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J. Plant Physiol. 161. 509–517 (2004)
http://www.elsevier.de/jplhp

JOURNAL OF PLANT PHYSIOLOGY

Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis

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Received July 22, 2003 · Accepted October 9, 2003

Summary

A Petri dish system in which development of oak (*Quercus robur* L.) microcuttings is stimulated by the late stage ectomycorrhizal (EM) fungus *Piloderma croceum* J. Erikss. & Hjortst. in a long pre-symbiotic stage was optimised to allow synchronous, rhythmic plant growth. Addition of indole-3-acetic acid or activated charcoal to the medium caused an early and more intensive EM formation coupled with suppression of most developmental effects of *P. croceum*. Leaf area, chlorophyll fluorescence, and content were compared in inoculated and uninoculated plants grown at two relative humidity levels (45 and 95 %) and under consideration of three possible answers to inoculation, that is, no or EM formation after the 1st or the 2nd shoot flush. The culture conditions for uninoculated plants were suboptimal, leading toward photochemical stress reflected by a non photochemical quenching (qE) increase and a reduced Chl content at the end of the assay. Prior to EM formation, inoculation itself enhanced the optimal (Fv/Fm) and effective (ΦPSII) quantum yield in leaves of the 1st shoot flush under reduced relative humidity. It also fully protected the plants against stress during the complete assays. The results indicate that inoculated plants only form EM once they have acquired a sufficient development level and C-providing capacity. However, the fungus actively improves the development and photosynthesis of plants up to the pre-mycorrhizal stage, helping them to reach this capacity.

Key words: *Quercus robur* – *Piloderma croceum* – activated charcoal – Chl fluorescence – ectomycorrhiza – indole-3-acetic acid – relative humidity – root/shoot development

Abbreviations: AC = activated charcoal. – CLSM = confocal laser scanning microscopy. – Fv/Fm = optimal quantum yield. – IAA = indole-3-acetic acid. – MR = mother roots. – ΦPSII = effective quantum yield. – qE = non-photochemical quenching. – r. h. = relative humidity. – SLA = specific leaf area. – SR = short roots. – SRL = specific root length. – SF = shoot flush

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0176-1617/04/161/05-509 $ 30.00/0

Introduction

In boreal, temperate, and some tropical regions, establishment and development of most trees are subjected to ectomycorrhizal (EM) symbioses. More than 6000 fungal taxa are involved in EM, and a wide functional diversity corresponds to this high number of mycobionts (Smith and Read 1997). One aspect of the functional diversity concerns the requirements for the onset of the EM formation itself. Early- and late-stage EM fungi have been distinguished and were shown to have different ecophysiological demands (Erland and Finlay 1992, Hutchinson and Piché 1995). In previous work, we established a system for controlled mycorrhization of rooted oak microcuttings under gnotobiotic conditions (Herrmann et al. 1998). We showed that the mycobiont *Piloderma croceum* has a prolonged pre-mycorrhizal phase during which it stimulates plant growth and development. This was not observed for another mycobiont, *Paxillus involutus*, which rapidly formed EM with the rooted oak microcuttings. The goal of the present work was to characterize the pre-mycorrhizal phase in cultures with *P. croceum* and to identify processes specific for this phase and different from those of EM differentiation.

Addition of exogenous substances like indole-3-acetic acid (IAA) (Slankis 1973, Gay et al. 1994, Rudawska and Kieliszewska-Rokicka 1997, Karabaghli-Degron et al. 1998, Niemi et al. 2002) or activated charcoal (AC) (Kottke et al. 1987, Roth-Bejerano et al. 1990) have been reported to promote EM formation. In pioneering work on Petri dish mycorrhization systems, Kottke et al. (1987) showed that optimal moisture around roots is a determinant for EM formation. Thus relative humidity (r. h.) in the air surrounding the growing shoots might also influence EM formation.

Besides developmental effects, EM are known to stimulate photoassimilation and to protect plants against stresses. The amount of chlorophyll and the quantum yield Fv/Fm are classically used to characterize the photosynthetic capacity of a plant. In addition, Chl fluorescence reflects both the photosynthetic carbon assimilation (described as the effective quantum yield [ΦPSII], cf. Seaton and Walker 1990) and the protection of the PSII against oxidative damages (measured as qE, the energy-dependent non-photochemical quenching, cf. Li et al. 2000). Here we extend our analysis of the oak – *P. croceum* interaction and demonstrate that the morphological effects introduced in our previous work (Herrmann et al. 1998) are accompanied by differences in the state and function of the photosynthetic apparatus.

Materials and Methods

Fungal and plant materials, mycorrhization system

The isolate TUMY N° 729 of *Piloderma croceum* J. Erikss. & Hjortst. was cultured, and the *Quercus robur* L. clone DF 159 was micropropagated using the methods described by Herrmann et al. (1998). For mycorrhization, rooted micropropagated oak plantlets were transferred into 90 mm diameter Petri dish rhizotrons. In this system, the roots grew two-dimensionally inside the dish and were inoculated with a nylon sheet carrying 7–8 fungal plugs (8 mm diameter), while the shoot developed outside. Between inoculation and transfer to the growth chambers, the plants were kept in plastic bags moistened with wet cotton sheets to reduce adaptation shocks for the shoots. The basic culture substrate was a modified MMN medium (Marx 1969) without carbohydrate source and 1/10 strength P and N.

Treatments

In a first experiment, AC (2 % w/v) or IAA (5.7 μmol/L) were added to the culture medium before or after autoclaving, respectively, in the absence or presence of *P. croceum* (AC-, IAA-, Pilo/AC-, Pilo/IAA-treatments). Two controls without AC or IAA and in the absence or presence of *P. croceum* (control-, Pilo-treatments) were also performed. The experiment was run over a 10-week period in growth chambers at 25 °C, 95 % r. h. and 16 $h \cdot d^{-1}$ illumination (70 $\mu mol\, m^{-2}\, s^{-1}$; Philips TLM 115W/33RS). A second experiment was performed without addition of AC or IAA. After three weeks of acclimatization, the r. h. was reduced from 95 to 45 % for half of the inoculated (Pilo/r. h. 95-, Pilo/r. h. 45-treatments) and uninoculated plants (control/r. h. 95-, control/r. h. 45-treatments).

Monitoring plant growth and EM formation

Detailed plant growth monitoring was performed in the first experiment. Root formation and elongation were recorded weekly for the first- and second-order mother roots and the first- and second-order short roots. Leaf area was recorded at the end of each shoot flush (SF) and at the end of the incubation period. The measurements of leaf area and root length were performed with a digitized image-analysis-system (Videoplan 2.2 software, Zeiss-Kontron, Germany). Root/shoot dry weight ratio, specific root length (SRL; root length/root dry weight), 1st order MR branching index [number of 1st order SR + 2nd order MR/length of 1st order MR] and specific leaf area (SLA; leaf area/leaf dry weight) were also determined at the end of the incubation period.

Mycorrhiza formation was recorded weekly by checking the appearance of the characteristic yellow hyphal mantle of *P. croceum*, which has previously been shown to correspond to the development of ectomycorrhizas with a Hartig net (Herrmann et al. 1998). Verification that no hyphal mantle or Hartig net were present in rootlets classified as pre-mycorrhizal was done by CLSM, which allows easy detection of these structures by autofluorescence (Mogge et al. 2000). Short roots were sampled and prepared directly on glass slides in IMMU-MOUNT Cat N° 9990402 (Shandon, U.S.A), a polyvinyl alcohol resin with glycerol, less than 1 % amino alcohol and cationic quaternary ammonium chloride. Preparations were dried in an oven at 37 °C for 30 minutes. The LSM 510 META was used on an Axioplan 2 imaging upright microscope (Zeiss Jena, Germany) equipped with an UV laser for excitation of autofluorescence at 364 nm. An oil immersion lens (Plan Apochromat, 100 ×, N. A. = 1.4) was used. For spectrally-resolved confocal measurements, emission was detected as λ stacks in the range between 404–564 nm in steps of 10.7 nm with the META multi-channel detector.

Chl fluorescence measurements and Chl *a* and *b* determination

The following analyses were performed in the second experiment: *In vivo* Chl fluorescence parameters were measured with a pulse amplitude-modulated fluorometer (PAM 101/103, Heintz Walz, Effeltrich, Germany). Single oak leaves, with a minimal area of 1 cm^2, belonging to the different SF were positioned under the emitter/detector unit without being sectioned from the mother plant. Measurements were performed as described by Pfannschmidt et al. (2001) and the following parameters were calculated: (i) optimal quantum yield given by Fv/Fm = (Fm-Fo)/Fm; (ii) effective quantum yield of photosystem II calculated as ΦPSII = (Fm′-Fs)/Fm′ with Fs = Ft-Fo′; (iii) non-photochemical quenching given by qE = 1-(Fm′-Fo′)/(Fm-Fo) [Fo = minimal fluorescence in the dark acclimated state, Fm = maximal fluorescence under saturating white light after dark adaptation, Fo′ = minimal fluorescence in the light acclimated state, Fm′ = maximal fluorescence of illuminated leaves until stable fluorescence level Ft is reached].

For Chl *a* and *b* determination, isolated leaves belonging to the different SF were harvested on ice, and Chl concentrations were determined according to Porra et al. (1989) after grinding of the material in liquid nitrogen and extracting the pigments with 80 % buffered acetone.

Statistical analyses

The data were analyzed by one-way ANOVA or one-way ANOVA on ranks depending on their distribution (SigmaStat® 3.2, Jandel). Mean values for a minimum of six individuals are given with standard errors. Differences among means were evaluated by a multiple comparison procedure using Student-Newman-Keul's or Dunn's tests at $P \leq 0.05$. Correlation coefficients (r) between parameters are given at $P \leq 0.05$.

Results

Effects of IAA and AC on development and mycorrhization of the microcuttings

In this experiment, the micropropagated oaks displayed a typical rhythmic development characterized by out-of-phase root and shoot flushes. The development of all plants began by a root flush at week 4.9 ± 0.7, and in the following shoot flush, the epinasty occurred synchronously. This synchronous plant development made statistical analyses of root formation and elongation possible. In the Pilo-treatment, *P. croceum* had considerable effects on plant development at the end of the incubation time manifested by an increase of total leaf area, total root length, SRL and length of 1st and 2nd order MR and 1st order SR (Table 1). In contrast, the fungus did not modify the 1st order MR branching index and reduced the R/S ratio only slightly. The strong development of 2nd order MR of at least 8 to 10 fold compared to the control was caused by a second shoot flush. The increase in dry weight correlated with the increase in the total leaf area ($r = 0.825$, $P < 0.0001$), and no modification of the SLA was observed.

IAA had no significant effect on the development of uninoculated plants compared to the control (Table 1). In the presence of AC, we observed a significant reduction of the 1st order MR branching index in both AC- and Pilo/AC-treated material. Since this occurred in none of the other treatments, 1st order MR branching was not further investigated in this study.

In the Pilo/IAA- and Pilo/AC-treatments, plant development was reduced compared to the Pilo-treatment. In particular,

Table 1. Combined effects of *Piloderma croceum,* and/or addition of AC, 2 % w/v or IAA, 5.7 µmol/L on root and shoot development of *Quercus robur* microcuttings after 10 weeks of co-cultivation. Means ± SE are given for n ≥ 6. MR1 and MR2, first and second order mother roots; SR1, first order short roots; SRL, specific root length; SLA, specific leaf area; R/S, root/shoot dry weight ratio. (*) Total root length corresponds to the sum of MR1, MR2 and SR1 plus the length of the principal root and second order short roots (SR2) which are not given in the table.

			AC		IAA	
	Control	Pilo	Control	Pilo	Control	Pilo
Root parameters						
Total length* (mm)	476±87 b	1293±104 a	374±99 b	704±125 b	557±45 b	1085±290 ab
MR1 length (mm)	342±58 b	678±56 a	330±83 b	558±97 ab	424±29 ab	481±283 ab
MR2 length (mm)	14.3±4.5 b	249±34 a	18.5±8.6 b	84.7±32.0 ab	37.0±16.5ab	293±104 a
SR1 length (mm)	108±24 b	254±32 a	25.0±9.4 c	69.1±14.7 b	88.6±8.9 b	154±50 ab
MR1 branch. ind.	0.26±0.05 a	0.26±0.02 a	0.05±0.01 b	0.11±0.02 b	0.20±0.02 a	0.25±0.04 a
SRL	24.9±2.6 b	40.0±3.0 a	20.4±3.1 b	27.7±3.5 ab	21.1±2.8 b	27.2±6.4 ab
Shoot parameters						
Leaf area (mm^2)	451±32 b	1356±109 a	622±145 b	758±68 b	513±43 b	597±145 b
SLA	17.2±1.3 a	17.9±0.5 a	15.1±0.6 a	18.1±1.4 a	15.1±0.6 a	17.9±1.2 a
Total plant parameters						
Plant d. wt (mg)	74.5±8.1 b	140.9±8.3 a	81.0±12.5 b	93.7±10.9 b	87.3±6.8 b	98.3±10.1 b
R/S	0.52±0.05 ab	0.38±0.02 b	0.42±0.07 ab	0.49±0.03ab	0.49±0.05ab	0.66±0.09a

Within each row a, b are significantly different at $P \leq 0.05$.

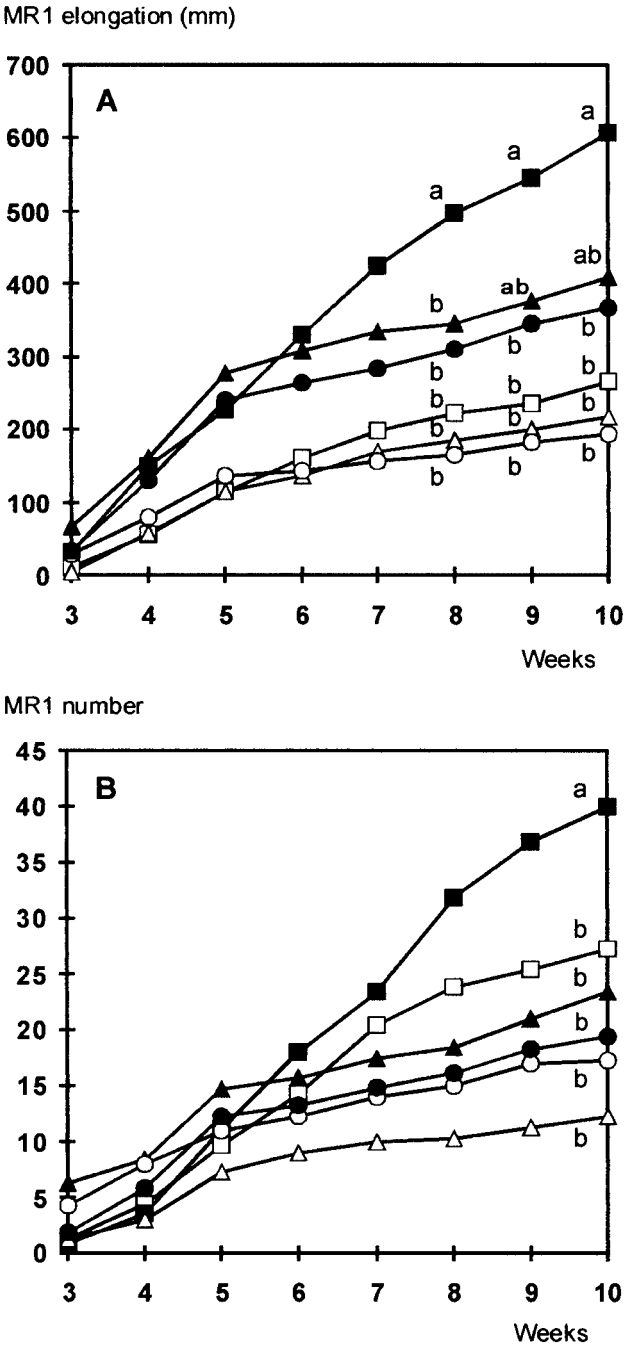

Figure 1. Cumulative elongation (mm) (**A**) and number (**B**) of MR1 (first order mother roots) of *Quercus robur* microcuttings between week 3 to 10 of culture in Petri-dish rhizotrons under combined inoculation with *Piloderma croceum* and/or addition of AC, 2 % w/v or IAA, 5.7 µmol/L. Significant differences (a, b) between treatments are given at $P \leq 0.05$. □ Control; △ Control + AC; ○ Control + IAA; ■ Pilo; ▲ Pilo + AC; ● Pilo + IAA.

application of AC or IAA totally suppressed the effects of *P. croceum* on plant dry weight and leaf surface. The increase of root development and SRL was also repressed, and either in the same order of magnitude, or at least not significantly different, from the controls (Table 1). However, a significant increase of 2nd order MR elongation in the Pilo/IAA-treatment was detectable, which was obvious up to week 8 (20.1 mm vs. 134.4 mm; $P < 0.001$), and which led to a high final value of the total root length in this treatment. Since the leaf area did not increase in a similar manner, we observed an increase in the R/S ratio in the Pilo/IAA- vs. Pilo-treatment (Table 1).

Both AC and IAA accelerated mycorrhization from week 9.06 ± 0.49 to week 5.12 ± 1.15 and 5.0 ± 1.06, respectively ($P < 0.001$). They also induced a significant increase of the mycorrhization rates (42.5 % ± 14.6 and 24.0 % ± 5.7, respectively) compared to the Pilo-treatment alone (5.87 % ± 2.57) ($P = 0.022$). In the Pilo/IAA-treatment, mycorrhization was diffuse and not restricted to the proximity of the inoculum plugs. To establish a relationship between the morphogenetic effects of *P. croceum* and the onset of EM formation, the development of 1st order MR and the 1st SF were investigated. Formation and elongation of 1st order MR appeared to be similar until week 5 in all three inoculated treatments (Fig. 1). However, up to the onset of EM formation in week 5 in the Pilo/AC- and Pilo/IAA-treatments, the slopes of both curves tended to level. This led to significant differences in week 8 for 1st order MR elongation ($P < 0.001$) and in week 10 for the number of formed 1st order MR ($P = 0.003$). On the other hand, the epinasty of the 1st shoot flush was reached after week 6.5 ± 0.16 in all inoculated treatments, that is, before the onset of mycorrhization in the Pilo-treatment and after it in both Pilo/IAA and Pilo/AC-treatments. However, total leaf area was significantly higher in the Pilo-treatment (753 ± 118 mm^2) than in the Pilo/AC- or Pilderma/IAA-treatments (253 ± 67 mm^2 and 237 ± 35 mm^2, ($P<0.001$). Thus, the AC or IAA mediated suppression of the effects of *P. croceum* on 1st order MR elongation and leaf area (Table 1) might be caused by an earlier onset of EM formation.

Influence of r. h. on plant survival and EM formation

At 45 % r. h., none of the control plants survived, while *P. croceum* provided resistance to most microcuttings (Table 2). Plant survival was much higher under 95 % r. h., but in this case as well, inoculation was beneficial (Table 2). In the three treatments (control/r. h. 95, Pilo/r. h. 95 and Pilo/r. h. 45) the surviving plants developed a 2nd SF after week 6–7. EM formation was monitored over the whole experiment, and three types of behaviour were observed: (i) no EM formation at the end of the assay; (ii) EM formation after the 2nd SF, that is, after week 6–7; (iii); EM formation already after the 1st SF, that is, between weeks 4 and 6–7. The distribution into these three categories of behaviour revealed an accelerated onset of EM formation under reduced r. h. (Table 2). In contrast, under 95 % r. h. more than half of the plants did not form any EM, and the minority of plants that formed EM did so only after the 2nd SF. EM formation was also higher under reduced r. h. con-

Table 2. Survival of plants and EM formation in microcuttings of *Quercus robur* inoculated with *Piloderma croceum* and grown at two levels of relative humidity (r. h.) compared to uninoculated plants (control). EM formation was assessed after formation of the 1st and the 2nd shoot growth flush (SF), respectively incubation week 6–7 and 9–10.

Treatment/r. h.	Surviving plants	Plants not forming EM		Plants forming EM after the 2nd SF		Plants forming EM after the 1st SF	
		number	percent	number	percent	number	percent
Pilo/r. h. 95	11/12	6	54.5 %	4	36.3 %	1	9.1 %
Control/r. h.95	6/12	–	–	–	–	–	–
Pilo/r. h. 45	9/12	1	11.1 %	3	33.3 %	5	55.5 %
Control/r. h. 45	0/12	–	–	–	–	–	–

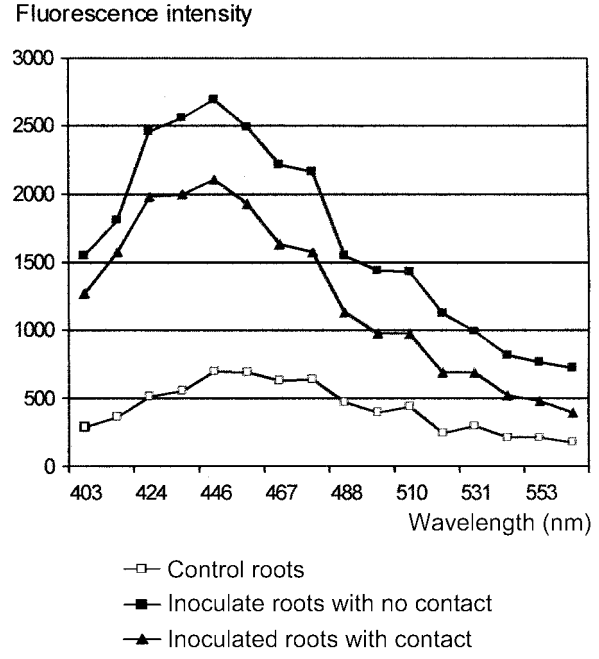

Figure 2. Spectral emission values of rhizodermic cells from short roots of *Quercus robur* microcuttings after stimulation with UV laser at 364 nm: Comparison of autofluorescence by confocal laser scanning microscopy in control roots and roots pre-mycorrhizal with *Piloderma croceum* and having or not having contact to fungal hyphae.

ditions, and in this case it remained much lower than in the case of IAA addition to the medium. However, the mycorrhization rates were highly variable and, due to the reduced number of plants in some of the distinguished categories, statistical analysis of the observed tendencies were not possible.

CLSM control of the pre-mycorrhizal state

The CLSM observations revealed no fungal structure in SR classified as pre-mycorrhizal. In inoculated plants, the wall of the rhizodermic root cells exhibited a strong autofluorescence, which was barely detectable in SR of control plants. Spectral analysis revealed five intensity maxima and the highest difference of fluorescence intensity was observed at 446 nm (Fig. 2). Fluorescence was in the same order of magnitude in SR having direct contact to the mycelial inoculum and in SR distant from any growing hyphae. Both SR categories had been distinguished by staining fungal hyphae with blue cotton.

In vivo Chl fluorescence (Figures 3 A, B)

In vivo Chl fluorescence was measured in the 2nd experiment after the 1st SF (week 6–7) and the 2nd SF (week 9–10). As uninoculated plants did not survive when incubated at 45 % r. h., only the control/r. h.95, Pilo/r. h.95 and Pilo/r. h.45 treatments were considered. An effect of r. h. itself could only be observed in week 6–7. At this stage, significantly higher values of Fv/Fm and ΦPSII were noticed in the Pilo/r. h.45-treatment compared to control/r. h.95 and Pilo/r. h.95 treatments (Fig. 3 A). No difference was found concerning qE. The effects at the end of the experiment were not related to r. h., but to inoculation. In general, inoculated plants maintained equivalent levels of Fv/Fm, ΦPSII, and qE, as in week 6–7, while for control plants, Fv/Fm and ΦPSII significantly decreased and qE was enhanced.

In order to better characterize this inoculation effect, the results were re-analysed by grouping the plants into the four categories according to EM formation (cf. Table 2): (i) uninoculated plants (only control/r. h.95-treatment); (ii) inoculated plants that did not form any EM within 10 week and which were incubated at 95 % r. h. except for one exemplar; (iii) inoculated plants that formed EM only after the 2nd SF and from which approximately equal individual numbers had been incubated at 95 and at 45 % r. h.; (iv) inoculated plants which already formed EM after the 1st SF, and which were incubated at 45 % r. h. expect one exemplar (Fig. 3 B). After the 1st SF, Fv/Fm approached 0.8 and ΦPSII was significantly higher in both plant groups that were able to form EM (iii and iv). No significant difference concerning qE was noticed between the four plant categories in week 6–7 (Fig. 3 B). At the end of the assay, Fv/Fm and ΦPSII were decreased and qE increased in control plants (Fig. 3 B), but was maintained at the same level in all three groups of inoculated plants.

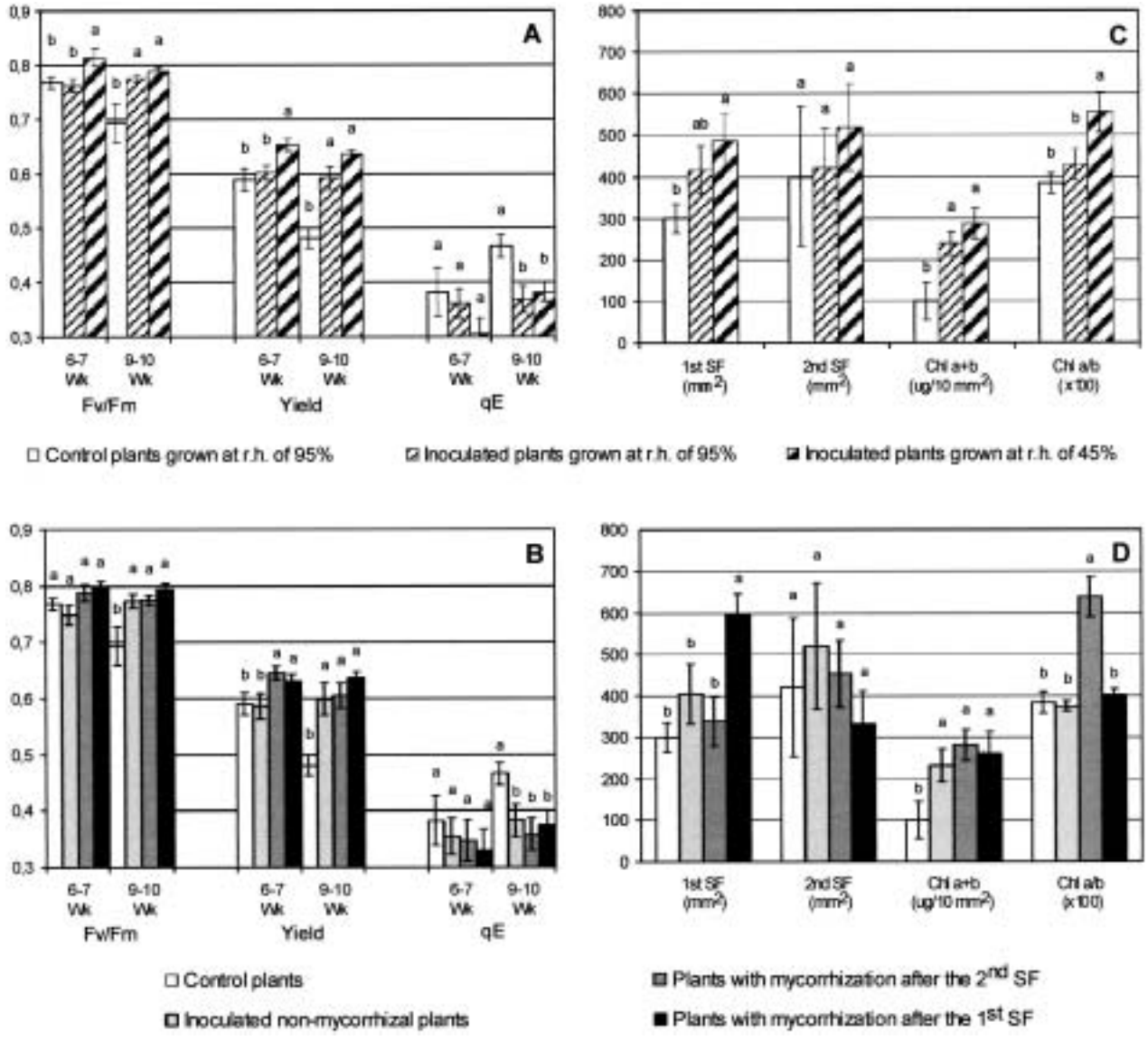

Figure 3. Physiological incidence of relative humidity (r. h.) (**A** and **C**) and of the onset on EM formation (**B** and **D**) in a mycorrhization system with microcuttings of *Quercus robur* inoculated with *Piloderma croceum*. In **A** and **B**, *in vivo* chlorophyll fluorescence was measured after the 1st shoot flush (SF) at week 6–7 and after the 2nd SF at week 9–10 (Fv/Fm, optimal quantum yield; yield (ϕPSII), effective quantum yield; qE, non-photochemical quenching). In **C** and **D**, total Chl *a* + *b* content, Chl *a/b*, as well as the total leaf area produced by each SF were measured at the end of the assay.

Leaf surface, Chl *a* + *b* and Chl *a/b* (Figures 3 C, D)

The leaf area produced by each SF, Chl *a* + *b* content, and the Chl *a/b* ratio were measured at the end of the experiment. Comparing the treatments control/r. h. 95, Pilo/r. h. 95, and Pilo/r. h. 45 showed differences in the final area of the leaves produced during the 1st SF (Fig. 3 C). This effect apparently resulted from a synergy between inoculation and reduced r. h., as the highest area was significantly produced in the Pilo/r. h. 45-treatment, while in Pilo/r. h. 95, the area increase was intermediate. Concerning the chlorophyll analyses, a similarly mixed influence of r. h. and inoculation was observed for Chl *a/b*, although here, both treatments at 95 % r. h. had a significantly reduced ratio. In contrast, a clear inoculation rather than an r. h. effect was detected for the Chl *a* + *b* concentration, which was increased in both inoculated treatments (Fig. 3 C).

The same data were compared among the four plant groups distinguished on the basis of their EM formation (cf. Table 2). It appeared that in the plant group that formed EM after the 1st SF, the final area of the leaves initiated during this flush increased significantly compared to the other three plant

groups (Fig. 3D). Concerning the chlorophyll values, a significant and equally high Chl *a* + *b* concentration was observed in all inoculated plants independent of whether they formed EM or not, while for the Chl *a/b*, a higher value was only noticed in plants that formed EM after the 2nd SF.

Discussion

In our previous work on pre-mycorrhizal effects of *P. croceum*, the oak microcuttings did not develop synchronously, and growth dynamics of each individual had to be analysed separately (Herrmann et al. 1998). In the present assays, maintaining high moisture around the shoot during transfer into the mycorrhization system allows one, not only to lower the material loss, but leads also to an earlier and synchronized 1st SF. In Pilo-treatments, this amelioration often led to formation of a 2nd SF. Despite this modification, the pre-mycorrhizal morphological effects were maintained, with an additional increase of the dry weight and thus a related lack of measurable effects on SLA.

The Pilo/AC and Pilo/IAA treatments were initiated to accelerate EM formation. They confirmed former observations that AC (Kottke et al. 1987, Roth-Bejerano et al. 1990) and IAA (Slankis 1973, Gay et al. 1994, Rudawska and Kieliszewska-Rokicka 1997, Karabaghli-Degron et al. 1998) enhance mycorrhization. In our system, both substances accelerated the mycorrhization onset by *P. croceum* by about 4 wks. AC is known for its ability to adsorb different types of substances that could also inhibit mycorrhization (see Duclos and Fortin 1983, Roth-Bejerano et al. 1990), but the present experiments were not designed to elucidate this effect. IAA, the second substance tested in this study, has a well-established effect on mycorrhization, and the involved cytological (Rincón et al. 2001, Laurans et al. 2001) and molecular (Charvet-Candela et al. 2002) mechanisms are currently under investigation. In our experiment, we observed two IAA effects, namely, an earlier mycorrhization and an extension of the spatial distribution of the formed EM. The latter effect is consistent with the suggestion by Rudawska and Kieliszewska-Rokicka (1997) that a certain level of fungal-released IAA is needed to induce EM symbiosis. In the Pilo-treatment, the minimal level would only be reached in the direct vicinity of the inoculation points, where mycelium density is high. In the Pilo/IAA-treatment, IAA addition would also allow EM formation far from the inoculation plugs by diffuse growing mycelium.

Almost all growth-promoting effects of *P. croceum* were depressed when an earlier mycorrhization was obtained through addition of AC or IAA. This could be interpreted as a consequence of exhausted nutrients in our system. In the case of early EM formation, this would result in a C depletion for the plant. A competitive C and N allocation leading to an intermediary reduction of plants growth after EM formation was shown on pine seedlings (Colpaert et al. 1996). A similar reaction might occur here, as *Piloderma* is known to be a mycobiont with high C demand (Hutchinson and Piché 1995). It is, however, important to note that in our experiments, in contrast to the observation of Colpaert et al. (1996), growth depression of early EM forming microcuttings was not coupled with an enhanced growth of extra-radical mycelium. This remained scarce in all treatments. Whether these results reflect the situation in nature or occur only under our experimental conditions remains to be determined.

In the additional manipulative extension of the system, r. h. around the shoot part was reduced to 45 % r. h. Microcuttings alone could not resist at this humidity, but when inoculated, most survived. Mycorrhizal plants are known to better resist soil drought (Dosskey et al. 1991 and citations therein). Apparently, such resistance is not related to an enhanced tolerance, but to an increased water conductance (Mushin and Zwiazek 2002, Querejeta et al. 2003), which also maintains photosynthetic activity (Morte et al. 2000). In our case, the drought protection was related to inoculation and not to EM formation itself, indicating early physiological effects of *P. croceum* in the pre-mycorrhizal stage. On the other hand, EM formation was accelerated under reduced r. h. The importance of adequate air humidity for EM formation is only considered in a few studies (e.g., Estrada et al. 1993). Soil drought was recently reported to not influence the number of EM (Fransson et al. 2000, Shi et al. 2002). However, the authors also mention shifts in the EM community, confirming that some mycobionts display a better EM formation under drought (Lambamedi et al. 1992).

For healthy plants, values for Fv/Fm over 0.8 are typical. The Fv/Fm levels measured in our experiments were usually slightly lower, indicating that plants are under suboptimal conditions in the mycorrhization system. In uninoculated plants, Fv/Fm levels under 0.7 toward the end of the experiment demonstrate that photosynthesis is suboptimal. This is also expressed by the lower Chl *a* + *b* content and is consistent with the decreased ΦPSII value. In parallel, the qE was increased, which is a sign of protective reaction against formation of oxidizing free radicals in a stress situation (Li et al. 2000). Inoculation with *P. croceum* had a protective effect against the increasing stress at the end of the experiment, and this effect was comparable in plants forming EM early or late, or in plants which stayed pre-mycorrhizal until the end of the experiment. This beneficial inoculation effect, also expressed by the Chl content and the decreased autofluorescence of SR rhizodermic cells of uninoculated plants in CSLM, clearly demonstrates physiologically beneficial effects of *P. croceum prior* to any differentiation of a mycorrhizal interface. This opens the opportunity to study early events of the interaction before mycorrhization reprograms the development in both organisms. The fact that the Chl *a/b* ratio is not stable in all inoculated treatments and in all plant categories separated on the basis of the EM formation dynamic, and the fact that plant development was highly affected by the delay until onset of EM formation, also demonstrates that the physiology in pre-mycorrhizal and mycorrhizal stages must differ.

In particular, variations of the Chl *a/b* ratios to the same magnitude as the ones observed here have been reported to reflect photosynthetic acclimation to varying growth irradiance (Baily et al. 2001).

To some extent, the presented data open a discussion on the interaction tuning involved in moving from the pre-mycorrhizal to the mycorrhizal stage. From this point of view, the results of the experiment with changing r. h. are of special interest. Especially noteworthy is the finding that plants that formed EM in our system showed a significantly higher ΦPSII, which is correlated to a higher photosynthetic carbon assimilation (Seaton and Walker 1990). Only vigorous plants with the highest leaves production in the 1st SF formed EM early. This suggests that, in accordance with the known higher C demand of late stage fungi (Hutchinson and Piché 1995), these would only differentiate EM interface on plants having a sufficient level of photoassimilation. In underdeveloped plants, the fungus would first proceed to stimulate growth and C assimilation during a prolonged pre-mycorrhizal phase. Forced reduction of this stage, for example with IAA and AC, would result in an unbalance for the system leading to plant growth reduction.

Acknowledgements. The present work was supported by the Deutsche Forschungsgemeinschaft (DFG, BU 941/1-1; 1-2 and 1-3). We are indebted to Dr. Solveig Hehl from the Carl Zeiss (Jena, Germany) for the CLSM facilities, to Barbara Bratfisch and Bernd Mäusezahl for technical assistance, and to anonymous colleagues for useful critics and suggestions on a first version of this article. Best thanks to Dr. Thomas Pfannschmidt for constructive discussion. Dr. Michael Kaldorf kindly revised the manuscript.

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