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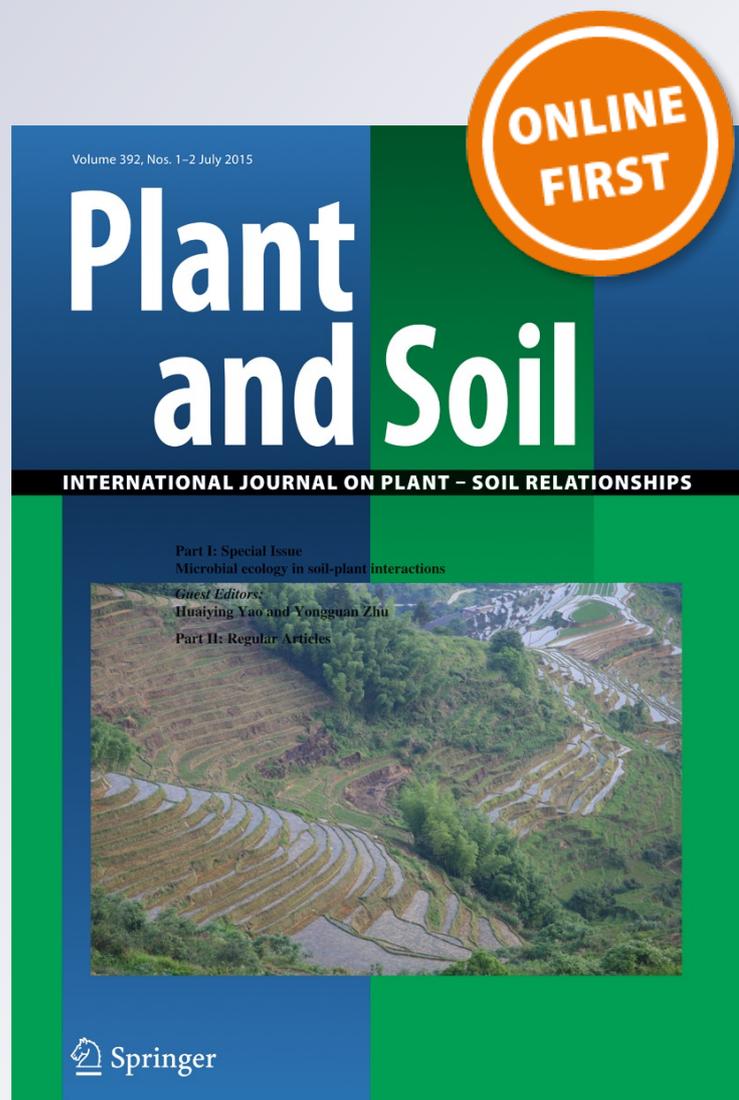
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# Plant tolerance of ammonium varies between co-existing Mediterranean species

Teresa Dias · Maria Amélia Martins-Loução ·  
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## Abstract

**Background** Previous studies showed that the two main Mediterranean plant functional groups, summer semi-deciduous and evergreen sclerophylls, differ in soil characteristics and nitrate ( $\text{NO}_3^-$ ) use strategies: even though summer semi-deciduous plants have higher  $\text{NO}_3^-$  availability than evergreen sclerophylls,  $\text{NO}_3^-$  reduction (i.e., nitrate reductase activity—NRA) is lower, and is not stimulated by substrate ( $\text{NO}_3^-$ ) availability. **Aims** Test if in *Cistus albidus* plants, a summer semi-deciduous species, ammonium ( $\text{NH}_4^+$ ) can inhibit NRA, despite the availability of  $\text{NO}_3^-$ , and whether *Olea europaea* plants, evergreen sclerophyll, are more tolerant of  $\text{NH}_4^+$  than the former. **Methods** One-year-old *C. albidus* and wild *O. europaea* potted plants were supplied with both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  at increasing levels (0.1; 0.2; 0.4; 0.8 and 1.6 % N). Tolerance of  $\text{NH}_4^+$  was evaluated using integrative (mortality and biomass accumulation) and plant

nitrogen metabolism parameters (in vitro NRA and concentrations of  $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) determined in roots and leaves.

**Results** *C. albidus* plants were consistently less  $\text{NH}_4^+$  tolerant than *O. europaea*, displaying: higher mortality; growth and NRA inhibition and  $\text{NH}_4^+$  accumulation above 0.2 %  $\text{NH}_4\text{NO}_3\text{-N}$  in the soil. In contrast, *O. europaea* plants seemed to buffer the full range of tested  $\text{NH}_4\text{NO}_3$  levels.

**Conclusions** *C. albidus* plants were less  $\text{NH}_4^+$  tolerant than *O. europaea*. The ecological implications of this contrasting  $\text{NH}_4^+$  tolerance are discussed.

**Keywords** Ammonium · *Cistus albidus* · Mediterranean · NRA · *Olea europaea* · Root shoot partitioning

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## Introduction

The alarming decline in global biodiversity (Sala et al. 2000; Rockström et al. 2009) is of paramount concern in biodiversity hotspots such as Mediterranean-type ecosystems (Myers et al. 2000; Klausmeyer and Shaw 2009). Although they cover only c.a. 2 % of the Earth's land area, they host c.a. 20 % of our planet's known vascular plant diversity (Cowling et al. 1996; Klausmeyer and Shaw 2009). Indeed, Mediterranean-type ecosystems are thought to be currently experiencing the greatest proportional change in biodiversity (Sala et al. 2000).

Despite its richness, complexity and diversity (Cowling et al. 1996), Mediterranean vegetation may be divided into two main plant functional groups: summer semi-deciduous and evergreen sclerophylls. These groups have been characterized in terms of their phenology (Correia 1988), water relations, carbon exchange properties (Werner et al. 1999), post-fire regeneration strategies (Trabaud and Lepart 1981; Keeley and Soderstrom 1986; Clemente et al. 2005) and their demographic patterns during a secondary post-fire succession (Clemente et al. 1996). In early successional stages, summer semi-deciduous species are more abundant, being progressively eliminated under canopies of evergreen sclerophyll species in the late successional stages. At intermediate stages, the two plant functional groups co-exist. More recently, a relation was shown between the two plant functional groups and the characteristics of the soils they influence (Cruz et al. 2008), and how this relation conditions plants' nitrate ( $\text{NO}_3^-$ ) use strategies (Dias et al. 2011): although summer semi-deciduous species occupied  $\text{NO}_3^-$ -richer soil patches and had more  $\text{NO}_3^-$  in their leaves than evergreen sclerophylls,  $\text{NO}_3^-$  reduction (i.e., nitrate reductase activity—NRA) in the former was lower than in the latter and responded less to increases in substrate ( $\text{NO}_3^-$ ) availability (Dias et al. 2011). One of the suggested hypotheses for the lack of stimulation of NRA by  $\text{NO}_3^-$  was that the summer semi-deciduous species were being inhibited, possibly by ammonium ( $\text{NH}_4^+$ ), as there appeared to be a threshold soil  $[\text{NH}_4^+]$  above which no summer semi-deciduous species occurred (Dias et al. 2011). This exclusion of summer semi-deciduous species (but not of evergreen sclerophylls) from soil patches with higher  $[\text{NH}_4^+]$  seems to occur despite the  $[\text{NO}_3^-]$ , as shown by Dias et al. (2014) in a field N-manipulation experiment.

All the factors that modulate NRA (e.g., concentration and composition of N compounds present in the plant material, hormone balance of leaves and roots, vacuolar pH, mitochondrial concentration of AMP, etc.—Kaiser and Huber 2001) are affected by the relative uptake and metabolization of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  (Britto and Kronzucker 2002). Indeed,  $\text{NH}_4^+$  can affect  $\text{NO}_3^-$  metabolism, because  $\text{NH}_4^+$ , or its metabolic products, can inactivate (Orebamjo and Stewart 1975a) and/or repress NRA (Orebamjo and Stewart 1975b). The objective of this study was therefore to test whether  $\text{NH}_4^+$  can indeed inhibit the stimulation of NRA by  $\text{NO}_3^-$  in summer semi-deciduous species. To that end, we conducted a pot experiment in which plants were

supplied with both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ( $\text{NH}_4\text{NO}_3$ ) at increasing levels, within the range they experience under natural conditions where they co-exist (Cruz et al. 2008; Dias et al. 2011). The inhibition of NRA by  $\text{NH}_4^+$  displayed by summer semi-deciduous species would be an indicator (together with  $\text{NO}_3^-$  and  $\text{NH}_4^+$  accumulation in plant material) of an overall sensitivity to / lower tolerance of  $\text{NH}_4^+$  (high plant mortality and low biomass accumulation). In contrast, evergreen sclerophylls would be more tolerant of a wider range of  $\text{NH}_4^+$  availabilities than summer semi-deciduous species.

This is especially relevant now that increased nitrogen (N) availability is a major threat to biodiversity (Bobbink et al. 1998, 2010); and specifically in Mediterranean type ecosystems (Sala et al. 2000; Phoenix et al. 2006; Ochoa-Hueso et al. 2011), and since  $\text{NH}_4^+$  is a driving force of Mediterranean ecosystems' structure and function (Dias et al. 2014). Of the five Mediterranean regions in the world, California and the Mediterranean Basin are those most threatened by increased N deposition (Ochoa-Hueso et al. 2011). While much is known of the impacts of increased N deposition in California, still very little is known of Mediterranean Basin ecosystems (Bobbink et al. 2010), where it is expected to increase threefold by 2050 (Galloway et al. 2004; Phoenix et al. 2006). We tested the contrasting  $\text{NH}_4^+$  tolerance of one species from each of the two plant functional groups, using two widespread Mediterranean Basin species that co-exist in many habitats (Werner et al. 2001; Cruz et al. 2008; Dias et al. 2011): *Cistus albidus* L., a summer semi-deciduous, and wild *Olea europaea* L., an evergreen sclerophyll. The implications of these species' tolerances of  $\text{NH}_4^+$  will be discussed in terms of secondary post-fire succession and the expected increases in N deposition in the Mediterranean Basin.

## Materials and methods

### Plant material and growth conditions

Soil and seeds from *Cistus albidus* L. and wild *Olea europaea* L. were collected in 2010 from the same Mediterranean maquis described in Dias et al. (2011), located in Serra da Arrábida, in the Arrábida Natural Park, south of Lisbon, Portugal (38°27'34"N, 9°0'20"W, a Natura 2000 site—PTCON0010 Arrábida/Espichel).

Background N deposition at the site is estimated to be  $<4 \text{ kg ha}^{-1} \text{ year}^{-1}$  (grid location:  $x=53$  and  $y=4$ , [http://www.emep.int/mscw/index\\_mscw.html](http://www.emep.int/mscw/index_mscw.html)).

The soil, collected in April 2010 under the canopies of the summer semi-deciduous plants, contained c.a. 9 % organic matter, 0.1 % total N, 33 ppm total P and 400 ppm K, and had pH (H<sub>2</sub>O) 6.5 (Cruz et al. 2008). In July 2010, two fruits (capsules) per plant were collected from ten randomly chosen *C. albidus* individuals and in October 2010, 20 fruits per plant were collected from ten randomly chosen individuals of wild *O. europaea*. The seeds from these fruits were then cleaned. Seeds of *C. albidus* were stored (4 °C) until December, while those of *O. europaea* were germinated immediately.

One hundred plants of each species were grown from seed at the Universidade de Lisboa (Portugal). Seedlings of both species were germinated in large trays, where they were kept for 1 month (*C. albidus*) or 3 months (*O. europaea*), after which 50 'homogenous' plants were transferred to pots (10 L capacity; 1 plant per plot—January 2011). One year later, pots with one plant each were randomly divided into 5 groups for each species. Each group received a defined amount of ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) to achieve the following levels of NH<sub>4</sub>NO<sub>3</sub>-N in the soil at the beginning of the experiment: 0.1 (the control group received no NH<sub>4</sub>NO<sub>3</sub>); and 0.2; 0.4; 0.8; and 1.6 % soil dry weight (DW). The NH<sub>4</sub>NO<sub>3</sub>-N was added in January 2012 in 2 equal applications 2 weeks apart. The defined concentrations of total N correspond to the full range found at the site where they were collected (Cruz et al. 2008; Dias et al. 2011).

Plants were grown for another 6 months (from January to the end of June 2012) in open air conditions, similar to those at the source site (Cruz et al. 2008; Dias et al. 2011), except that they were watered regularly to maintain soil water content at c.a.  $0.16 \text{ m}^3 \text{ H}_2\text{O m}^{-3}$  soil (time domain reflectometry—TDR). During the experimental period, the air temperature varied between 5 and 36 °C, air relative humidity between 48 and 100 %, maximum day light intensity between 340 and  $1600 \mu\text{E m}^{-2} \text{ s}^{-1}$ , and soil temperature between 8.5 and 22.5 °C.

#### Harvesting and sample collection

At the end of June 2012 plants were harvested. Soil samples for determination of inorganic N availability were collected from each pot and consisted of three

cores, with 2 cm diameter and 10 cm length each, taken within 1 cm of each other and 5 cm from the plant stem.

To ensure detection of maximum nitrate reductase activities (NRA), the third and fourth youngest pairs of leaves were collected between 10:00 and 14:00 (Dias et al. 2011) from all the potted plants and the fresh weight recorded. Leaves were immediately frozen in liquid N and later used to determine leaf NRA and the concentrations of nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>).

At harvesting, roots and the remaining shoots were separated and fresh weights determined. Roots were sampled, weighed, immediately frozen in liquid N and later used to determine NRA and the concentrations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>. The ratio between fresh and dry weight was determined by drying the roots and shoots of five plants (per species and per NH<sub>4</sub>NO<sub>3</sub>-N treatment) at 60 °C until constant weight. The leaf and root samples collected for chemical analysis were taken into consideration in the calculation of total accumulated biomass.

#### Chemical determinations

The concentrations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> in the soils and plant material (roots and leaves) were determined. Soil extracts were prepared as described in Dias et al. (2014) and plant extracts as described in Cruz and Martins-Loução (2000). NO<sub>3</sub><sup>-</sup> was determined by electrophilic substitution of salicylate acid (Matsumura and Witjaksono 1999) and NH<sub>4</sub><sup>+</sup> was determined using a modified Berthelot reaction (Cruz and Martins-Loução 2000).

NRA was determined in vitro in roots and leaves of *C. albidus* and *O. europaea* plants according to Kaiser et al. (2000), as described in Dias et al. (2011). The potential NRA (under non-limiting conditions) and the physiological reaction (under limiting conditions) were determined. NRA was expressed as  $\mu\text{mol g}^{-1} \text{ FW h}^{-1}$ . The enzyme activation state was determined as the ratio between the potential NRA and the physiological NRA.

#### In vitro effect of increasing [NH<sub>4</sub><sup>+</sup>] on NRA

Roots and leaves of the plants grown with 0.2 % NH<sub>4</sub>NO<sub>3</sub> were collected and transferred to vials so that the roots and petioles were immersed in an incubation medium: 25 ml of 25 % modified Crone solution (Cruz et al. 1991). With the exception of the control plants, the incubation medium was supplemented with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

to obtain the following final  $[\text{NH}_4^+]$ : 0; 0.1; 0.2; 0.3; 0.4; and 0.5 mM  $\text{NH}_4^+\text{-N}$ . The media were gently aerated to maintain adequate oxygen supply and minimize boundary layers around the plant material. Five replicates per plant part and  $[\text{NH}_4^+]$  treatment were prepared. Roots and leaves were incubated for 8 h, roots in the dark. Each pool of leaves and roots was then washed with deionised water three times for 30 s, and the water discarded. At the end of the incubation period NRA (potential and physiological) and  $[\text{NH}_4^+]$  were determined in the roots and leaves as described above.

### Calculations and statistics

The in vitro effect of the  $[\text{NH}_4^+]$  on NRA (potential and physiological—Supplementary data, Table S1) was expressed as the % of change at a given  $[\text{NH}_4^+]$  (Pot. NRA<sub>x</sub> and Phys. NRA<sub>x</sub>;  $x=0.1; 0.2; 0.3; 0.4$  and 0.5 mM) in relation to the absence of  $\text{NH}_4^+$  from the incubation medium (Fig. 4), calculated as follows:

$$\text{NRA inhibition}(\%) = \frac{\text{NRA}_{[\text{NH}_4^+]=x} - \text{NRA}_{[\text{NH}_4^+]=0}}{\text{NRA}_{[\text{NH}_4^+]=0}} \times 100$$

Effects of the N treatments on plant mortality were investigated using a generalized linear model (GLM) with binomial distribution and a logit link function (binary variable) for each plant species separately. Whenever significant differences were found ( $p < 0.05$ ), post-hoc comparisons were performed with pairwise *t*-tests with Holm adjustment. Summary statistics of soil and plant concentrations of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  and plant NRA of the  $\text{NH}_4\text{NO}_3$  treatments were compared. Two-way ANOVA was applied to determine if there were significant interactions between species and  $\text{NH}_4\text{NO}_3$  level for soil and plant variables (Table S1). In most cases there were significant interactions between factors so that differences between  $\text{NH}_4\text{NO}_3$  levels in NRA (potential, physiological and activation state) and soil, root and leaf  $[\text{NO}_3^-]$  and  $[\text{NH}_4^+]$  were examined for each species (LSD test;  $p < 0.05$ ). The effect of  $[\text{NH}_4^+]$  in the incubation medium in NRA inhibition (potential and physiological) and leaf  $[\text{NH}_4^+]$  were compared (two-sided *t*-test,  $p < 0.05$ ) for the different  $[\text{NH}_4^+]$  in the incubation medium. In all cases, analyses were performed to ensure that the assumptions regarding the tests' applications were not violated. SPSS software, version 20.0, was used for all tests.

### Results

Quantification of the pools of nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) in the soils at the end of the experiment showed that the  $\text{NH}_4\text{NO}_3$  treatments increased the availability of both  $\text{NO}_3^-$  and  $\text{NH}_4^+$ , the concentrations of which increased according to the  $\text{NH}_4\text{NO}_3$  levels (Tables S1 and 1).

Despite the presence of  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  did inhibit the stimulation of NRA by  $\text{NO}_3^-$  in *C. albidus* but not in *O. europaea*. The contrasting tolerances of  $\text{NH}_4^+$  displayed by the two plant species were evident when analysing integrative- (e.g., mortality, biomass accumulation) and plant N metabolism-related parameters (NRA,  $[\text{NO}_3^-]$  and  $[\text{NH}_4^+]$ ).

Tolerance of  $\text{NH}_4^+$  differed between *C. albidus* and *O. europaea*, and indeed the  $\text{NH}_4\text{NO}_3$  treatments caused greater mortality of plants of *C. albidus* (Wald estimate=103.33;  $p=0.000$ ) plants than of *O. europaea* (Wald estimate=5.40;  $p=0.249$ —Table 2): under the highest  $\text{NH}_4\text{NO}_3$  treatments (0.8 and 1.6 %) all the *C. albidus* plants died, whereas only a few *O. europaea* plants did. Biomass accumulation in response to the  $\text{NH}_4\text{NO}_3$  treatments also differed between the two plant species (Table S1 and Fig. 1): biomass accumulation by *C. albidus* increased at up to 0.2 %  $\text{NH}_4\text{NO}_3$ , whereas further increases in  $\text{NH}_4\text{NO}_3$  drastically reduced biomass accumulation (0.4 %) and led to plant death (0.8 and 1.6 %—Table 2). In contrast, biomass accumulation in *O. europaea* plants did not respond to the  $\text{NH}_4\text{NO}_3$  treatments.

The in vitro NRA assays showed that both roots and leaves of both plant species contributed to  $\text{NO}_3^-$  reduction (Fig. 2). However the relative contribution of each plant part to the total  $\text{NO}_3^-$  reduction varied according to the  $\text{NH}_4\text{NO}_3\text{-N}$  dose and between species (Table S1). The main differences between the plant species' response to the  $\text{NH}_4\text{NO}_3$  treatments were in the leaf response patterns. As expected, increasing  $\text{NH}_4\text{NO}_3$  availability had a negative impact on the NRA (potential and physiological) and the enzyme activation state of *C. albidus* plants but not of *O. europaea*, which remained almost unchanged (Fig. 2) despite the increased  $\text{NH}_4\text{NO}_3$  availability.

Species-specific responses to the  $\text{NH}_4\text{NO}_3$  treatments were also evident in terms of the inorganic N pools in the plants: in *O. europaea* roots and especially leaves, the  $[\text{NH}_4^+]$  remained low despite the N treatments, while in *C. albidus* grown under 0.4 %  $\text{NH}_4\text{NO}_3$

**Table 1** Effect of  $\text{NH}_4\text{NO}_3$  treatments (0.1; 0.2; 0.4; 0.8; and 1.6 % N) on the concentrations of ammonium ( $[\text{NH}_4^+]$ ) and nitrate ( $[\text{NO}_3^-]$ ) in the soils of the pots where the *Cistus albidus* and *Olea europaea* plants grew. There were significant interactions between treatment and species (Table S1). Different letters (Latin for

*C. albidus* and Greek for *O. europaea*) refer to significant differences for soil  $[\text{NH}_4^+]$  and  $[\text{NO}_3^-]$  between  $\text{NH}_4\text{NO}_3$  treatments (ANOVA  $p < 0.05$  followed by a LSD test) for each species. Values represent the mean  $\pm$  SD ( $n = 10$  pots)

$\text{NH}_4\text{NO}_3$ treatments	<i>Cistus albidus</i>		<i>Olea europaea</i>	
	Soil $[\text{NH}_4^+]$ ( $\mu\text{g g}^{-1}$ )	Soil $[\text{NO}_3^-]$ ( $\mu\text{g g}^{-1}$ )	Soil $[\text{NH}_4^+]$ ( $\mu\text{g g}^{-1}$ )	Soil $[\text{NO}_3^-]$ ( $\mu\text{g g}^{-1}$ )
0.1 %	15 $\pm$ 1 e	11 $\pm$ 1 d	12 $\pm$ 1 $\epsilon$	10 $\pm$ 1 $\epsilon$
0.2 %	39 $\pm$ 2 d	11 $\pm$ 1 d	18 $\pm$ 1 $\delta$	15 $\pm$ 1 $\delta$
0.4 %	154 $\pm$ 3 c	86 $\pm$ 3 c	38 $\pm$ 2 $\chi$	135 $\pm$ 4 $\chi$
0.8 %	283 $\pm$ 6 b	255 $\pm$ 7 b	156 $\pm$ 5 $\beta$	281 $\pm$ 6 $\beta$
1.6 %	312 $\pm$ 8 a	281 $\pm$ 9 a	337 $\pm$ 8 $\alpha$	327 $\pm$ 8 $\alpha$

the  $[\text{NH}_4^+]$  more than doubled in the roots and tripled in the leaves in comparison to the 0.1 %  $\text{NH}_4\text{NO}_3$  plants (Table S1 and Fig. 3).

*C. albidus* NRA was more strongly inhibited by  $\text{NH}_4^+$  added to the incubation medium than *O. europaea* (Figs S1 and 4) except for the physiological NRA determined in the leaves (Fig. 4d). Addition of  $\text{NH}_4^+$  had almost no effect on the potential NRA determined in the roots of *O. europaea*, while 0.3 mM  $\text{NH}_4^+$  caused at least a 50 % inhibition in *C. albidus* (Fig. 4a). A 50 % inhibition of the potential NRA in the leaves of *C. albidus* was attained with 0.3 mM  $\text{NH}_4^+$ , but much less in *O. europaea* (Fig. 4b). The difference in inhibitory effect of  $[\text{NH}_4^+]$  in the incubation medium on the physiological NRA in plant roots was even more pronounced: that in *C. albidus* was 50 % inhibited by 0.1 mM  $\text{NH}_4^+$ , while even 0.5 mM  $\text{NH}_4^+$  had no such negative effect on physiological NRA in roots of *O. europaea* (Fig. 4c).

Analysis of the same extracts used for determining NRA, showed that the roots of *C. albidus* accumulated

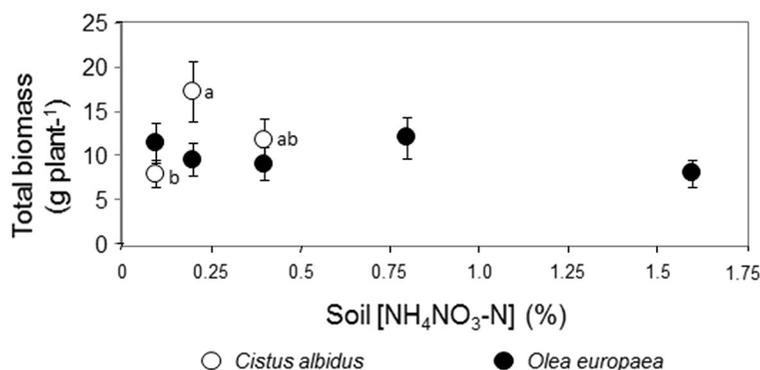
more  $[\text{NH}_4^+]$  than *O. europaea* when incubated with the higher  $[\text{NH}_4^+]$  (0.2; 0.3, 0.4 and 0.5 mM—Fig. 5a), while for the leaves there were no differences between the two species in accumulation in the leaves (Fig. 5b).

## Discussion

As hypothesized, despite the presence of  $\text{NO}_3^-$  (Table 1),  $\text{NH}_4^+$  did inhibit the stimulation of nitrate reductase activity (NRA) by  $\text{NO}_3^-$  in *Cistus albidus* but not in *Olea europaea*. The two co-existing plant species displayed contrasting tolerances of  $\text{NH}_4^+$  that were evident when analysing integrative- (e.g., mortality, biomass accumulation) and N metabolism-related parameters (NRA,  $[\text{NO}_3^-]$  and  $[\text{NH}_4^+]$ ) in both roots and shoots. It has been suggested that sensitivity to  $\text{NH}_4^+$  is a universal phenomenon, with the threshold at which symptoms manifest differing widely between plant species (Britto and Kronzucker 2002). The symptoms of  $\text{NH}_4^+$  toxicity include chlorosis of leaves, overall growth suppression and even death. Thus, depending on the parameter, different thresholds for  $\text{NH}_4^+$  toxicity could be identified. For instance, based on our results, using death as an indicator, the threshold concentration for  $\text{NH}_4^+$  toxicity to *C. albidus* could be set at  $< 0.4$  %  $\text{NH}_4^+$ , since all plants grown with 0.4 %  $\text{NH}_4^+$  (0.8 %  $\text{NH}_4\text{NO}_3$ ) died (Table 2). Based on biomass accumulation, the toxicity to *C. albidus* would be  $< 0.2$  %  $\text{NH}_4^+$ , since the biomass accumulation of plants grown with 0.2 %  $\text{NH}_4^+$  (0.4 %  $\text{NH}_4\text{NO}_3$ ) was lower than that of those grown with 0.1 %  $\text{NH}_4^+$  (0.2 %  $\text{NH}_4\text{NO}_3$ —Fig. 1). On the other hand, comparison of the potential NRA in the leaves (Fig. 2b) of the *C. albidus* plants

**Table 2** Effect of  $\text{NH}_4\text{NO}_3$  treatments (0.1; 0.2; 0.4; 0.8; and 1.6 % N soil) on the mortality of *Cistus albidus* and *Olea europaea*. Different letters refer to significant differences between the  $\text{NH}_4\text{NO}_3$  treatments (GLM) for a given species ( $p < 0.05$ ). Values represent the number of dead plants out of 10

$\text{NH}_4\text{NO}_3$ treatment (%)	<i>C. albidus</i>	<i>O. europaea</i>
0.1	3 <sup>b</sup>	0
0.2	2 <sup>b</sup>	0
0.4	2 <sup>b</sup>	0
0.8	10 <sup>a</sup>	1
1.6	10 <sup>a</sup>	3

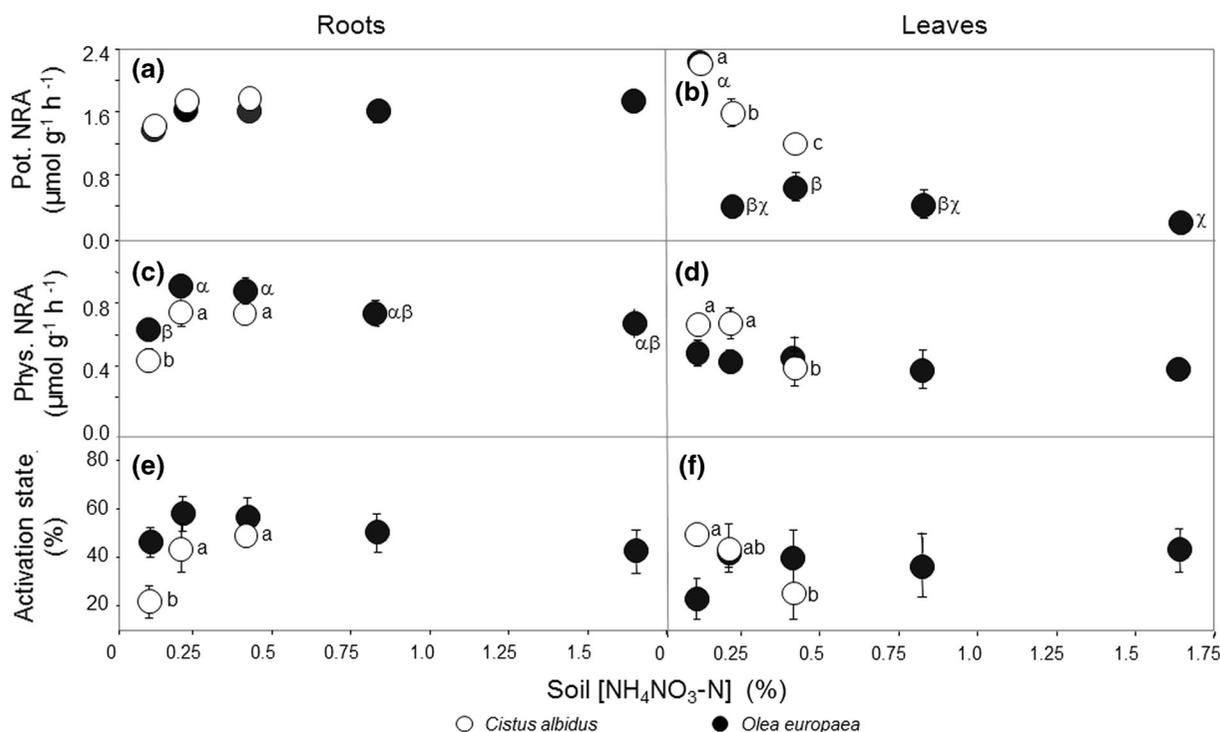


**Fig. 1** Effect of NH<sub>4</sub>NO<sub>3</sub> treatments (0.1; 0.2; 0.4; 0.8; and 1.6 % N soil) on the biomass accumulation of *Cistus albidus* (white circles) and *Olea europaea* (black circles) plants. There were significant interactions between treatment and species (Table S1).

Different letters (Latin for *C. albidus* and Greek for *O. europaea*) refer to significant differences between NH<sub>4</sub>NO<sub>3</sub> treatments (ANOVA  $p < 0.05$  followed by a LSD test) for each species. Symbols represent the mean  $\pm$  SD ( $n = 5$  plants)

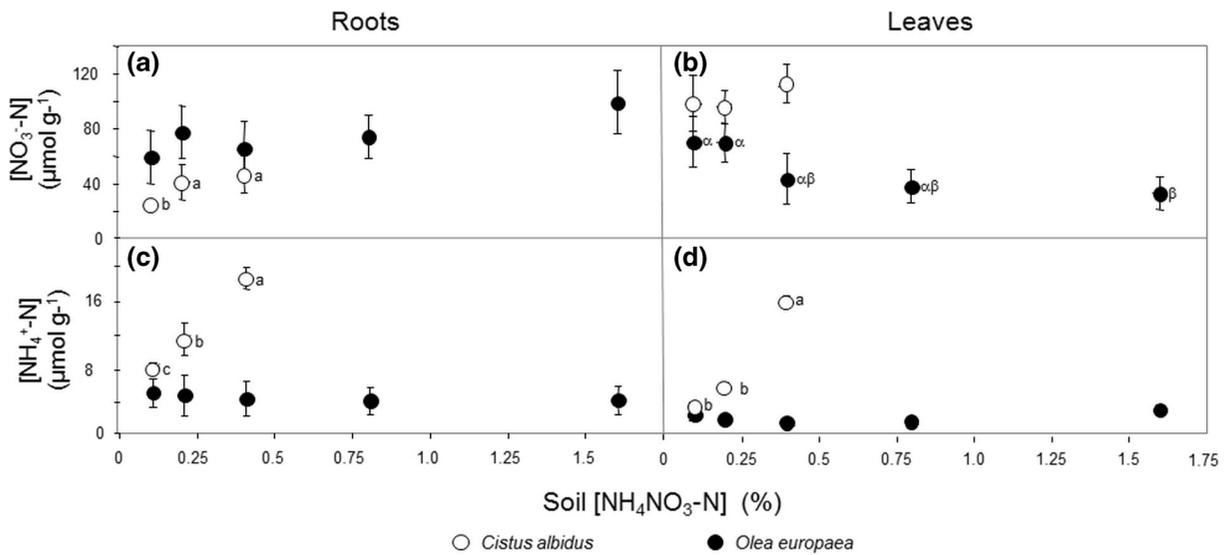
grown with 0.1 % and 0.2 % NH<sub>4</sub>NO<sub>3</sub> (0.05 and 0.1 % NH<sub>4</sub><sup>+</sup> respectively), would set the threshold for NH<sub>4</sub><sup>+</sup> toxicity at <0.1 % NH<sub>4</sub><sup>+</sup>. We suggest that the threshold for NH<sub>4</sub><sup>+</sup> toxicity to *C. albidus* plants should be based on biomass accumulation, i.e., <0.2 % NH<sub>4</sub><sup>+</sup>. In

contrast, based on biomass accumulation alone the threshold for NH<sub>4</sub><sup>+</sup> toxicity to *O. europaea* plants would be >0.8 % NH<sub>4</sub><sup>+</sup> (1.6 % NH<sub>4</sub>NO<sub>3</sub>), since no effect of the NH<sub>4</sub>NO<sub>3</sub> treatments was observed for the tested range (Fig. 1).



**Fig. 2** Effect of the NH<sub>4</sub>NO<sub>3</sub> treatments (0.1; 0.2; 0.4; 0.8; and 1.6 % N soil) on the potential NRA (a and b), physiological NRA (c and d) and the NR activation state (e and f), determined in the roots and leaves of *Cistus albidus* (white circles) and *Olea europaea* (black circles) plants. There were significant interactions

between treatment and species (Table S1). Different letters (Latin for *C. albidus* and Greek for *O. europaea*) refer to significant differences between NH<sub>4</sub>NO<sub>3</sub> treatments (ANOVA  $p < 0.05$  followed by a LSD test) for each species. Symbols represent the mean  $\pm$  SD ( $n = 10$  plants)

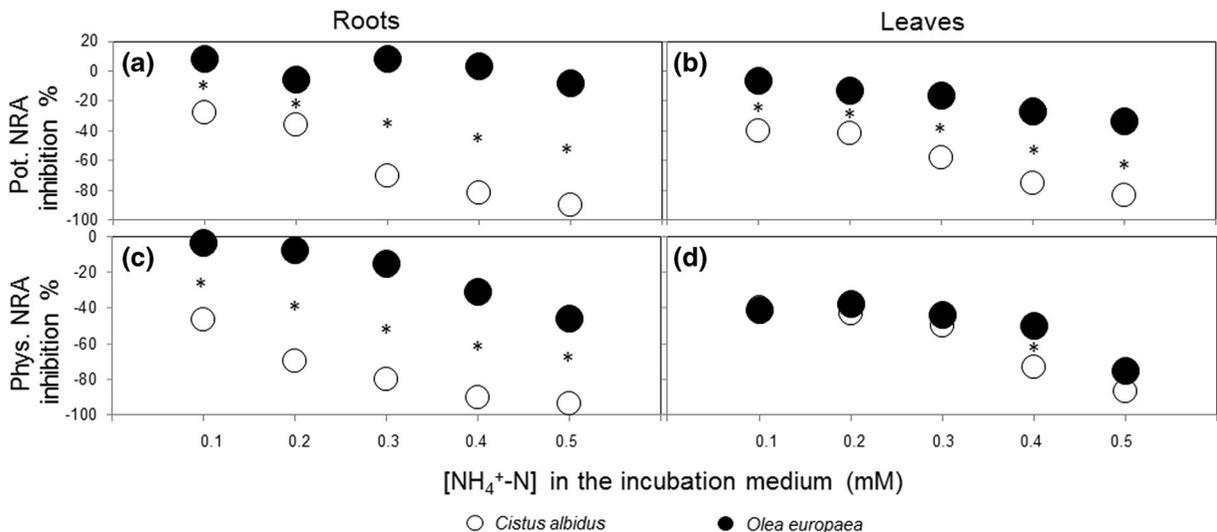


**Fig. 3** Effect of the  $\text{NH}_4\text{NO}_3$  treatments (0.1; 0.2; 0.4; 0.8; and 1.6 % N soil) on the  $[\text{NO}_3^-]$  (a and b) and  $[\text{NH}_4^+]$  (c and d) determined in the roots (a and c) and leaves (b and d) of *Cistus albidus* (white circles) and *Olea europaea* (black circles) plants. There were significant interactions between treatment and species

(Table S1). Different letters (Latin for *C. albidus* and Greek for *O. europaea*) refer to significant differences between  $\text{NH}_4\text{NO}_3$  treatments (ANOVA  $p < 0.05$  followed by a LSD test) for each species. Symbols represent the mean  $\pm$  SD ( $n = 10$  plants)

If  $\text{NH}_4^+$  was not having a toxic effect then, as  $\text{NO}_3^-$  availability increased ( $\text{NH}_4\text{NO}_3$  treatments), NRA potential should have also increased, especially in the leaves, due to the increasing rate of  $\text{NO}_3^-$  transport to the shoots. However, *O. europaea* plants were not affected by increasing  $\text{NO}_3^-$  availability (Table 1) in terms

of either NRA (Fig. 2) or the  $\text{NO}_3^-$  transported to the shoots; in fact  $\text{NO}_3^-$  tended to accumulate in their roots but not their leaves (Fig. 3). Despite the tendency of *C. albidus* plants to accumulate  $\text{NO}_3^-$  in both their roots and leaves, NRA was not stimulated, rather it was inhibited.  $\text{NH}_4^+$  accumulated in the roots and leaves of



**Fig. 4** Inhibitory effect of increasing  $[\text{NH}_4^+]$  in the incubation medium on the potential (a and b) and physiological NRA (c and d) determined in the roots and leaves of *Cistus albidus* (white circles) and *Olea europaea* (black circles) plants. Roots and leaves were collected from the plants grown with 0.2 %  $\text{NH}_4\text{NO}_3$ , then

incubated for 8 h in media containing increasing concentrations of ammonium sulphate (0; 0.1; 0.2; 0.3; 0.4 and 0.5 mM of N). Asterisks (\*) refer to statistically significant differences between species ( $t$ -test  $p < 0.05$ ). Symbols represent the mean  $\pm$  SD ( $n = 5$  plants)

*C. albidus* plants more than  $\text{NO}_3^-$ , suggesting that this species cannot control the uptake and transport of  $\text{NH}_4^+$ , which is crucial for survival (Britto and Kronzucker 2002; Kronzucker et al. 2003; Britto and Kronzucker 2013), and that  $\text{NH}_4^+$ , or an related  $\text{NH}_4^+$ -metabolite, inhibited NRA. The  $[\text{NH}_4^+]$  determined in the leaves of *C. albidus* may indicate whether the availability of  $\text{NH}_4^+$  is adequate for a given plant species as, under field conditions: i) the  $[\text{NH}_4^+]$  determined in the leaves of three evergreen sclerophyll species (*Arbutus unedo*, *O. europaea* and *Quercus coccifera*) ranged between 1 and  $3 \mu\text{mol g}^{-1}$  (unpublished), similar to the concentrations determined in *O. europaea* plants grown under the full range of  $\text{NH}_4\text{NO}_3$  treatments (Fig. 3); and ii)  $[\text{NH}_4^+]$  in three summer semi-deciduous species (*C. albidus*, *C. salvifolius* and *Rosmarinus officinalis*) ranged between 2 and  $7 \mu\text{mol g}^{-1}$  (unpublished), similar to the concentrations determined in the 0.1 and 0.2 %  $\text{NH}_4\text{NO}_3$  *C. albidus* plants (Fig. 3). The plants subjected to 0.4 %  $\text{NH}_4\text{NO}_3$  that accumulated  $\sim 16 \mu\text{mol g}^{-1}$  of  $\text{NH}_4^+$  would already be suffering from  $\text{NH}_4^+$  toxicity, suggesting that the  $[\text{NH}_4^+]$  in the leaves, of at least summer semi-deciduous species ( $< 16 \mu\text{mol g}^{-1}$ ) may be used as an indicator of 'adequate'  $\text{NH}_4^+$  availability.

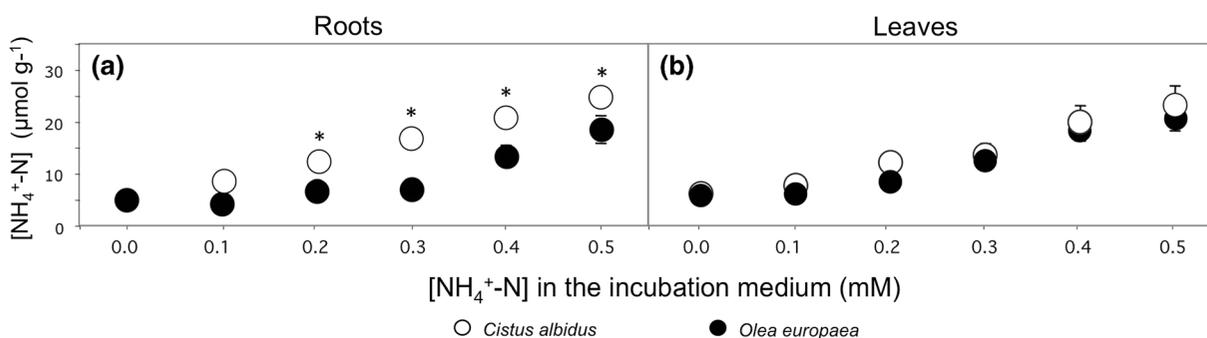
The inhibitory effect of  $\text{NH}_4^+$  on NRA despite the presence of its substrate ( $\text{NO}_3^-$ ) was observed by Botella et al. (1993) when testing the effects of different  $\text{NH}_4^+/\text{NO}_3^-$  ratios on NRA in shoots and roots of wheat seedlings. The direct inhibitory effect of  $\text{NH}_4^+$ , or an  $\text{NH}_4^+$ -related metabolite, on the synthesis and activity of NR was confirmed by incubating roots and leaves of plants grown under 0.1 %  $\text{NH}_4^+$  (0.2 %  $\text{NH}_4\text{NO}_3$ ) under increasing  $[\text{NH}_4^+]$  (Fig. 4). The results clearly show that

$\text{NO}_3^-$  reduction in the leaves, and especially the roots, of *C. albidus* is much more sensitive to inhibition by  $\text{NH}_4^+$  than that in *O. europaea*, thus showing a clear difference in the susceptibility of the two plant species' NRA to  $\text{NH}_4^+$ .

Our data suggest that it is not just a case of maintaining adequate  $[\text{NH}_4^+]$ , as even when there were no significant differences between the two plant species in the  $\text{NH}_4^+$  accumulated in the plant material (e.g., the roots of plants incubated with 0.1 %  $\text{NH}_4^+$ —Fig. 5), NRA was inhibited much more in *C. albidus* than in *O. europaea* (e.g., 20–50 % and 0 % respectively—Fig. 4). Altogether, it seems that the inability of *C. albidus* plants to prevent the uptake of  $\text{NH}_4^+$  from reaching toxic levels negatively impacts its metabolism (Fig. 2), and ultimately leads to plant death (Table 2). *O. europaea* plants showed a surprisingly high capacity to buffer the wide range of  $\text{NH}_4^+$  (and  $\text{NO}_3^-$ ) applications tested. This may be due to many non-exclusive factors including chemical, physical and spatial compartmentation of  $\text{NH}_4^+$ .

#### Potential ecological implications

Since *C. albidus* and *O. europaea* occupy distinct nitrogenous environments (Cruz et al. 2008; Dias et al. 2011), and differ in their patterns of NRA (Dias et al. 2011), the difference in their tolerance of  $\text{NH}_4^+$  availability was not surprising. Along a post-fire secondary succession, besides the shift in dominance from summer semi-deciduous (e.g., *C. albidus*) to evergreen sclerophylls (e.g., *O. europaea*), there is also a shift in the predominant form of inorganic N;  $\text{NO}_3^-$  is more



**Fig. 5** Effect of increasing  $[\text{NH}_4^+]$  in the incubation medium on  $[\text{NH}_4^+]$  determined in the roots (a) and leaves (b) of *Cistus albidus* (white circles) and *Olea europaea* (black circles) plants. Roots and leaves were collected from the plants grown with 0.2 %  $\text{NH}_4\text{NO}_3$ , then incubated for 8 h in media containing increasing

concentrations of ammonium sulphate (0; 0.1; 0.2; 0.3; 0.4 and 0.5 mM of N—as in Fig. 4). There were significant interactions between treatment and species (Table S1). Asterisks (\*) refer to statistically significant differences between species (*t*-test  $p < 0.05$ ). Symbols represent the mean  $\pm$  SD ( $n = 5$  plants)

abundant in the early stages, and  $\text{NH}_4^+$  later on (Cruz et al. 2003). When these species co-exist, *C. albidus* and other summer semi-deciduous species being relatively  $\text{NH}_4^+$ -intolerant (Kronzucker et al. 2003) only occur in soil patches with low  $[\text{NH}_4^+]$  and higher  $[\text{NO}_3^-]$ , while *O. europaea* and other evergreen sclerophylls being more tolerant of  $\text{NH}_4^+$  (Kronzucker et al. 2003) occur irrespective of the  $[\text{NH}_4^+]$ , in soil patches where nitrification (biological conversion of  $\text{NH}_4^+$  into  $\text{NO}_3^-$ ) is inhibited (Cruz et al. 2008; Dias et al. 2011). Nitrification inhibition under the influence of *O. europaea* and other evergreen sclerophylls would make  $\text{NH}_4^+$  availability toxic to *C. albidus* and other summer semi-deciduous, which could contribute to explain why summer semi-deciduous species are progressively eliminated under the canopies of evergreen sclerophylls along a secondary post-fire succession.

The impacts of increasing the  $\text{NH}_4^+$  availability (despite that of  $\text{NO}_3^-$ ) on *C. albidus* plants, and in particular their mortality (Table 2), suggest that the predicted increase in N deposition for the Mediterranean Basin (Galloway et al. 2004; Phoenix et al. 2006) will narrow the ecological niche occupied by this species. In contrast, *O. europaea* plants seem to tolerate and buffer a wide range of  $\text{NH}_4^+$  availabilities, suggesting that their ecological niche is unlikely to be affected. This may imply that the early successional species, functionally equivalent to *C. albidus*, are more likely to be negatively impacted by increased N deposition (if  $\text{NH}_y$  deposition increases) than the late successional ones, functionally equivalent to *O. europaea*. This change in the demographic patterns of plant species and plant functional groups in post-fire Mediterranean successions may have important implications in terms of ecosystem structure (number of species and composition) and functioning (e.g., N retention, organic matter decomposition, carbon sequestration and soil protection—Dias et al. 2013, 2014). Data from an N-manipulation field experiment (manipulating N dose and form) in a Mediterranean maquis dominated by another Cistaceae (*Cistus ladanifer*), where evergreen sclerophylls also co-exist suggest, that the predominant form of N being deposited has differing effects on ecosystem structure and function (Dias et al. 2014). Thus, shifts in the  $\text{NH}_4^+/\text{NO}_3^-$  ratio can differentially influence species and modify successional trajectories, thus being a driving force for ecological succession.

## Conclusions

Our hypothesis that despite the presence of  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  would inhibit the stimulation of NRA by  $\text{NO}_3^-$  in *Cistus albidus* but not in *Olea europaea* was confirmed. *C. albidus* plants were less tolerant of  $\text{NH}_4^+$  than *O. europaea* ones, with the thresholds for  $\text{NH}_4^+$  toxicity being set at  $<0.2\%$   $\text{NH}_4^+$  for *C. albidus* and  $>0.8\%$   $\text{NH}_4^+$  for *O. europaea*. Data show that NRA may or may not be inhibited by  $\text{NH}_4^+$  (or an  $\text{NH}_4^+$ -related metabolite) which opens up the possibility that  $\text{NH}_4^+$  (or an  $\text{NH}_4^+$ -related metabolite) may differently impact co-existing plant species namely when considering: i) the natural changes that occur in the predominant form of soil inorganic N along a post-fire Mediterranean succession; and ii) the anthropogenic increases in N deposition, especially if N is depositing predominantly in the reduced form ( $\text{NH}_y$ ).

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