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Research

Hormone concentrations in tobacco roots change during arbuscular mycorrhizal colonization with *Glomus intraradices**

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Orna Shaul-Keinan[1], Vijay Gadkar[1], Idit Ginzberg[1], José M. Grünzweig[2], Ilan Chet[3], Yigal Elad[1], Smadar Wininger[1], Edi Belausov[1], Yuval Eshed[1], Nir Atzmon, Yossi Ben-Tal[1] and Yoram Kapulnik[1]

[1]Agricultural Research Organization (ARO), The Volcani Center, Bet Dagan 50 250, Israel; [2]Present address, Department of Environmental Sciences and Energy Research, Weizmann Institute of Science, Rehovot 76 100, Israel; [3]Department of Plant Pathology and Microbiology, Faculty of Agriculture The Hebrew University of Jerusalem, Rehovot 76 100, Israel

Author for correspondence:
Yoram Kapulnik
Tel: +1 972 3 968 3488
Fax: +1 972 3 966 9642
Email: Kapulnik@agri.huji.ac.il

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Summary

• Phytohormones are known to play a pivotal role in various developmental processes in plants and in arbuscular–mycorrhizal (AM) fungal–host symbiosis. This study focuses on characterizing the changes in the concentrations of auxins, cytokinins and gibberellins in tobacco (*Nicotiana tabacum*) during the early stages of colonization by *Glomus intraradices*, using advanced analytical detection techniques.

• High-pressure liquid chromatography analysis followed by radioimmunoassay detection revealed that AM colonization induced the accumulation of specific zeatin riboside-like and isopentenyl adenosine-like compounds in both roots and shoots.

• Use of the gas chromatography–mass spectrometry technique on the same developmental stage revealed that gibberellins (GA) of the earl-13-hydroxylation biosynthetic pathway (GA_1, GA_8, GA_{19} and GA_{20}) were significantly more abundant in roots, but not shoots, of AM inoculated plants than in those of nonmycorrhizal plants. Indoleacetic acid concentrations (total and free) remained unaltered by AM colonization.

• This study demonstrates that hormonal changes do occur during AM symbiosis with tobacco, before the fungal benefits manifest.

Key words: auxins, cytokinins, gibberellins, phytohormones, arbuscular mycorrhizal fungi, tobacco, *Nicotiana tabacum*.

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Introduction

Arbuscular–mycorrhizal (AM) fungi are obligate symbiotic soil fungi, which colonize the roots of vascular plants (Mosse, 1957). A conservative estimate suggests that 80% of terrestrial land plants are potential host to these fungi (Bonfante-Fasolo, 1987). This symbiosis typically results in enhanced host vigor, most frequently demonstrated in increased uptake of immobile nutrients, principally phosphorus, from the soil (Harrison, 1999). Among the many other benefits conferred by this symbiosis, resistance to drought and pest tolerance are among the most notable ones (Nelsen & Safir, 1982). This complex cellular relationship between the host roots and the AM fungi requires a continuous exchange of signals, that in turn regulate the mutual modification of metabolic processes and development during the course of the symbiotic association (Gianinazzi-Pearson, 1996). One of the key endogenous plant signals, the phytohormones, which are known to play a pivotal role in various developmental processes in the plant, are now being recognized as also involved in this unique symbiosis (Smith & Gianinazzi-Pearson, 1988; Barker & Tagu, 2000).

The developmental regulation of the mycorrhizae may be achieved through modification of endogenous phytohormone levels, or through the regulation of phytohormone receptors in the fungus and/or the host (Bee & Anderson, 1998). Assessments of mycorrhizal development following exogenous

*This paper is dedicated to the memory of Orna Shaul-Keinan, who unexpectedly passed away while this research project was in progress. This work constituted a part of her PhD thesis.

applications of plant growth regulators were recently reviewed by Ludwig-Müller (2000). Endogenous modifications of phytohormone levels, associated with AM infection have also been described in specific host–fungus associations: mycorrhizal blue grama grass (*Bouteloua gracilis*) exhibited enhanced cytokinin contents in roots and leaves (Allen *et al.*, 1980) as well as altered levels of gibberellin-like substances and abscisic acid (ABA; Allen *et al.* 1982). Increased cytokinin levels were also measured following AM infection of *Plantago major* ssp. *pleiosperma* and *Zea mays* (Baas & Kuiper, 1989; Danneberg *et al.*, 1992), and enhanced contents of gibberellin-like substances were found in mycorrhizal *Linum usitatissimum* (Dugassa *et al.*, 1996). However, in dicotyledons, no thorough investigation of hormonal balance that included cytokinins, auxins and gibberellins has been carried out in the same experimental system. In most cases, evaluation of hormonal balance in mycorrhizal plants was carried out at various symbiotic stages in which morphological differences between mycorrhizal and nonmycorrhizal plants could be detected (Dugassa *et al.*, 1996). In such circumstances, plant hormonal changes caused by the establishment of the symbiosis could be confused with those arising from plant growth, and the effects detected would not be a true reflection of the hormonal changes, if any, brought about by the AM fungi *per se*. Furthermore, the nature of the assay system used to detect the changes is a key factor in achieving thorough understanding of the hormonal variations. For example, Danneberg *et al.* (1992), using the classical pea epicotyl bioassay, did not detect any variation in auxin content in maize roots infected by *Glomus* species, whereas Ludwig-Müller *et al.* (1997), using a sensitive isotope dilution analysis in the same experimental system, detected differences in the conjugate indole-3-butyric acid (IBA) levels.

The present study, using an advanced analytical detection system, sought to characterize the effects of the AM fungus, *Glomus intraradices* on the levels of the auxins, cytokinins and gibberellins in tobacco roots and shoots. Measurements were done before the colonization by the AM fungus had elicited any visible benefits to the tobacco plant.

Materials and Methods

Plant material and growth conditions

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown under microbiologically controlled conditions in 0.5-kg pots (three plants per pot) containing autoclaved sand, supplemented with 0.15 g commercial super phosphate per pot before autoclaving. Plants were grown in greenhouse (16 h/8 h day/night cycle and 27/22°C day/night temperature). Natural daylight (April–June) was extended with incandescent illumination (5 $\mu E\ m^{-2}\ s^{-1}$) at plant level. Inoculation with *G. intraradices* spores was performed as described by David *et al.* (1998). Briefly, about 80 spores per plant were placed in a layer 3 cm below the soil surface in the pot before seeding to ensure localized infected zone in the roots. The pots were watered twice a week with P-free modified Johnson solution (Johnson *et al.*, 1957) containing 8 mM NH_4NO_3. In a P-supplemented treatment, 0.3 mM KH_2PO_4 was added to the nutrient solution of noninoculated plants from the third week of growth. Measurements and analyses for most phytohormones were performed twice with 5-wk-old-plants; gibberellin evaluation was carried out once in 5-wk-old plants. Experiment was carried with two main treatments (inoculated and noninoculated) with four replicates for each treatment. Each replicate included eight pots with three plants each.

At sampling, no obvious differences in plant biomass between mycorrhizal and nonmycorrhizal plants, were found in any of the experiments described in the present paper. The plants were harvested and the mycorrhizal colonization of inoculated plants was determined by staining with Trypan blue solution (Phillips & Hayman, 1970) and inspecting under a dissecting microscope (WILD M8, Heerburrg, Switzerland). The percentage infection was quantified according to the gridline intersection method as described by Giovannetti & Mosse (1980). For hormonal analysis, samples of the youngest fully exposed leaf of each plant (for shoots) or the upper 4 cm of the roots (to include the localized infected zone) were excised from each treatment. Plant samples of eight pots were composed to a unified tissue sample, for each replicate, in all assays described.

Auxin detection

Quantitative analyses of the plant hormone indole-3-acetic acid (total IAA and free IAA) were carried out according to McDougall & Hillman (1978), with high-performance liquid chromatography (HPLC) and full-scan gas chromatography–mass spectrometry (GC-MS), using internal standards. Samples (approximately 1 g of fresh tobacco roots or leaves) were purified and prepared for analysis according to Chen *et al.* (1988).

Cytokinin detection

Zeatin riboside (ZR) and isopentenyl adenosine (iPA) compounds were extracted from 1 g of fresh tobacco leaves (first fully expanded leaf) or roots according to van Rhijn *et al.* (1997). Briefly, samples were separated by HPLC in an RP-18 column and were UV-analysed with an on-line spectrophotometric detector. Chemical standards were used to determine the ZR or iPA retention time (RT) by means of the HPLC running protocol. All fractions (at 1-min intervals) were collected and analysed by radioimmunoassay (RIA), as described by van Rhijn *et al.* (1997). Fractions that eluted at similar retention times to those of the zeatin, zeatin glucoside and zeatin riboside standards were regarded as 'ZR-like compounds', whereas fractions eluted at similar retention times to those of the isopentenyl and isopentenyl adenosine standards were regarded as 'iPA-like compounds'. Results were calculated per gram fresh weight (f. wt) of the tested tissue.

Gibberellin detection

Leaf (20 g) and root (10 g) tissues were ground in liquid nitrogen, and the gibberellins (GAs) were extracted overnight in 10 ml of cold 80% methanol g^{-1} f. wt. Extracts were spiked with [17,17-^{2}H2]GA_1, [17,17-^{2}H2]GA_4, [17,17-^{2}H2]GA_8, [17,17-^{2}H2]GA_9, [17,17-^{2}H2]GA_{19}, and [17,17-^{2}H2]GA_{20} as internal standards (kind gift from Prof. L. N. Mander, Australian National University, Canberra, Australia). Extracts were then purified and fractionated according to Grünzweig *et al.* (1997). The HPLC fractions were methylated and trimethylsilylated, and the gibberellins were identified by GC-MS, according to Gaskin & MacMillan (1991) and Grünzweig *et al.* (1997). Quantification of the gibberellins was performed using GC-MS selected-ion monitoring (Grünzweig *et al.*, 2000). The endogenous concentrations of GA_1, GA_4, GA_8, GA_9, GA_{19} and GA_{20} were calculated from the peak area ratios of the ion pairs *m/z* 508/506, 286/284, 596/594, 300/298, 436/434 and 420/418, respectively, by reference to calibration curves. The values obtained for each concentration of gibberellins in leaves and roots of mycorrhizal and control plants were analysed statistically at $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$ confidence limits by means of Student's *t*-test.

Results

Plant growth and AM colonization

To study changes in hormonal levels of plants during AM establishment, tobacco plants were grown with (+AM) or without (–AM) *G. intraradices* spores. Five weeks after planting, mycorrhizal plants showed 40–57% root infection (in the various experiments), with no significant differences between their growth rates and that of the nonmycorrhizal plants. Biomass accumulation amounted to 3.6 ± 0.4 g and 3.8 ± 0.4 g (mean ± standard error) of f. wt per plant, in inoculated and uninoculated plants, respectively. Similarly, development of P-treated plants did not differ statistically from that of the controls up to the fifth week of growth (data not shown).

Evaluation of IAA

Quantitative analysis of total and free IAA in roots and leaves of tobacco plants revealed no significant differences between mycorrhizal and nonmycorrhizal plants. Free IAA levels in roots of mycorrhizal and nonmycorrhizal plants were 10 ± 1.8 ng g^{-1} f. wt and 11 ± 1.2 ng g^{-1} f. wt, respectively, whereas total IAA levels were 45 ± 8 ng g^{-1} f. wt and 42 ± 6 ng g^{-1} f. wt, respectively. Free IAA levels in leaves of mycorrhizal and nonmycorrhizal plants were 16 ± 3 ng g^{-1} f. wt and 13 ± 3 ng g^{-1} f. wt, respectively, and their total IAA levels were 84 ± 11 ng g^{-1} f. wt and 114 ± 15 ng g^{-1} f. wt, respectively.

Table 1 Total cytokinins (ng g^{-1} f. wt) in leaf and root tissues of nonmycorrhizal (control) and mycorrhizal (AM) tobacco plants on P-free solution, and nonmycorrhizal plant with P amendment (0.3 mM)

Plant material	Treatment	Total ZR-like	Total iPA-like
Leaves	Control (–P)	1.1 ± 0.1	0
	+AM	1.5 ± 0.2	4.2 ± 0.5
	Control (+P)	1.8 ± 0.2	5.2 ± 0.6
Roots	Control (–P)	1.6 ± 0.1	0
	+AM	1.9 ± 0.3	15.5 ± 0.9
	Control (+P)	2.0 ± 0.3	1.9 ± 0.2

Values are mean ± SE.

Evaluation of cytokinins

The HPLC analysis revealed that all ZR-like standards were eluted at RTs of 0–19 min, whereas iPA-like compounds were eluted at 20–40 min (data not shown). Similar total amounts of ZR-like compounds were found in both roots and leaves of nonmycorrhizal control and mycorrhizal plants (Table 1). However, the two treatments differed markedly in their contents of iPA-like compounds: whereas these cytokinins were undetectable in the nonmycorrhizal controls, inoculation with the fungus resulted in the presence of detectable amounts of iPA-like compounds. It is important to note that mycorrhizal roots contained 370% higher levels of iPA-like compounds than leaves of the same plant.

In order to examine whether the modified pattern of cytokinin composition in roots and leaves of mycorrhizal plants resulted from the biotic supply of P through the mycorrhizal symbiosis, nonmycorrhizal plants were watered with 0.3 mM P. This P application did not affect the total concentrations of ZR-like cytokinins in roots and leaves of either nonmycorrhizal or mycorrhizal plants (Table 1). However, iPA-like compounds, which were not detectable in nonmycorrhizal control plants, were present following P supply. The P treatment raised the total concentration of iPA-like cytokinins in leaves of nonmycorrhizal plants to a level similar to that measured in leaves of mycorrhizal plants, whereas their concentration in roots of nonP-treated mycorrhizal plants was significantly higher than in those of P-treated nonmycorrhizal plants. These findings indicate an additive symbiotic contribution of iPA-like molecules to AM plants, over and above the effect of the mineral P nutrition.

A more detailed analysis revealed differences among the above-mentioned treatments, in the retention-time profiles of the cytokinin compounds. Mycorrhizal roots contained multiple nonspecific ZR-like compounds, which were also detected in the nonmycorrhizal control plants (RTs of 8–14 min); P-treated plants contained only the 8-min RT molecule (Fig. 1). Only mycorrhizal roots contained a specific ZR-like compound that eluted at a RT of 18 min (Fig. 1). It is important to note that leaves of mycorrhizal plants did not contain any specific ZR-like molecules.

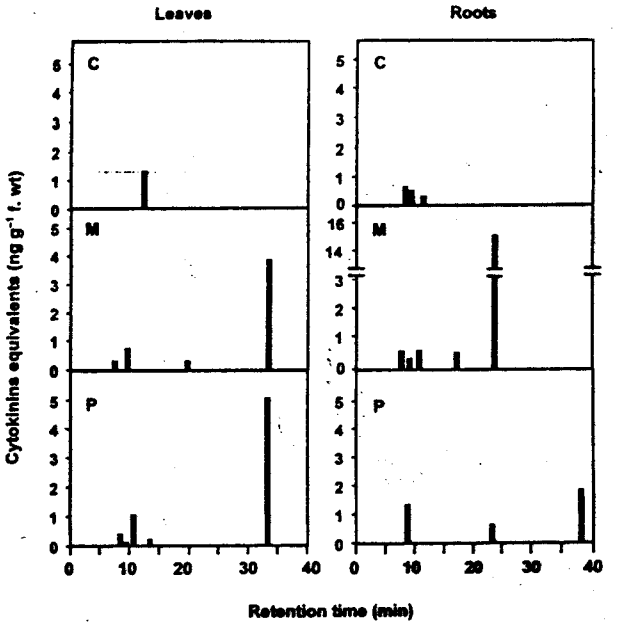

Fig. 1 Retention time (RT) profile of ZR-like compounds in the leaves and roots in nonmycorrhizal control (C); mycorrhizal (M); and plants following phosphorus (P) fertilization (0.3 mM P).

In order to examine the specific contribution of the mycorrhizal treatment to the iPA-like molecules we evaluated the effect of a high P nutritional level on their profile. The P-treated plants contained two detectable iPA-like molecules, of which only RT 24 could be detected in mycorrhizal roots and none was detected in the nonmycorrhizal control roots. In plant leaves, two iPA-like molecules could be detected in mycorrhizal plants (RT 21 and 33), but the molecule eluted at RT 21 seems to have been specific to the mycorrhizal treatment (Fig. 1) whereas the molecule eluted at RT 33 may have been induced in both the high P mycorrhizal treatments.

Evaluation of gibberellins

Concentrations of several endogenous gibberellins in roots were significantly increased by mycorrhizal colonization (Table 2). Mycorrhizal colonization caused most of the GAs of the earl-13-hydroxylation biosynthetic pathway – GA1, GA_8, GA_{19} and GA_{20} – to be increased in root samples of 5-wk-old plants relative to those in the nonmycorrhizal controls. However, no significant differences in GA compounds could be detected in leaves of mycorrhizal or nonmycorrhizal plants (Table 2).

Discussion

This study addressed the relative concentrations of three major plant growth regulators (cytokinins, auxin and gibberellins) in mycorrhizal plants, during the early stages of AM-tobacco symbiosis. Concentrations of the hormones detected were evaluated 5 wks after germination and before the symbiotic processes, *per se*, could elicit any noticeable growth difference in the inoculated plants. This approach enabled us to evaluate early hormonal events in mycorrhizal plants and to ascertain the hormonal balance that reflects specific host–fungus interactions rather than better plant growth.

Under low P concentrations, total iPA-like compounds were found at significantly higher concentrations in mycorrhizal roots and leaves than in the nonmycorrhizal controls (Table 1). These results are consistent with those of several previous studies (Allen *et al.*, 1980; Edriss *et al.*, 1984; Dixon *et al.*, 1988; Baas & Kuiper, 1989; Danneberg *et al.*, 1992; Drüge & Schönbeck, 1992). It is also important to note that the higher cytokinin levels in leaves of mycorrhizal plants could be attributed to translocation from the roots, where the synthesis of the hormone may be stimulated either by the plant or the AM fungus, rather than to *de novo* synthesis in the leaves (Dixon *et al.*, 1988). Most importantly, the present study facilitated the evaluation of cytokinin compounds in a quantitative manner. For example, no iPA-like compounds could be detected in either roots or leaves of nonmycorrhizal plants, whereas mycorrhizal plants accumulated high concentrations of these compounds. Differences between mycorrhizal and nonmycorrhizal plants in total ZR-like compounds have been reported previously by us (van Rhijn *et al.*, 1997; Ginzberg *et al.*, 1998) and by others (Drüge & Schönbeck, 1992). However, our approach in the present study enabled us to detect new forms of ZR-like compounds that were found to be associated with the presence of the mycorrhizal fungus and could not be elicited by mineral P nutrition treatment (Fig. 1). Both previously and in the present study, mycorrhizae

Table 2 Endogenous concentrations (ng g^{-1} fresh weight) of gibberellins (GA) in leaves and roots of nonmycorrhizal (control) and mycorrhizal (AM) tobacco plants

Plant material	Treatment	*n*	GA_1	GA_4	GA_8	GA_9	GA_{19}	GA_{20}
Leaves	Control	3	0.88 ± 0.06	0.17 ± 0.02	0.15 ± 0.02	0.14 ± 0.02	0.73 ± 0.06	0.20 ± 0.01
	AM	3	0.99 ± 0.06	0.20 ± 0.02	0.15 ± 0.02	0.13 ± 0.01†	0.80 ± 0.10	0.23 ± 0.02
Roots	Control	2	0.08 ± 0.02	–[1]	0.09 ± 0.03	n.d.[2]	0.62 ± 0.01	0.06 ± 0.01
	AM	2	0.38 ± 0.10†	0.06[1]	0.60 ± 0.16†	n.d.	3.47 ± 0.33*	0.10 ± 0.01*

†,*: Differences between control and AM are statistically significant at $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$, respectively, according to Student's *t*-test. Values are mean ± SE. [1]Extraneous contamination of *m/z* 284 reduced *n* to 0, 1, 1 and 2 for GA_4 in control roots [2]n.d., Not detected.

and P nutrition have both been shown to induce the accumulation of cytokinin molecules in roots and leaves (Baas & Kuiper, 1989; Torelli *et al.*, 2000). The present results indicate that, following AM fungal colonization, specific cytokinin compounds were being synthesized irrespective of the improvement of P level associated with AM symbiosis. The fact that these results were obtained in the early stages of symbiosis implies either that the AM fungus modifies the hormonal production processes of the host or that the fungus synthesizes cytokinins in the root tissue, as previously suggested by Barea & Azcon-Aguilar (1982) for *Glomus mosseae* spores. Interestingly, ectomycorrhizas have previously been shown to produce cytokinin (Crafts & Miller, 1974).

Exogenous application of IAA has been found to improve mycorrhizal development namely, arbuscule frequency (Gunze & Hennessy, 1980) and fungal colonization (Bareen *et al.*, 1988). Uninfected roots that had been treated with auxin showed morphological characteristics similar to those of mycorrhizal ones (Ludwig-Müller, 2000), therefore it was suggested that auxins play an integral role in the symbiotic relationship (Meyer, 1974). However, in the present study we demonstrated that endogenous levels of auxins (total and free), in either leaves or roots, did not differ significantly between mycorrhizal and nonmycorrhizal tobacco plants. Similarly, Danneberg *et al.* (1992) and Torelli *et al.* (2000) did not find that the auxin contents in maize and leek roots differed according to whether or not they were infected with a *Glomus* species. The criticism was raised that the latter result was obtained with a classical low-sensitivity bioassay (Frankenberger & Arshad, 1995) that could not distinguish between different forms of IAA in the sample. In the present study we have used GC-MS together with stable-isotope-labeled standards (Cohen *et al.*, 1986; Sutter & Cohen, 1992) to reconfirm that no conspicuous differences could be found between the concentrations of free and conjugated IAA in mycorrhizal tobacco roots and those in nonmycorrhizal controls. These data confirm previous findings that maize varieties with differing endogenous levels of IAA were uniformly colonized by *G. mosseae* (Ludwig-Müller *et al.*, 1997). The reason for the lack of appreciable changes in the level of IAA could be that this particular phytohormone does not play a major role during the AM symbiosis. Baser *et al.* (1987) showed that addition of exogenous IAA to black oak seedlings did not stimulate the early stages of ectomycorrhizal infection, suggesting that this phytohormone plays a minor role.

Not much work has been reported on the interactions between gibberellins and mycorrhizal fungi. Allen *et al.* (1982) observed that infection with the mycorrhizal fungus *Glomus fasciculatus* resulted in a significant increase of GA-like activity in the leaves of *Bouteloua gracilis*, while gibberellin levels in the roots tended to decrease. Also, in *L. usitatissimum*, gibberellin levels were higher in leaves of mycorrhizal than in those of nonmycorrhizal plants (Dugassa *et al.*, 1996). Clapperton *et al.* (1985) used GC-MS analysis to compare GAs levels in *Agropyron thrachycaulum* infected with a *Glomus* species with those in uninfected plant tissues; they found, as we did, that infected roots had more GAs than control roots. This contrasts with the findings of Allen *et al.* (1982). These differences may be species related but are more likely to be due to the different methods employed for analysis. We used GC-MS and found that the concentrations of gibberellins were increased by the colonization of the AM fungus in the roots (Table 2). Most significantly, mycorrhizal colonization increased root concentrations of GA_1, which is the major biologically active gibberellin; it is known to be involved in plant growth regulation (Phinney, 1984; Ross *et al.*, 1993; Spray *et al.*, 1996), and increased GA_1 concentrations have been correlated with increased plant growth (Grünzweig *et al.*, 2000). Since no gibberellin-related growth effect could be seen in the present study, the alteration in gibberellin levels may be associated with the fungal colonization (establishment processes) rather than with the improved growth characteristics imparted by symbiosis (e.g. through mineral acquisition processes). An AM-induced increase in gibberellin production has been suggested to be involved in strengthening the carbohydrate sink of the fungi, expression of sugar-related enzymes and the secretion pathway (Bee & Anderson, 1998). It is therefore possible that the higher gibberellin concentrations observed in our AM-treated plants indicate mycorrhizal activity related to the fungal carbohydrate requirements.

Our present findings contrast with results obtained by others in *B. gracilis* (Allen *et al.*, 1982) and in *L. usitatissimum* (Dugassa *et al.*, 1996). However, in both of those studies, bioassays were used to monitor GA_3-like activity, which introduced uncertainties concerning the type of gibberellins detected in the assay procedure and the intrinsic sensitivity of detection procedure. It is well known that different bioassays are sensitive to different GAs (Crozier *et al.*, 1970). Moreover, Dugassa *et al.* (1996) reported that shoot growth in *L. usitatissimum* was already improved by AM fungi at plant sampling therefore, in that species and possibly also in *B. gracilis*, increased gibberellin-like activity might have been a consequence of larger plant size rather than a symbiosis-specific signal. The fact that increased gibberellin-like activity was detected in leaves, but not in roots by Allen *et al.* (1982) supports this hypothesis, as growth responses triggered in the soil often appear first in leaves and later in roots (Grünzweig *et al.*, 1993). The development in AM-colonized roots of increased cytokinin and gibberellin concentrations with unchanged auxin concentrations, as found in the present study, effectively changed the auxin–cytokinin and auxin–gibberellin ratios. These changes could, *per se*, be developmental signals in AM–plant symbiosis. Changes in the ratios between phytohormones have been shown to affect different stages in plant development, such as plant growth, root proliferation, shoot development and fruit development (Barker & Tagu, 2000), some of which are also associated with mycorrhizal symbiosis.

To conclude, the enhancement of mineral uptake by fungal hyphae is well recognized as a primary mechanism responsible for plant growth stimulation by AM fungi (Saito, 2000). Whether the enhancement in plant hormones has a direct effect on the establishment of AM symbiosis is not yet known. The present study was intended to provide a better comprehension of the role of phytohormones in the biology of mycorrhizae during the phase of the symbiosis in which no growth differences between mycorrhizal and nonmycorrhizal plants are observed. It was found that mycorrhizal symbiosis altered gibberellin and cytokinin concentrations in tobacco roots, both quantitatively and qualitatively, but that auxin (IAA) concentrations were not altered significantly. The present study supports the notion that a new balance of plant phytohormones is associated with early symbiotic events, and is the direct result of the symbiotic event. These changes in the hormonal balance may be necessary for the plant to regulate the colonization of the fungus, or it could be a perturbation of the endogenous balance in the plants, brought about by the invading fungus. Future research directed at the analysis of these early events would help in elucidating the exact biological significance of this phenomenon.

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