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Effect of Phosphinothricin Herbicide on Nitrogen Metabolism in *Pinus radiata* and *Laccaria bicolor*

By

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Key words: Ammonia, CO_2 assimilation, GS activity, *Laccaria bicolor*, L-amino acid oxidase, mycorrhiza, *Pinus radiata,* phosphinothricin.

Summary

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The phosphinothricin (PPT) herbicide inhibited the glutamine synthetase activity (GS) extracted from *Laccaria bicolor* (transferase and semibiosynthetic activities). In pine needle PPT had an inhibitory effect in the transferase assay, while it acted as an activator for the semibiosynthetic activity. The close relationship observed between CO_2 assimilation rate, Fv/Fm ratio and ammonia is a good indicator of the interrelation between nitrogen metabolism and photosynthetic carbon assimilation. The capacity to deaminate PPT by L-amino acid oxidase seems to confer *Laccaria bicolor* quite tolerance to this herbicide, since the increment in GS activity allow the adaptation of the fungus to grow in presence of this herbicide.

Introduction

The mycorrhiza *Pinus radiata-Laccaria bicolor* is an interesting association for forestry in The Basque Country. The high annual rainfall and the warm temperatures in this geographic area make not only the yield of the economic plantations considerable (Romanyà & Vallejo 1996), but also it favours the spread out of weeds, which represents one of the main problems during the growth of plants in nursery and during the first years after outplanting. At this stage, the mycorrhizae are a potential factor to improve the physiological quality of plants destined to reforestations (Duñabeitia & al. 1996).

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Phosphinothricin (L-PPT), an analogue of glutamate, is an inhibitor of Glutamine synthetase activity (GS, EC. 6.3.1.2). PPT is a non selective herbicide and the susceptibility of the plant to this compound depends on several factors: absorption by the plant, sensitivity and regulation of the target enzyme (GS), and carbon and nitrogen metabolism interaction (reassimilation of ammonia released from photorespiration pathway and from nitrogen compound catabolism). *Pinus radiata* is a C3 plant, in which high photorespiration rates can make the PPT effect be more drastic.

PPT is considered as a friendly herbicide, because it is degraded by soil microorganisms. The presence of specific enzyme activities (L-amino acid oxidase, PPT-acetylating enzymes) confers some microorganisms resistance to this inhibitor. The unicellular green eukaryotic alga *Chlamydomonas reinhardtii* is able to deaminate this herbicide to the corresponding keto acid (PPO, 4-methylphosphinico-2-oxobutanoic acid), which allows this alga to utilize PPT as nitrogen source for growth (FRANCO & al. 1996). Although the PPT metabolism has been studied in bacteria, information about ectomycorrhizal fungi are unknown.

In order to check the possibility of using PPT as herbicide in nurseries of *Pinus radiata*, we have analyzed the possible effect of PPT on ammonia assimilation in both partners of the mycorrhizal symbiosis.

Material and Methods

Plant material: Pine plants were grown for 5-6 months in a mixture of *Sphagnum* peat and vermiculite (1:1) and watered with deionized water. Nutrient solution was supplied every two weeks to fertilize pine plants. PPT treatment was carried out by spraying pine seedling with 10 mL of an aqueous solution of 0, 0.25 and 0.5 mM of a commercial preparation of glufosinate (phosphinothricin, Hoëchst Iberica SA, Barcelona, Spain).

Fungal material and growth: *Laccaria bicolor* S238 and S238N strains correspond to a subculture from the INRA fungal collection (Nancy, France). For the determination of enzymatic activities the fungal mycelium was grown for 21 days in liquid modified Melin-Norkrans medium (MMN, pH 5.5), containing ammonia and glucose as nitrogen and carbon source, respectively, and where phosphinothricin (L-homoalanine-4-yl (methyl) phosphinic acid) had been supplemented.

Enzymatic activities and physiological measurements: The fungal extractions were obtained as described by LACUESTA & al. 1989. The semibiosynthetic GS activity was assayed according to LACUESTA & al. 1989. Enzymatic pine extraction was obtained and semibiosynthetic GS activity assayed as described by PÉREZ-SOBA & al. 1994. The transferase assay from both, fungal mycelium and pine, was determined according to SHAPIRO & STADTMAN 1970. L-amino acid oxidase activity was measured according to PIEDRAS & al. 1992. The protein content was determined by the Coomassie Blue method (BRADFORD 1976). Ammonia content in pine needles was quantified with glutamate dehydrogenase (VÉZINA & al. 1992).

The CO_2 assimilation was measured with a portable IRGA fitted to an open system (IRGA, ADC LCA-4, Analytical Development Co. Ltd. Hoddesdon, Herts, UK). Chlorophyll fluorescence parameters, the variable (Fv) and maximum (Fm) fluorescence, were determined with a Plant Stress Meter (Biomonitor AB, Umea, Sweden). The maximum quantum yield of PSII in dark-adapted leaves (30 min) was estimated by the fluorescence ratio Fv/Fm. Values are the mean of 4 independent experiments.

Result and Discussion

PPT is the active ingredient of a non selective herbicide BASTA (AgrEvo, Frankfurt am Main, Germany) and an inhibitor of GS activity from many organisms. We observed a strong inhibition of GS transferase activity when pine needle extract was incubated with PPT (PPT in vitro effect), so the activity was practically nil at PPT concentrations higher than 350 µM, and an I_{50} value of 14 µM PPT was estimated (Fig. 1). However, surprisingly, this pattern was not observed when the GS semibiosynthetic activity was registered since PPT acted as an activator (Fig. 1).

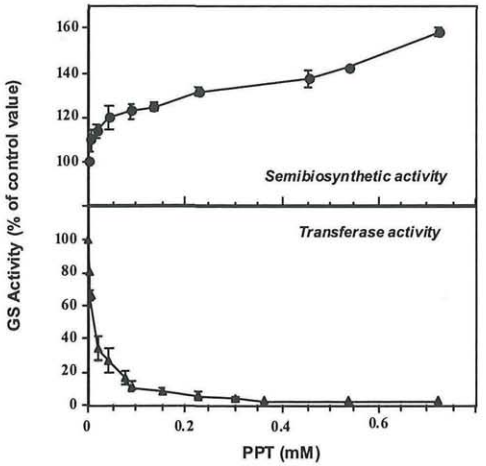

Fig. 1. PPT in vitro effect on GS activity extracted from pine. The GS activity was determined by semibiosynthetic assay (control activity 31 ± 9.4 µmol γ-GHM mg^{-1} prot h^{-1}) and transferase assay (control activity 68.48 ± 8.8 µmol γ-GHM mg^{-1} prot h^{-1}).

A significant accumulation of ammonia was observed in PPT-sprayed seedlings (Fig. 2), indicating the mode of action of PPT in pine involves the inhibition of GS and that the ammonia assimilation was really interrupted in treated pines, despite the semibiosynthetic activity remained constant over 4 days after initiating the treatment (data not shown). ÁVILA & al. 1998 have also reported an early activation of GS activity in *Pinus sylvestris* cotyledons after a few hours of supplying PPT, and an induction of cytosolic GS isoform was observed later. GS plays a key role in the reassimilation of photorespiratory ammonia released during the deaminating condensation of two glycine molecules to yield one serine molecule. The interruption of photorespiratory ammonia assimilation provokes a secondary effect on CO_2 assimilation (GONZÁLEZ-MORO & al. 1993, 1997a, 1997b), and this can also be observed in pine when the decline in photosynthetic activity was represented versus ammonia levels (Fig. 3). This fact has also been described for another GS inhibitor such as methionine sulfoximine (PLATT & RAND 1982). However, despite this close relationship, the effect of PPT on CO_2 assimilation is not mediated by an excess of ammonia levels, it is rather caused by the accumulation

of intermediates (glycolate and glyoxylate) as consequence of the blockage of the photorespiratory cycle (GONZÁLEZ-MORO & al. 1997a, 1997b). The decrease in the CO_2 assimilation rate and the Fv/Fm ratio reflected that pine metabolism was sensitive to PPT.

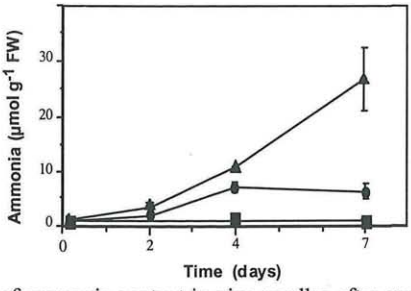

Fig. 2. Variation of ammonia content in pine needles after spraying with: 0.25 mM PPT (●), 0.5 mM PPT (▲) and control with water (■).

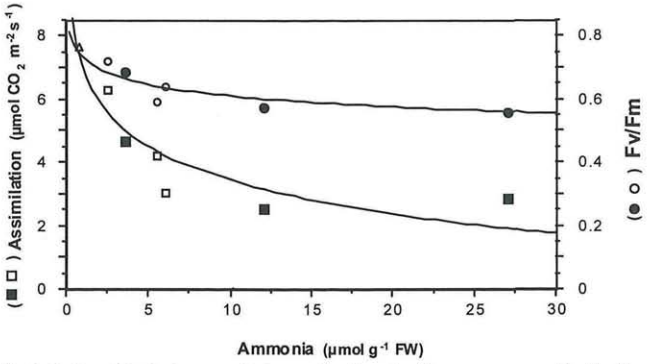

Fig. 3. Relationship between photosynthesis rate, fluorescence ratio Fv/Fm and ammonia content in pine needles at 2nd, 4th and 7th days after spraying with: 0.25 mM PPT (□, ○), 0.5 mM PPT (■,●) and control with water (△).

GS activity from *Laccaria bicolor* was inhibited by PPT, but never an inhibition higher than 60 % was observed in the semibiosynthetic assay (Fig. 4). *Pinus radiata* can be mycorrhized by several fungi (*Boletus pinophilus, Paxillus involutus, Scleroderma citrinum, Laccaria bicolor, Pisolithus tinctorius, Rhizopogon luteolus, Rhizopogon vulgaris*), but only *Laccaria bicolor* grew in the culture medium with PPT (IRIBERRI & al. 1998). Mycelia of S238 and S238N strains presented higher GS activities when increasing PPT concentrations were supplied to the growing medium compared with the control mycelium (without PPT) (Fig. 5). An increment of protein content per dry weight in these conditions was also reported (IRIBERRI & al. 1998). It is well established that many microorganisms have the ability to deaminate PPT to the corresponding keto acid (FRANCO & al. 1996), which allow these microorganisms to employ PPT as nitrogen and carbon source.

Both strains of *Laccaria* presented considerable activities of L-amino acid oxidase (Table 1), being the strain with the higher deaminating activity (S238N) the one which showed higher growth (IRIBERRI & al. 1998) and a linear increment in GS activity (Fig. 5) when PPT was supplied to the growing medium. The ammonia from PPT deaminated by L-amino acid oxidase could provoke de novo appearance of GS proteins in *Laccaria*, as described for another species when the ammonia was accumulated (PÉREZ-GARCÍA & al. 1995). The L-amino acid oxidase activity seems to confer *Laccaria bicolor* tolerance to PPT.

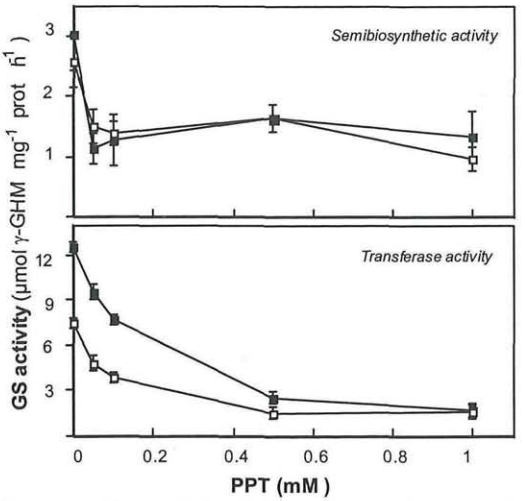

Fig. 4. PPT in vitro effect on GS activity from *Laccaria bicolor* S238 (□) and S238 N (■) mycelium at 21th day of growth in MMN liquid medium.

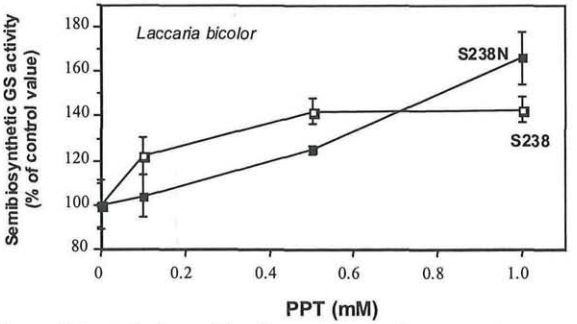

Fig. 5. GS semibiosynthetic activity from *Laccaria bicolor* cultures at 21th of growth in liquid MMN medium supplied with PPT. The semibiosynthetic GS activity in control mycelium, growing without PPT, was the same as in Fig. 4.

Table 1. In vitro effect of 0.5 and 1 mM PPT on L-amino acid oxidase activity from *Laccaria bicolor* S238 and S238 N strains grown during 21 days in liquid MMN medium.

In vitro L-aminoacid oxidase activity (nmol mg^{-1} prot h^{-1})			
Ectomycorrhizal fungi	CONTROL	0.5 mM PPT	1 mM PPT
Laccaria bicolor S238	1.890 ± 0.153 a	2.035 ± 0.517 a	1.914 ± 0.370 a
Laccaria bicolor S238N	3.843 ± 1.117 b	3.079 ± 0.762 b	3.002 ± 0.806 b

In conclusion, the activation of GS in pine and the high remaining semibiosynthetic activity in *Laccaria* indicates this target enzyme would have particular kinetic properties in both organisms. Ammonia accumulated coming from photorespiration in pine or released from L-amino acid oxidase activity in *Laccaria* could induce new GS polypeptides, which would contribute to confer tolerance to PPT.

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