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Original article

Release of oxalate and protons by ectomycorrhizal fungi in response to P-deficiency and calcium carbonate in nutrient solution

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Abstract – The release of oxalate and H^+ by six ectomycorrhizal isolates (*Hebeloma cylindrosporum* 1 and 9, *Paxillus involutus*, *Suillus collinitus* 2 and 22, *Rhizopogon roseolus*), able to grow *in vitro* with NO_3^- as the sole source of N, was measured in response to orthophosphate (Pi) concentration (20, 100 and 500 µM Pi) and $CaCO_3$ (+500 µM Pi) in the solution. Without $CaCO_3$, all isolates excepted *H. cylindrosporum* ones released oxalate. For each isolate, oxalate production was not related to P-deficiency but was strongly enhanced by $CaCO_3$. Whatever the medium composition, *H. cylindrosporum* alkalinised the solution. Among oxalate-producing fungi, *S. collinitus* 22 and *R. roseolus* exhibited an important proton efflux that could be related to transport phenomena for oxalate excretion. $CaCO_3$ enhanced both oxalate and proton efflux, increasing the chemical action exerted by the fungi on the mineral.

oxalate production / pH / ectomycorrhizal fungi / growth / mineral nutrition

Résumé – Libération d'oxalate et de protons par les champignons mycorhiziens en réponse à une carence en P et en carbonate de calcium dans la solution nutritive. La libération d'oxalate et de protons par six isolats fongiques ectomycorhiziens (*Hebeloma cylindrosporum* 1 et 9, *Paxillus involutus*, *Suillus collinitus* 2 et 22, *Rhizopogon roseolus*), capables de se développer sur NO_3^- comme seule source d'azote, a été mesurée en présence de différentes concentrations en orthophosphate (Pi) (20,100 et 500 µM) et de $CaCO_3$ (+500 µM de Pi) dans le milieu de culture. En absence de $CaCO_3$, tous les isolats libèrent de l'oxalate, excepté ceux de *H. cylindrosporum*. Pour chaque isolat, la production d'oxalate ne dépend pas de la déficience en P mais est fortement augmentée par la présence de $CaCO_3$. Quelle que soit la composition du milieu, *H. cylindrosporum* alcalinise la solution. Parmi les isolats produisant de l'oxalate, *S. collinitus* 22 et *R. roseolus* présentent un fort efflux de protons qui pourrait être lié aux phénomènes de transport accompagnant l'excrétion d'oxalate. La présence de $CaCO_3$ stimule les efflux d'oxalate et de protons, augmentant ainsi l'action chimique exercée par les champignons sur le minéral.

production d'oxalate / pH / champignons ectomycorhiziens / croissance / nutrition minérale

1. INTRODUCTION

Many soil fungi are able to produce and excrete oxalate. However, as pointed out by Dutton and Evans [5] and Gadd [6], numerous studies were carried out on saprophyte or pathogenic fungi. In contrast, studies dealing with mycorrhizal fungi are fewer and restricted to some species such as the basidiomycete *Paxillus involutus.* In pure culture conditions, it was demonstrated that the different forms of N affect oxalate synthesis by this fungal species. Compared to ammonium, nitrate supply favours markedly oxalate production [7, 16]. Besides the effect of N source, bicarbonate ions also enhance oxalate production by *P. involutus* [16].

Released in the external medium, carboxylic acids will complex multivalent cations of insoluble minerals and thus promote their dissolution. This was observed for calcium phosphate [18] and gypsum [7], two minerals that were dissolved after culture of *P. involutus* grown in NO_3^- agar gel and releasing oxalate. Similarly, oxalate concentrations were measured in water extracted from peat after culture of *Pinus sylvestris* associated with *Suillus variegatus.* Variations in these concentrations were correlated to the dissolution of a fluorapatite used as the phosphorus source [26]. In forest soil conditions, oxalate production occurring in hyphal mats of the ectomycorrhizal fungi *Hysterangium crassum* [3, 8], *Hysterangium setchelli* and *Gautiera monticola* [9] was observed simultaneously with an intense weathering of soil minerals and increased concentrations of inorganic ions in soil solution. These data indicate that oxalate produced by ectomycorrhizal fungi can play an important role in the weathering of P-containing minerals, thus promoting desorption of solid phase P into the soil solution.

However, in natural conditions such as in calcareous soils, where nitrate is considered as the main N source, the magnitude

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of the release of organic anions and/or protons by ectomycorrhizal fungi can rely on other environmental factors such as P deficiency or presence of $CaCO_3$. Indeed, in plants, several studies showed that some species grown with nitrate are able to release carboxylates and protons in response to phosphorus deprivation [4, 11, 12, 21, 22] whereas others are not [21]. Such variability can also exist in ectomycorrhizal fungi but specific information is unavailable. Similarly, no information is available about the effect of the presence of $CaCO_3$ on the growth and the capacities of ectomycorrhizal fungi to release protons and carboxylic anions. Nevertheless, such information will be of great importance in determining the potential role of ectomycorrhizal symbiosis for P mobilization and subsequent P nutrition of the ectomycorrhizal host-plant in soil conditions. The objective of this work was therefore to quantify the release of oxalate and/or protons by six ectomycorrhizal isolates belonging to 4 species grown in pure culture in response to orthophosphate (Pi) supply level and $CaCO_3$ in the nutrient solution. The isolates, two of *Hebeloma cylindrosporum*, one of *Paxillus involutus*, two of *Suillus collinitus* and one of *Rhizopogon roseolus*, were chosen because of their ability to grow with NO_3^- as the sole source of N. The ability of these fungi to produce oxalate when associated with the host plant will be investigated in a further paper (Casarin et al., submitted).

2. MATERIALS AND METHODS

2.1. Fungal cultures

Fungal cultures were always isolated from sporocarps of corresponding species. Both isolates of *Hebeloma cylindrosporum* Romagn. (1 and 9) were obtained from sporocaps harvested in acidic sandy soil. Other fungal species, the isolate of *Paxillus involutus* (Batsch: Fr.) Fr, both isolates of *Suillus collinitus* (Fr.) Kuntze (2 and 22) and the isolate of *Rhizopogon roseolus* (Corda) Th. Fr. were obtained from sporocarps harvested in the organic horizon of a calcareous soil. Stock cultures were grown at 24 °C in the dark, in Petri dishes containing an agar (15 $g \cdot L^{-1}$) medium in the following nutrient solution (N6): 6 mM KNO_3, 4 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$ $7H_2O$, 100 $\mu g \cdot L^{-1}$ thiamine-HCl, 10 $mg \cdot L^{-1}$ ferric citrate, 0.2 $mL \cdot L^{-1}$ of Morizet and Mingeau microelements solution [20] and 110 mM glucose. Before each experiment, fungi were transferred to agar N6 medium containing a soluble P concentration of 100 µM instead of 1 mM. After 3 weeks of growth, fungal plugs of 8 mm in diameter were cut from the edges of the colony and used to inoculate liquid or agar medium.

Liquid cultures were carried out in 125 mL glass bottles containing 40 mL of N6 solution previously autoclaved for 30 min at 120 °C. An agar plug was held at the surface of the solution with a nichrome wire (Ref. 41000013, Sté Labover, France) and the fungus was allowed to grow in the dark for 3 weeks at 24 °C in stagnant condition. Four culture media were made up from the basic N6 solution without soluble P. Three media contained 20, 100 or 500 µM of soluble P as NaH_2PO_4. These media were called P20, P100 and P500, respectively. The fourth medium, referred to as Pi + $CaCO_3$ medium, contained 500 µM NaH_2PO_4 and 1 $g \cdot L^{-1}$ of $CaCO_3$ solid phase instead of $CaCl_2$. The mineral was a reagent grade commercial product (Merck 2066) and was added to each flask before sterilisation. Calculations of solubility equilibrium show that P cannot precipitate as calcium phosphates in P20, P100 and P500 media because of their low pH values (5.2). In Pi + $CaCO_3$ medium, P could precipitate as hydroxylapatite. The resulting P concentration would then range from 50 to 500 µM, depending on CO_2 partial pressure in culture bottle [2]. To reveal local pH changes in this Pi + $CaCO_3$ medium, other cultures were performed in Petri dishes with 15 $g \cdot L^{-1}$ agar added to the Pi + $CaCO_3$ medium.

2.2. Sample preparation and extraction methods

After culture in liquid medium, mycelia were rinsed with distilled water and dried at 80 °C for 24 h. The dry matter was separated into 2 parts. After weighing, the first one was used to measure phosphorus contents after dry matter hydrolysis with $HClO_4$ (220 °C, 10 min). The second part was used to analyse calcium and oxalate associated with the fungus, after extraction of dry matter for 10 min at 100 °C in 1 M HCl. Measurements of pH and NO_3^- concentrations were performed directly in the liquid medium. To analyse oxalate and other organic acids excreted by fungi in the culture medium, a known volume of solution or suspension of $CaCO_3$ was evaporated to dryness in an oven at 60 °C for 12 h and extracted by the same volume of 1 M HCl for 10 min at 100 °C. After dilution, organic acids were analysed in the acidic extract.

2.3. Methods of analysis

Nitrate concentration was measured in an autoanalyser where NO_3^- was reduced in a column containing activated cadmium [10]. The nitrite produced was colorimetrically determined with 0.5% (w/v) sulfanilamide in 3M HCl and 0.1% (w/v) N-naphtyl-(1)-ethylenediammonium-dichloride. Phosphate concentration was determined by colorimetry of the phosphomolybdate complex after reduction according to the Taussky and Shorr method [25]. Calcium concentration was determined by atomic absorption spectrophotometry after dilution in 0.1M HCl containing 36 mM $LaCl_3$. Oxalate and other organic anions were assayed by High Performance Ionic Chromatography (DIONEX 4000i) with a column of anionic resin (AS11 type). Anions were eluted with a gradient of NaOH made up from solutions of NaOH at 0.75 mM (elutant 1) and 100mM (elutant 2) respectively, with the following steps: 0–3 min, 100% of elutant 1; 18 min, 70% of elutant 1 and 30% of elutant 2. Calibrations for retention times and peak areas were carried out with standard solutions containing oxalic acid or other organic anions as acids or salts of sodium or potassium. Peaks were apparent and quantified when the concentration of oxalate in the solution injected into the column was 10 µM.

2.4. pH measurements

In liquid culture pH was measured with a glass electrode. Microelectrodes were used to measure values of pH in agar medium after the fungi had grown for 3 weeks in presence of $CaCO_3$. Microelectrodes (reference and H^+-selective) were pulled from filament-containing borosilicate glass capillaries (Clark, GC 150F) with a vertical puller. After pulling, the tips of the reference ones were broken before front-filling them with 2% agar solution of 2 M KCl. H^+-microelectrodes were made as described previously [23]. Before and after pH measurement in agar, H^+-microelectrodes were calibrated in buffered solutions of pH 4 and 7 (Titrisol, Merck ref 1.09884 and 1.0987, respectively). The agar from Petri dishes was inverted and measurements were carried out in a Faraday cage by inserting both electrodes to a depth of 1 mm. The pH was measured directly above the initial plug and at 0.5 cm intervals to the edge of the dish.

2.5. Statistics

All results given are means and standard deviations from five replicates. When indicated, data were analysed by ANOVA and significant differences between treatments determined by Scheffe's F-test using Statview® software (Abacus Concepts, USA) at $P = 0.05$.

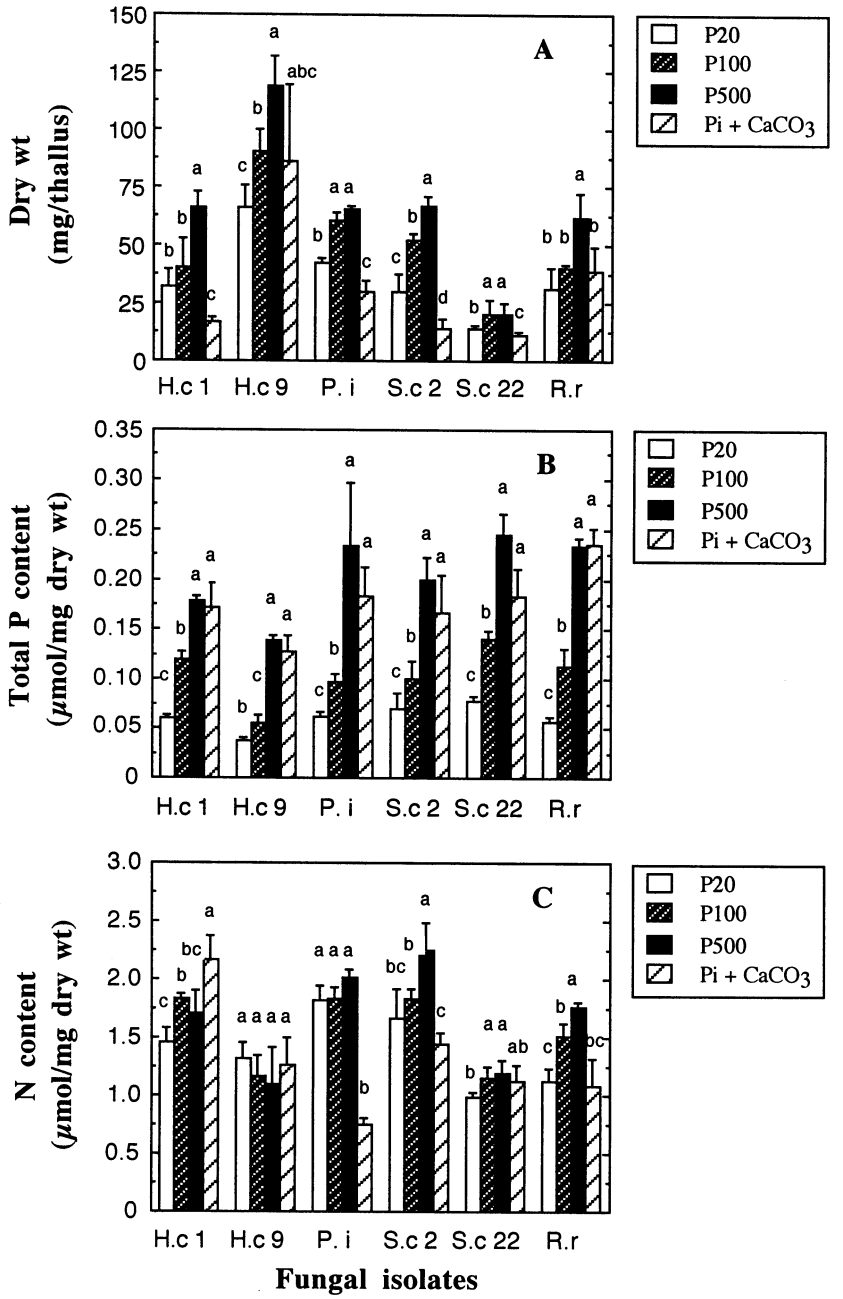

Figure 1. Growth, P and N contents of six ectomycorrhizal isolates (*H.c* 1, *H.c* 9*: Hebeloma cylindrosporum* 1 and 9; *P.i: Paxillus involutus*, *S.c* 2, *S.c* 22*: Suillus collinitus* 2 and 22; *R.r: Rhizopogon roseolus*) cultivated in nitrate solution containing different P supply levels or $CaCO_3$. The mycelia were grown for 21 d in solution containing 20 (P20), 100 (P100), 500 (P500) µM Pi or 500 µM Pi + 10 mM $CaCO_3$ (Pi + $CaCO_3$). A: dry weight, B: total P contents, C: total N contents. Bars are means ($n = 5$) with standard deviation. Within each isolate, different letters indicate significant differences between treatments at $P = 0.05$ (ANOVA, Scheffe's F-test).

3. RESULTS

3.1. Growth and mineral nutrition

The effects of P supply level and $CaCO_3$ addition in the nutrient solution on dry weights, P and N contents of fungi are shown in Figure 1. In all treatments, *H. cylindrosporum* 9 isolate gave the greatest dry weights and *S. collinitus* 22 isolate the lowest ones. In the absence of $CaCO_3$ and for a given isolate, effects of Pi concentration in nutrient solution were noticeable. For all isolates, dry weight amounts and P contents were lower in P20 treatment than those measured in P500 treatment (Figs. 1A and 1B). The ratios between P contents measured in mycelia supplied with 500 and 20 µM of P ranged from 2.3 (*H. cylindrosporum* 1) to 4 (*R. roseolus*). These data indicated that fungi were P-stressed in P20. In contrast, N contents, measured from NO_3^- depletion from the medium, were either not modified by P concentration in the medium in both isolates of *H. cylindrosporum* and *P. involutus* or decreased by low P supply in both isolates of *S. collinitus* and *R. roseolus* (Fig. 1C). However, the decreasing of N contents with P starvation was lower than that of P contents, with ratios between N contents of mycelia from P500 and P20 media of 1.2, 1.3 and 1.5 for *S. collinitus* 22, *S. collinitus* 2 and *R. roseolus*,

Table I. Contents of oxalate, total or fungus associated, and calcium from six ectomycorrhizal isolates (*H.c* 1, *H.c* 9*: Hebeloma cylindrosporum* 1 and 9; *P.i: Paxillus involutus*, *S.c* 2, *S.c* 22*: Suillus collinitus* 2 and 22; *R.r: Rhizopogon roseolus*) cultivated in nitrate solution containing different Pi supply levels or $CaCO_3$. The mycelia were grown for 21 d in solution containing 20 (P20), 100 (P100), 500 (P500) µM Pi or 500 µM Pi + 10 mM $CaCO_3$ (Pi + $CaCO_3$). Values are means ($n = 5$) ± standard deviation. Within each isolate, different letters indicate significant differences between treatments at $P = 0.05$ (ANOVA, Scheffe's F-test).

Isolate	Treatment	Contents (µmol mg^{-1} dry wt) of		
		Total oxalate	Fungus associated oxalate	Calcium
H.c 1	P20	ND*	ND	0.03 ± 0.006^a
	P100	ND	ND	0.03 ± 0.007^a
	P500	ND	ND	0.03 ± 0.010^a
	Pi + CaCO3	0.25 ± 0.08	0.04 ± 0.01	0.06 ± 0.017^b
H.c 9	P20	ND	ND	0.03 ± 0.005^a
	P100	ND	ND	0.03 ± 0.006^a
	P500	ND	ND	0.03 ± 0.06^a
	Pi + CaCO3	0.1 ± 0.03	0.04 ± 0.01	0.06 ± 0.027^b
P.i	P20	0.75 ± 0.23^a	0.43 ± 0.10^a	0.35 ± 0.05^a
	P100	0.61 ± 0.21^a	0.39 ± 0.09^a	0.33 ± 0.10^a
	P500	0.85 ± 0.25^a	0.52 ± 0.15^a	0.40 ± 0.12^a
	Pi + CaCO3	2.43 ± 0.80^b	1.60 ± 0.35^b	1.56 ± 0.30^b
S.c 2	P20	0.44 ± 0.10^a	0.30 ± 0.08^a	0.27 ± 0.05^a
	P100	0.57 ± 0.18^a	0.32 ± 0.10^a	0.31 ± 0.07^a
	P500	0.64 ± 0.15^a	0.37 ± 0.09^a	0.36 ± 0.10^a
	Pi + CaCO3	1.63 ± 0.40^b	1.15 ± 0.30^b	1.50 ± 0.28^b
S.c 22	P20	0.75 ± 0.21^a	0.52 ± 0.15^a	0.42 ± 0.08^a
	P100	0.79 ± 0.23^a	0.51 ± 0.12^a	0.45 ± 0.07^a
	P500	1.06 ± 0.26^a	0.65 ± 0.17^a	0.56 ± 0.10^a
	Pi + CaCO3	2.39 ± 0.58^b	1.40 ± 0.39^b	1.50 ± 0.10^b
R.r	P20	0.85 ± 0.23^a	0.52 ± 0.14^a	0.43 ± 0.08^a
	P100	0.91 ± 0.26^a	0.49 ± 0.13^a	0.42 ± 0.09^a
	P500	1.11 ± 0.27^a	0.51 ± 0.15^a	0.45 ± 0.08^a
	Pi + CaCO3	3.10 ± 0.82^b	1.60 ± 0.35^b	1.90 ± 0.17^b

* ND: not detectable (< 0.02 µmol·mg^{-1} dry wt).

respectively. Compared to P500 treatment, the addition of $CaCO_3$ in nutrient solution containing 500 µM P decreased the growth of all fungal isolates but one (*H. cylindrosporum* 9) (Fig. 1A). The addition of $CaCO_3$ did not modify P contents (Fig. 1B) whereas it had variable effects on N contents of fungi (Fig. 1C).

3.2. Oxalate production and calcium content

Analysis of organic anions extracted from the mycelia or accumulated in culture solution showed that oxalate was always the main organic anion (> 90%) produced by the fungi studied, with minor quantities of citrate, succinate, malate and tartrate (data not shown). Measurements of total oxalate amounts, corresponding to the sum of the amount excreted in the medium and the amount associated with the mycelium, depending on the Pi level in the solution and the addition of $CaCO_3$, showed that the fungi can be divided into two groups (Tab. I). First the two isolates of *H. cylindrosporum* presenting a very low or undetectable oxalate production and second the four other isolates presenting a significant production. Regarding the effect of Pi supply level without $CaCO_3$, it can be noticed that the limiting P20 level neither induced oxalate production in *H. cylindrosporum* isolates nor increased production in other isolates compared to P100 and P500 treatments. The addition of $CaCO_3$ significantly enhanced total oxalate production per dry weight unit which became measurable in *H. cylindrosporum* and increased by 2 to 3 times compared to P500 treatment in *P. involutus*, *S. collinitus* and *R. roseolus.* However as $CaCO_3$ also depressed the fungal growth, oxalate production by the mycelia was only increased in *R. roseolus*.

The contents of calcium assayed in the mycelia varied greatly between the isolates (Tab. I). Whatever the nutrient solution, both *H. cylindrosporum* isolates presented very low Ca contents that were 8 to 15 and 25 to 30 times lower than those assayed in the four oxalate producing isolates in absence and in presence of $CaCO_3$, respectively. In these oxalate producing isolates, calcium contents were of the same order of magnitude as those of oxalate associated with the mycelia, suggesting that calcium is bound to oxalate. This hypothesis is supported by scanning electron microscopy observations showing the occurrence of numerous bipyramidal quadratic crystals, characteristic of weddelite (CaC_2O_4, $2H_2O$) at the surface of hyphae (data not shown).

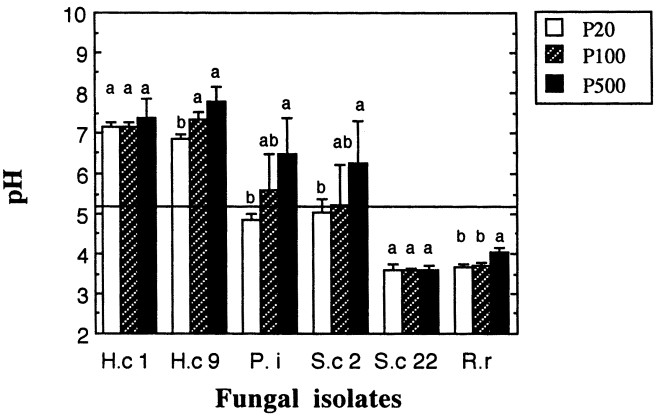

Figure 2. Final pH values measured in medium after culture of six ectomycorrhizal isolates (*H.c* 1, *H.c* 9*: Hebeloma cylindrosporum* 1 and 9; *P.i: Paxillus involutus*, *S.c* 2, *S.c* 22*: Suillus collinitus* 2 and 22; *R.r: Rhizopogon roseolus*) cultivated in nitrate solution containing different P supply levels. The mycelia were grown for 21 d in solution containing 20 (P20), 100 (P100), 500 (P500) µM Pi. Bars are means ($n = 5$) with standard deviation. Within each isolate, different letters indicate significant differences between treatments at $P = 0.05$ (ANOVA, Scheffe's F-test).

3.3. Nutrient solution pH

In the absence of $CaCO_3$, studied isolates produced different effects on final pH value of the nutrient solution that was initially set at 5.2 (Fig. 2). Both *H. cylindrosporum* isolates

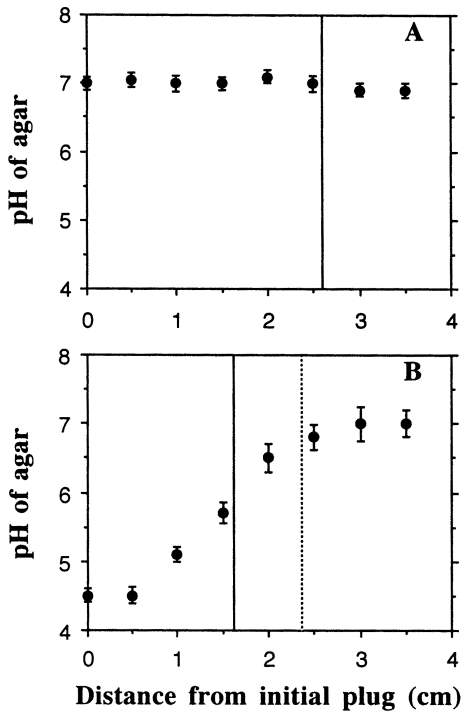

Figure 3. Final pH values of agar medium after culture of *Hebeloma cylindrosporum* 9 (A) or *Rhizopogon roseolus* (B). The mycelia were grown for 21 d in Petri dishes containing 6 mM NO_3^-, 0.5 mM Pi and 10 mM $CaCO_3$ and pH was measured with H^+-selective microelectrodes. The continuous line represents the growth limit of the mycelium and the dashed line the $CaCO_3$ dissolution zone limit. Each point is the mean with standard deviation ($n = 5$).

increased the solution pH by around 2 units, *S. collinitus* 22 and *R. roseolus* decreased it by 1.2 to 1.7 unit whereas *P. involutus* and *S. collinitus* 2 slightly decreased or increased it depending on the P supply levels. In the presence of $CaCO_3$, the solution pH ranged between 7 and 7.5 due to the solubility equilibrium in the system $CaCO_3 - CO_2 - H_2O$ with the CO_2 partial pressure occurring in the atmosphere of the culture bottle. The buffering effect of the carbonate phase on pH value of the bulk solution does not however exclude local pH variations near fungal hyphae. In order to reveal these possible local pH variations, two isolates representing alkalinising and acidifying species (*H. cylindrosporum* 9 and *R. roseolus*) were grown in agar medium containing $CaCO_3$. After a 21-day culture, the agar medium was not modified by the growth of *H. cylindrosporum,* whereas, the agar becoming transparent, a zone of $CaCO_3$ dissolution was observed 1 cm beyond the colony edge of *R. roseolus* (Fig. 3). Measurements of pH with microelectrodes showed no acidification of agar medium after culture of *H. cylindrosporum* and a strong acidification near and under the mycelia of *R.. roseolus* (Fig. 3). Values of pH dropped from about 7.0 in the bulk medium to values below 4.5 under the centre of the mycelium. These data demonstrated that *R. roseolus* exerted strong chemical actions on culture medium resulting in dissolution of $CaCO_3$ and, furthermore, acidification of agar.

4. DISCUSSION

Our results showed that the fungal species we used differed considerably according to their ability to produce oxalate. In the absence of $CaCO_3$, no oxalate production was detected in both *H. cylindrosporum* isolates whereas an important one was measured in *P. involutus*, *S. collinitus* and *R. roseolus* isolates. The synthesised oxalate is partly excreted in the culture medium, the other part (50 to 70% of total, see Tab. I) remained bound to hyphae. The results of our study indicated clearly that oxalate production did not depend on P starvation, contrary to observations reported in plants such as rape [11], *Lupinus albus* [4, 21], tomato [12, 21] or Proteaceae [24] dealing with other carboxylates as malate or citrate.

In contrast to Pi supply level, the presence of $CaCO_3$ always increased oxalate production. A low production was detected in both *H. cylindrosporum* isolates, suggesting that these fungi have the enzymes necessary for the synthesis of oxalate. In other isolates the production was increased by 2 to 3 times. This effect of $CaCO_3$ was previously observed in white-rot fungi (see [5]). In ectomycorrhizal fungi, it was demonstrated that increasing concentrations of $NaHCO_3$ in culture medium enhanced oxalate production in *P. involutus* [16] by incorporation of HCO_3^- ions during oxalate biosynthesis [15]. In nutrient solutions with $CaCO_3$, carbonate solid phase and CO_2 arising from fungal respiration react together to produce HCO_3^- in solution. Indeed, in our culture conditions in closed bottles, we measured CO_2 partial pressures ranging from 0.05 to 0.1 atmosphere. At the solubility equilibrium of $CaCO_3$, such CO_2 partial pressures determine concentrations of HCO_3^- of 6 to 8 mM that are high enough to enhance oxalate synthesis [16]. Nevertheless, the effect of $CaCO_3$ might also be due to the resulting high pH value in culture medium that might inhibit the activity of enzymes for oxalate degradation [5]. However, the favouring effect of $CaCO_3$ on oxalate production will also depend on the effect of $CaCO_3$ on the fungal growth. Indeed, we showed that the presence of $CaCO_3$ depressed the fungal growth so that oxalate production per mycelium is increased to a lesser degree than oxalate production per unit of dry weight. Finally, with $CaCO_3$, a significant increase of oxalate production per mycelium was only observed in *R. roseolus*.

It was demonstrated that oxalate excreted by fungi can precipitate as Ca oxalate crystals, which partly remained bound to the surface of hyphae [1, 17], thus apparently increasing calcium contents of fungi. This phenomenon may account for the variations in apparent calcium contents observed in our experiments. Fungus associated calcium was low in *H. cylindrosporum* isolates but increased in oxalate producing fungi, specially in the presence of $CaCO_3$. Expressed as $CaC_2O_4{,}2H_2O$, the quantities of calcium and oxalate associated with the hyphae may then represent 20 to 25% of the fungal dry matter.

In the absence of $CaCO_3$, the effect of P starvation on pH of the solution depended on the fungal isolate (Fig. 2). Whatever the P level supply, both *H. cylindrosporum* isolates alkalinized the medium. This decrease of proton concentration in the solution can be explained by the OH^-/NO_3^- exchange required to maintain the ionic balance in conditions of NO_3^- nutrition [19]. However, in the same conditions, *S. collinitus* 22 and *R. roseolus* always acidified the medium while *P. involutus*

Table II. Molar ratios of total oxalate production (μmoL·mg^{-1} dry wt) to nitrate uptake (μmoL·mg^{-1} dry wt) in four oxalate producing fungal isolates (*P.i: Paxillus involutus*, *S.c* 2, *S.c* 22*: Suillus collinitus* 2 and 22; *R.r: Rhizopogon roseolus*) cultivated in nitrate solution containing different P supply levels or $CaCO_3$. The mycelia were grown for 21 d in solution containing 20 (P20), 100 (P100), 500 (P500) μM Pi or 500μM Pi + 10 mM $CaCO_3$ (Pi + $CaCO_3$).

Isolate	Total oxalate production / NO_3^- uptake in treatment			
	P20	P100	P500	Pi + $CaCO_3$
P.i	0.41	0.33	0.42	3.20
S.c 2	0.26	0.31	0.28	1.12
S.c 22	0.76	0.69	0.90	2.08
R.r	0.75	0.60	0.63	2.87

and *S. collinitus* 2 showed intermediate effects according to the P supply level. We showed that these four fungal isolates released important amounts of oxalate outside the fungal cells, either bound to the hyphae or free in the medium. The efflux of oxalate through fungal cell membranes occurs as anion transport because the cytosolic pH (around 7) is higher than the pK of oxalic acid (pK for oxalate$^-$/oxalate^{2-} is 4.19) [13, 14], meaning that the organic acid is actually present as organic anion in the cytosol. Therefore, as underlined by Roelofs et al. [24], when carboxylates are exuded as anions, their charge could be balanced by a cation efflux, or alternatively, by an anion influx. The exchanges that can be hypothesised are $C_2O_4^{2-}$/2 K^+ or $C_2O_4^{2-}$/2 NO_3^- with no acidifying effect or $C_2O_4^{2-}$/2 H^+ with an acidifying effect. Because of the increase of K^+ concentration and pH in the external solution, Roelof et al. [24] proposed potassium as the accompanying cation of citrate release by roots of Proteaceae. Such a K^+ efflux with oxalate could explain the pH increase observed after culture of *P. involutus* and *S. collinitus* 2 in P100 and P500 treatments. Unfortunately, we cannot check this hypothesis because in our culture conditions, potassium was supplied in excess (around 6 mM), preventing us from measuring any variations of K^+ concentration. However, from our data, it is possible to calculate the value of the molar ratio of excreted oxalate to nitrate taken up by each isolate. Values below 0.5 indicate that nitrate uptake is sufficient to compensate for oxalate output. Values above 0.5 indicate that the compensation for oxalate output furthermore requires a symport $C_2O_4^{2-}$/2 H^+. The ratios of excreted oxalate to nitrate taken up by the oxalate producing fungal isolates as a function of the composition of the culture medium are shown in Table II. In the absence of $CaCO_3$, ratio values are below 0.5 in *P. involutus* and *S. collinitus* 2. A weak acidification is only observed for both isolates in P20 experiment. On the contrary, in *R. roseolus* and *S. collinitus* 22 isolates, ratios are above 0.5 and a strong acidification is observed in P20, P100 and P500 experiments which indicates a high H^+ release. These calculations suggest that the pH increase in the solution observed after culture of *P. involutus* and *S. collinitus* 2 could be due to the oxalate efflux balanced by the NO_3^- influx.

In the presence of $CaCO_3$ high values of the ratio in the four oxalate producing fungi denote either another mechanism for transport of $C_2O_4^{2-}$ ions such as an antiport $C_2O_4^{2-}$/2 HCO_3^- or an enhancement of $C_2O_4^{2-}$/2 H^+ symport. A significant HCO_3^- input in fungal cell is suggested by the utilisation of these ions in oxalate synthesis as demonstrated by Lapeyrie [15]. However our experiment with *R. roseolus* in agar medium containing $CaCO_3$ showed a carbonate dissolution zone which extended beyond the colony limits and an important decrease in pH value under the colony. Both these observations indicate an important H^+ release by the fungus and then support an enhancement of symport $C_2O_4^{2-}$/2 H^+ in the presence of $CaCO_3$.

In conclusion this study showed that some species of ectomycorrhizal fungi are able to produce and excrete oxalate whereas others are not. Oxalate production is not related to a phosphorus deficiency but is favoured by the presence of calcium carbonate. One could think to rely the ability to produce oxalate with ecological conditions of soils where the fungal species were harvested. Our results do not enable us to analyse thoroughly the effect of these soil conditions. Nevertheless, we showed that high concentrations of bicarbonate ions, characterising calcareous soils, increased drastically the enzyme reactions responsible for oxalate synthesis. On the other hand, it was shown that high pH values in these soils depressed the enzyme reactions responsible for oxalate degradation [5]. These two properties of calcareous soils tend to increase the net oxalate production observed in *P. involutus*, *S. collinitus* and *R. roseolus* isolates. On the contrary the low ability of both *H. cylindrosporum* isolates could be due to either a low enzyme synthesis or a high enzyme degradation of oxalate. These behaviours could result from acquired properties in the conditions of harvest sites.

In oxalate producing fungi, some species also exhibit an important proton efflux that is probably related to transport phenomena accompanying oxalate excretion. In the presence of $CaCO_3$ both oxalate excretion and proton efflux are enhanced, which increase the chemical action exerted by the fungi on the mineral. This could play an important role for fungal mobilisation of P in calcareous soils.

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