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Original article

Growth, carbon dioxide assimilation capacity and water-use efficiency of *Pinus pinea* L seedlings inoculated with different ectomycorrhizal fungi

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Summary – Three months after sowing, seedlings of *Pinus pinea* L grown in a nursery on a perlite-Sphagnum peat mixture were inoculated with different ectomycorrhizal fungi: *Rhizopogon roseolus* and *Suillus collinitus* (2 strains: 1 and 2). The growth medium was maintained well-watered and was fertilized with a dilute Coïc-Lesaint (N, P, K; 3, 2, 7.5 g l^{-1}) solution. Fertilization was stopped at the end of the first growing season (October) and growth and gas exchange parameters of the seedlings were assessed prior to the beginning of their second growth season. Inoculation with the 2 *S collinitus* strains led to the greatest plant elongation, but biomass growth was greatest with *R roseolus*. Whole plant CO_2 assimilation capacity in the *R roseolus* treatment was 1.83 times that in the control treatment and 1.38 times that in the *S collinitus* 2 treatment. The plants infected by *R roseolus* and *S collinitus* 1 had similar whole plant CO_2 assimilation capacities, but root and total plant biomass were significantly higher in the *R roseolus* treatment. This difference could be due partly to greater carbon diversion by the fungal associate in the case of *S collinitus* 1. Mean water-use efficiency (*WUE* = CO_2 assimilation rate/transpiration rate) of the inoculated seedlings (pooled mean value 7.29 mol $kmol^{-1}$) was significantly ($P < 0.05$) higher than that of the controls (5.06 mol $kmol^{-1}$). This is linked to the double tendency, neither being statistically significant, of the infected plants to exhibit higher CO_2 assimilation rates and lower transpiration rates than the controls.

***Pinus pinea* / ectomycorrhiza / growth / CO_2 assimilation / water-use efficiency**

Résumé – Croissance, capacité d'assimilation de CO_2 et efficience de l'eau de plants de *Pinus pinea* L inoculés par différents champignons ectomycorhiziens. *Des plants de* Pinus pinea *L âgés de 3 mois et cultivés en pépinière sur un subtrat à base de perlite et de*

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tourbe blonde de Sphaigne, ont été inoculés avec différents champignons ectomycorhiziens : Rhizopogon roseolus *et* Suillus collinitus *(2 souches, 1 et 2). Le substrat était maintenu en permanence à un niveau hydrique non limitant et était fertilisé à l'aide d'une solution diluée de type Coïc-Lesaint (N, P, K ; 3, 2, 7.5 g* l^{-1}*). La fertilisation a été interrompue à la fin de la première saison de végétation des plants (octobre). On a mesuré les caractéristiques de taille et de biomasse des plants ainsi que les échanges gazeux de* CO_2 *et* H_2O *avant le début de la seconde saison de végétation (février). La hauteur des plants était la plus forte pour les plants inoculés avec les 2 souches de* S collinitus, *mais la croissance pondérale état la plus élevée dans le cas des plants inoculés avec* R roseolus. *La capacité totale d'assimilation de* CO_2 *des plants inoculés par* R roseolus *représentait 183 % par rapport à la capacité des plants non mycorhizés et 138 % par rapport au traitement* S collinitus *2. Les plants inoculés par* R roseolus *et* S collinitus *1 étaient caractérisés par des capacités totales d'assimilation de* CO_2 *similaires, mais la biomasse racinaire ainsi que la biomasse totale des plants étaient plus élevées dans le cas du traitement* R roseolus. *Cette différence pourrait être liée, du moins partiellement, à une utilisation plus importante du carbone assimilé, par l'associé fongique, dans le cas de* S collinitus *1. L'efficience de l'eau (*WUE = *taux d'assimilation de* CO_2*/taux de transpiration) moyenne des plants mycorhizés (valeur moyenne générale 7.29 mol* $kmol^{-1}$*) était significativement supérieure (*$P < 0.05$*) à celle des plants non mycorhizés (5.06 mol* $kmol^{-1}$*). Cela est à relier à la double tendance, non statistiquement significative pour chacune des 2 composantes considérées séparément, des plants mycorhizés à présenter des valeurs moyennes de taux d'assimilation de* CO_2 *(A) plus élevées et de taux de transpiration (E) plus faibles que les plants non mycorhizés.*

Pinus pinea / *ectomycorhize* / *croissance* / *assimilation de* CO_2 / *efficience de l'eau*

INTRODUCTION

Ectomycorrhizal infection is generally accompanied by alterations in the host plant CO_2 assimilation capacity with effects on both leaf area and assimilation rate (A) (Ekwebelam and Reid, 1983; Harley and Smith, 1983; Paul *et al*, 1985; Jones and Hutchinson, 1988). Part of the C fixed, 4% to 17% as reported by Paul *et al* (1985), is diverted towards the fungal associate to meet its metabolic requirements (Martin *et al*, 1987). Despite this specific C cost, the increase of CO_2 assimilation provided by mycorrhizal infection is often sufficient to achieve enhanced plant growth (Ekwelebam and Reid, 1983; Harley and Smith, 1983). The mechanisms most commonly proposed for explaining enhanced photosynthesis in mycorrhizal plants involve aspects of P and N nutrition, source-sink regulation and hormones (Harley and Smith, 1983).

Some authors have also shown that fungi can directly affect plant water relations. Duddrige *et al* (1980) demonstrated that the mycelium of *Suillus bovinus* could absorb tritiated water which was then transported through the mycelial network to the host plant. Brownlee *et al* (1983) and Boyd *et al* (1986) found that physiologically significant quantities of water were being transported through such mycelia, since the cutting of mycelial strands connecting plants to moist peat led to a rapid decrease in leaf water potential, transpiration and photosynthesis of the host plant. Jones and Hutchinson (1988) observed higher transpiration rates in *Betula papyrifera* seedlings inoculated with *Scleroderma flavidum* than in non inoculated seedlings.

Little attention has been paid to examining the effects of mycorrhizas on

water-use efficiency (WUE = ratio of CO_2 assimilation to transpiration) of host plants, yet WUE constitutes a major aspect of plant growth limitation in dry conditions and is subject to physiological regulation involving ontogenic adaptation (Wong *et al*, 1985; Guehl *et al*, 1988) and to short term changes in response to environmental factors (Cowan and Farquhar, 1977; Guehl and Aussenac, 1987).

The purpose of the present study was to assess growth, CO_2 assimilation capacity and WUE in different ectomycorrhizal *Pinus pinea* seedlings under non-limiting water supply conditions.

MATERIALS AND METHODS

Plant inoculation and growing conditions

Isolates of the following ectomycorrhizal fungi were obtained from basidiocarps harvested in a *Pinus pinea* stand established on a calcareous sandy soil (La Grande Motte, Hérault, France): *Suillus collinitus* (ss. Flury *nec* ss. Sr.; 2 strains, 1 and 2) and *Rhizopogon roseolus* (Corda *in* Sturn). Mycelial inocula were grown in aseptic conditions for 7 weeks on a perlite-peat mixture (4:1, v/v) moistened with a Pachlewski (Pachlewski, 1967) solution.

At the end of the winter 1986, seeds of *Pinus pinea* L. were germinated in a heated greenhouse on a perlite-Sphagnum peat mixture (1:1, v/v) in 500 cm^3 anti-coiling containers with 2 easily removable and replaceable sides (Riedacker, 1978). Three months after sowing, each seedling was inoculated with 50 ml inoculum brought into contact with the roots by temporarily removing the 2 sides of the containers. The growth medium was maintained in a well watered state (pF < 1.5) during the whole growth period. Before inoculation the containers were watered with water at pH 8.3, which adjusted the growth medium to pH 6.2. After inoculation the containers were fertilized every other week with a dilute Coïc-Lesaint solution containing major (N, P, K; 3, 2, 7.5 10^{-2}g l^{-1}) and trace elements. Uninoculated and inoculated plants received the same fertilization (Moussain *et al*, 1988).

After inoculation, the plants were grown outside in uniform nursery conditions in Southern France (mediterranean climate) with 60% of the natural incident radiation at shoot level. Five months after inoculation the root colonization by the mycorrhizal fungi was assessed. The proportion of plants colonized by the inoculated fungi was 91, 78 and 9% in *S collinitus* 1 and 2 and *R roseolus*, respectively. The mycorrhizal index (index ranging from 0 to 5 and representing the frequency of mycorrhizal tips *versus* the total number of root apices) of the colonized plants was 3.0 in the 2 treatments inoculated with *S collinitus* and 2.5 in the *R roseolus* treatment, control plants were nonmycorrhizal.

At the end of the growing season, in October 1986, fertilization was stopped and the plants were left in full sunlight conditions as is usual in forestry practice. In February 1987, 30 plants (only mycorrhizal plants for the 3 inoculated treatments and nonmycorrhizal control plants) were taken at random within each of the 4 treatments and transferred to Nancy (Northeastern France) where their gas exchange, biomass and size characteristics were assessed in controlled standardized conditions. Gas exchange measurements made at this time of year provide an estimation of the physiological status of the plant just prior to planting-out (Guehl *et al*, 1989). All the plants of the different treatments were dormant at the period of gas exchange measurements.

Gas exchange and growth measurements

Carbon dioxide and H_2O gas exchange were measured with an open gas exchange system consisting of 3 assimilation chambers (28 × 15 × 33 cm^3) connected in parallel and through which air was passed at a flow rate of 150 l h^{-1}. Air temperature in the chambers was maintained at 22.0 ± 0.5 °C. Photosynthetic photon flux density (400–700 nm) at shoot level was 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and was provided by high pressure sodium lamps (Sont, Philips). The CO_2 molar fraction of the air entering the chamber was measured continuously with an ADC-225 MK2 IR-

GA and was adjusted to 350 ± 5 $Pa \cdot MPa^{-1}$. The difference in CO_2 molar fraction between the airs entering and leaving the chambers was measured with a differential ADC-225 MK3 IRGA, alternately for periods of 3 min for the 3 chambers by means of an automated switching system. The dewpoint of the airs entering the chambers and of the different airs leaving the chambers was measured concurrently with the CO_2 measurements with a dewpoint hygrometer (System 1 100 DP, General Eastern). The air entering the chambers was maintained at 1 380 ± 40 Pa water vapour pressure, leading to leaf-to-air vapour molar fraction differences (ΔW) in the chambers of between 7.0 and 10.0 $Pa \cdot kPa^{-1}$, depending on the intensity of plant transpiration. Because transpiration, in turn, depends on ΔW, and in order to permit comparisons between plants, corrections were made using appropriate formulae (Caemmerer and Farquhar, 1981) to set each value to a constant ΔW of 8.5 $Pa \cdot kPa^{-1}$. Gas exchange calculations were made on a needle dry-weight basis, giving CO_2 assimilation rates (A) in $nmol \cdot g^{-1} \cdot s^{-1}$ and transpiration rates (E) in $\mu mol \cdot g^{-1} \cdot s^{-1}$ Measurements of gas exchange rates were taken as the steady-state values after a period of 1–2 h adjustment by the seedlings to the assimilation chamber conditions.

After gas exchange measurements, the plants were separated into their different components (whole root system, needles, nonphotosynthetic aerial parts), oven dried at 80 °C for 48 h, and the different dry-weights were assessed. There were 9 replicates for the uninoculated treatment (controls), 13 for the *R roseolus* treatment which had the highest biomass growth, and 6 for each of the 2 *S collinitus* treatments. In addition, 5 *S collinitus* 2 infected plants were used only for whole plant gas exchange measurements. In 5 individuals of each of the controls *R roseolus*, and *S collinitus* 1 treatments of the total projected needle area of the plants was also determined with an image analysis system (TAS) in order to assess the specific dry-weight of the needles (dry weight/area ratio). For these different types of measurements, samples were taken randomly within the different treatments. For all the variables assessed, differences between treatments were tested by means of Scheffe's multiple comparison test.

RESULTS

Size and biomass growth

Maximum height growth of the plants (table I) occurred with the treatments *S collinitus* 1 and *S collinitus* 2 with values significantly greater than those of the control treatment. Growth in height of the *R roseolus* plants was not

Table I. Growth characteristics of one year old *Pinus pinea* seedlings inoculated with ectomycorrhizal fungi. Means within a column not sharing a common letter differ significantly ($P < 0.05$) by Scheffe's multiple comparison test.

Treatment and nb. of replicates	*Root collar diameter* (mm)	*Height* (mm)	*Root dry weight* (g)	*Shoot dry weight* (g)	*Total dry weight* (g)	*Root : shoot ratio*	*Needle : shoot ratio*
Control (9)	4.24 a	172 b	1.47 b	1.83 b	3.30 b	0.81 a	0,66 a
R roseolus (13)	4.48 a	206 a b	1.98 a	2.49 a	4.47 a	0.81 a	0,66 a
S collinitus 1 (6)	4.05 a	220 a	1.18 b	2.01 a b	3.19 b	0.59 b	0,67 a
S collinitus 2(6)	4.36 a	210 a	1.55 a b	2.14 a b	3.70 a b	0.72 a b	0,65 a

significantly different from that of the controls. No significant treatment effects were found for root collar diameter of the plants. The highest total dry weight occurred in the treatment *R roseolus*, with a value significantly greater than those of the *S collinitus* 1 and the control treatments, but not than that of *S collinitus* 2. At the individual level, total plant dry weight (*TDW*, g) was poorly correlated with plant height (*H*, mm) (r = 0.32, n = 34, $P < 0.05$), and better correlated with root collar diameter (*D*, mm) (*TDW* = 1.33D–1.96, $r = 0.78$, n = 34, $P < 0.05$) and with $H \times D^2$ ($TDW = 6.35\ 10^{-4}\ HD^2 + 1.369$, r = 0.83, $n = 34$, $P < 0.05$). Significant differences between treatments were found for the root/shoot ratio of the plants, with *S collinitus* 1 having the lowest value (0.59). This low value was primarily due to low root dry weight in the *S collinitus* 1 treatment, the estimated mean value being even less than in the control plants. The plants infected by *S collinitus* 2 and *R roseolus* had ratios not significantly different from that of the controls.

The *R roseolus* infected plants had needle dry weights and areas significantly greater than those of the control plants (tables I and II), the values for the 2 treatments inoculated with the *S collinitus* strains being intermediate. There was no treatment effect on needle/shoot ratio (table I). The needles of the mycorrhizal plants had lower specific needle dry weights (table II, *S collinitus* 2 was not measured) than the control plants.

Carbon dioxide assimilation capacity

There was no significant treatment effect relative to A (table III) though large differences were measured among treatments. However, significant treatment effects were noticed relative to whole plant CO_2 assimilation capacity, the capacity of the *R roseolus* plants (50.5 nmol·s^{-1}) being 1.81 times greater than that of the control plants and 1.38 times greater than that of the *S collinitus* 2 infected plants. There was no close relationship between the mean treatment values of total plant dry weight (table I) and whole plant CO_2 assimilation capacity measured at the end of the growing season (table III), since the *S collinitus* 2 infected plants had higher dry weights than the *S collinitus* 1 infected plants, but lower CO_2 assimilation capacities.

Table II. Needle area, dry weight and specific dry weight of 1-year old *Pinus pinea* seedlings inoculated with ectomycorrhizal fungi. Means within a column not sharing a common letter differ significantly ($P < 0.05$) by Scheffe's multiple comparison test.

Treatment and no. of replicates	*Needle area* (10^{-2} m^2)	*Needle dry weight* (g)	*Specific needle dry weight* (10^2 g· m^{-2})
Control (5)	0.83 b	1.02 b	1.22 a
R. roseolus (5)	1.38 a	1.62 a	1.18 a b
S. collinitus 1 (5)	1.15 a b	1.34 a b	1.17 b

Table III. CO_2 and H_2O gas exchange parameters of one year old *Pinus pinea* seedlings inoculated with ectomycorrhizal fungi. Means within a columnn not sharing a common letter differ significantly ($P < 0.05$) by Scheffe's multiple comparison test.

Treatment and No. of replicates	*Needle dry weight (g)*	*CO_2 assimilation rate (A, nmol· g^{-1}· s^{-1})*	*Transpiration rate E, µmol g^{-1}· s^{-1}*	*Total plant CO_2 assimi-lation (nmol ·s^{-1})*	*Water use efficiency A/E_3 (10^{-3})*
Control (9)	1.21 b	23.3 a	4.62 a	27.9 b	5.06 b
R roseolus (13)	1.65 a	30.8 a	4.47 a	50.5 a	7.21 a
S collinitus 1 (6)	1.34 a b	32.4 a	4.24 a	44.0 a b	7.63 a
S collinitus 2 (6)	1.40 a b	27.3 a	3.89 a	36.5 b	7.04 a

In fig 1a the individual total dry weight values of the plants are plotted against their total CO_2 assimilation capacities; there was only a weak linkage between these 2 variables. No relationship was observed between the total dry weight of the plants and their A values (fig 1b), thus indicating that the weak dependence noticed in fig 1a is attributable solely to the correlation between total dry weight and needle dry weight of the plants (fig 1c).

Water-use efficiency

The mean transpiration rates of the mycorrhizal plants (table III) were not significantly different from those of the control plants. However, *WUE* in the control plants (5.06 mol $kmol^{-1}$) was markedly and significantly lower than that of the infected plants (pooled mean value = 7.29 mol $kmol^{-1}$). This is to be associated with the double tendency, neither being statistically significant, of the infected plants to exhibit higher A and lower E values (table III) than the controls. Fig 2a gives an interesting insight into the *WUE* regulation at the individual level: the individual variability of the points rela-

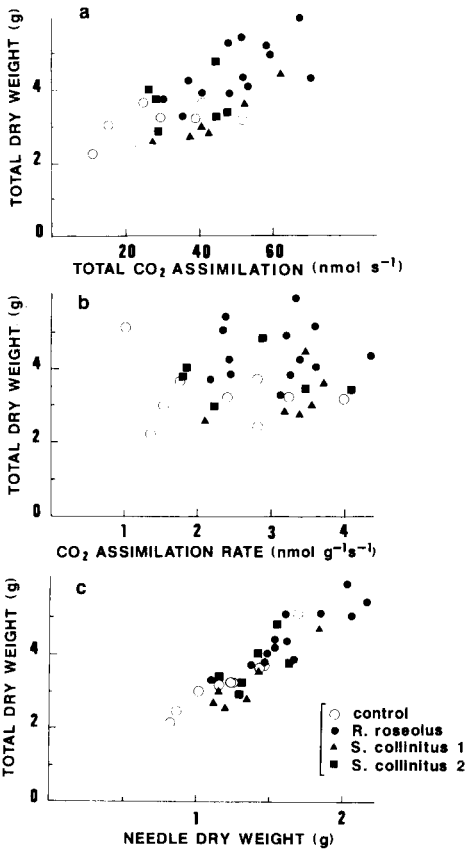

Fig 1a, b, c. Individual values of total dry-matter weight of *Pinus pinea* seedlings inoculated with different ectomycorrhizal fungi plotted against (a) their total assimilation capacity, (b) their CO_2 assimilation rate (A) and (c) their needle dry weight.

tive to the infected treatments (all treatments pooled) appears to be ordered along a unique linear relationship expressing almost proportionally between CO_2 assimilation and transpiration (constant *WUE*), since the Y-axis intercept of the regression line ($Y = 5.57X+6.50$, $r = 0.82$) was not signifi-

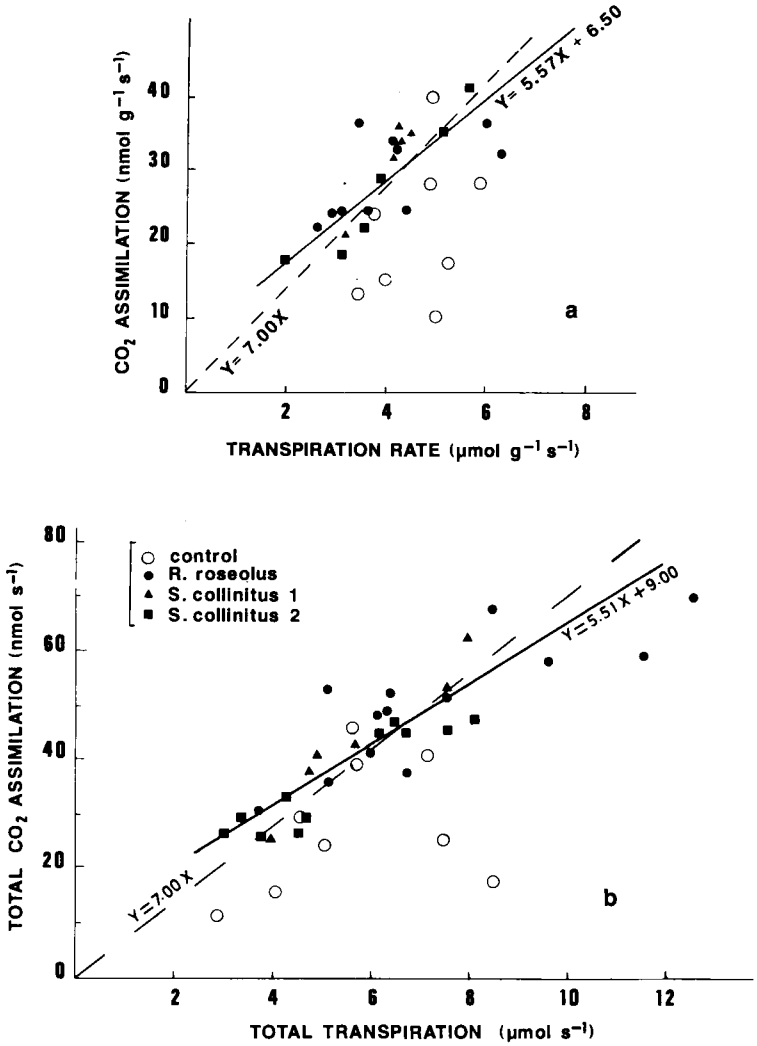

Fig 2a, b. (a) Carbon dioxide assimilation rate (A) of *Pinus pinea* seedlings inoculated with different ectomycorrhizal fungi in relation to transpiration rate (E) ; (b), total plant CO_2 assimilation in relation to total transpiration. Regression lines drawn in the figure refer to inoculated treatments only ; full line, orthogonal least square regression ; broken line, regression line forced through the origin. *S collinitus* 2, $n = 6$ in fig 2a and $n = 11$ in fig 2b.

cantly different from the origin. A regression line forced through the origin ($Y = 7.00\ X$) has also been represented in fig 2a. The control plants did not exhibit such a control of *WUE*: 4 individuals out of 9 had *WUE* values identical to those of the inoculated plants, but 5 individuals had markedly lower *WUE* values, thus providing a clear discrimination between uninoculated and inoculated plants in figure 2a. The data in fig 2b show the same discrimination in a total plant assimilation *vs* transpiration graph.

DISCUSSION

Ectomycorrhizal infection by *R roseolus* had a significant positive effect on biomass growth of *Pinus pinea* seedling raised over 1 growing season in nursery conditions, whereas there was no enhancing effect in seedlings infected by the 2 *S collinitus* strains. Ekwebelam and Reid (1983), Harley and Smith (1983), Tyminska *et al* (1986) have reported similar results indicating that the extent to which growth was affected by the infection will depend on the fungal species and strain used as mycobiont. It should be stressed here that mycorrhizal infection had differential effects on shoot height growth and biomass growth, since the *S collinitus* 1 treatment produced the tallest plants without increasing the total plant biomass compared to the control plants. This can be somewhat misleading in field experiments in which height growth is often taken as an indicator of plant vigour.

The present study also provides some information regarding the biomass distribution between the different plant components and its modulation by mycorrhizal infection. In their review paper, Harley and Smith (1983) reported that in most cases ectomycorrhizal infection will reduce the root: shoot ratio. These authors noted that in the examples where the root/shoot ratio was found to be slightly enhanced by infection, the increase may be accounted for by the fungal sheath biomass if this were to comprise 20% of the weight of the roots. Our results (table I) are consistent with these general findings, the root/shoot ratio of the infected plants being lower than (*S collinitus* 1 treatment) or equal to (*R roseolus* and *S collinitus* 2 treatments) that of the control plants.

Whole plant CO_2 assimilation was highest in the *R roseolus* infected plants. Relatively high (though not significantly different from the controls) values were also found in the *S collinitus* 1 and 2 treatments, but biomass- and especially root biomass-growth was not enhanced in these latter treatments as compared to the controls. Whole plant CO_2 assimilation did not exhibit significant differences between the *R roseolus* and *S collinitus* 1 treatments, but root and whole plant biomass were lower in the *S collinitus* 1 treatment. Differential seasonal courses of growth and CO_2 assimilation cannot be eliminated as an explanation for these discrepancies. These results may also suggest that in the *S collinitus* infected plants C allocation to the vegetative sinks of the host plant could be curtailed because of important C diversion to the mycobiont metabolic requirements (Paul *et al*, 1985; Martin *et al*, 1987). Further evidence for such an interpretation is provided by the low specific needle dry weights found in the *S collinitus* 1 plants (table II), probably reflecting low needle carbohydrate contents (Ehret and Jolliffe, 1985) and high C sink activity (Harley and Smith,

1983). The greater growth efficiency of the *R roseolus* infected seedlings could be linked to lower fungal C requirements (Harley and Smith, 1983; Paul *et al* 1985; Tyminska *et al*, 1986; Marshall and Perry, 1987) *R roseolus* appears to be a very efficient fungus, worth selecting for practical applications.

Enhanced whole plant CO_2 assimilation capacity at the end of the growing season in the inoculated seedlings was probably due to higher values of both needle dry-weight and A (table III), though the differences in assimilation rate were not statistically significant. In the absence of foliar nutrient determinations, it is not possible to assess here whether these effects and the large variability of A and E within the treatments are due to varying N or P nutritional status or to other factors.

Regardless of the physiological processes responsible for the high variability of CO_2 assimilation both at the treatment (table III) and individual (fig 2) levels, CO_2 assimilation and transpiration of the infected seedlings, measured under standard conditions, were in nearly constant proportion (figure 2). Such a coupling, reflecting near constancy of *WUE*, has been reported for variations due to mineral nutrition (Wong *et al*, 1985; Guehl *et al*, 1989).

A main result of the present study is the observation of the absence of coupling between CO_2 assimilation and transpiration, as well as lower *WUE* in the control plants (fig 2). It might be suggested that this lack of stomatal control is linked to a low orthophosphate (Pi) level in the needles of the nonmycorrhizal plants. Mousain (unpublished results) found very low Pi concentrations in the needles of juvenile nonmycorrhizal *Pinus pinaster seedlings. Harris et al* (1983) found that in leaf discs of *Spinacia oleracea* low Pi led to a loss of stomatal control and wide stomatal apertures, while high Pi induced stomatal closure. In the same species, Herold (1978) observed that mannose and deoxyglucose induced wilting by metabolically sequestring Pi. Further investigations are required to test this hypothesis in the case of coniferous species.

The results obtained in the present study might be of relevance to forestry practice. Guehl *et al* (1989) have observed that whole plant CO_2 assimilation capacity was an important physiological determinant of survival after planting-out in *Cedrus atlantica* seedlings. Low CO_2 assimilation capacities, plus lower and more variable *WUE* in non-inoculated seedlings, may, at least partly, explain the poor survival and initial growth after planting-out commonly observed in different plantation systems around the world in non-inoculated as compared to inoculated seedlings (Marx *et al*, 1977; Le Tacon *et al*, 1987).

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