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# Indole compounds released by the ectendomycorrhizal fungal strain MrgX isolated from a pine nursery

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**Abstract.** The production and metabolism of indole compounds in pure cultures of the ectendomycorrhizal strain MrgX, a common symbiont of Scots pine in forest nurseries, were investigated. Different indole compounds produced by this fungus were purified and identified by thin-layer chromatography, high-performance liquid chromatography and mass spectrometry. Indole-3-acetic acid (IAA) and indole-3-carboxylic acid were the most abundant. Although MrgX is able to synthesize IAA when cultivated on a medium without tryptophan, much higher IAA production was obtained when 1 mM tryptophan was added. Buffering of the medium at pH 5.8 was shown to be essential for IAA accumulation in the culture filtrate. In vitro IAA-synthesizing activity of the enzymes extracted from the mycelia of MrgX was also maximal when mycelia were grown in a buffered, tryptophan-supplemented medium. The hydrogen ion concentration strongly affected in vivo activity of IAA-synthesizing enzymes. This activity was rather weak at acid pH and was stimulated by increase in pH up to 8.5. These results and their possible significance for ectendomycorrhizal symbiosis are discussed with reference to the hormonal metabolism of ectomycorrhizal fungi and ectomycorrhizae.

**Key words:** Ectendomycorrhizae - Indole-3-acetic acid - Indole-3-carboxylic acid - IAA-synthesizing activity - High-performance liquid chromatography - Mass spectrometry

## Introduction

The mycorrhizal populations in nurseries usually differ greatly from those of natural forest soil. This is due to differences in soil properties such as pH or organic matter content but also to nitrogen fertilization, which in modern nursery techniques often greatly exceeds the optimal level for mycorrhizae (Mikola 1988). A prevalent nursery fungus is the ectotrophic symbiont *Telephora terrestris* Ehrh. ex Fr. (Mikola 1988). Other strains forming mycorrhizae with the features of both ecto- and endotrophic associations are also found. Only limited studies have been carried out on the species of fungi involved in ectendomycorrhizae and their significance to tree growth (Laiho 1965, 1986; Mikola 1965, 1988; Wilcox 1971; Wilcox et al. 1974; Wilcox and Wang 1987). In Poland, ectendomycorrhizal mycelia were isolated from Scots pine nurseries and their high mycorrhizal ability was confirmed by in vitro mycorrhizal syntheses (Pachlewski and Pachlewska 1971). Pachlewski (1983) isolated an ectendomycorrhizal strain designated MrgX (Mycelium radicis group X). The characteristics of mycorrhizae formed by this strain seemed to be similar in most respects to those of the E-strain fungus on *Pinus sylvestris* L. described by Mikola (1965) and Laiho (1965) and BDG-58 described by Wilcox (1971) for nursery seedlings of *P. resinosa* Ait. Ultrastructural analysis of the transverse septa of the strain MrgX by Pachlewski and Kocon (1985) showed that the fungus belongs to the Ascomycetes. Recently, the taxonomy of several E-strain fungi responsible for the formation of ectendomycorrhizae has received considerable attention (Danielson 1982; Thomas and Jackson 1982; Yang and Wilcox 1984; Yang and Korf 1985a, b). Detailed structural characteristics of ectendomycorrhizae synthesized between roots of *P. resinosa* and the E-strain fungus *Wilcoxina mikolae* var. *mikolae* have also been published by Piché et al. (1986).

Very little information is available on the physiology of ectendomycorrhizal fungi of forest nurseries. Mikola (1965) studied the metabolism of different ectendomycorrhizal strains. Their carbon, nitrogen and pH requirements resembled those of ectomycorrhizal fungi in general, and cellulose was not used by any strain. Considering these results and especially the lack of evidence on the use of carbon polymers, Harley and Smith (1983) emphasized the absence of an explanation of why ectendomycorrhizal strains penetrate cortical cells so readily.

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The ability of ectomycorrhizal fungi to produce indole-3-acetic acid (IAA) in pure culture has been demonstrated by several authors (Moser 1959, 1962; Ulrich 1960; Tomaszewski 1974; Rudawska 1983; Ek et al. 1983), and it has often been suggested that auxins may be an additional factor affecting mycorrhiza formation (Slankis 1973; Meyer 1974; Mudge 1987; Nylund 1988). Slankis (1973) claimed that auxin in root cultures causes anatomical and morphological changes similar to those appearing during ectomycorrhizae formation. Unestam and Stenström (1989) found that IAA treatment of roots led to marked production of mycorrhiza. A very recent observation by Durand et al. (1992) on the abundant fungal mantle and multi-layered Hartig-net formation by a high-IAA-producing mutant of *Hebeloma cylindrosporum* confirmed a role for IAA as a mediator of symbiotic activity in ectomycorrhizal fungi. However, doubts about the significance of auxin in ectomycorrhiza formation have been expressed by Wallander et al. (1992).

Whilst much research has been done on the production and significance of IAA in ectomycorrhizae, no data exist on auxin synthesis by ectendomycorrhizal strains. The following study was undertaken in order to define the IAA metabolism of an ectendomycorrhizal strain and to understand better the position of ectendomycorrhizae in a forest ecosystem. Indole compounds released by the strain MrgX in pure culture were first purified and identified. The optimal pH conditions for in vivo IAA-synthesizing activity were determined, and in vitro IAA-synthesizing activity of the fungus was demonstrated.

## Materials and methods

### *Source and culture of fungal isolate*

The strain MrgX was isolated in 1975 from ectendomycorrhizae formed by 1-year-old Scots pine (*P. sylvestris* L.) nursery seedlings and supplied to us by Professor R. Pachlewski, Department of Soil Science and Fertilization, Forestry Research Institute, Warsaw-Sekocin, Poland. The culture was maintained in Petri dishes on an agar medium as described previously (Rudawska 1980).

### *Culture conditions*

Mycelia were cultivated in 100-ml Erlenmeyer flasks containing 40 ml of $P_3$ liquid medium (Gay 1990) buffered with 50 mM 2-(*N*-morpholino) ethane sulphonic acid (MES) (pH 5.8) and autoclaved for 25 min at 115° C. Filter-sterilized tryptophan solution was added to the autoclaved medium to a final concentration of 1 mM. Two 4-mm inocula cut from the margin of fast-growing cultures obtained on the agar medium were transferred to each flask. The cultures were grown at 22 ± 1° C in darkness for 5 or 6 weeks. Four different combinations of the media were established: unbuffered with or without tryptophan, and buffered with or without tryptophan.

### *Estimation of fungal growth*

The cultures were harvested by filtration through a glass-fibre filter and rinsed with distilled water; the fresh weight of mycelium was determined immediately before further analysis.

### *Identification of indole compounds by high performance liquid chromatography and mass spectrometry*

IAA was only analysed in culture filtrates taken from buffered media. At the end of the culture period, the pH of culture filtrates was adjusted to pH 3 with 2 N HCl and partitioned three times against an equal volume of ethyl acetate. The ethyl acetate phase containing the acidic indoles was then evaporated to dryness under vacuum (30 ± 1° C) and further purified by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), using a modification of the method described by Rouillon et al. (1986).

### *Chromatography conditions*

Analytical TLC was performed with glass plates (20 × 20 cm) coated with Silica Gel G 60 Merck without fluorescence indicator. Methyl acetate–isopropanol–25% ammonium hydroxide (45 : 35 : 20 by volume) was used as solvent. After chromatographic development, guide spots were sprayed with Salkowski or Van Urk reagent (Pilet 1961). The zone corresponding to IAA and indole-3-carboxylic acid (ICA) was scraped off the plates and eluted with methanol. The UV-absorption spectrum of 0.5 ml of methanolic extract was determined and compared with the IAA reference standard. The residue was reduced to dryness prior to further HPLC analysis.

For preparative TLC of samples to be identified by mass spectrometry, mycelia were cultured in 100-ml Erlenmeyer-flasks on $P_3$ buffered (50 mM MES) medium supplemented with 1 mM tryptophan. After 7 weeks of culture, the indole compounds were extracted from the filtrate as described above. Preparative TLC was carried out by centrifuge TLC chromatotrone on round plates covered with silica gel and eluted with a step gradient of ethyl acetate in hexane followed by a linear gradient of methanol in ethyl acetate into 20 fractions. Fractions containing indole compounds were collected, further purified by HPLC and reduced to dryness prior to analysis by mass spectrometry.

HPLC analysis was performed using a Waters Associates instrument with a wavelength detector set at 280 and 292 nm and a Lichrosorb RP-18 (7 µm) column (250 mm × 4 mm). The column was eluted with water-acetonitrile-$CH_3COOH$ (74.9 : 25 : 0.1 by volume) at a flow rate of 1 ml $min^{-1}$. Indole compounds were tentatively identified by comparing their retention times and UV spectra with the reference standards.

### *Mass spectrometry*

The mass spectrometric identification of indole compounds was performed with a VG MM 305 instrument linked to a VG 200 computer. The operating conditions were direct introduction, an ionization potential of 70 eV and an ionizing-source temperature of 200° C.

### *The kinetics of IAA accumulation in culture filtrates*

Mycelia were cultivated as previously described on $P_3$ liquid medium supplemented or not with 1 mM tryptophan. Filtrate samples of 1 ml were regularly taken in sterile conditions throughout the culture period and IAA was quantified according to Pilet and Collet (1962) using a modified Salkowski reagent (Pilet 1957) which does not react with ICA or indole-3-aldehyde (IAld) (Rouillon et al. 1986). For analysis, 250 µl of culture filtrate was incubated with 1 ml of the Salkowski reagent and incubated at 24° C in the dark. After 30 min, the absorbance was measured at 528 nm

and compared with an IAA standard. The specificity of this method was demonstrated by HPLC and mass spectrometry.

### *In vivo activity of the IAA-synthesizing enzymes*

After 6 weeks of growth on the buffered, tryptophan-supplemented medium, mycelia were separated from the filtrates and rinsed with distilled water. Mycelia obtained from one flask were placed in a Petri dish containing 10 ml of 10 mM tryptophan and incubated at 24±1° C in the dark. To determine the effect of pH on in vivo activity of the tested enzymes, the pH of the incubation mixture was adjusted from 3.5 to 8.5 at intervals of approximately one pH unit using 10 mM MES or 3-(*N*-morpholino)-propane-sulphonic acid as buffers.

The IAA-synthesizing activity of the mycelia was determined from the amount of IAA released into the incubation solution as measured by the Salkowski reagent (Pilet 1957), and expressed as micromoles of IAA synthesized per milligram of fungal fresh weight after 1 h.

### *In vitro IAA-synthesizing activity of the mycelia*

After a 5-week culture period on the buffered or unbuffered culture medium, supplemented or not with 1 mM tryptophan, enzymes were extracted and IAA-synthesizing activity was determined as described by Gay (1986a, b). The enzyme activity was expressed as the amount of IAA produced per milligram of fungal fresh weight. Five replicates for each set of analyses were made and the experiments were repeated two or three times.

## Results

### *The effect of tryptophan and buffering of the medium on the mycelial growth*

The ectendomycorrhizal strain MrgX was able to synthesize the indole ring and subsequently tryptophan since it could grow on $P_3$ medium without indole compounds (Table 1). Both pH and tryptophan affected mycelial growth of this fungus during a 5-week culture. When the fungus was cultured on unbuffered medium, the pH of the filtrate decreased from 5.8 to 3.0 and 2.1 in flasks with and without 1 mM tryptophan, respectively. In contrast, when the medium was buffered with 50 mM MES, the pH drop throughout the culture period was less than 0.3 pH unit. Regardless of the presence of tryptophan, the mycelial fresh weight was nearly twofold higher on the buffered media. Tryptophan at a concentration of 1 mM significantly reduced mycelial growth of the fungus cultivated on both buffered and unbuffered media.

**Table 1.** Effect of 1 mM tryptophan and buffering [50 mM 2-(*N*-morpholino)ethane-sulphonic acid (MES), pH 5.8] of the culture medium on growth of 5-week-old MrgX mycelium in $P_3$ liquid medium

| | Unbuffered medium (initial pH 5.8) | | Buffered medium (+50 mM MES; pH 5.8) | |
|---|---|---|---|---|
| | +1 mM tryptophan | without tryptophan | +1 mM tryptophan | without tryptophan |
| Fungal growth (mg fresh wt. per culture) | 96±12[a] | 300±13 | 245±16 | 585±26 |
| Decrease in medium pH | 2.8±0.2 | 3.7±0.3 | 0.3±0.05 | 0.3±0.04 |

[a] Mean±standard deviations of the mean

### *IAA identification in culture filtrates*

Indole compounds were extracted from filtrates of 5-week-old buffered cultures. Analytical TLC separated a number of Salkowski-positive spots in the tryptophan-supplemented medium, one of which had the same $R_f$ value as synthetic IAA. This spot, which probably included various indole compounds, was the only one detected in fungal filtrates cultured in the absence of tryptophan. Spots were scraped from the plate and eluted with methanol. Their UV absorption spectra suggested they might contain IAA and ICA and they were further analysed by TLC and HPLC. In the case of filtrates obtained in the presence of tryptophan, the elution pattern from the HPLC column included two main peaks (Fig. 1A) with retention times of 8 min 48 s and 10 min 48 s, i.e. identical to those of synthetic ICA and IAA. A fur-

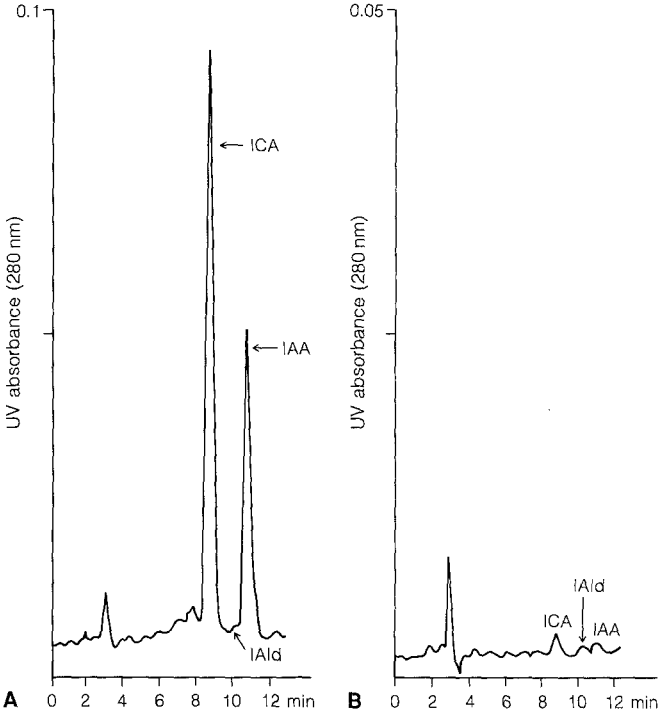


**Fig. 1A, B.** Elution pattern of indole compounds from the HPLC during analysis of TLC purified extracts from 5-week-old MrgX culture filtrates. **A** Culture filtrate obtained in the presence of 1 mM tryptophan. **B** Culture filtrate obtained in the absence of tryptophan. Sample injection: 10 µl; flow rate 1 ml $min^{-1}$; UV detection at 280 nm. Absorbance unit full scale: 0.1 (**A**) and 0.05 (**B**). *IAA*, indole-3-acetic acid; *ICA*, indole-3-carboxylic acid; *IAld*, indole-3-aldehyde

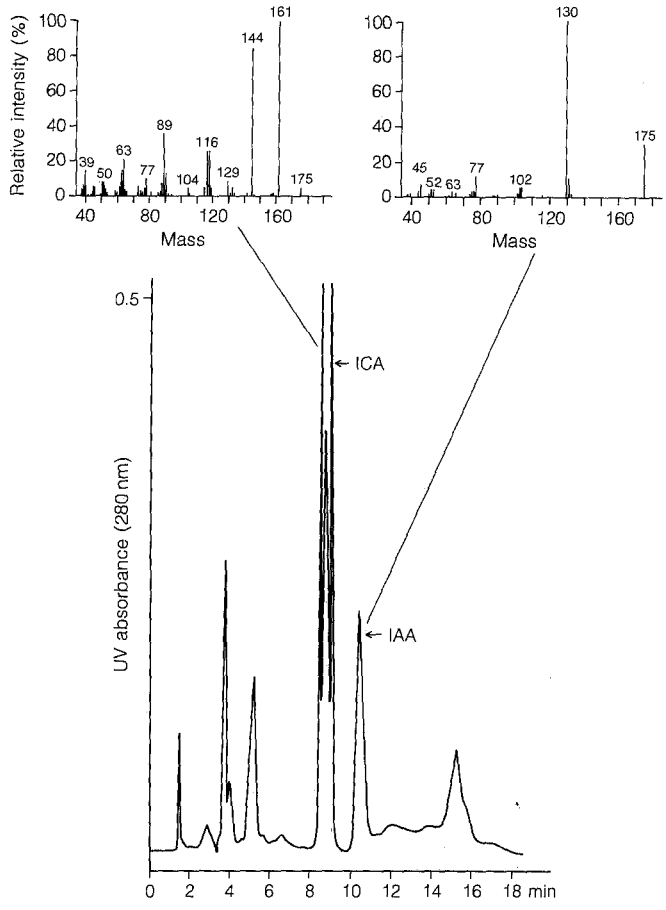


**Fig. 2.** HPLC analysis of indole-compound-containing fraction from TLC-chromatotrone purification corresponding to IAA and ICA and their electron-impact mass spectra. HPLC conditions as in Fig. 1

ther very small peak with a retention time of 10 min 12 s may correspond to IAld. These three peaks were also detected in filtrates obtained in the absence of tryptophan (Fig. 1B), but at approximately 50-fold lower concentration.

The indole compounds corresponding to the two main peaks detected in filtrates in the presence of tryptophan were identified by mass spectrometry. The mass spectrum of the compound with an HPLC retention time of 8 min 48 s was identical to that of synthetic ICA. Likewise, the compound with an HPLC retention time of 10 min 48 s gave a mass spectrum corresponding to that of synthetic IAA (Fig. 2).

### *The kinetics of IAA accumulation in culture filtrates*

The MrgX strain produced only trace amounts of IAA when cultured on unbuffered medium without tryptophan (Fig. 3B), but detectable amounts of auxin when 1 mM tryptophan was added (Fig. 3A). Buffering of the medium had a significant effect on IAA accumulation in the culture filtrates. IAA was easy detectable as early as day 10 of culture, even in the absence of tryptophan. At the end of the culture period, an Erlenmeyer flask ini-

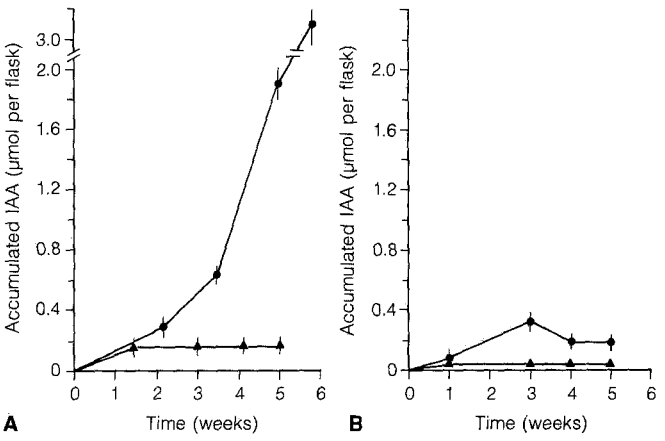


**Fig. 3A, B.** Kinetics of IAA accumulation in MrgX culture filtrates. **A** Culture medium buffered with 50 mM 2-(*N*-morpholino)ethane-sulphonic acid (MES), pH 5.8. **B** Unbuffered culture medium. ●—●, Medium supplemented with 1 mM tryptophan; ▲—▲, culture medium not supplemented with tryptophan. *Vertical lines* represent standard deviations of the mean

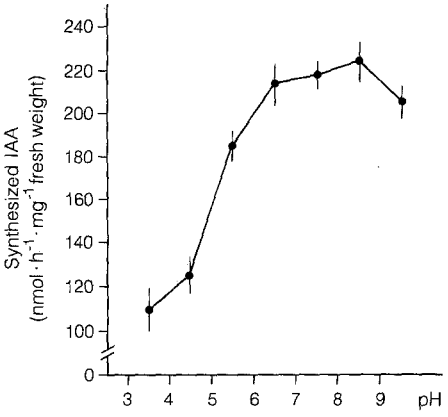


**Fig. 4.** Effect of incubation medium pH on in vivo IAA-synthesizing activity of 5-week-old MrgX mycelia. *Vertical lines* represent standard deviations of the mean

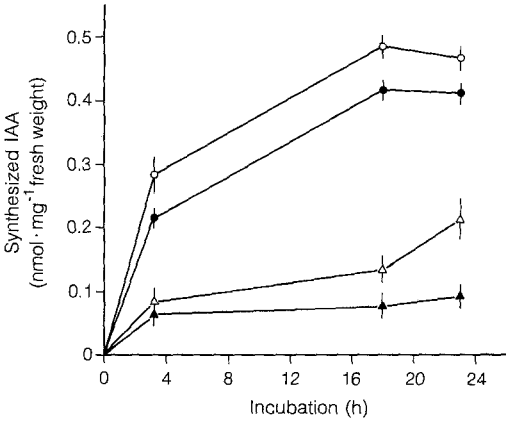


**Fig. 5.** Effect of culture conditions on in vitro IAA-synthesizing activity of 5-week-old MrgX mycelia. Mycelia were grown in the presence of 1 mM tryptophan (○, ●) or in the absence of tryptophan (△, ▲). *Open symbols*, Culture medium buffered with 50 mM MES (pH 5.8); *closed symbols*, unbuffered culture medium

tially supplemented with 40 µmol tryptophan contained ca. 3 µmol IAA, whereas less than 0.3 µmol IAA was detected in the absence of tryptophan. The amount of IAA in 5-week-old culture filtrates was of the same order in unbuffered medium supplemented with 1 mM tryptophan as in buffered medium without the precursor.

*The effect of pH on in vivo activity of the IAA-synthesizing enzymes*

Hydrogen ion concentration strongly affected the in vivo activity of IAA-synthesizing enzymes, which was lowest at acid pH and was stimulated by a pH increase up to 8.5 (Fig. 4).

*In vitro IAA-synthesizing activity of the mycelia*

The IAA-synthesizing enzymes appear to be constitutive since mycelia cultivated in the absence of tryptophan exhibited IAA-synthesizing activity (Fig. 5). However, the enzyme activity of the mycelia was dependent on the culture conditions. In mycelia grown in a buffered nutrient solution, enzyme activity was always slightly higher than in mycelia obtained on unbuffered media, the presence of tryptophan in the nutrient solution increased the IAA-synthesizing activity of the mycelia several fold (Fig. 5).

## Discussion

The ectendomycorrhizal symbiont of Scots pine MrgX, like most ectomycorrhizal fungi (Moser 1959; Ulrich 1960; Ek et al. 1983; Rudawska 1983; Gay and Debaud 1987), is able to synthesize IAA. As demonstrated in the case of ectomycorrhizal fungi (Moser 1959; Horak 1964; Strzelczyk et al. 1977; Gay et al. 1989), tryptophan appeared to be a very effective precursor of IAA in this fungus. The MrgX strain can be considered to be a rather active IAA producer since a 5-week-old culture initially supplemented with 40 µmol tryptophan contained 3 µmol IAA. Thus during the culture period, the fungus metabolized at least 7.5% of the tryptophan to IAA. This is of the same order as that recorded by Gay et al. (1989) in the case of the ectomycorrhizal fungus *Hebeloma hiemale*. Under the same conditions, strains of the ectomycorrhizal fungi *Suillus bovinus* or *Rhizopogon luteolus* released lower amounts of IAA (M. Rudawska, unpublished work). IAA was also detected in culture filtrates obtained in the absence of exogenous tryptophan. This indicates that MrgX, like some other ectomycorrhizal fungi (Tomaszewski and Wojciechowska 1974; Rudawska 1983), is able to metabolize endogenous tryptophan to IAA. However, it must be emphasized that IAA production by many ectomycorrhizal fungi is dependent on exogenously supplied tryptophan (Moser 1959, 1962). Since tryptophan is not commonly available to microorganisms in the soil (Strzelczyk 1975), it can be postulated that fungi able to metabolize endogenous tryptophan to IAA (e.g. MrgX) are better IAA producers under the field conditions where this precursor is not readily available. Whether this confers some advantage to these fungi in competition for mycorrhizae formation remains an open question.

Buffering of the medium appeared especially favourable for fungal growth and IAA release. During the culture period, the pH dropped about 3 units in unbuffered media, reducing fungal growth by half and IAA production by an order of magnitude. A parallel can be drawn between these results and the high buffering capabilities of forest soil colloids, which make pH drops of 2 or 3 pH units unlikely in the natural mycorrhizosphere, although this is frequently recorded in unbuffered culture media of most ectomycorrhizal fungi (Hung and Trappe 1983). Hence, it can be assumed that numerous results on IAA production by ectomycorrhizal fungi are underestimated due to the lack of pH control in the culture medium.

The effect of pH on fungal IAA production was further examined by studying in vivo activity of IAA-synthesizing enzymes. This activity appeared to be pH dependent, being low at acid pH and markedly increasing with increase of pH. This is probably one of the reasons for the relatively low amount of IAA in unbuffered medium, where significant decrease of pH took place. Such observations are in general agreement with the chemiosmotic hypothesis of IAA transport in higher plant cells (Rubery and Sheldrake 1974; Raven 1975; Rubery 1980), and confirm previous results (Gay 1986b) recorded with the ectomycorrhizal fungus *H. hiemale*. According to this theory, extracellular alkaline pH promotes passive IAA release by the cell.

Two other indole compounds in addition to IAA were identified in MrgX culture filtrates namely ICA and IAld. These two compounds possibly originate from IAA degradation by IAA oxidases.This seems to confirm previous reports (Tomaszewski and Wojciechowska 1974; Strzelczyk et al. 1977; Rudawska 1980; Rouillon et al. 1986) that mycorrhizal fungi are able to both produce and destroy IAA, and that the amount of IAA accumulated in culture filtrates is the resultant of IAA synthesis by the fungus and its degradation in the culture medium. In this regard, the IAA detected in cultures of several mycorrhizal fungi (including Mrg) indicates that compounds able to protect auxin against IAA oxidase are probably synthesized by the fungus. Polyphenolic pigments have been demonstrated to act as auxin protectors both in mycelial cultures and on pine root surfaces (Tomaszewski and Wojciechowska 1974; Rudawska 1980). Hence, the physiological significance of substances which can inhibit the IAA oxidative activity of either the fungus or host roots is higher than previously concluded. Thus, hyperauxiny of mycorrhizae seems to result from the production by the fungus of both IAA and phenolic compounds able to inhibit IAA oxidase of the fungus or the host root itself. Phenolic compounds synthesized by the plant in response to mycorrhizal infection may also be involved (Sylvia and Sinclair 1983).

Together with the results of the previous paper (Rudawska and Gay 1989), these findings demonstrate that ectendomycorrhizal fungi are able to release IAA and that the hormonal status in ectendomycorrhizae is probably comparable to that present in ectomycorrhizae. The question remains why ectendomycorrhizal fungi penetrate host cortical cells so readily (Harley and Smith 1983), especially in nitrogen-fertilized nursery soils generally considered to be unfavourable for mycorrhiza formation. Further experiments in progress on the nitrogen metabolism of this fungus may solve this problem.

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