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Original article

Effect of simulated acid rain on mycorrhizae of Aleppo pine (*Pinus halepensis* Miller) in calcareous soil

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Summary – Mycorrhiza formation and plant growth, in particular root development, of *Pinus halepensis* were studied in relation to the influence of pH from simulated rain in pot cultures. Four treatments of water (7.5, 6.0, 4.5 and 3.0) were established by adding a mixture of sulphuric and nitric acids (2:1, v/v) or 10% NaOH to distilled water. Three experiments were carried out: i) seedlings growing in calcareous forest soil; ii) 2-year-old naturally mycorrhizal seedlings, transplanted into vermiculite in order to differentiate old and new-formed roots; and iii) seedlings growing in peat vermiculite, inoculated with mycelial inoculum of *Suillus collinitus*. Although no visible effects on the aerial part were observed, a reduction of root length in the most acidic treatment was noted. Enhancement of ectomycorrhizae formation was also recorded in this treatment in the three experiments. In substrata of neutro-basic pH, short-term exposures to acid rain positively affected ectomycorrhizal fungi, in particular, *Suillus* species.

acid rain / mycorrhizae / pH / *Pinus halepensis* / plant growth

Résumé – Effet du pH d'une pluie acide simulée sur les mycorhizes de pin d'Alep (*Pinus halepensis* Miller) sur sol calcaire. *L' effect du pH d' une pluie simulée sur la formation des mycorhizes et sur la croissance de* Pinus halepensis *a été étudié. Les traitements de pH de l' eau (7,5, 6,0, 4,5 et 3.0) on été établi par l'addition à l' eau distillée de* H_2SO_4 *et* HNO_3 *(2:1, v:v) ou de NaOH (10%). Trois essais ont été conduits : i) des plants sur sol forestier calcaire ; ii) plants agés de 2 ans naturellement mycorhizés, transplantés dans la vermiculite pour différencier les racines préexistantes des racines nouvellement formées et iii) plants mycorhizés avec* Suillus collinitus. *Les résultats montrent une réduction de la longueur des racines et une amélioration de la formation des ectomycorhizes sous le traitment le plus acide. Sur substrat neutro ou basique (calcaire) une courte exposition à la pluie acide peut améliorer la formation d' ectomycorhizes, en particulier avec des spèces de* Suillus.

pluie acide / mycorhizes / pH /* Pinus halepensis */ croissance

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INTRODUCTION

Due to the implication of acid rain as a contributing factor to forest decline in central Europe and North America, many studies have been made recently on the influence of acid precipitation on alpine and temperate forests and on different plant species. Mycorrhizae, as a component of the forest ecosystem of indisputable importance, could be affected directly, and thereby affect the tree, or be indirectly affected by the tree (Dighton et al, 1988).

In this context, the impact of natural or simulated acid rain has been studied, among others, on mycorrhizae of *Betula papyrifera* (Keane and Manning, 1988), *Picea abies* (Blaschke, 1988; Blaschke and Weiss, 1990), *Picea rubens* (Meier et al, 1989) *Quercus rubra* (Reich et al, 1985) and *Quercus alba* (Walker and McLaughin, 1991). More attention has been focused on pine species: *Pinus strobus* (Stroo et al, 1988), *P taeda* (Shafer et al, 1985; Walker and McLaughin, 1991; Edwards and Kelly, 1992), *P sylvestris* (Dighton and Skeffington, 1987; Dighton, 1988), *P banksiana* (McAfee and Fortin, 1987) and *P thunbergii* (Maheara et al, 1993).

Acid rain events have been reported from Mediterranean areas such as Greece (Samara et al, 1992), Italy (Camufo et al, 1991) and Spain (Bellot and Escarré, 1988; Carratalá, 1993; Carratalá et al, 1994). Howewer, except for some studies in California on *Pinus ponderosa* (Temple et al, 1993), little information exists on the influence of acid rain on Mediterranean forests.

Aleppo pine (*Pinus halepensis* Miller) is a widely distributed species in Mediterranean forests. Some previous reports have revealed that this plant species is affected by atmospheric pollutants, such as SO_2 and O_3 (Sánchez-Gimeno et al, 1992; Velissariou et al, 1992; Inclán et al, 1993; Wellburn and Wellburn, 1994; Anttonnen et al, 1995). Díaz et al (1996) reported reductions in the percentage of mycorrhizal colonization and a change in mycorrhizal species composition in seedlings treated with SO_2 and O_3. However, we are not aware of any report of the influence of acid rain on mycorrhizae of *P halepensis*.

The study reported here concerns the effect of acid deposition as simulated acid rain on the formation and development of mycorrhizae in *P halepensis* seedlings in neutral and calcareous soils.

MATERIALS AND METHODS

The simulated rain was applied twice weekly by watering the seedlings with a spray nozzle. Four pH treatments were established: 7.5, 6.0, 4.5 and 3.0. The acid treatments were obtained by adding a mixture of sulphuric and nitric acids (2:1 v/v; 1/100) in the appropiate amounts to distilled water. These acids were selected because SO_4 and NO_x are contaminants commonly associated with acid precipitations. The pH 7.5 treatment was obtained by adding a solution of 10% NaOH. This treatment was included in the range of pH values to compare its effects to those of acid ones, due to the fact that neutro-basic pH are frequent on rain water in Mediterranean ecosystems.

The pH for each rain event was determined in advance with a CRISON 507 pH meter and monitored throughout the exposure. No additional watering or fertilization were supplied during the experiments. In each exposure, each plant received an average of 3.5 mL of simulated rain at appropriate pH.

Plants were grown in a greenhouse under natural day/night light conditions. Three different experiments were carried out.

Experiment 1

The objectives of this experiment were to test the influence of simulated acid rain on plant growth and natural mycorrhiza formation by a variety of mycorrhizal fungi.

Seeds of *P halepensis* were surface-sterilized in hydrogen peroxide (30%) for 30 min and then rinsed three times with sterile water. They were sown into 125 mL polyethylene containers filled with a mixture (1:1 v/v) of vermiculite and natural soil (which contained mycorrhizal propagules) collected from an Aleppo pine stand at Foz de Calanda, Teruel (Spain). This soil had a pH (KCl) of 7.73, 28.50 g kg^{-1} organic C, 2.81 g kg^{-1} total N, 488 g kg^{-1} carbonates and 0.84 dS/m electrical conductivity. Plants were thinned to two per cavity 2 weeks after germination. Sixty replicates per treatment were established.

Simulated rain treatments were applied 3 months after germination, when the formation of secondary roots was noted, and lasted 33 weeks. Seedlings received a total of 231 mL of simulated rain during the experiment.

At the end of the experiment, the height of all plants was determined. Eight randomly-selected seedlings were used to determine aerial and root biomass (80 °C, 16 h). The total length of the root system was estimated by the gridline intersect method (Marsh, 1971). The entire root system was examined for the presence of ectomycorrhizae. The total number of short roots and the number of mycorrhizal short roots was determined. Results were expressed as percentage of ectomycorrhizae and number of ectomycorrhizae per unit length. Although no attempts to quantify each distinct mycorrhizal morphotype were made, characterization of the main morphotypes was made following the criteria of Agerer (1987–1995).

Experiment 2

The objective of this experiment was to differentiate between the effects of simulated rain on mycorrhizae formed before or after the application of rain.

For this purpose, 2-year-old seedlings, naturally mycorrhizal, were transplanted into pots filled with sterilized vermiculite. The seedlings, growing from seed in plastic bags commonly used in forest practices and containing untreated forest soil from Sierra Cresta del Gallo, Murcia (Spain), were provided by EL Valle tree nursery. The soil had a pH (KCl) of 7.30, 1.14% organic matter, 19.4 g kg^{-1} total N, 5.92% $CaCO_3$ and 0.63 dS/m electrical conductivity. As roots grow through the plastic, the original plastic bag was not discarded in order to facilitate the differentiation between old and new roots. The root systems of five replicates were previously studied and their percentage of mycorrhizae was determined. There were five seedlings per treatment that received a total of 252 cc of simulated rain.

After 42 weeks, the seedlings were harvested and root biomass and the percentage of mycorrhizae were determined, both in old and new roots. Moreover, root length and the number of short roots were determined in the new roots. These determinations were made as in experiment 1.

Experiment 3

To study the influence of the pH on mycorrhiza formation by a particular mycorrhizal fungus, a third experiment was carried out. The selected fungus was *Suillus collinitus* (Fr) O Kuntze, which is very common on Aleppo pine stands and is known to form mycorrhizal association with it (Torres et al, 1991; Torres and Honrubia, 1994).

Fruit bodies of *S collinitus* strain 157ED came from Foz de Calanda, Teruel (Spain), the same site where soil was collected for experiment 1. Isolations from carpophore tissue were made on MMM medium (Marx, 1969) and then fragments of mycelia were subcultured on liquid medium in bioreactor (Byostat® B) at 23 °C, pH 5.5, 50 rpm and 58% pO_2. The mycelium obtained was then grown at 23 °C for 6 weeks in a mixture of peat:vermiculite (1:4) sterilized twice at 120 °C and additioned with MMN liquid medium. This inoculum was added to a substrate in a proportion of 1:10 (v/v) and carefully mixed with it. The substrate consisted of a mixture of peat:vermiculite:sand (1:1:1 v/v) sterilized by autoclaving twice at 120 °C.

Four-month-old seedlings of *P halepensis*, free of mycorrhizae, provided by Las Rejas nursery, Albacete (Spain), were transplanted into 125 mL polyethylene containers filled with the substrate. There were 25 replicates per treatment.

The experiment lasted 13 weeks, during which time plants received a total of 91 cc of simulated rain. At the end of the experiment all plants were harvested and the percentage of mycorrhization determined. Due to the short duration of the experiment in comparison with the age of the seedlings, no determinations on plant growth were made.

pH substrata in KCl 1N was determined before and after the three experiments.

Data were subjected to an analysis of variance (ANOVA) and differences between media were established by Duncan's test.

RESULTS

Experiment 1

No differences in plant height or aerial/root biomass were noted among the treatments.

No visible symptoms of foliar injury were detected. However, a slight reduction in the length of the root system and in the number of short roots per plant was observed in the pH 3 treatment.

With respect to mycorrhization, a slight increase in the amount of ectomycorrhizae was recorded at pH 3, which became clearer when expressed as number per unit length (table I). The dominant morphotype observed was of the *Suillus* kind. No variation of the substrate pH was observed after the 33 weeks of the experiment (table II).

Experiment 2

Root formation and growth were negatively affected by low pH, so root length and biomass were significantly lower with the most acidic treatment. Although in contrast with experiment 1, the great number of short roots/cm occurred at pH 3, no differences were noted in the total number of short roots per plant among treatments, due to the reduction of root length in this treatment.

The roots formed before the experiment were not seemingly affected by acidity, and showed similar percentages of mycorrhization among treatments, which were also similar to those recorded before starting the application of simulated rain. In contrast, as in experiment 1, mycorrhiza formation was enhanced by acidity (pH 3) in the new roots (table III). The dominant morphotype observed was of the *Suillus* kind. No differences in the substrate pH were noted at the end of the experiment (table II).

Experiment 3

Although the percentages of mycorrhization obtained by inoculation were not high enough to draw definitive conclusions, some remarks can be made. The effectiviness of inoculation was not apparently affected by rain pH, and similar amounts of

Table I. Effect of pH of simulated rain on plant growth and mycorrhization of *Pinus halepensis* seedlings growing in calcareous forest soil (experiment 1).

	Simulated rain pH			
	7.5	*6.0*	*4.5*	*3.0*
Aerial biomass (mg)	486.6[a]	457.1[a]	433.4[a]	409.2[a]
Root biomass (mg)	411.0[a]	417.8[a]	365.0[a]	406.9[a]
Height (cm)	10.7[a]	10.1[a]	10.2[a]	10.4[a]
Root length (cm)	421.5[a]	374.6[ab]	411.6[a]	307.8[b]
Short roots/cm	1.3[a]	0.98[a]	0.91[a]	1.02[a]
Short roots/plant	420[a]	365[ab]	390[ab]	307[a]
Ectomycorrhizae (%)	39.1[a]	33.5[a]	37.4[a]	44.6[a]
Ectomycorrhizae/cm	0.36[ab]	0.35[ab]	0.33[ab]	0.47[b]

Date are means of eight replicates. Numbers in a row followed by the same letter are not significantly different (Duncan's test, $P < 0.05$).

Table II. pH values of the substrata used before and after the experiments.

	Before experiment	*Simulated rain pH*			
		7.5	*6.0*	*4.5*	*3.0*
Experiment 1	7.80	7.97	7.86	7.89	7.90
Experiment 2	7.81	7.82	7.78	7.83	7.79
Experiment 3	6.50	6.60	6.63	6.52	6.72

Numbers in a row followed by the same letter are not significantly different (Duncan's test, $P < 0.05$).

mycorrhizal plants were recorded in all treatments. However, mycorrhizae development was enhanced at pH 3 (table IV). The percentage of mycorrhization was higher, always being greater than 35%, and mycorrhizae appeared with a well-developed mantle and abundant external mycelium. No differences among treatments in terms of pH substrate were observed (table II).

DISCUSSION

Simulated acid rain had little influence on *P halepensis* seedlings. Visible effects on the aerial part were inexistent. However, a certain influence on the root system towards a clear trend of reduction of root length due to acidity was noted in the experiments.

The reduction or inhibition of root growth in response to acidity is a fact repeatedly observed in similar experiments. Stroo et al (1988) reported a reduction in the number of short roots/lateral on *Pinus strobus*, and Walker and McLaughin (1991) noted reductions in the length of the lateral roots of *P taeda*. In axenic conditions, Maehara et al (1993) observed a decrease in the total number of short roots. Similar effects have

Table III. Effect of pH of simulated rain on root growth and mycorrhization of *Pinus halepensis* seedlings aged 2 years (experiment 2).

	Simulated rain pH			
	7.5	*6.0*	*4.5*	*3.0*
New roots				
Root biomass (mg)	763.0^a	643.0^a	693.0^a	475.0^b
Growth (%)	47.4^a	31.4^a	23.6^a	32.5^a
Root length (cm)	596.0^a	599.0^a	605.0^a	470.0^b
Short roots/cm	0.85^a	0.96^a	0.81^a	1.40^b
Short roots/plant	522.0^a	563.0^a	491.0^a	626.0^a
Ectomycorrhizae (%)	22.4^a	30.0^a	24.1^a	42.3^a
Ectomycorrhizae/cm	0.20^a	0.29^{ab}	0.17^a	0.62^b
Old roots. Mycorrhization before the experiment 53.5%				
Root biomass (mg)	$1\,918^a$	$1\,930^a$	$2\,050^a$	$2\,036^a$
Ectomycorrhizae (%)	51.0^a	50.2^a	47.0^a	43.1^a

Date are means of eight replicates. Numbers in a row followed by the same letter are not significantly different (Duncan's test, $P < 0.05$).

Table IV. Effect of pH of simulated rain on mycorrhiza formation by *Suillus collinitus* on *Pinus halepensis* seedlings (experiment 3).

	Simulated rain pH			
	7.5	*6.0*	*4.5*	*3.0*
Mycorrhizal seedlings (%)	39	39	25	39
Mycorrhization (%)	40.2^a	36.1^a	28.8^a	54.6^b

Numbers in a row followed by the same letter are not significantly different (Duncan's test, $P < 0.05$).

also been reported in field experiments: Rudawska et al (1994) by comparison of contaminated and uncontaminated forests; and Dighton (1988) in *P sylvestris* stands treated for 2 years with acid rain. The possible mechanisms by which acid rain adversely affected root growth are not clear. One possibility is that soil acidification and the mobilization of Al has an inhibitory effect on root development, as has been reported by McQuattie and Schier (1992). However, this does not seem likely for our findings, because of the physical and chemical characteristics of the soil used in the experiment.

The most obvious conclusion that can be drawn from the data presented here is that no negative effect of acidity was apparent on mycorrhization. On the contrary, ectomycorrhizae were slightly favoured in the most acid treatment (pH 3) in the three experiments carried out, this tendency being clearer in the third experiment.

There is no consistency in the literature on the effects on acid deposition on ectomycorrhiza. Many papers report no response of ectomycorrhizal fungi (McAfee and Fortin, 1987; Meier et al, 1989; Blaschke and Weiss, 1990; Edwards and Kelly, 1992). The studies that show a negative influence of acidity refer to acid substrata or a specific fungus. For example, Shafer et al (1985) reported inhibition of mycorrhization by *Thelephora terrestris* and *Laccaria laccata*; Stroo et al (1988) by *Pisolithus tinctorius* on soils of pH 4.1–5.7; and Maheara et al (1993) also with *Pisolithus tinctorius* in axenic conditions. Dighton and Skeffington (1987) and Dighton (1988) showed similar results on a coraloid morphotype. In natural soils, with a community of mycorrhizal fungi, Blaschke (1988) and Rudawska et al (1996) in acid soil also reported a reduction on mycorrhizae. From these studies it can be deduced that responses to acidity vary depending on host plant, fungus species and, above all, the soil characteristics and duration of exposure.

Hung and Trappe (1983), among others, reported the preference of ectomycorrhizal fungi for slightly acid media. In particular, *S collinitus* 157ED, the strain used for experiment 3, has been observed to have similar colony diameter when cultured in vitro at a range of pH values of 3.5–7.5 (1 point intervals), although a slight reduction in mycelium biomass was noted at 4.5, 3.5 and 2.5. Other *Suillus* species (*S granulatus, S luteus* and *S variegatus*) showed similar behaviour. On the other hand, the tolerance of these species at pH 7.5 has also been demonstrated (Honrubia et al, 1995, 1996). *Suillus* species are very common in *P halepensis* forests and fruit bodies have been found in the site where soil for experiment 1 was collected (Sánchez et al, 1996). Moreover, isolations of fungal symbionts from ectomycorrhizae of seedlings from bioassays of this soil revealed that mycelia obtained were of the *Suillus* type (Honrubia et al, 1995). These facts support that the ectomycorrhizae in experiments 1 and 2 were mainly formed by *Suillus* species. Although the behaviour of fungi in axenic conditions does not exactly reflect their ability to form ectomycorrhizae, the above-mentioned reasons suggest that ectomycorrhizal fungi from the experiments presented here could grow at acid pH. Liming, increased pH and increased soil Ca concentration have been reported to have a negative influence on mycorrhizae of *Picea abies* (Lehto, 1994). Taking into account the high pH of the substrata used, it is possible that the application of rain at pH 3 could favour mycelia growth and mycorrhiza formation.

Moreover, we can hypothesize that acid rain could have an indirect effect on mycorrhiza formation in calcareous soils. The enhancement of mycorrhizal colonization could be due to a mobilization of nutrients in the soil by the acid treatment, or even to the extra amount of N added in this treatment. On the contrary, in acid soils the decreased mycorrhizal colonization due to acid rain has been related to the increased N availability in the soil as a result of high N inputs in the acidic rain treatments (Reich et al, 1985). Further studies to dilucidate the effect of the N incorporated with the acid rain on the plant–soil system and its possible influence at a critical level on mycorrhizae formation should be conducted. In any case, as these authors hypothesized, the negative effect of acid rain on mycorrhiza formation occurs when it is more acidic than the soil.

Enhancements of mycorrhiza formation by low pH have been previously reported. Walker and McLaughin (1991) observed the greatest ectomycorrhizal development of *Pisolithus tinctorius* on loblolly pine treated with the most acidic of the simulated rain, suggesting a depression of the pH of the growing medium. However, we did not observe acidification of the substrata. In experiments 1 and 2, this could be due to the high amount of carbonates in the soil and their consequent buffering action. In experiment 3, an acidification could be expected, but the time of exposure was probably too short. Little information exists about acidification of substrate by acid rain. Dighton (1988) reported the decrease of pH by about 0.4–0.6 of a pH unit in an humoferric podsol treated 2 years with acid rain (pH 3). In contrast, Edwards and Kelly (1992) did not find soil acidification after 3 years of treatment at pH 3.

On the other hand, effects of acid deposition on ectomycorrhizal fungi can be explained without acidification. Maehara et al (1993) reported that acid mist adversely affected the plant transpiration rate and lowered the extractable phosphorus content, and suggested that the retarded mycorrhiza formation was due to alteration of seedling physiological activities, but without affecting the soil.

We can deduce from these findings that in calcareous soils of neutro-basic pH, long-term exposures to acid rain would be necessary to produce slight acidification and, even so, no damage to ectomycorrhizal fungi would be produced. Partial neutralization of rain acidity has been already reported in Mediterranean areas due to the presence of calcareous soils (Camufo et al, 1991; Samara et al, 1992), which suggests the buffering mechanisms of the ecosystem and their importance in relation to stress phenomena such as acid rain.

In conclusion, our results indicate that in calcareous soils of neutro-basic pH, short-term exposures to acid deposition did not negatively affect ectomycorrhizal fungi, in particular the *Suillus* species, and that they even responded favourably to acidity.

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