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Biochemical approaches to the study of plant-fungal interactions in arbuscular mycorrhiza

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Abstract. This communication compares some biochemical methods for quantifying colonization by arbuscular mycorrhizal (AM) fungi. The degree of mycorrhizal colonization can conveniently be measured by determining fungal specific sterols. AM-colonized plants show a specific synthesis of 24-methylene cholesterol and an enhanced level of campesterol (= 24-methyl cholesterol). A gene probe for nitrate reductase, the key enzyme for nitrogen assimilation, has been developed, which allows the monitoring of the distribution of this enzyme in fungi. Among the phytohormones tested, only abscisic acid (ABA) is found at a considerably higher level in AM-colonized plants than in controls. The concentration of ABA is about twenty times higher in spores and hyphae of the AM fungus *Glomus* than in maize roots. Other phytohormones (auxins, cytokinins) do not show such alterations after mycorrhizal colonization. The roots of gramineous plants become yellow as a result of mycorrhizal colonization. The yellow pigment(s) formed is (are) deposited in larger quantities in the vacuole(s) of the root parenchyma and endodermis cells during the development of the gramineous plants. A substance isolated from such roots has now been identified as a C-14 carotenoid with two carboxylic groups, and named mycorradicin.
Key words. Arbuscular mycorrhiza; abscisic acid; carotenoid; *Glomus*; nitrate reductase; mycorradicin; sterols; yellow pigment in mycorrhiza.

Introduction

A wide variety of plant taxa forms symbioses with arbuscular mycorrhizal (AM) fungi of the order Glomales. AM-colonized plants often (but not always) have thicker roots with fewer root hairs, whereas roots of non-mycorrhizal plants tend to be thin and are abundantly covered with root hairs[1,29]. Mycorrhizal fungi form typical structures within the roots, like internal hyphae, arbuscules and vesicles (the latter with the exception of the genera *Scutellospora* and *Gigaspora*; therefore the term 'vesicular' has now been abandoned for this type of symbiosis[28]). The thin extraradical hyphae of the fungi penetrate into the soil over a wide area and can apparently exploit nutrients more efficiently than the plant roots. It has been amply demonstrated in pot experiments that colonization of roots by AM fungi can enhance plant growth and crop productivity when nutrients such as phosphorus are limiting[12,17,20,29]. In natural ecosystems, there is apparently no such clear-cut correlation between mycorrhizal colonization and soil fertility or water availability. However, as has been stated for perennial species, colonization by AM fungi enables plants to occupy a wider range of habitats[23]. There is also a lot of experimental evidence that AM-colonized plants are more resistant to attack by pathogenic fungi[10,23] and exposure to soil toxins[7,21,25]. Thus AM fungi have the potentiality to enhance crop productivity and plant health. Any wider commercial application of arbuscular mycorrhiza is still hampered by the fact that the fungi cannot be grown independently of the host. The obligate biotrophic nature of the symbiosis might also explain why knowledge of the physiology and biochemistry of mycorrhiza is still in its infancy despite its importance. The present paper summarizes recent results from this laboratory and compares them with data from the literature.

Biochemical methods to assess the degree of mycorrhizal colonization

The extent of mycorrhizal colonization, both in the field and in laboratory experiments, may vary. It is generally assessed by microscopic counting which is time-consuming and somewhat subjective, even with the gridline intersect method[11,19]. A simple biochemical method to quantify mycorrhizal colonization would, therefore, be desirable. Some of the biochemical methods described were recently examined[24]. One method is based on the fact that the fungal cell wall contains chitin, which can be determined by HPLC after acid hydrolysis and formation of the phenylisothiocyanate derivative. The data showed that the chitin content correlates fairly well with the degree of mycorrhizal colonization in general, and with the amount of vesicles in particular. However, prior to HPLC analysis, the heavy salt load in the samples to be assayed has to be removed. This step could not easily be performed quantitatively in our hands[24]. Thus this method is likely to be of limited value for assessing mycorrhizal colonization.

The degree of mycorrhizal colonization correlates better with the fungal specific sterol content of the samples[24].

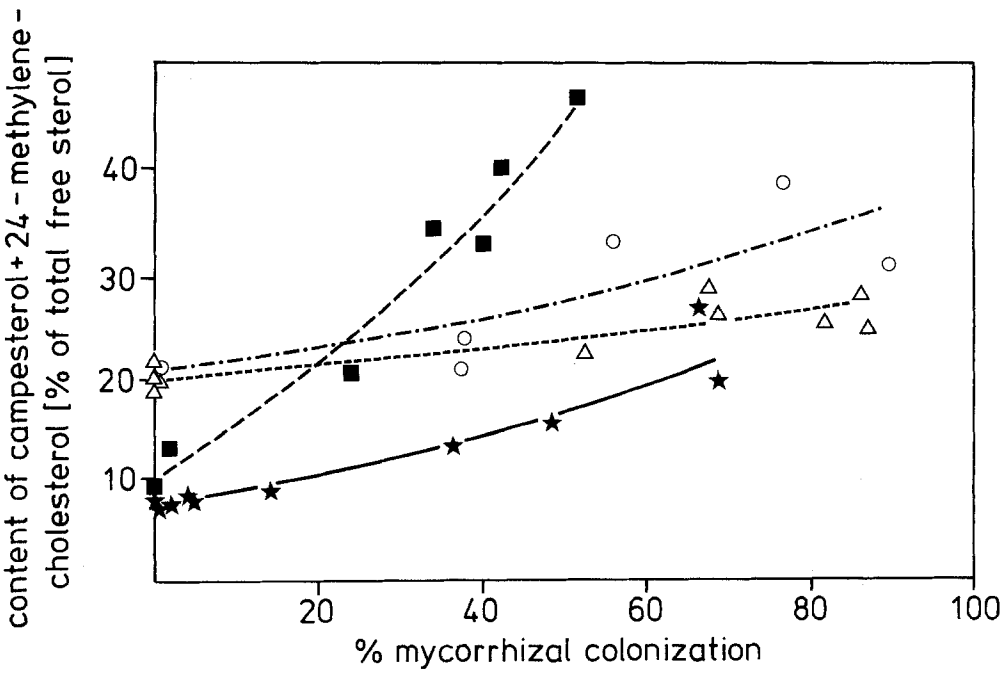


Figure 1. The correlation between arbuscular mycorrhizal colonization and the content of campesterol and 24-methylene cholesterol in four different plants (adapted from ref. 24).
★——★ *Tagetes*
△---△ Corn
○-·-·-○ *Sorghum*
■- - -■ Onion

Plants colonized by AM fungi specifically synthesize 24-methylene cholesterol and have an increased level of campesterol (= 24-methyl cholesterol). Campesterol and 24-methylene cholesterol can quantitatively be separated from each other by a combination of HPLC and gas chromatography but such a separation is rather time-consuming for routine analysis. The extent of mycorrhizal colonization can more easily be determined by measuring the total content of campesterol plus 24-methylene cholesterol using gas chromatography only. Figure 1 shows that mycorrhizal colonization and the content of these two sterols are closely correlated in the case of onion and *Tagetes*, whereas with corn and *Sorghum* the correlation exists, but is not so clear-cut. Among the other sterols, we could not detect a specific increase in the content of ergosterol, which is in agreement with the literature (refs 3, 22 but see 8). There is no real disagreement between our findings and those of the Wädenswil laboratory[8]. This group agrees that the amount of ergosterol represents less than 0.5% of the total sterol fraction of AM-colonized plants. It is questionable whether such a compound, occurring at best in small amounts, is suitable for assessing mycorrhization. In our experiments, ergosterol was particularly seen when the plants grown in the greenhouse were cross-infected by pathogenic fungi (*Pythium*, *Olpidium*). Thus the level of ergosterol could possibly indicate the degree of infection by pathogenic fungi, and may also be a characteristic feature in ectomycorrhiza. These suggestions need to be clarified in much more detail experimentally.

The development of a gene probe for arbuscular fungi

Mycorrhizal fungi can supply plants more efficiently with water and mineral salts than roots. This has been amply demonstrated for phosphorus[12,29], but data for nitrogen are also available[26]. The greater part of the nitrogen incorporated into plants is taken up as nitrate from the soil. It has not been demonstrated as yet whether the fungi themselves reduce nitrate to nitrite and ammonia, and synthesize an amino acid which is then transferred to the host by active transport. Alternatively, since arbuscules are continuously synthesized and degraded within the life span of the symbiosis, e.g. 11 times during the growing season (110 days) of maize plants[27], proteins and/or amino acids could be made available for the plant with each degradation of an arbuscule within the root. A third alternative is that nitrate could be directly transferred to the plants, which then reduce it and supply the symbiotic fungus with nitrogen.

The key enzyme for nitrate assimilation is nitrate reductase. This laboratory recently started to monitor the distribution of this enzyme between fungal structures and plant root cells by in situ hybridizations. For this purpose, a gene probe coding for part of the apoprotein of this enzyme had to be developed[14]. Published se-

quences of the enzyme were compared for conserved regions by computer analysis. One 26mer and one 29mer primer from such regions were synthesized, which allowed a gene segment of about 1 Kb to be amplified in several fungi. Sequence comparison and/or DNA-DNA hybridizations indicated that the PCR amplificates did indeed come from the organisms from which the DNA had been isolated. This part of the assimilatory nitrate reductase could be amplified from the fungi *Aspergillus nidulans*, *Pythium intermedium*, *Phytophthora infestans* and *Phytophthora megasperma*, and also from the AM fungus *Glomus* D13. A DNA hybridization experiment with the digoxigenine labeled PCR-segment and DNA isolated from about 0.5 million *Glomus* spores confirmed that this mycorrhizal fungus possesses a nitrate reductase gene[14]. The data indicated that the parasitic fungi tested might possess the enzyme. This somewhat surprising result is generally in accord with older enzyme activity measurements (for the literature see ref. 14). No amplificates could be obtained with DNA from *Saprolegnia ferax* and the saprophytic *Mucor mucedo*, and these results also agree with the older activity measurements. Studies to localize nitrate reductase within the fungal structures are currently underway.

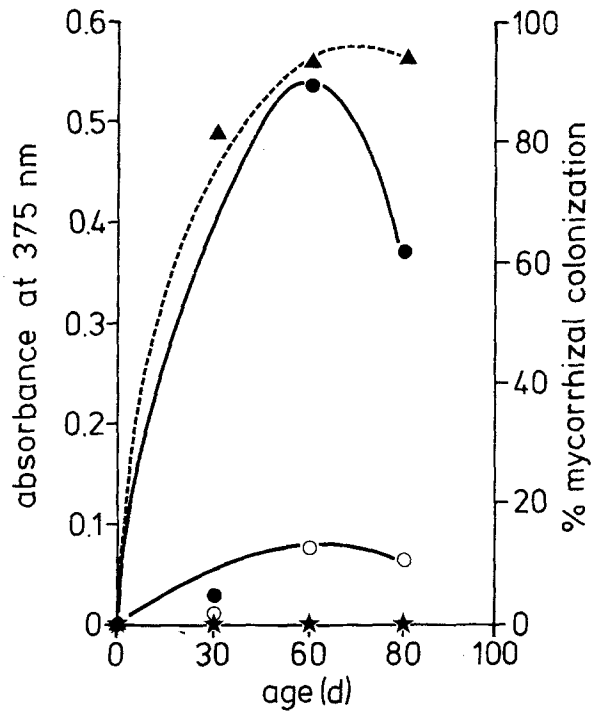
Phytohormone balances in AM-colonized and control plants

As mentioned above, roots colonized by AM fungi are often thicker and carry fewer root hairs. Such changes in morphology are expected to be under phytohormonal control. This laboratory analyzed the levels of plant phytohormones in maize both by ELISA, using polyclonal antibodies, and by conventional assays[4]. Abscisic acid (ABA) was found to be considerably enhanced in both roots and shoots of AM plants as compared to non-mycorrhizal controls. In contrast, the levels of auxins and cytokinins (zeatin riboside) were essentially the same in colonized and non-colonized plants[4]. Methods are now available to isolate spores and hyphae in quantities sufficient for biochemical assays[6,13]. Indirect ELISA tests with polyclonal antibodies against ABA showed that the level of this phytohormone is at least twenty times higher in spores and hyphae than in roots of maize at all stages of plant development[6]. It is difficult to draw definitive conclusions from such analytical data. As the arbuscular fungi cannot be grown independently of the host, it cannot be demonstrated directly that the fungi synthesize and excrete ABA, as several microorganisms including non-mycorrhizal fungi have been shown to do (for the literature see ref. 6). It is unlikely that ABA is originally synthesized by the maize cells, since the fungi would then have to enrich this phytohormone from the apoplasm by active transport. It is more probable that the fungus exerts control over the morphology of the roots, and ABA could play an important role in this. This is, however, speculation, and the exact nature of the phytohormonal control by ABA is difficult to find out. ABA exerts multiple phytohormonal effects in plants, both of a short-term and a long-term nature. Among the latter, ABA is involved in the regulation of solute fluxes within plants, which could also happen in the AM symbiosis. Our findings do not rule out the possibility that other phytohormones could be involved in controlling the symbiosis between AM fungi and plants. Cytokinins have recently been described for the flax (*Linum usitatissimum*) symbiosis[5]. This system could be totally different from the AM-colonized maize of our studies, although the accuracy with which the ELISAs were performed in that study was somewhat surprising to us.

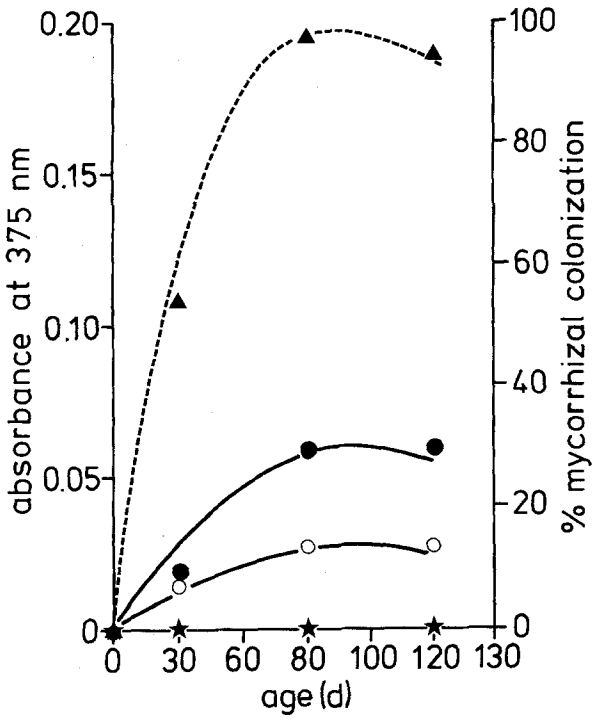
The identification of a yellow pigment formed in AM-colonized gramineous plants

Many plants form yellow roots upon mycorrhizal colonization whereas non-colonized roots have a more brownish appearance. The yellow pigment can easily be extracted with hot water and utilized to assess mycorrhizal colonization quantitatively[2,9,24]. The method is, however, somewhat coarse and can hardly be used to quantify early stages of the colonization process. Surprisingly, the formation of the yellow pigment has not been investigated cytologically in much detail, and the chemical identity of the yellow pigment(s) is also unknown. A detailed study was therefore undertaken in this laboratory[15,16], with some surprising results. In maize, the yellow pigment can first be seen as yellow droplets after 3–4 weeks of growth of the symbiosis. In unfixed roots, the droplets were observed particularly in the vicinity of the arbuscules (but definitely not in the arbuscular structures themselves). They showed Brownian movement, indicating that they had been deposited in the vacuole(s) of the plant cells. During the course of the development of the maize plants, the cells of the root parenchyma tissues became more and more densely filled with the yellow pigment, and the vacuoles were the only space where such a component could be deposited in larger amounts. In the case of grasses, e.g. wheat (fig. 2A), maize, barely and millet[15], the formation of the yellow pigment paralleled the degree of mycorrhizal colonization. In contrast, under the conditions employed[15], no yellow pigment could be extracted with methanol from onion (*Allium cepa*, fig. 2B), leek (*Allium porrum*) or garden bean (*Phaseolus vulgaris*), although the roots of these plants also showed yellow pigmentation. This possibly indicates that such plants contained modified or different pigment(s).

Pigment formation was apparently independent of the arbuscular mycorrhizal isolate used (fig. 3). Detailed studies with phytopathogenic fungi could not be per-



(a)



(b)

Figure 2. The extraction of the yellow pigment from the roots of two different plants: (a) wheat, (b) onion. The mycorrhizal colonization was counted microscopically, and the yellow pigment extracted with methanol was quantified by its absorbance at 375 nm, at different plant ages.
▲- - -▲ Degree of colonization by *Glomus* D13 counted microscopically.
★- - -★ Mycorrhizal colonization of the non-inoculated plant.
●——● Absorbance at 375 nm of the AM-colonized plant.
○——○ Absorbance at 375 nm of the non-colonized plant.

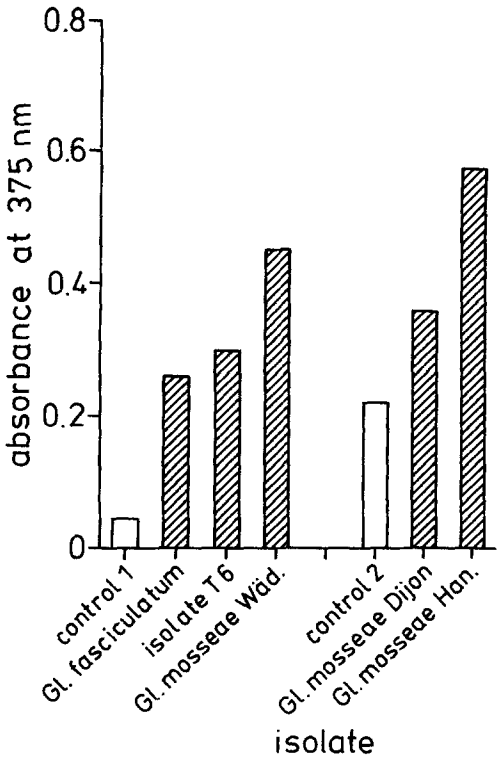


Figure 3. The concentration of the yellow pigment(s) in maize with different arbuscular mycorrhizal isolates.
Maize plant were grown with different *Glomus* isolates, as indicated on the abscissa, for 80 days. The yellow pigment was then extracted with methanol and quantified by its absorbance at 375 nm. Two sets of different experiments were performed. Controls were non-inoculated plants. The degree of mycorrhizal colonization was about 70% (see ref. 15). The three different *Glomus mosseae* isolates were from Dijon, Hannover and Wädenswil, *Glomus* T6 came from Dr. Dehne, Leverkusen and *Glomus fasciculatum* was supplied by Dr. Moawad, Göttingen (for details see ref. 15).

formed as yet. However, control maize plants in the greenhouse occasionally got cross-infected by phytopathogenic fungi (*Pythium*, *Olpidium*), and such plants did not show signs of formation of a yellow pigment. Thus the synthesis of the methanol-extractable pigment appears to be specifically induced by arbuscular mycorrhizal fungi, and was observed only in grasses among the plants tested. It should be noted that the endodermis cells, i.e. plant cells which are never colonized by mycorrhizal structures[12], were filled with the yellow pigment(s) at the end of the vegetation period.
Methanol extracts of AM-colonized maize roots showed a distinct absorption spectrum, with broad maxima at 375 and 400 nm which were not seen in the same preparation of non-colonized roots (fig. 4). The yellow pigment(s) can also be monitored by fluorescence emissions with maxima around 498 and 533 nm, which are also not present in control plants. Purification from colonized roots was guided by these typical absorbances and resulted in the isolation of a compound with a UV-visible spectrum showing maxima at 375 and

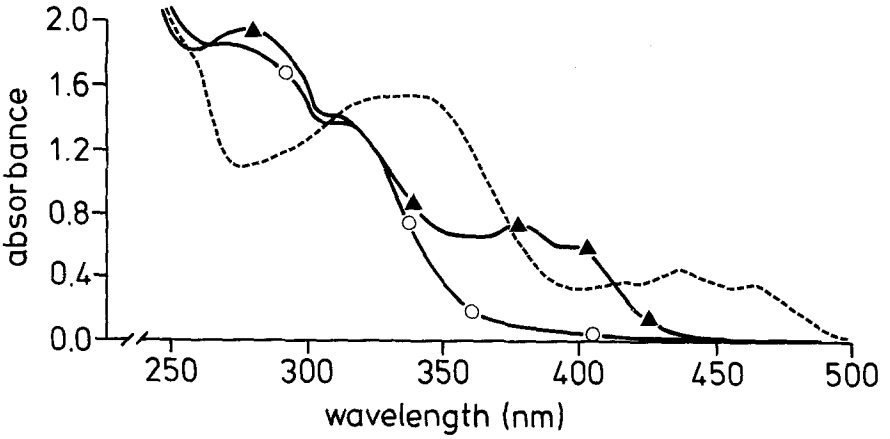


Figure 4. Absorbances of methanol extracts from roots of 80-day-old maize plants. For the extraction, 5 g (fresh weight) of either colonized or control plants was used:
line marked with triangles: AM-colonized plants
line marked with circles: non-colonized controls
dashed line: extract from 50 stigmas and styles from *Crocus* garden hybrids.

395 nm and a shoulder at 359 nm, and with fluorescence emission maxima at 493 and 528 nm. This yellow substance crystallized out in methanol solution as spherical, partly amorphous material. The chemical analysis[16] identified it as being a C-14 carotenoid with one carboxylic group at each end (all-E-4,9-Dimethyl-2,4,6,8,10-pentaene-1,12 dicarboxylic acid; fig. 5). This substance has never been described before in biological material to our knowledge. It was named mycorradicin. Its chemical relatedness to crocetin ('safran') and azafrin is obvious (fig. 5). Crocetin can easily be isolated from the stigmas and styles of *Crocus* garden hybrids, and the UV-visible spectrum in crude extracts from these organs shows close similarities to the spectrum of extracts from AM-colonized maize; however, the maxima are shifted by about 60 nm towards longer wavelengths in the case of crocetin (fig. 4).

Biosynthetically, mycorradicin could well be related to azafrin (fig. 5). This compound occurs in the roots of *Escobedia scabrifolia* and *E. linearis* of the Scrophulariaceae[30]. It was suggested already in 1932 by Kuhn and Winterstein[18] that azafrin is formed by degradation of a C-13 unit from one end of a C-40 carotenoid. Likewise, mycorradicin could be synthesized by oxidative cleavage of 2 × 13 C-atoms being split off from each end of a C-40 precursor. A direct synthesis of mycorradicin from an isoprenoid could hardly explain the occurrence of four carbon atoms between the two side chain methyl groups in the *trans* configuration (fig. 5). On the other hand, a closely related substance was described as occurring in mycelia of the ectomycorrhizal fungus *Corticium* (*Piloderma*) *croceum*, and termed corticrocin[5a]. This substance, with the possible formula HOOC—(CH=CH)$_6$—COOH, lacks the two side-chain methyl groups and has one π-bond more than mycorradicin[5a].

The deposition of mycorradicin into the plant vacuole appears to be quite unusual. Its transfer into this compartment should be facilitated by the presence of the two terminal carboxylic groups. As the substance is formed only after colonization by arbuscular mycorrhizal fungi, and as it is also found in plant cells which are not colonized by fungal structures, a 'signal' from the fungus could cause the degradation of a C-40 carotenoid in root plastids of the plants, and/or a transfer across the plastid envelope and the tonoplast into the vacuole. Alternatively, mycorradicin could be synthesized by the fungus. However, signs of its presence in fungal cells could not be detected at any of the developmental stages of the symbiosis.

The biological meaning of this pigment formation is not yet understood. Irrespective of its formation in either plant or fungal cells, the deposition of such a C-14 carotenoid in the plant vacuoles in larger quantities is at the cost of organic carbon which is then lost for syntheses by the plants. Although experimental evidence is totally lacking currently, it is nevertheless tempting to speculate that the formation of such a carotenoid is connected with the increased resistance of the plants to fungal attack and/or to toxins. When deposited in the plant vacuole, mycorradicin is unlikely to play such a role, but intermediates in its synthesis may well do so.

Concluding remarks

Due to the obligate biotrophic nature and the length of time needed for the cultivation of this symbiosis, any biochemical work with arbuscular mycorrhiza is time-consuming. A major recent breakthrough came from the development of methods which allow external hyphae and spores to be isolated for biochemical analyses[6,13]. The system is thus now amenable to molecular biological

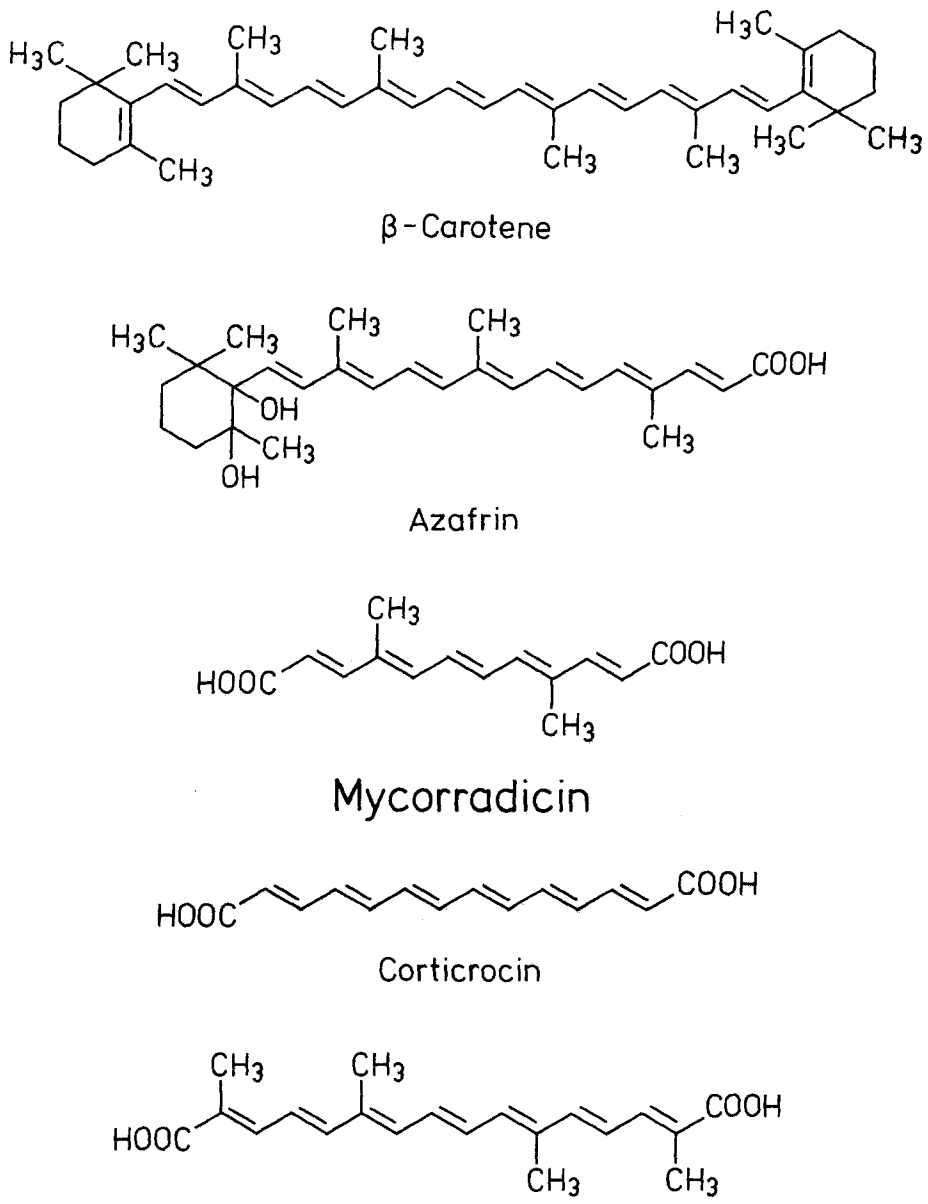


Figure 5. The formula of the yellow pigment isolated from AM-colonized roots ('mycorradicin') in comparison with related substances.

studies. The development of fungal and plant-specific gene probes (e.g. for nitrate reductase) will allow the study of gene expression in both partners. The identification of genes specifically expressed by either of the partners during the symbiosis is to be expected in the near future. The symbiosis between higher plants and arbuscular mycorrhizal fungi is probably the most important symbiosis in nature, and its study can provide unexpected results, as exemplified by the identification of the yellow pigment in the present investigation.

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1 Bayliss, G. T. S., The magnoloid mycorrhiza and mycotrophy in root systems derived from it, in: Endomycorrhizas, pp. 373–389. Eds F. E. Sanders, B. Mosse and P. B. Tinker, Academic Press, London 1975.

2 Becker, W. N., and Gerdemann, J. W., Colorimetric quantification of vesicular-arbuscular mycorrhizal infection in onion. New Phytol. *78* (1977) 289–295.

3 Beilby, J. P., Fatty acid and sterol composition of germinated spores of the vesicular-arbuscular mycorrhizal fungus *Acaulospora laevis*. Lipids *15* (1980) 949–952.

4 Danneberg, G., Latus, C., Zimmer, W., Hundeshagen, B. Schneider-Poetsch, Hj., and Bothe, H., Influence of vesicular arbuscular mycorrhiza on phytohormone balances in maize (*Zea mays* L.). J. Pl. Physiol. *141* (1992) 33–39.

5 Drüge, U., and Schönbeck, F., Effect of vesicular arbuscular mycorrhizal infection on transpiration, photosynthesis and growth of flax (*Linum usitatissimum* L.) in relation to cytokinin levels. J. Pl. Physiol. *141* (1992) 40–48.

5a Erdtman, H., Corticrocin, a pigment from the mycelium of a mycorrhiza fungus. Acta chem. scand. *2* (1948) 209–219.

6 Esch, H., Hundeshagen, B., Schneider-Poetsch Hj., and Bothe, H., Demonstration of abscisic acid in spores and hyphae of the arbuscular mycorrhizal fungus *Glomus* and in the N_2-fixing cyanobacterium *Anabaena variabilis*. Pl. Sci. (1994) in press.

7 Fitter, A. H., Costs and benefits of mycorrhizas: Implication for functioning under natural conditions. Experientia *47* (1991) 350–355.

8 Frey, B., Buser H.-R., and Schüepp, H., Identification of ergosterol in vesicular-arbuscular mycorrhizae. Biol. Fert. Soils *13* (1992) 229–234.

9 Fyson A., and Oaks A., Rapid method for quantifying VAM fungal infections in maize roots. Pl. Soil *147* (1992) 317–319.

10 Garcia-Garrido, J. M., and Ocampo, J. A., Interaction between *Glomus mosseae* and *Erwinia carotovora* and its effects on the growth of tomato plants. New Phytol. *110* (1988) 551–555.

11 Giovanetti, M., and Mosse, B., An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. *84* (1980) 489–500.

12 Harley J. L., and Smith, S. E., Mycorrhizal Symbiosis. Academic Press, London 1983.

13 Horn, K., Hahn, A., Pausch F., and Hock, B., Isolation of pure spore and hyphal fractions from vesicular-arbuscular mycorrhizal fungi. J. Pl. Physiol. *141* (1992) 28–32.

14 Kaldorf, M., Zimmer, W., and Bothe, H.,Genetic evidence for the occurrence of assimilatory nitrate reductase in arbuscular mycorrhizal and other fungi. Mycorrhiza (1994) in press.

15 Klingner, A., Hundeshagen, B., Kernebeck H., and Bothe H., Localisation of the yellow pigment formed in roots of gramineous plants colonized by arbuscular fungi. Protoplasma (1994) in press.

16 Klingner, A., Wray, V., Bothe H., and Marner, F. J., Identification of a yellow pigment formed in maize roots upon mycorrhizal colonization. Phytochemistry (1994) submitted.

17 Kothari, S. K., Marschner H., and Römheld, V., Direct and indirect effects of VA mycorrhizal fungi and rhizosphere microorganisms on acquisition of mineral nutrients by maize (*Zea mays* L.) in a calcerous soil. New Phytol. *116* (1990) 637–645.

18 Kuhn R., and Winterstein, A., Die Dihydroverbindung der isomeren Bixine und die Elektronenkonfiguration der Polyene. Ber. dt. chem. Ges. *65* (1932) 1873–1875.

19 McGonigle, T. P., Miller, M. H., Evans, D. G., Fairschild, G. L., and Swan, J. A., A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. *115* (1990) 495–501.

20 Miranda, J. C. C., Harris P. J., and Wild, A., Effects of soil and plant phosphorus concentrations on vesicular-arbuscular mycorrhiza in sorghum plants. New Phytol. *112* (1989) 405–410.

21 Moser M., and Haselwandter, K., Ecophysiology of mycorrhizal symbiosis, in: Encyclopedia of Plant Physiology, New Series, vol. 12, pp. 392–421. Eds O. L. Lange. P. S. Nobel, C. B. Osmond and H. Ziegler. Springer, Berlin-Heidelberg-New York 1983.

22 Nordby, H. E., Nemec, S., and Nagy, S., Fatty acids and sterols associated with *Citrus* roots mycorrhizae. J. Agric. Food Chem. *29* (1981) 396–401.

23 Peat, H. J., and Fitter, A. H., The distribution of arbuscular mycorrhizas in the British flora. New Phytol. *125* (1993) 845–854.

24 Schmitz, O., Danneberg, G., Hundeshagen, B., Klingner, A., and Bothe, H., Quantification of vesicular-arbuscular mycorrhiza by biochemical parameters. J. Pl. Physiol. *139* (1991) 106–111.

25 Schüepp, H., Dehn, B., and Sticher, H., Interaktionen zwischen VA-Mykorrhiza und Schwermetallbelastungen. Angew. Bot. *61* (1987) 85–96.

26 Tobar, R., Azcon, R., and Barea, J. M., Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stress conditions. New Phytol. *126* (1994) 119–122.

27 Toth, R., and Miller, R. M., Dynamics of arbuscule development and degeneration in a *Zea mays* mycorrhiza. Am. J. Bot. *71* (1984) 449–460.

28 Walker, C., Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales) – a possible way forward. Agronomie *12* (1992) 887–897.

29 Xiao-Lin, L., George, E., and Marschner, H., Phosphorus depletion and pH decrease at root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytol. *119* (1991) 397–404.

30 Zechmeister, L., Carotinoide, Julius Springer, Berlin 1934.