mixture of compounds eluted from all chromatographic systems and no separation was achieved by applying different (including acidic) solvent systems. The separation was considerably improved, however, by derivatization of the compounds. Since the yellow pigment showed acidic behaviour, being slightly soluble in aqueous base and extractable from acidic solutions, esterification of the substance was tried. Indeed, after treatment of the extract with diazomethane, a much better separation was obtained, and only one peak of the mixture, now resolved by RP-HPLC, showed the characteristic chromophore also observed in crude extracts. Subsequently, the esterified compound was isolated by flash chromatography on silica gel and final purification by RP-MPLC.

EI- and negative CI-mass spectra of the compound showed $[M]^+$ at m/z 276. The substance was always accompanied by small amounts (5–10%) of an impurity, which eluted slightly prior to the main component from the HPLC column. This minor constituent was presumably formed by isomerization of the yellow pigment during chromatography, as it had a very similar UV spectrum with λ_{max} = 380 nm which, however, was missing the former's fine structure. The isomeric nature of the substance was confirmed by HPLC/ion spray mass spectrometry, where it showed the same spectrum as the major constituent with $[M+1]^+$ at m/z 277 and $[2M+1]^+$ at m/z 553. In the ^{13}C NMR spectrum signals of



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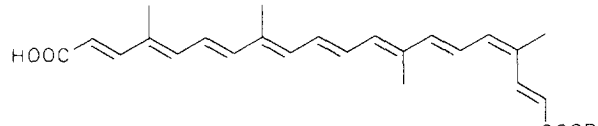
2 R = H



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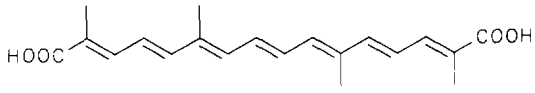

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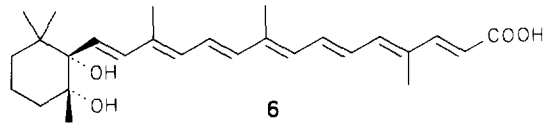

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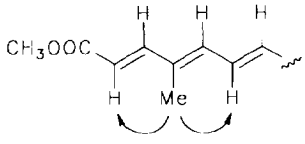

Fig. 1. NOE interactions.

eight carbons were observed. These were assignable to the following groups: one carbonyl, four CH = , one C=, one methoxyl and one methyl. Considering the mass spectrum, a highly symmetric compound with the molecular composition $C_{16}H_{20}O_4$ had to be present. From the long-range C/H correlation, it was obvious that the two ends of the component were formed by MeO–CO–CH = CH–C(CH_3) = groupings. In the ^{1}H NMR spectrum the two olefinic protons gave rise to doublets at δ5.94 and 7.37, respectively. A vicinal coupling of 15.6 Hz pointed to an *E*-configuration of the double bond. These two fragments were connected by a chain of four olefinic CH groups, the protons of which appeared as an AA'XX' system in the ^{1}H spectrum at δ6.51 and 6.75, the former signal being broadened by allylic coupling to the neighbouring methyl group. Analysis of the AA'XX' system revealed a vicinal coupling constant of 14.5 Hz for the protons of the central double bond, thus establishing its *E* configuration. The *J* values calculated were in perfect agreement with data published for an identical partial structure [7] and were confirmed by computer simulation. The geometry of the –C(Me) =CH– sequence was elucidated from ^{1}H difference NOE's. Upon irradiation of the CH_3 group positive NOE's were observed for the resonances at δ5.94 and 6.75 (Fig. 1), proving the all-*E* configuration of the pentaene moiety. Thus, the compound is the dimethyl ester **1** and the structure of an all-*E*-4,9-dimethyldodeca-2,4,6,8,10-pentaenedioic acid (**2**) is established for the yellow pigment formed in the plant cells. As it is synthesized in roots of gramineous plants only upon mycorrhizal colonization [6], it was named mycorradicin. The formation of the byproduct is presumably due to the *E*/*Z* isomerization of one or more double bonds which may lead to a loss of fine structure in the absorption spectrum. The same behaviour has frequently been observed for carotenoids [8]. As reported recently for a comparable system [9], this isomerization may be induced by white light in methanol. It has been observed in the present study that compound **2** exhibits a pronounced sensitivity to light [6] which may have led to a considerable loss during the isolation.

Mycorradicin has not been found as a natural product so far. However, synthetic **1** has been used for the structure elucidation of an azafrin degradation product [10]. To date only a few examples of similar substances, i.e. the longer homologues bixin (**3**), and norbixin (**4**) from *Bixa orellana* [7], crocetin (**5**) from *Crocus sativus* [11] and the monocarboxylic acid azafrin (**6**) from *Escobedia scabrifolia* and *E. linearis* [12], have been found in nature. Nothing is known as yet about the biosynthesis of these acids, but they may originate from oxidative degradation of a carotenoid from one (**6**) or both ends. It will be interesting to find out more about the formation and transport of mycorradicin within the root cells of gramineous plants. Carotenoids are typical components of plastids in plants, whereas mycorradicin is deposited into the vacuole of root parenchyma cells and is observed only in members of the grass family (Poaceae).

EXPERIMENTAL

General. HPLC: Kontron model 200, column: LiChrocart RP 18 (125 mm, Merck); solvent: MeOH–H_2O (4:1) (5 min), lin. gradient to 100% MeOH (30 min), 100% MeOH (5 min), flow: 1 ml min^{-1}; Hewlett-Packard 1040A diode-array detector. TLC: silica gel 60 F_{254} (Merck); solvent: petrol–Et_2O (7:3); RP18 F_{254}, 0.25 mm (Merck); solvent: MeOH–H_2O. (4:1); Flash CC: silica gel 60, 30–60 μ (ICN Biochemicals); solvent: petrol–Et_2O (7:3); MPLC: LiChroPrep RP_{18} 40–60 μ (Merck), column: 30 cm × 1.2 cm i.d., solvent: MeOH–H_2O (4:1). UV: MeOH. Fluorescence spectra: MeOH. MS: Finnigan-MAT 4510 GC/MS, solid probe (EI: 70 eV, NCI: NH_3); HPLC-MS: Perkin Elmer Sciex API III, ion spray, column: Superspher 60RP-Select B (2 mm × 125 mm, Merck), solvent: see HPLC, flow: 100 μl min^{-1} (30 μl min^{-1} to MS). NMR: Bruker AM-600 (^{1}H: 600 MHz, ^{13}C: 150 MHz); solvent: $CDCl_3$.

Extraction and isolation. The frozen roots (liquid N_2) were ground in a mortar and extracted with MeOH–H_2O (9:1, 10 ml g^{-1} fr. wt) for 2 hr with stirring at 50°. After filtration, the extraction was repeated at room temp. overnight. The MeOH of the combined filtrate was evapd *in vacuo* and the residue applied to RP_{18}-material (40–60 μ, 40 g/50 ml extract), which had been equilibrated with 50% MeOH. The fraction eluting with 50% MeOH was discarded. The fraction eluting with 100% MeOH contained the yellow pigment. After addition of an excess of CH_2N_2 in Et_2O, the soln was evapd to dryness. Polar impurities were removed by filtration over silica gel (Et_2O). Subsequent flash CC on silica gel (petrol–Et_2O; 7:3), followed by MPLC on RP18 (MeOH–H_2O, 4:1) yielded the dimethyl ester **1** (7 mg from 1200 g roots).

*all-*E*-4,9-Dimethyldodeca-2,4,6,8,10-pentaenedioic acid, dimethyl ester* (**1**). UV λ_{max}^{MeOH} nm: 395, 375, 359 (sh); Fluorescence emission spectrum (excitation at 375 nm, MeOH) λ_{max} nm: 493, 528; EI-MS *m/z* (rel. int.): 276 $[M]^+$ (50), 245 $[M-OMe]^+$ (6), 217 $[M-CO_2Me]^+$ (38), 185 (45), 157 (100), 142 (59), 115 (39), 91 (53), 77 (55), 59 (92); CI-MS (neg.) *m/z* 276 $[M]^-$; HPLC-MS (ion spray) *m/z* 553 $[2M+1]^+$, 277 $[M+1]^+$; ^{1}H NMR: δ7.37 (2H, *d*, J = 15.6 Hz, H-3 and H-10), 6.75 (2H, AA′XX′, $J_{AA'}$ = 14.5 Hz, J_{AX} = 11.7 Hz, $J_{AX'}$ = −1 Hz, $J_{XX'}$ = 0 Hz, H-6 and H-7), 6.51 (2H, AA′XX′, H-5 and H-8), 5.94 (2H, *d*, J = 15.6 Hz, H-2 and H-11), 3.77 (2 OMe, *s*), 1.95 (2 Me, *s*). ^{13}C NMR: 167.7 (*s*, C=O), 148.5 (*d*, C-3 and C-10), 138.3 (*d*, C-5 and C-8), 135.8 (*s*, C-4 and C-9), 132.4 (*d*, C-6 and C-7), 117.4 (*d*, C-2 and C-11), 51.6 (*q*, 2 OMe), 12.7 (*q*, 2 Me).

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