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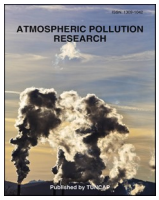

Rhizobia inoculation alleviates ozone-induced foliar damage and root biomass loss for *Robinia pseudoacacia* L.

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A B S T R A C T

Tropospheric ozone (O_3) is an air pollutant with phytotoxic effects on plants. This research aimed to evaluate the impacts of O_3 on *Robinia pseudoacacia* L., a tree species introduced worldwide due to its ability for symbiotic nitrogen fixation, and to assess if rhizobia root inoculation could alleviate the effects from O_3. Using a free-air O_3 exposure system, plants either inoculated with *Mesorhizobium* or left uninoculation (Inoculated *vs.* Control) were subjected to ambient, 1.5 × , and 2 × ambient O_3 levels over a 129-day period. Measurements were made of visible foliar injury (VFI), leaf color index (SPAD), biomass growth and stomatal O_3 uptake. In the presence of elevated O_3, rhizobia inoculation significantly decreased VFI and fine root biomass loss. This protective mechanism was associated with a 22 % reduction in maximum stomatal conductance (g_{max}), which restricted stomatal O_3 uptake. Dose-response relationships showed that flux-based indices (POD_y) better explained VFI and biomass development than exposure-based indices (AOT40). Accordingly, critical levels (CLs) for O_3 were set at 24.5 mmol m^{-2} POD_0 causing the first appearance of VFI, and 7.1 mmol m^{-2} POD_4 resulting in a 4 % reduction in biomass. These CLs are higher than those for O_3-sensitive species, suggesting moderate O_3 tolerance of *R. pseudoacacia* to O_3. Overall, the present results highlight that rhizobia inoculation can increase tree resistance to O_3 stress, possibly by greater limitation of stomatal O_3 uptake and by improving tolerance to oxidative stress. As climate change intensifies abiotic stressors, further research should investigate the combined stress mitigation potential of rhizobial symbioses.

1. Introduction

Tropospheric ozone (O_3) is widely recognized as one of the most deleterious air pollutants to plant growth and development (Grulke and Heath, 2020). With global background O_3 levels on the rise, O_3 pollution poses a significant threat to terrestrial ecosystems, both currently and in the decades to come (De Marco et al., 2022; Mills et al., 2018).

One of the primary symptoms of O_3 stress in plants is visible foliar injury (VFI), which reflects oxidative damage and often correlates with declines in photosynthetic capacity and overall plant health (Moura et al., 2022). Eventually, O_3 has negative effects on plant growth, with impairment of physiological and biochemical processes in leaves and acceleration of leaf senescence (Grulke and Heath, 2020). These aboveground effects lead to decreased carbon assimilation, which limits carbon allocation to the roots and results in a decline in fine-root biomass (Agathokleous et al., 2016; Arab et al., 2022).

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Robinia pseudoacacia L. (common name: black locust) is a pioneer woody species of the *Fabaceae* family native to the eastern United States. It has been introduced to other regions due to its rapid growth (reaching up to 25 m tall) and strong ability to control soil erosion (Cierjacks et al., 2013; Du et al., 2019; Hu et al., 2017). Plant species in the *Fabaceae* family are capable of biologically fixing nitrogen through a symbiotic relationship with nitrogen-fixing bacteria known as rhizobia, enabling them to meet their own nutritional demand as well as support those of surrounding vegetation (Du et al., 2019; Hansen et al., 2017). Recent studies have highlighted the potential of symbiotic microorganisms such as rhizobia to enhance plant resilience to abiotic stress (Liu et al., 2025). Rhizobia inoculation not only improves nitrogen availability but also modulates plant antioxidant defenses and stress signaling pathways (Hu et al., 2025). However, despite these potential benefits, the role of rhizobial symbiosis in mitigating O_3-induced damage remains poorly understood.

Designing an effective O_3 risk assessment strategy is a significant challenge for future policies on precursor emission reductions (CLRTAP, 2017). Although exposure-based indicators, such as the Accumulated exposure index of O_3 exceeding over a Threshold of 40 ppb (AOT40), are still widely used to assess the harmful impacts of O_3, there has been a growing shift toward stomatal flux-based approaches. This change reflects the scientific understanding that O_3 primarily affects plants by entering through the stomata. As result, cumulative O_3 uptake, so-called Phytotoxic O_3 Dose above a stomatal-flux threshold y (POD_y, nmol m^{-2} s^{-1}), is now recommended for a more accurate assessment of O_3-induced damage to vegetation (CLRTAP, 2017). According to the dose-response relationships, a critical level (CL) of 5.2 mmol m^{-2} POD_1 has been suggested corresponding to 4 % reduction in biomass for sensitive tree species such as beech and birch (CLRTAP, 2017). Additionally, Sicard et al. (2016) proposed flux-based thresholds for the appearance of VFI of 19.3 and 17.6 mmol m^{-2} POD_0 in O_3-sensitive conifers and broadleaved trees, respectively.

This study investigated whether rhizobia inoculation affected O_3-induced VFI, biomass development and the dose-response relationships of *R. pseudoacacia*. To establish the O_3 dose-response relationships, both linear and non-linear regression models were employed. We hypothesized that rhizobia-inoculated plants may exhibit reduced O_3 injury due to enhanced physiological resistance, providing insight into a potential biological strategy for improving tree resilience in O_3-polluted environments. Recent studies indicate that dose-response relationships are often nonlinear, as low O_3 doses may have negligible or even stimulatory effects, whereas higher doses result in detrimental effects (Agathokleous et al., 2019; Hoshika et al., 2024).

2. Materials and methods

2.1. Experimental site and plant material

Experiments were carried out at the Free-air O_3 eXposure (FO_3X) facility, located in the National Research Council (CNR) experimental field near Florence, Italy (Latitude: 43°48′59″ N, Longitude: 11°12′01″ E; Elevation: 55 m a.s.l.). Details about the exposure system can be found in Paoletti et al. (2017). *R. pseudoacacia* DB214 plants (Hu et al., 2025) were propagated *in vitro* on solid Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) at 23 °C under a 16-h photoperiod (120 μE m^{-2} s^{-1}). In May 2023, the plants were transferred to 12 cm × 12 cm × 20 cm pots filled with a soil–sand mixture (3:1). In April 2024, the two-year-old *R. pseudoacacia* plants, either rhizobia-inoculated (referred to as "Inoculated") or non-inoculated ("Control"), were transported to the CNR experimental field (see section 2.2 for the inoculation treatment). The plants were transplanted into 10-liter plastic pots containing a substrate composed of 95 % washed river sand and 5 % vermiculite. At the time of potting, the average plant height (mean ± SD) was 100 ± 16 cm, and the mean stem diameter (mean ± SD) was 5.6 ± 1.0 mm. From 17 May to September 23, 2024 (129 days of exposure), three O_3 treatments were applied: ambient O_3 concentration (AA), 1.5 times ambient O_3 (1.5 ×), and twice ambient O_3 (2 ×). Elevated O_3 treatments were applied during whole day (24 h). Each O_3 level was replicated across three separate plots (5 m × 5 m × 2 m per plot), comprising all combinations of the two inoculation treatments and three O_3 levels (3 plants × 2 inoculation treatments × 3 O_3 levels × 3 plots = 54 plants in total). Ozone concentrations and meteorological parameters were continuously recorded during the experiments and mean values were specified in Appendix Table S1. Every two weeks, each pot received 250 mL of a modified Hoagland nutrient solution containing: 0.5 mM KH_2PO_4, 0.05 mM K_2SO_4, 0.01 mM Ferric citrate, 0.25 mM $MgSO_4{\cdot}7H_2O$, 1 mM $CaCl_2$, 2 mM KNO_3, 0.4 mM NH_4NO_3, 2 μM H_3BO_3, 0.5 μM $ZnSO_4{\cdot}7H_2O$, 0.2 μM $CuSO_4{\cdot}5H_2O$, 1 μM $MnSO_4{\cdot}H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2H_2O$, 0.1 μM $CoSO_4{\cdot}7H_2O$ (Flemetakis et al., 2006). Plants were irrigated daily to avoid any water stress (2 L per day).

2.2. Rhizobia inoculation treatment

After one month of acclimatization in a greenhouse, they were moved to an outdoor area of the Botanical Garden in Braunschweig, Germany (Lower Saxony, 52°16′13″ N, 10°32′01″ E; 70 m above sea level). Half of the plants were inoculated with *Mesorhizobium robiniae*, kindly provided by Prof. Michael Pester (DSMZ Braunschweig). The strain *Mesorhizobium robiniae* (DSM 100022) was obtained from Leibniz Institute DSM, Microorganisms and Cell Cultures collections in Germany (Braunschweig, Germany); this bacterium was first isolated from root nodules of *R. pseudoacacia* at Yangling, Shaanxi Province in China (Zhou et al., 2010). The rhizobial strain was cultivated in liquid YMA medium for 48 h at 28 °C on a shaker, then centrifuged for 10 min at 25 °C and 4000×*g*. The pellet was subsequently dissolved in sterile distilled water to produce a bacterial suspension adjusted to an OD_{600} of 1.0. One milliliter of this suspension was applied to the roots of each *R. pseudoacacia* plant, and the inoculation was repeated twice to ensure effective nodulation.

2.3. Measurement of visible foliar injury and leaf color index

Visible foliar injuries caused by O_3 exposure were assessed every two to four weeks by two trained observers, both of whom had completed an inter-calibration course under the ICP-Forests program (Schaub et al., 2016). The assessments included a calculation of the proportion of leaves with symptoms per plant (LA) and the percentage of affected leaf area within those symptomatic leaves (SA). These evaluations were carried out using a 10 × hand lens and standardized photoguides (Innes et al., 2001; Paoletti et al., 2009). To quantify injury at the whole-plant level, the Plant Injury Index (PII) was calculated by combining the two parameters using the following formula (Calatayud et al., 2007; Paoletti et al., 2009):

$$PII = (LA \times SA) \,/\, 100 \quad (1)$$

In addition, a SPAD meter (Konica Minolta, Tokyo, Japan) was used to assess the leaf color index as a proxy of chlorophyll content. Measurements were taken at three time points: T_0 (15 May, prior to O_3 exposure), T_1 (8 July, after 52 days of exposure), and T_2 (26 August, after 101 days of exposure). For these measurements, three leaves for each plant were selected from the 3rd to 5th position below the shoot apex.

2.4. Parameterization of the stomatal conductance model

The flux-based approach requires modeling of stomatal conductance. A measurement campaign of stomatal conductance for water vapor (g_{sw}) for 3rd to 5th ordered leaves (used for SPAD measurements) of all target plants was conducted using an open flow-through differential porometer (LI-600, Lincoln, NE, USA). Measurements were made for 16 days (at least once a month) during the experiment (954 g_{sw} data in total). The

dataset was utilized to parameterize a multiplicative model as follows (Jarvis, 1976; Hoshika et al., 2020a):

$$g_{sO3} = g_{max} \cdot f_{light} \cdot \max\{ f_{min}, \left(f_{temp} \cdot f_{VPD}\right) \} \quad (2)$$

where g_{sO3} is stomatal conductance for O_3 (= g_{sw} × 0.663, Grünhage et al., 2012), g_{max} is the maximum stomatal conductance (mmol O_3 m^{-2} Projected Leaf Area [PLA] s^{-1}), f_{min} is the minimum conductance, and other functions indicate the response of stomata to environmental factors, i.e., stomatal response to photosynthetic photon flux density (PPFD) (f_{light}), temperature (f_{temp}) and vapor pressure deficit (VPD) (f_{VPD}). Further information on these functions can be found in Hoshika et al. (2020a). Parameterization was achieved using the boundary line approach (Braun et al., 2010; Hoshika et al., 2012). Following the methodology used by Bičárová et al. (2019) and Hoshika et al. (2020a), g_{max} and f_{min} were assigned to the 95th and 5th percentiles of the dataset, respectively.

2.5. Measurement of biomass

At the end of the experiment, all plants were harvested and separated into shoots, leaves and roots (diameter ≤2 mm for fine and >2 mm for coarse roots). The organs were oven-dried at 80 °C for seven days and then weighed.

To estimate dry mass production, an additional set of plants (6 plants for Control and 6 plants for Inoculated) was harvested at the start of the experiment in May 2024 to obtain baseline dry mass data. Total biomass production during the experiment was then determined by comparing the average biomass at the beginning and at the end.

In addition, before O_3 exposure and at the end of the experiment, nodules were separated from the roots, gently washed with tap water, and the fresh weight was recorded.

2.6. Calculation of ozone indices deriving dose-response relationships

The exposure-based (AOT40) and flux-based indices (POD_y) were calculated according to the methodology given by CLRTAP (2017). AOT40 was determined by using hourly O_3 concentrations recorded during daylight periods when shortwave radiation exceeded 50 W m^{-2}. It is given by:

$$\text{AOT40} = \sum_{i=1}^{n} max([O_3]_i - 40, \quad 0) \quad (3)$$

where $[O_3]_i$ is the O_3 concentration (ppb) measured at the ith hour, with $i = 1 \ldots n$, and n is the total number of hours considered in the calculation.

Phytotoxic O_3 dose above a threshold y (PODy; mmol m^{-2}) was determined by summing the hourly stomatal O_3 uptake values that exceed the threshold:

$$\text{POD}_y = \sum_{i=1}^{n} \max(F_{st\_i} - y, \quad 0) \quad (4)$$

where F_{st_i} represents the O_3 uptake via stomata at the ith hour (nmol O_3 m^{-2} PLA s^{-1}) calculated by considering g_{sO3}, leaf boundary layer resistance and ambient O_3 concentration (see Appendix Method S1 for detail). n is the total number of hours in the calculation period, and y is a species-specific threshold (nmol O_3 m^{-2} PLA s^{-1}) for stomatal O_3 uptake. Different thresholds for POD_y (y = 0–10 nmol O_3 m^{-2} PLA s^{-1}) were examined to identify the best POD_y index for explaining O_3 effects on PII and relative biomass development. According to the approach by Paoletti et al. (2017), the relative biomass development was calculated as total biomass production divided by the hypothetical reference biomass production without O_3 impacts. Linear regressions between total biomass production and 24-h average concentration (M24) were applied to obtain the hypothetical reference biomass production for each inoculation treatment at M24 of 20 ppb as the baseline level at which O_3 may begin to cause adverse effects on plants (Grünhage et al., 2001; Pleijel et al., 2002; Hoshika et al., 2024) considering the pre-industrial O_3 level (10–15 ppb: Fowler et al., 2008; Tarasick et al., 2019). Critical levels (CLs) were calculated as the POD_y and AOT40 values corresponding to: (1) the onset of VFI, being defined as a PII score ≥0.5 (Calatayud et al., 2007; Hoshika et al., 2020a), and (2) a 4 % biomass reduction, following the recommendation by CLRTAP (2017).

2.7. Data analysis

Statistical analyses were carried out in R 4.3.1 (R Core Team, 2023). Data were tested for normal distribution (Kolmogorov-Smirnov D-test) and homogeneity of variance (Levene's test). Since data for biomass and SPAD were normally distributed, we applied a full-factorial split-plot analysis of variance (ANOVA) considering O_3 and inoculation treated as fixed factors and plot included as a random factor nested within O_3. When significative interaction was found (O_3 × Inoculation), Tukey's post-hoc test was applied. On the other hand, given that the non-normal distribution was found for the PII data effect of treatments was evaluated by the Kruskal-Wallis analysis of variance with Mann-Whitney U tests for pairwise comparisons. Based on the O_3 VFI and physiological data, linear and quadratic regressions for exposure- and flux-based dose-response relationships were compared by the Akaike information criterion (AIC). When the regression lines were statistically significant in each inoculation treatment, the statistical comparisons were made. For the linear regression lines, a covariance analysis (ANCOVA) was conducted to examine if the regression lines differed statistically. On the other hand, a F-test was applied to examine if the two quadratic regressions were statistically coincident. Results were considered significant at $p \leq 0.05$.

3. Results

3.1. Visible foliar injury and leaf color index

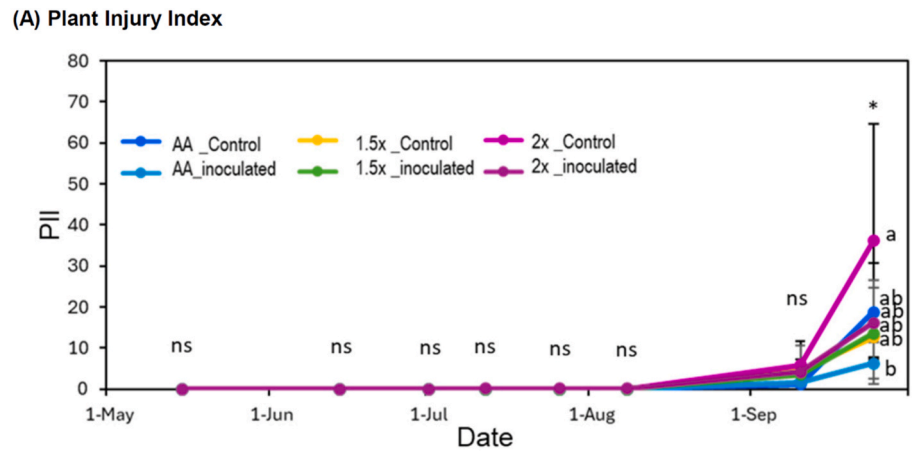
The first appearance of symptoms was found for Inoculated plants grown with 2 × O_3 treatment on 12 July, although the PII was low (=0.09) (Fig. 1A). In September, symptoms developed across all O_3 treatments, with more severe injuries recorded in Control plants compared to Inoculated ones. The PII values for Control plants were 18.8, 12.7, and 36.2 under AA, 1.5 × , and 2 × O_3 treatment, respectively, whereas Inoculated plants showed lower PII values of 6.3, 13.6, and 16.1 for the same treatments. Ozone-induced VFI appeared as reddish stippling on the upper leaf surface (Fig. 1B).

At T_0, rhizobia inoculation had a positive effect on SPAD values (Table 1). However, SPAD values did not differ between Inoculated and Control plants (i.e., non-inoculated) during mid-summer (T_1). At the late summer (T_2), on the other hand, O_3 exposure caused a decline in SPAD values, with the reduction being more evident in Control plants.

3.2. Biomass

The average plant biomass at the beginning was 9.9 ± 3.8 g, and there was no difference in the initial biomass between Control and Inoculated plants (data not shown). After the 4-month experiment, plants showed biomass increases. Neither O_3 nor inoculation effects were statistically significant on most biomass parameters (Table 2). However, there was an interactive effect of O_3 and inoculation on fine root biomass, where the negative effect on this parameter was observed only in Control plants grown under 1.5 × compared to AA-treated plants, with a reduction of approximately 49 %.

The fresh weight of nodules was enhanced by inoculation at the beginning of the experiment (Appendix Table S2). The color of nodules was white to light brown at the beginning whereas it seems that the



(B) Photographs of *Robinia* leaves showing different levels of O_3-VFI:
a: Leaf without visible injury; b: Leaf with low percentage of O_3-VFI on the adaxial surface; c: Leaf with high percentage of O_3-VFI on the adaxial surface; d: Leaf with O_3-VFI absent on the abaxial surface.

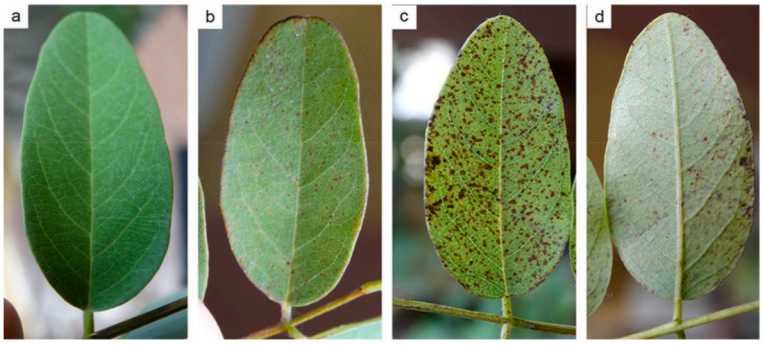


Fig. 1. (A) Plant Injury Index (PII) and (B) Photographs of visible foliar injury of *R. pseudoacacia* plants grown at three O_3 levels (AA, ambient O_3 concentration, 1.5 × , 2 ×) with or without rhizobia inoculations (Inoculated *vs.* Control). Data are mean ± SD (n = 6 to 9 plants, 3 replicated plots). Asterisks show the significance of Kruskal-Wallis test: *$p \leq 0.05$, ns denotes not significant. Different letters show significant differences among treatments ($p \leq 0.05$, Mann-Whitney U test) at each measurement time.

Table 1
Leaf color index (SPAD) of *R. pseudoacacia* plants grown at three O_3 levels (AA, ambient O_3 concentration, 1.5 × , 2 ×) with or without rhizobia inoculation (Inoculated *vs.* Control). Data are mean ± SD (n = 9 plants, 3 replicated plots). Asterisks show the significance of ANOVA: *$p \leq 0.05$, **$p \leq 0.01$, ns denotes not significant. When the interaction was found, Tukey's post-hoc test was applied.

O_3 × inoculation treatment	SPAD		
	T_0	T_1	T_2
	Before exposure	52 days exposure	101 days exposure
Control			
AA	18.2 ± 2.1	27.6 ± 3.5	26.5 ± 1.6
1.5 ×	19.3 ± 2.2	28.5 ± 4.6	23.6 ± 2.0
2 ×	20.2 ± 2.8	28.8 ± 3.9	23.1 ± 2.1
Inoculated			
AA	23.9 ± 3.8	29.7 ± 3.6	26.6 ± 1.1
1.5 ×	23.9 ± 3.3	27.3 ± 1.7	26.9 ± 2.3
2 ×	24.1 ± 3.6	28.2 ± 2.9	24.8 ± 1.7
ANOVA results			
O_3	ns	ns	*
Inoculation	***	ns	**
O_3 × Inoculation	ns	ns	ns

color of some nodules became more dark brown in plants exposed to O_3 at the end of the experiment (data not shown). At the harvest stage, although nodule biomass was higher in Inoculated plants under AA conditions, elevated O_3 treatment inhibited such an increase in nodule biomass.

3.3. Parameterization of the stomatal conductance model

The g_{max} value was 222 mmol O_3 m^{-2} PLA s^{-1} in Control plants while a relatively low g_{max} was found in Inoculated plants (173 mmol O_3 m^{-2} PLA s^{-1}) (Fig. 2). Other model functions were similar for both Control and Inoculated plants, although the response of g_s to PPFD (f_{light}) indicates a slightly lower a value in Inoculated plants relative to Control plants (Appendix Table S3). The optimal temperature for stomatal opening (T_{opt}) was found at approximately 30 °C in this species. A VPD higher than around 3 kPa induced stomatal closure regardless of the inoculation treatments.

3.4. Dose-response relationship for visible foliar injury and biomass development

Stomatal flux-based or exposure-based O_3 dose-response relationships for PII of *R. pseudoacacia* are shown in Fig. 3 and Appendix Table S4. For both exposure-based and flux-based indices, a nonlinear pattern of dose-response curve was obtained for PII according to the results of AIC. Considering the R^2 value reflecting how accurately the regression model represents the observed data, the flux-based index was more effective than the exposure-based one in explaining the variation of PII under O_3 exposure. Among the O_3 indices, POD_0 was the best flux-based indicator for PII. According to the regression line, the CL corresponding to the appearance of VFI was estimated to 24.5 mmol m^{-2}

Table 2
Dry mass of plant organs per *R. pseudoacacia* plants grown at three O_3 levels (AA, ambient O_3 concentration, 1.5 × , 2 ×) with or without rhizobia inoculations (Inoculated *vs.* Control). Data are mean ± SD (n = 7 to 9 plants, 3 replicated plots). Asterisks show the significance of ANOVA: $^{**}p \leq 0.01$, ns denotes not significant. When the interaction was found, Tukey's post-hoc test was applied. Different letters show significant differences among treatments.

O_3 × inoculation treatments	Leaf biomass (g)	Shoot biomass (g)	Fine root biomass (≤2 mm) (g)	Coarse root biomass (>2 mm) (g)	Total biomass (g)
Control					
AA	4.0 ± 1.2	19.4 ± 1.3	4.1 ± 0.7 a	11.2 ± 1.6	38.8 ± 2.2
1.5 × AA	4.0 ± 1.9	17.8 ± 7.7	2.1 ± 1.4 b	8.7 ± 3.1	32.6 ± 12.7
2.0 × AA	2.8 ± 1.4	18.2 ± 9.2	2.7 ± 1.0 ab	9.4 ± 3.2	33.1 ± 13.5
Inoculated					
AA	3.7 ± 2.0	21.9 ± 7.9	3.2 ± 1.2 ab	11.7 ± 4.2	40.5 ± 13.2
1.5 × AA	5.8 ± 3.9	21.9 ± 9.6	3.9 ± 1.3 a	10.8 ± 4.5	42.5 ± 18.3
2.0 × AA	3.0 ± 1.4	21.9 ± 5.7	2.9 ± 1.1 ab	9.8 ± 2.6	37.7 ± 8.5
ANOVA results					
O_3	ns	ns	ns	ns	ns
Inoculation	ns	ns	ns	ns	ns
O_3 × Inoculation	ns	ns	**	ns	ns

POD_0. On the other hand, for biomass development, both flux- and exposure-based dose-response relationships were linear, although the R^2 values between linear and quadratic functions were similar (Fig. 4; Appendix Table S4). The best model fit was found in POD_4 where the CL corresponding to a 4 % reduction of biomass development was estimated to be 7.1 mmol m^{-2} POD_4. The AOT40-based dose-relationship was not statistically significant.

Statistical results also indicated that no significant difference was found between the regression lines for PII when comparing the dose-response relationships between two inoculation treatments (Table S5). On the other hand, for the relative biomass, the comparison of the regression lines between two inoculation treatments was not made because all regression lines were not statistically significant when separately analyzing the data in each inoculation treatment because of the small sample size (n = 3).

4. Discussion

Elevated O_3 concentration caused VFIs in *R. pseudoacacia* plants, although the symptoms were found only in the late summer season. This may be attributed to the increasing cumulative O_3 uptake and the corresponding rise in the demand for O_3 detoxification and repair, which eventually led to O_3-induced injury (Li et al., 2021). Given that several sensitive tree species have shown VFIs already in June or July under 2 × O_3 treatments (Hoshika et al., 2020a), *R. pseudoacacia* was a relatively tolerant species to O_3. In fact, no significant effect of O_3 on total biomass accumulation was found for this species. Although the presence of VFIs may not directly lead to impairment of plant growth (Davison and Barnes, 1998; Marzuoli et al., 2019), the amount of VFIs found in summer can be an indicator of the biomass reduction due to O_3 exposure (Paoletti et al., 2022). Some minor effects of O_3 were, however, observed in fine root biomass production. Fine roots, which are known as a crucial interface between plants and soil, play a key role in nutrient and water uptake (Andersen, 2003). The negative effect of O_3 is often highlighted in below-ground rather than above-ground biomass (Agathokleous et al., 2016; Arab et al., 2022). Despite the single-season duration of this study, O_3 impact on fine-root growth could lead to general growth defects in the subsequent years (Hayes et al., 2011; Agathokleous et al., 2020).

Rhizobia inoculation did not produce a statistically significant increase in biomass in *R. pseudoacacia*. Similar findings were also reported in several plant species (Holatko et al., 2024; Pulido-Suárez et al., 2021; Simbine et al., 2021), probably because the response was species-specific and the effects were often non-significant or marginal when the plants received a certain amount of soil nutrition (Holatko et al., 2024). Nonetheless, the inoculation reduced VFIs of *R. pseudoacacia* plants grown under elevated O_3 concentrations, although such treatments did not fully mitigate O_3 injuries on leaves. In addition to the VFI, we found some mitigation effect of rhizobia

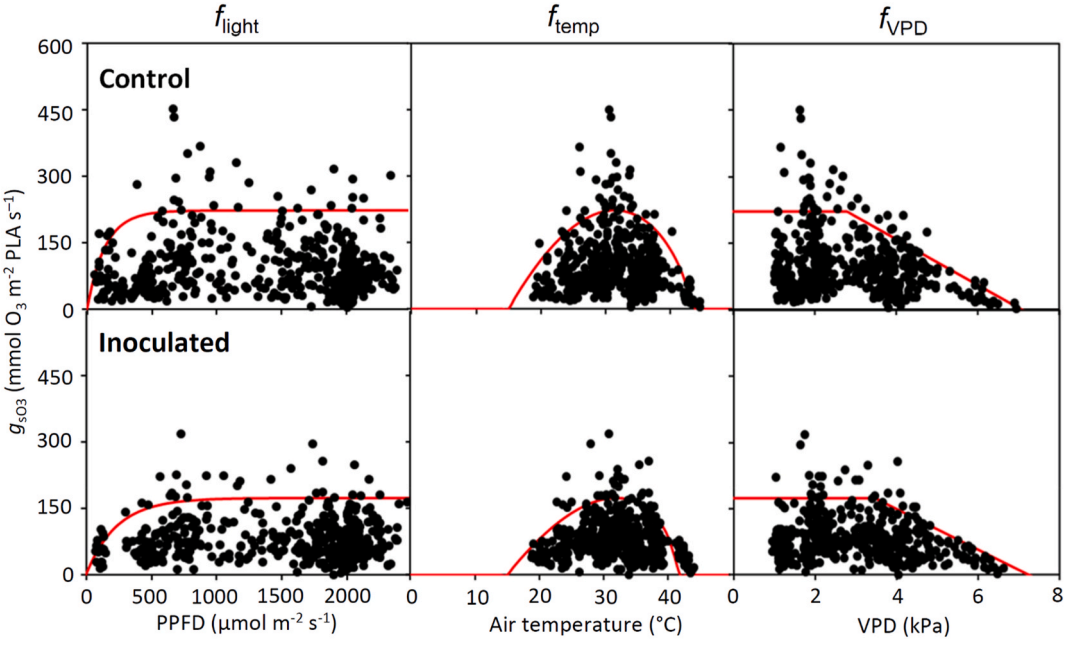
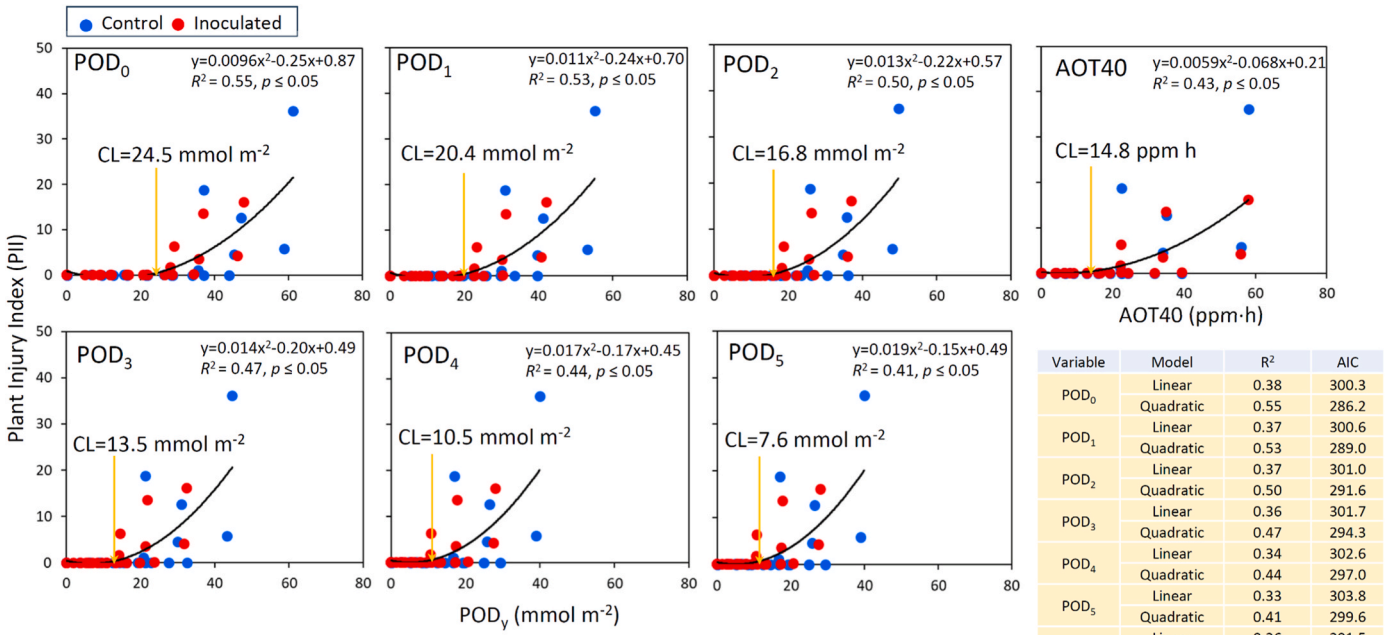
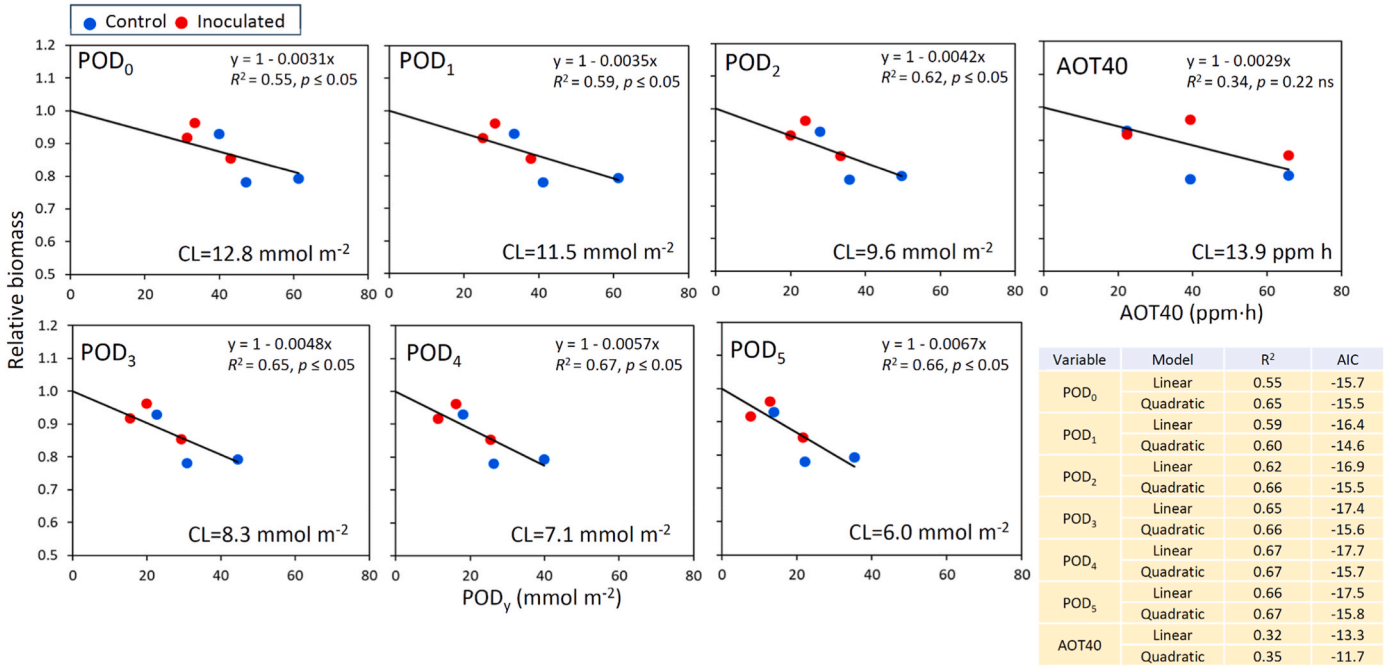


Fig. 2. Parameterization of stomatal response functions (f_{light}, f_{temp}, and f_{VPD}) of *R. pseudoacacia* plants with or without rhizobia inoculations (Inoculated *vs.* Control). The red lines illustrate the model's predicted functions, whereas the black points correspond to the experimentally measured stomatal conductance.



Variable	Model	R^2	AIC
POD_0	Linear	0.38	300.3
	Quadratic	0.55	286.2
POD_1	Linear	0.37	300.6
	Quadratic	0.53	289.0
POD_2	Linear	0.37	301.0
	Quadratic	0.50	291.6
POD_3	Linear	0.36	301.7
	Quadratic	0.47	294.3
POD_4	Linear	0.34	302.6
	Quadratic	0.44	297.0
POD_5	Linear	0.33	303.8
	Quadratic	0.41	299.6
AOT40	Linear	0.36	301.5
	Quadratic	0.43	298.0

Fig. 3. Dose-response relationships based on Phytotoxic O_3 Dose (POD) with different stomatal ozone uptake threshold y (y = 0–5 nmol m^{-2} s^{-1}) and AOT40 against Plant Injury Index (PII) of *R. pseudoacacia* plants grown at three O_3 levels (AA, ambient O_3 concentration, 1.5 × , 2 ×) with or without rhizobia inoculation (Inoculated *vs.* Control). The critical levels (CLs) corresponding to the occurrence of visible foliar injury are also shown for each O_3 index. We chose either a linear or quadratic function according to the AIC, which was applied to compare both functions to select the better method for the dose-response functions. The result for other thresholds for POD_y (y = 6–10 nmol O_3 m^{-2} PLA s^{-1}) are shown in Appendix Table S4.



Variable	Model	R^2	AIC
POD_0	Linear	0.55	-15.7
	Quadratic	0.65	-15.5
POD_1	Linear	0.59	-16.4
	Quadratic	0.60	-14.6
POD_2	Linear	0.62	-16.9
	Quadratic	0.66	-15.5
POD_3	Linear	0.65	-17.4
	Quadratic	0.66	-15.6
POD_4	Linear	0.67	-17.7
	Quadratic	0.67	-15.7
POD_5	Linear	0.66	-17.5
	Quadratic	0.67	-15.8
AOT40	Linear	0.32	-13.3
	Quadratic	0.35	-11.7

Fig. 4. Dose-response relationships based on Phytotoxic O_3 Dose (POD) with different stomatal ozone uptake threshold y (y = 0–5 nmol m^{-2} s^{-1}) and AOT40 against the relative biomass development of *R. pseudoacacia* plants grown at three O_3 levels (AA, ambient O_3 concentration, 1.5 × , 2 ×) with or without rhizobia inoculation (Inoculated *vs.* Control). The critical levels (CLs) corresponding to a 4 % reduction of biomass were also shown for each O_3 index. We chose either a linear or quadratic function according to the AIC, which was applied to compare both functions to select the better method for the dose-response functions. The results for other thresholds for POD_y (y = 6–10 nmol O_3 m^{-2} PLA s^{-1}) are shown in Appendix Table S4.

inoculation on O_3 damage to fine root biomass production. This result can be explained by the limited stomatal O_3 uptake of rhizobia-inoculated plants due to a reduction of stomatal conductance, i.e., g_{max} was reduced by 22 %. The effect of rhizobia inoculation on the stomatal conductance of legume plants is species-specific. rhizobia inoculation increased stomatal conductance for cowpea (*Vigna unguiculata* (L.) Walp), while a reduction of stomatal conductance was found in rhizobia inoculated soybean (*Glycine* max (L.) Merril) and Bambara

groundnut (*Vigna subterranea* (L.) Verdc.) (Matiru and Dakora, 2005). Although underlying mechanisms are still under investigation for the effect of rhizobia inoculation on stomatal response, Matiru and Dakora (2005) suggested that the decrease in stomatal conductance may be related to the production of lumichrome, which is released by rhizobia, showing abscisic acid (ABA)-like effects such as stomatal closure. In addition, as nitrogen becomes available via the rhizobia symbiosis, rhizobia-inoculated plants may realize a fine regulation of stomatal opening to optimize CO_2 uptake with preventing excess water loss to match internal nitrogen status (Jaiswal and Dakora, 2025). In fact, *R. pseudoacacia* plants, which are the early-successional tree species with fast-growing, are known to be water consuming (Wu et al., 2015), as confirmed by a relatively high g_{max} compared to water saving Mediterranean evergreen plants (*Arbutus unedo*, 95 mmol O_3 m^{-2} PLA s^{-1}; *Phillyrea angustifolia*, 135 mmol O_3 m^{-2} PLA s^{-1}: Moura et al., 2022). Moreover, O_3 induces slower or less efficient stomatal control, i.e., stomatal sluggishness, which may be related to ethylene emission due to O_3 exposure (Hoshika et al., 2019; Paoletti, 2005). It should be noted that some rhizobia strains produce an enzyme called 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which can reduce ethylene levels (Ma et al., 2002). Therefore, rhizobia inoculation might reduce the O_3 damage to stomatal function, which results in improving water use efficiency.

Rhizobia inoculation may also have positive effects on the physiological tolerance against abiotic stress. Mushtaq et al. (2021) reported that symbiotic interaction increased the activities of key antioxidant enzymes, including catalase, superoxide dismutase and peroxidases for chickpea (*Cicer arietinum* L.), thereby helping to scavenge reactive oxygen species (ROS) generated under salinity stress conditions. Rhizobia-inoculated plants can better tolerate environmental stresses such as heavy metal exposure, by reducing oxidative damage and maintaining redox balance (Liu et al., 2024). Therefore, rhizobia-inoculated plants with enhanced antioxidant capacity may have more efficiently coped with the O_3-induced damage through detoxification and repair processes. However, the dose-response relationships for VFI between two inoculation treatments showed no statistical difference, indicating that the sensitivity per unit of O_3 flux was not significantly modified by the rhizobia inoculation. Achieving deeper understandings of the biochemical defense capacity of inoculated plants against O_3 damage will be a next step.

According to the dose-response relationships, the flux-based approach rather than AOT40 better explained the variation of VFI and biomass development under elevated O_3 conditions. As a result, this study established the flux-based CLs for *R. pseudoacacia*: 24.5 mmol m^{-2} POD_0 for the onset of VFI and 7.1 mmol m^{-2} POD_4 corresponding to a 4 % reduction in biomass development. In comparison, forest monitoring data from Sicard et al. (2016) suggested a lower flux-based CL for the onset of VFI to 17.6 mmol m^{-2} POD_0 for sensitive broadleaf species such as *Fagus sylvatica* and *Fraxinus excelsior*. For biomass reduction, a CL of 5.2 mmol m^{-2} POD_1 was reported for sensitive tree species (CLRTAP, 2017), which is lower than the corresponding CL for *R. pseudoacacia* (11.5 mmol m^{-2} POD_1), when assuming a y-threshold of 1 nmol m^{-2} s^{-1} for stomatal flux. In contrast, very high CLs were reported for O_3-resistant Mediterranean evergreen oak species, reaching 47.3 mmol m^{-2} POD_1 (CLRTAP, 2017). Thus, the CLs for both VFI and biomass reduction suggest that *R. pseudoacacia* is a less sensitive deciduous tree species to O_3 exposure compared to other broadleaf species, but more sensitive compared to evergreen species.

The threshold y is supposed to reflect the plant capacity to detoxify a given dose of O_3 (Pleijel et al., 2007). Interestingly, the best stomatal flux threshold (y) differed in the present study between the two target responses: 0 nmol m^{-2} s^{-1} for VFI and 4 nmol m^{-2} s^{-1} for biomass production. Agathokleous et al. (2019) argued that using a fixed threshold index ignores the biological acclimation response to O_3 exposures. Supporting this assumption, Conte et al. (2021) observed seasonal variation in O_3 thresholds, suggesting that detoxification capacity and repair mechanisms are influenced by plant phenology and environmental conditions. Ozone susceptibility may differ for biomass development and VFI, as crops yield is particularly sensitive during the flowering stage while their VFI appears at the vegetative stage. For temperate deciduous trees, biomass accumulation mainly occurs in early summer and typically ceases by late August, as plants shift from active growth to carbohydrate storage in roots and stems for winter (Larcher, 2003). Conversely, VFI often becomes more apparent in late summer, when repair and defense capacities decline due to reduced photosynthetic activity and limited assimilate supply (Paoletti et al., 2019).

Inoculated plants had greater nodule biomass, compared to the Control (non-inoculated) plants under the AA O_3 condition. However, in the present study, the roots of Inoculated plants exposed to elevated O_3 did not show such a development of nodulation upon harvest. This observation was consistent with previous studies, where it has been reported that exposure to O_3 can damage root nodules and decrease nodule biomass (Hewitt et al., 2016; Biancari et al., 2021). Interestingly, the Control plants did not show such an O_3-induced decrease in nodule biomass. Given that the experiment was performed in an open-air facility (FO_3X), this discrepancy might be due to random introduction of undefined rhizobia during plant cultivation, e.g., by rain events. There is, however, also a possibility where metabolically active nodules in the Inoculated plants could be more susceptible to O_3. Considering the development of O_3 induced injury, changes in nodule activity with target rhizobia strains in *Robinia* plants will be explored in future research.

5. Conclusions

The present study highlights the mitigation effect of rhizobia inoculation on O_3-induced damage in *R. pseudoacacia* plants, particularly in terms of O_3 VFI and fine root biomass production. This protective effect was primarily attributed to reduced stomatal O_3 uptake. Given that the O_3 damage was closely linked to actual O_3 uptake, stomatal flux-based metrics proved to be more reliable than exposure-based metrics for the O_3 risk assessment. For the first time, CLs for the O_3-induced VFI and biomass reduction in *R. pseudoacacia* plants were proposed: 24.5 mmol m^{-2} POD_0 for the onset of VFI and 7.1 mmol m^{-2} POD_4 corresponding to a 4 % reduction in biomass development. Beyond O_3 pollution, future climate change may increase the risk of other abiotic stresses such as drought (De Marco et al., 2022). The combined effects of O_3 and drought can trigger severe oxidative stress, leading to further physiological impairment in plants (Hoshika et al., 2020b). As rhizobia inoculation enhances root functions such as nutrient and water uptake, future studies should focus on whether rhizobia-inoculated plants may better cope with multiple stress factors, such as O_3 and drought, that are highly relevant in current and future climate change.

CRediT authorship contribution statement

Yasutomo Hoshika: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft. **Barbara Baesso Moura:** Formal analysis, Investigation, Writing – review & editing. **Robert Haensch:** Data curation, Investigation, Writing – review & editing. **Jacopo Manzini:** Investigation, Writing – review & editing. **Andrea Viviano:** Investigation, Writing – review & editing. **Elena Marra:** Investigation, Writing – review & editing. **Cesare Garosi:** Investigation, Writing – review & editing. **Matheus Casarini Siqueira:** Investigation, Writing – review & editing. **Ryoji Tanaka:** Investigation, Writing – review & editing. **Bin Hu:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Heinz Rennenberg:** Conceptualization, Formal analysis, Writing – review & editing. **Elena Paoletti:** Conceptualization, Funding acquisition, Writing – review & editing.

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability statement

All new data generated are presented in this article. Therefore, data sharing is not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apr.2025.102811.

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