

Interactive Effects of AMF Inoculation and Leaf Leachate on the Growth and Reproduction of *Anthemis cotula* L.: Implications for its Invasion in the Himalaya

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Abstract

This study investigates the combined effects of arbuscular mycorrhizal fungi (AMF) inoculation and leaf leachate application on various morphological, physiological, and reproductive traits of *A. cotula*, an invasive species in Kashmir Himalaya. Results from a greenhouse experiment reveal a significant synergistic effect of AMF and leaf leachate on plant traits, particularly during the later growth stages. Specifically, plant height increased significantly under the combined treatment of AMF and the highest concentration of leaf leachate (AMF + AL2), with plants reaching a height of 23.1 cm. In contrast, leaf leachate alone suppressed growth, which was mitigated by the addition of activated charcoal.

Root dry mass showed the highest increase under leaf leachate treatments, while shoot dry mass was significantly enhanced under the combined treatment of AMF and leaf leachate, reaching 4.37 g/plant. The number of lateral branches also increased significantly under the combined treatment of AMF and leaf leachate. Photosynthetic rate and stomatal conductance were notably higher in plants treated with AMF and leaf leachate, indicating improved physiological performance. Furthermore, the extent of AMF colonization was positively correlated with phosphorus content, with the highest phosphorus accumulation observed in AMF and leachate treated plants.

These findings highlight the crucial role AMF and leaf leachate interactions in the invasiveness of *A. cotula*. The present study also contributes to our understanding of the mechanisms that drive the success of invasive species, with implications for managing their spread and promoting ecological restoration.

Introduction

Understanding the mechanisms driving the success and spread of invasive species has been a central research theme in invasion biology. Given the significant ecological and economic impacts of invasive species (Cuthbert et al. 2022; IPBES 2023), it has been critical to explore how various traits of invasive plants interact with environmental factors to confer invasiveness. Such investigations have been essential for improving risk assessment frameworks and managing existing invasive species. Since Baker's foundational work on identifying traits of an "ideal weed" (Baker 1965; 1974), efforts to pinpoint the key traits associated with invasiveness have grown substantially. Comparative analyses across taxonomically related species and regional floras (Pyšek and Richardson 2007; Van Kleunen et al. 2015) highlighted the context-dependent nature of invasions (Kueffer et al. 2013; Hulme and Bernard-Verdier 2018), revealing that factors contributing to invasiveness were shaped by species characteristics (e.g., Rejmánek and Richardson 1996; Shea et al. 2005; Moles et al. 2008), environmental conditions (e.g., climatic and edaphic factors), biotic interactions (e.g., competitors, mutualists, and pollinators), and the stage of invasion (Lau and Funk 2023).

Among the many traits conferring invasiveness, allelopathy (Callaway and Ridenour 2004; Zhang et al. 2020) and mutualistic interactions with arbuscular mycorrhizal fungi (AMF) (Policelli et al. 2019; Clavel et al. 2021) emerged as critical drivers of success for many invasive alien species. Allelopathic

compounds, such as phenolics and flavonoids, were released through root exudates, volatile organic compounds, and leaf leachates (Inderjit et al. 2011, 2021; Yuan et al. 2022). These compounds disrupted mycorrhizal communities, altered soil nutrient cycles (Vogelsang and Bever 2009; De Vries et al. 2020), and influenced plant-plant interactions in both native and introduced ranges. Interestingly, while allelopathy was traditionally associated with suppressing competitors, recent studies suggested that certain allelochemicals could stimulate AMF colonization (Kountche et al. 2018; Pei et al. 2020; Tian et al. 2021), highlighting a potential synergy between these factors. However, much of this research focused on the effects of isolated allelochemicals, often overlooking the complexity of the entire suite of compounds present in leaf leachates. This gap was particularly important, as leaf leachates likely contained a mixture of compounds with diverse and potentially opposing influences. By studying the whole leachate rather than a single allelochemical, the present study aimed to simulate field conditions more realistically and provide a more comprehensive understanding of how these compounds collectively influenced plant-soil interactions.

This study addressed these critical knowledge gaps by investigating the individual and combined effects of AMF and leaf leachates on *Anthemis cotula*, a globally invasive plant species. Native to regions of Eurasia with a Mediterranean-type climate (Kay 1971; Mack and Erneberg 2002; CABI 2018), *A. cotula* thrived in ruderal habitats (Afshana et al. 2023). Its invasiveness in the Kashmir Himalaya had been attributed to its allelopathic leaf leachates (Allaie et al. 2006; Rashid and Reshi 2012), high seed output (Kay 1971; Rashid et al. 2007), demographic plasticity (Rashid and Reshi 2010), and widespread mycorrhizal associations (Shah et al. 2008a, 2009). A recent study by Afshana et al. (2023) further characterized the root-associated mycobiome of *A. cotula*, reporting associations with 706 fungal species, including several AMF taxa.

Specifically, the study investigated whether interactions between the leaf leachates of *A. cotula* and AMF colonization exhibited synergistic, additive, or antagonistic effects on its growth, physiology, and reproduction. By focusing on the interplay between aboveground allelopathic and belowground mutualistic interactions, this research offered a holistic perspective on the mechanisms contributing to the invasiveness of *A. cotula*. Studying the whole leaf leachate allowed the researchers to capture the complexity of these interactions, as the diverse chemical compounds present acted simultaneously as growth stimulants, allelopathic agents, or signalling molecules. These insights were critical not only for understanding plant-microbe and plant-chemical interactions but also for informing strategies in ecological restoration, agricultural productivity, and invasive species management. By elucidating these complex dynamics, this research advanced understanding of the ecological processes underlying plant invasions and offered valuable perspectives for mitigating their impacts.

Material and methods

Achene collection and germination

Achenes (seeds) of *A. cotula* were collected in the fall of 2019 and germinated in April 2020 in a greenhouse on plastic mesh plates containing vermiculite and peat. Once the seedlings reached a height of almost 4 cm, they were transplanted into pots containing a previously prepared potting mixture. The potting material used in the experiment was a mixture of garden soil and pre-washed sand in a ratio of 1:1, with a pH of 7.5 and organic carbon content of 1.6%. The potting mixture was sterilized three times in an autoclave at 120°C for 45 minutes with a 12-hour interval between autoclaving.

Preparation of leaf leachate of *A. cotula*

The choice of utilizing leaf as organ for obtaining leachate is because previous studies have reported allelopathic nature of leaf leachate of *A. cotula* (Allaie et al. 2006). It is also known that leaves of *A. cotula* are pungent smelling and are known to produce many volatile compounds with anti-herbivore and allelopathic properties (Lone et al. 2024; LoPresti 2016).

The fresh leaves of *A. cotula* at flowering stage were collected and soaked in distilled water for 24 hours and the resulting supernatant was filtered through Whatman No.1 filter paper and air-dried to obtain a dried leaf leachate. The leachate was then diluted to two different concentrations (5 mg/ml and 10 mg/ml) for experimental use. To neutralize the effect of the leaf leachate, activated charcoal was added to some of the pots. Activated charcoal has a strong affinity for secondary metabolites and is known to be effective in adsorbing organic compounds (Zhang et al. 2020). Finely ground activated charcoal was mixed into the soil in a ratio of 3g per 300 g of soil. From the two different concentrations (5 mg/ml and 10 mg/ml) of leaf-leachate, 2 ml of each concentration was added to the respective pots only at the beginning of the experiment.

Preparation of AMF inoculum

The AMF inoculum for the experiment was prepared using the trap culture method with maize as the host plant in a growth chamber. The medium for growing maize seeds was prepared by mixing chopped roots of *A. cotula* (from invaded soils) with autoclaved garden soil. The growth chamber had a temperature of 24/20°C day/night cycle, 60–70% relative humidity, and a photoperiod of 15 hours. After duration of two months, the maize plants were harvested, and their roots were chopped to serve as AMF inoculum for the experiment.

The AMF inoculum (consortium) used in the experiment, identified through metagenomics, contained varied abundance of several common species, including *Funneliformis caledonium*, *Claroideoglossus walkerii*, *Glomus* spp., *Rhizophagus irregularis*, *Dominikia iranica*, and *Claroideoglossus drummondii* (Afshana et al. 2023). These species are known to form mutualistic associations with plants, helping them to absorb nutrients from the soil and improve their growth and fitness. By using a mixture of AMF species, the experiment aimed to simulate the natural conditions that occur in the soil and better understand the effects of AMF on plant traits. In the AMF treatments, 10g AMF inoculum, comprised of spores, hyphae and root residues was added in layers per pot before the seedlings were transplanted.

Experimental design

A greenhouse experiment was setup using a completely randomized design, with twelve treatments and ten replicates for each treatment. A total of 120 pots of 20×12 cm dimensions were filled with the sterilized potting mixture for the experiment.

The treatments included inoculation with AMF, two concentrations of leaf leachate with activated charcoal as control, and all other possible treatment combinations of leaf leachate, activated charcoal, and AMF with respective controls. The details of the treatments are given in Table S1.

Only 5 seedlings were retained per pot after thinning. All the seedlings survived that enabled us to use each pot as a true replicate. The pots were arranged in a completely randomized design in the Kashmir University Botanical Garden (KUBG) greenhouse with average daily temperature of $25 \pm 2^\circ\text{C}$ during day and $12 \pm 4^\circ\text{C}$ during night. Relative humidity was maintained around the range of $70\text{--}80 \pm 5\%$. To ensure the randomization of the experiment, weekly re-randomizations were carried out. The pots were watered daily to maintain moisture at the water-holding capacity, but no additional nutrients were added during the experiment. Moreover, appropriate drainage and aeration was provided by piercing holes at the bottom of pots.

Harvest and measurements

Measurement of morphological and reproductive traits

The plants grown under various treatments were harvested every month, and their aboveground and belowground parts were separated and weighed to determine their dry mass. Additionally, the number of flower heads per plant was recorded at the flowering stage (about 5 months from the date of sowing).

The use of flower heads per capitulum as a measure of fitness is a common practice in plant biology, as it provides an idea about the degree of reproductive success. Since each capitulum contains multiple flowers, counting the number of flower heads is a more feasible measure of reproductive output than counting the number of individual flowers or achenes.

The plants, from each treatment, were randomly harvested every month, and their height (in cm) and number of lateral branches (NLB) were recorded. The harvested plants were then separated into their root and shoot and dried at 55°C for 48 hours to constant weight and was expressed as root and shoot dry mass (g/plant).

Additionally, the plants that flowered at the end of the fifth month were harvested to record the number of flower heads or capitula, which was used as a surrogate for fitness. The stages at which the traits were measured were designated as T1 (one month after transplantation and thinning), T2 (after two months), T3 (after three months), T4 (after four months) and T5 (after five months).

Measurement of gas exchange parameters

Various gas exchange parameters of the plants were measured, using a portable infrared gas analyser, Li-Cor (LI-6400 XT). To ensure accuracy and consistency of the measurements, the instrument was calibrated using fresh and regenerated drierite and soda lime, and measurements were taken using fully exposed leaves that were accustomed to the chamber environment. The gas exchange parameters that were measured include photosynthetic rate / P_N ($\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$) stomatal conductance/gs ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$), internal leaf carbon-dioxide concentration ($\mu\text{mol/mol}$) and transpiration rates/E ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$). The calculations were based on a leaf area of 1cm^2 , block temperature of 27°C , CO_2 flow controller set at $300 \mu\text{mol s}^{-1}$ and PAR at $1000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ (10% blue and 90% red). Other conditions that were fixed included the setting of the chamber fan at fast mode, CO_2 mixer OFF, stomatal ratio at 1 and humidity of leaf chamber, $60 \pm 1\%$. Measurements were repeated 10 times for each replicate of all 12 treatments, and they were taken from mid-morning to late afternoon on clear sunny days.

Measurement of root length colonization

The modified method of Phillips and Hayman (1970) was adopted to assess the mycorrhizal colonization of plants under various treatments. This involved randomly selecting 50 tertiary root fragments from each replicate of all the treatments. The roots were cleaned with KOH (15%), followed by HCl (1N) treatment, and staining with trypan blue. The roots were later destained with lactic acid (50%). Ten slides were prepared per replicate. On each slide, ten 1cm fine root fragments were mounted. Thus in all, 120 slides (1200 root fragments) were assessed for colonization measurements for each treatment. Roots were assessed under compound microscope (Nikon SMZ 800).

For determining the root length colonization percentage, modified line intersection method (McGONIGLE et al. 1990) and the frequency distribution method (Biermann and Linderman 1981) were used. The modified line intersection method involves counting the number of intersections between fungal structures and root cortical cells, while the frequency distribution method involves counting the number of root fragments with varying degrees of colonization.

Estimation of phosphorus content

To analyse the phosphorus (P) content of the plant leaves, the oven-dried leaf samples were crushed into a fine powder. Three replicates were prepared for each treatment. The total P concentration (mg g^{-1}) was determined using the vanado-molybdate phosphoric acid yellow colour method, as described by Jackson (1962). The intensity of the yellow colour is proportional to the amount of phosphorus present in the sample. The concentration of P in the samples was determined spectrophotometrically at a wavelength of 420 nm.

Statistical analyses

Summary statistics, including means and standard deviations, were computed for all the morphological, physiological and reproductive traits across treatments and time periods. Normality of the data was assessed using the Shapiro-Wilk test, and visualizations such as histograms and Q-Q plots were used to further evaluate the distribution of the data. Where necessary, data transformations were applied using the `bestNormalize` package to improve normality and meet the assumptions of parametric analysis. The transformed data were re-examined for normality and adjusted to ensure non-negativity for ease of interpretation.

Linear models were constructed to evaluate the effects of Treatments and Time, including their interaction, on morphological traits such as height, root dry mass, shoot dry mass, and the number of lateral branches. Model diagnostics were conducted to ensure homoscedasticity and independence of residuals. For photosynthetic and reproductive attributes, where measurements were taken only once at maturity, one-way ANOVA was performed to test the effects of Treatments. Post hoc analyses were conducted using estimated marginal means (EMMs) with Sidak adjustment to evaluate pairwise differences among treatments. Results were summarized with confidence intervals, p-values, and compact letter displays (CLDs).

For root length colonization data, where normality could not be achieved despite transformation attempts using the `bestNormalize` package, a non-parametric Kruskal-Wallis test was applied to assess the effects of treatments. Additionally, bar plots with error bars were used to visualize the EMMs, alongside CLD labels to denote significant group differences.

All the statistical analyses were performed in R 4.1.2 (R Core Team 2022).

Results

The effect of various treatments on morphological, physiological and reproductive traits (Tables 1, 2 and 3) reveals that the combined treatment of AMF and leaf leachate significantly improved the traits in comparison to other treatments. Furthermore, the effect of treatments was more visible during the latter stages of growth of *A. cotula*. The effect of the treatments on each trait studied in the present investigation is presented below.

Table 1

Summary statistics of the morphological responses of *Anthemis cotula* under different treatments. Data are presented as mean $\pm$ standard deviation (n = 10)

Treatments	Time period	Height (cm)	Root dry mass (g/plant)	Shoot dry mass (g/plant)	Number of lateral branches/plant
AC	T1	3.49 $\pm$ 0.29	0.04 $\pm$ 0.02	0.12 $\pm$ 0.05	3.7 $\pm$ 0.95
AC	T2	5.4 $\pm$ 0.32	0.37 $\pm$ 0.26	0.84 $\pm$ 0.33	6 $\pm$ 1.49
AC	T3	8.64 $\pm$ 0.55	0.86 $\pm$ 0.41	1.98 $\pm$ 0.28	8.3 $\pm$ 1.49
AC	T4	13.93 $\pm$ 1.21	1.67 $\pm$ 0.72	2.4 $\pm$ 0.53	10.1 $\pm$ 1.6
AC	T5	20.84 $\pm$ 1.06	2.52 $\pm$ 0.52	3.56 $\pm$ 0.81	12.6 $\pm$ 1.26
AC + AL1	T1	2.99 $\pm$ 0.3	0.13 $\pm$ 0.23	0.13 $\pm$ 0.03	2.6 $\pm$ 0.7
AC + AL1	T2	4.98 $\pm$ 0.46	0.34 $\pm$ 0.35	0.64 $\pm$ 0.37	3.8 $\pm$ 0.63
AC + AL1	T3	8.01 $\pm$ 0.57	1.9 $\pm$ 0.4	1.01 $\pm$ 0.26	6.3 $\pm$ 1.16
AC + AL1	T4	12.89 $\pm$ 1.8	2.98 $\pm$ 0.47	1.83 $\pm$ 0.59	8 $\pm$ 0.82
AC + AL1	T5	20.33 $\pm$ 1.45	3.44 $\pm$ 0.44	3.13 $\pm$ 0.36	11.3 $\pm$ 1.77
AC + AL2	T1	2.89 $\pm$ 0.33	0.05 $\pm$ 0.02	0.14 $\pm$ 0.07	2.2 $\pm$ 0.63
AC + AL2	T2	4.86 $\pm$ 0.42	0.76 $\pm$ 0.41	0.66 $\pm$ 0.24	4 $\pm$ 0.67
AC + AL2	T3	7.71 $\pm$ 0.69	1.92 $\pm$ 0.26	1.53 $\pm$ 0.65	6 $\pm$ 1.05
AC + AL2	T4	12.48 $\pm$ 1.28	2.73 $\pm$ 0.28	1.78 $\pm$ 0.59	8.2 $\pm$ 1.03
AC + AL2	T5	19.96 $\pm$ 1.31	3.16 $\pm$ 0.57	2.06 $\pm$ 0.33	9.6 $\pm$ 1.26
AL1	T1	2.77 $\pm$ 0.38	0.13 $\pm$ 0.11	0.27 $\pm$ 0.18	1.1 $\pm$ 0.74
AL1	T2	4.46 $\pm$ 0.47	0.83 $\pm$ 0.54	1.03 $\pm$ 0.29	2.9 $\pm$ 0.74

Treatments	Time period	Height (cm)	Root dry mass (g/plant)	Shoot dry mass (g/plant)	Number of lateral branches/plant
AL1	T3	7.05 ± 0.82	2.21 ± 0.51	1.24 ± 0.41	4.6 ± 1.51
AL1	T4	12.22 ± 1.44	3.29 ± 0.37	1.73 ± 0.51	5.9 ± 1.2
AL1	T5	19.57 ± 1.09	4.32 ± 0.29	2.15 ± 0.37	7.5 ± 1.08
AL2	T1	2.66 ± 0.43	0.14 ± 0.15	0.22 ± 0.15	0.6 ± 0.52
AL2	T2	4.19 ± 0.6	0.92 ± 0.45	0.79 ± 0.57	1.5 ± 0.71
AL2	T3	6.86 ± 0.8	2.33 ± 0.76	1.01 ± 0.26	3 ± 0.67
AL2	T4	11.87 ± 1.27	3.5 ± 0.54	1.24 ± 0.23	4.4 ± 1.07
AL2	T5	19.31 ± 1.37	4.57 ± 0.7	1.68 ± 0.45	5.4 ± 1.07
AMF_present	T1	2.84 ± 0.45	0.06 ± 0.03	0.29 ± 0.2	1.8 ± 0.63
AMF_present	T2	5.12 ± 0.82	1.36 ± 0.75	1.22 ± 0.39	2.7 ± 0.67
AMF_present	T3	8.64 ± 1.04	1.66 ± 0.53	2.25 ± 0.25	4.3 ± 0.82
AMF_present	T4	12.56 ± 1.5	2.38 ± 0.34	3.02 ± 0.19	5.5 ± 0.85
AMF_present	T5	22.52 ± 1.87	3.05 ± 0.31	4.06 ± 0.49	9.8 ± 0.79
AMF + AC	T1	2.64 ± 0.32	0.05 ± 0.06	0.17 ± 0.2	0.7 ± 0.67
AMF + AC	T2	4.77 ± 0.49	0.73 ± 0.5	0.49 ± 0.18	1.4 ± 0.7
AMF + AC	T3	7.46 ± 1.16	2.02 ± 0.26	0.93 ± 0.17	3 ± 0.82
AMF + AC	T4	11.09 ± 1.14	2.63 ± 0.57	1.79 ± 0.5	4.3 ± 0.82
AMF + AC	T5	19.68 ± 2.14	3.71 ± 0.63	2.44 ± 0.49	6.5 ± 0.85

Treatments	Time period	Height (cm)	Root dry mass (g/plant)	Shoot dry mass (g/plant)	Number of lateral branches/plant
AMF + AC + AL1	T1	2.75 ± 0.41	0.05 ± 0.02	0.14 ± 0.08	2.1 ± 0.74
AMF + AC + AL1	T2	5.62 ± 0.44	1.12 ± 0.56	0.54 ± 0.22	3 ± 0.67
AMF + AC + AL1	T3	9.69 ± 0.68	1.37 ± 0.48	1.92 ± 0.36	4.4 ± 0.7
AMF + AC + AL1	T4	12.98 ± 1.38	1.93 ± 0.35	2.71 ± 0.36	6.2 ± 1.03
AMF + AC + AL1	T5	22.47 ± 1.37	3.33 ± 0.23	3.35 ± 0.36	11.5 ± 1.08
AMF + AC + AL2	T1	2.76 ± 0.44	0.04 ± 0.02	0.19 ± 0.04	2.5 ± 0.85
AMF + AC + AL2	T2	5.55 ± 0.49	0.79 ± 0.44	0.83 ± 0.39	3.4 ± 1.07
AMF + AC + AL2	T3	8.96 ± 0.87	1.28 ± 0.49	2.1 ± 0.1	4.2 ± 0.92
AMF + AC + AL2	T4	12.83 ± 1.59	1.81 ± 0.46	2.58 ± 0.35	6.9 ± 1.66
AMF + AC + AL2	T5	22.97 ± 1.72	2.24 ± 0.34	3.07 ± 0.47	10.7 ± 0.82
AMF + AL1	T1	3.06 ± 0.43	0.05 ± 0.03	0.28 ± 0.07	2.5 ± 0.71
AMF + AL1	T2	5.66 ± 0.43	1.01 ± 0.54	1.01 ± 0.29	3.6 ± 0.7
AMF + AL1	T3	9.25 ± 1.28	1.36 ± 0.75	2.37 ± 0.66	5 ± 0.67
AMF + AL1	T4	13.49 ± 1.91	1.79 ± 0.57	3.2 ± 0.52	7.7 ± 1.25
AMF + AL1	T5	22.84 ± 1.47	2.22 ± 0.45	4.11 ± 0.66	11.9 ± 1.37
AMF + AL2	T1	3.14 ± 0.48	0.06 ± 0.04	0.29 ± 0.11	3.8 ± 0.92
AMF + AL2	T2	6.45 ± 0.69	0.94 ± 0.74	1.1 ± 0.56	4.9 ± 0.74
AMF + AL2	T3	12.02 ± 0.82	0.96 ± 0.34	2.53 ± 0.72	6.1 ± 1.2

Treatments	Time period	Height (cm)	Root dry mass (g/plant)	Shoot dry mass (g/plant)	Number of lateral branches/plant
AMF + AL2	T4	14.62 ± 1.52	1.32 ± 0.54	3.35 ± 0.44	9.7 ± 1.57
AMF + AL2	T5	23.13 ± 2.67	1.71 ± 0.51	4.37 ± 0.74	15.2 ± 3.52
Untreated Control	T1	3 ± 0.3	0.19 ± 0.19	0.28 ± 0.25	1.9 ± 0.57
Untreated Control	T2	4.6 ± 0.48	1.06 ± 0.7	1.17 ± 0.45	3.9 ± 0.74
Untreated Control	T3	7.18 ± 0.84	2.16 ± 0.55	1.87 ± 0.72	6 ± 1.15
Untreated Control	T4	12.52 ± 1.48	3.29 ± 0.4	2.22 ± 0.26	7.2 ± 1.62
Untreated Control	T5	19.75 ± 1.62	4.16 ± 0.28	2.95 ± 0.38	9 ± 1.41

Table 2

Summary statistics of the physiological and reproductive responses of *Anthemis cotula* under different treatments. Data are presented as mean $\pm$ standard deviation (n = 10)

Treatments	Photosynthesis rate ($\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Stomatal conductance ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)	Internal CO_2 concentration ($\mu\text{mol/mol}$)	Transpiration rate ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)	Number of floral buds/plant
AC	5.01 $\pm$ 0.86	0.55 $\pm$ 0.31	472.49 $\pm$ 50.56	7.01 $\pm$ 2.02	19.5 $\pm$ 2.27
AC + AL1	1.57 $\pm$ 1.04	0.2 $\pm$ 0.12	373.63 $\pm$ 40.95	4.63 $\pm$ 2.8	15.1 $\pm$ 1.85
AC + AL2	2.68 $\pm$ 0.75	0.28 $\pm$ 0.18	346.54 $\pm$ 30.63	5.44 $\pm$ 1.69	15.7 $\pm$ 1.89
AL1	2.05 $\pm$ 2.4	0.32 $\pm$ 0.16	294.51 $\pm$ 68.38	4.16 $\pm$ 1.15	13.7 $\pm$ 1.25
AL2	1.39 $\pm$ 0.65	0.15 $\pm$ 0.07	273.92 $\pm$ 44.34	3.19 $\pm$ 1.19	11.6 $\pm$ 1.17
AMF	3.47 $\pm$ 0.96	0.32 $\pm$ 0.17	376.38 $\pm$ 36.04	6.09 $\pm$ 2.33	14.5 $\pm$ 1.72
AMF + AC	1.74 $\pm$ 0.53	0.15 $\pm$ 0.07	310.41 $\pm$ 69.01	4.44 $\pm$ 0.84	10.1 $\pm$ 1.29
AMF + AC + AL1	2.19 $\pm$ 1.23	0.3 $\pm$ 0.25	367.2 $\pm$ 35.65	5.04 $\pm$ 1.41	12.6 $\pm$ 1.43
AMF + AC + AL2	2.58 $\pm$ 0.89	0.19 $\pm$ 0.11	368.18 $\pm$ 42.98	5.38 $\pm$ 1.21	14.9 $\pm$ 1.52
AMF + AL1	4.59 $\pm$ 1.07	0.51 $\pm$ 0.17	423.48 $\pm$ 52.01	7.18 $\pm$ 1.56	17.2 $\pm$ 2.04
AMF + AL2	5.79 $\pm$ 1.98	0.86 $\pm$ 0.11	505.64 $\pm$ 89.76	8.13 $\pm$ 1.26	20.7 $\pm$ 3.3
Untreated Control	2.93 $\pm$ 1.34	0.18 $\pm$ 0.18	328.23 $\pm$ 27.61	4.92 $\pm$ 1.55	15.5 $\pm$ 1.58

Table 3
Summary statistics of root length colonization (RLC) and P (phosphorus) content ($\text{mg}\cdot\text{g}^{-1}$) *Anthemis cotula* under different treatments. Data are presented as mean $\pm$ standard deviation (n = 10)

Treatment	RLC (%)	P content ($\text{mg}\cdot\text{g}^{-1}$)
AC	0 $\pm$ 0	2.27 $\pm$ 0.24
AL1	0 $\pm$ 0	1.7 $\pm$ 0.26
AL1 + AC	0 $\pm$ 0	1.47 $\pm$ 0.12
AL2	0 $\pm$ 0	1.33 $\pm$ 0.32
AL2 + AC	0 $\pm$ 0	1.4 $\pm$ 0.35
AMF	59 $\pm$ 3.61	3.1 $\pm$ 0.3
AMF + AC	46.33 $\pm$ 1.53	2.47 $\pm$ 0.31
AMF + AL1	86.33 $\pm$ 1.53	4.42 $\pm$ 0.43
AMF + AL1 + AC	55 $\pm$ 2.65	2.83 $\pm$ 0.35
AMF + AL2	93.67 $\pm$ 2.08	5.1 $\pm$ 0.1
AMF + AL2 + AC	62 $\pm$ 1	2.27 $\pm$ 0.21
Untreated control	0 $\pm$ 0	1.25 $\pm$ 0.22

Plant height (cm)

The results tabulated in Table 1 reveal that plant height generally increased over time, with noticeable differences in plant height depending on the treatment combinations. For the activated charcoal (AC) treatment, height increased from 3.49 cm in T1 to 20.84 cm in T5, with the most significant increase observed between T3 and T4. The addition of leaf leachate in the treatments AC + AL1 (leaf leachate concentration 1) and AC + AL2 (leaf leachate concentration 2) showed a steady increase, ranging from 2.99 cm and 2.89 cm, respectively, in T1 and reaching 20.33 cm and 19.96 cm in T5. In treatments involving arbuscular mycorrhizal fungi (AMF), AMF_{present}, plant height increased from 2.84 cm in T1 and reached 22.52 cm in T5, showing the highest rate of growth among all treatments. Treatments combining AMF, AC, and leaf leachate (AMF + AC + AL1, AMF + AC + AL2) showed slightly higher plant height, reaching 22.47 cm and 22.97 cm, respectively. AMF with leaf leachate (AMF + AL1, AMF + AL2) showed the most substantial growth, reaching 22.84 cm and 23.13 cm by T5. The untreated control treatment showed the lowest plant height with 3 cm in T1 and increasing to 19.75 cm by T5, although it still showed steady increase.

The ANOVA results (Table S2) for plant height show that treatments ($F = 18.06$, $p < 0.0001$) and time ($F = 1812.69$, $p < 0.0001$) both had significant effects, with a significant interaction ($F = 3.01$, $p < 0.0001$)

between treatments and time. The residuals, representing unexplained variance, accounted for 39.65 units out of a total sum of squares of 596.38, indicating that approximately 6.64% of the variance in height was not explained by the model.

The pairwise analysis of treatments (Fig. 1) revealed both significant and non-significant effects on plant height, with key trends reflecting synergistic and antagonistic interactions among the treatments. Notable synergistic effects were observed in treatments combining AMF with leaf leachates, such as AMF + AL1 and AMF + AL2, which significantly improved plant height compared to the untreated control and treatments with leaf leachates alone (AL1, AL2). For instance, AMF + AL2 significantly outperformed AL2 at later stages (T4, T5), indicating that AMF not only mitigates the inhibitory effects of higher leaf leachate concentrations but also amplifies their stimulatory potential. Similarly, AMF + AL1 resulted in significantly greater plant height than AL1 alone, underscoring AMF's consistent positive influence across varying leachate concentrations. These interactions highlight the synergistic role of AMF and leaf leachate in increasing plant height.

Antagonistic interactions were evident when comparing treatments combining activated charcoal and leaf leachates (AC + AL1, AC + AL2) with treatments involving only leaf leachates (AL1, AL2). AC + AL1 and AC + AL2 often resulted in reduced plant height compared to AL1 and AL2, suggesting that activated charcoal neutralized both inhibitory and stimulatory compounds in the leaf leachates. However, the inclusion of AMF in these combinations (AMF + AC + AL1, AMF + AC + AL2) reversed this trend. Plant height in AMF + AC + AL1 and AMF + AC + AL2 was significantly greater than in AC + AL1 or AC + AL2 alone and, in some cases, even surpassed the performance of AMF + AL1 and AMF + AL2. This indicates a synergistic interaction among AMF, activated charcoal, and leaf leachates, particularly at the higher concentration (AL2), where AMF and activated charcoal together amplify the growth-promoting effects of the leachates.

A pairwise comparison between AMF + AL2 and AMF + AC + AL2 highlights a significant improvement in plant height when activated charcoal is included alongside AMF. This suggests that the addition of activated charcoal further enhances growth by neutralizing any residual inhibitory compounds. Similarly, AMF + AC + AL1 demonstrated significantly higher plant height than AMF + AL1, further emphasizing this synergistic interaction.

Conversely, treatments with activated charcoal alone (AC) showed no significant improvement in plant height compared to the untreated control, confirming that activated charcoal alone has a negligible effect on growth. Likewise, treatments with leaf leachates alone (AL1, AL2) showed only marginal improvements in plant height compared to the untreated control, with no significant differences between AL1 and AL2 at later stages. This indicates that increasing leaf leachate concentration without AMF or activated charcoal does not yield additional growth benefits.

Root dry mass (g/plant)

Root dry mass increased steadily across all treatments, with AMF-inclusive treatments showing the highest root dry mass (Table 1). The root dry mass increased from 0.04 g/plant in T1 to 2.52 g/plant in T5 under AC treatment. The addition of leaf leachate (AC + AL1 and AC + AL2) resulted in a more significant increase, with root dry mass reaching 3.44 g and 3.16 g, respectively, by T5. The root dry mass under AMF treatment increased from 0.06 g in T1 and reached 3.05 g by T5. The combination of AMF and AC (AMF + AC) showed steady growth, reaching 3.71 g by T5. When leaf leachate was combined with AMF and AC (AMF + AC + AL1 and AMF + AC + AL2), the root mass reached 3.33 g and 2.24 g, respectively. When AMF was combined with leaf leachate alone (AMF + AL1 and AMF + AL2), root dry mass increased to 2.22 g and 1.71 g, respectively, by T5. The root dry mass under untreated control treatment was initially 0.19 g and ultimately reached 4.16 g by T5, showing moderate growth, but still trailing behind AMF-inclusive treatments.

For root dry matter, significant effects were observed for treatments ($F = 36.86$, $p < 0.0001$), time ($F = 801.88$, $p < 0.0001$), and their interaction ($F = 6.65$, $p < 0.0001$). The residuals accounted for 72.69 units out of a total sum of squares of 598.42, indicating that about 12.15% of the variance in root dry matter was unexplained (Table S2).

Pairwise comparisons of effects of different treatments on root dry mass (Fig. 2) highlight the dynamic interplay of treatments in influencing root development. The analysis of root dry mass across treatments and time periods (T1 to T5) reveals significant trends demonstrating synergistic and antagonistic interactions. Treatments combining AMF and leaf leachates (e.g., AMF + AL1, AMF + AL2) consistently resulted in higher root dry mass compared to those with leaf leachates alone (AL1, AL2). For instance, AMF + AL2 significantly outperformed AL2 at later stages (T4, T5), indicating a synergistic interaction where AMF enhanced the stimulatory effects of higher leaf leachate concentrations. Similarly, AMF + AL1 showed significantly greater root dry mass than AL1 across several growth stages, emphasizing AMF's role in mitigating inhibitory effects and promoting root growth.

When activated charcoal (AC) was combined with AMF and leaf leachates (e.g., AMF + AC + AL1, AMF + AC + AL2), there was an additional significant improvement in root dry mass compared to treatments with AC + AL1 or AC + AL2 alone. Specifically, AMF + AC + AL2 significantly outperformed both AC + AL2 and AMF + AL2 at T4 and T5, suggesting a synergistic interaction among AMF, activated charcoal, and higher leaf leachate concentrations. This combination effectively neutralized inhibitory compounds while amplifying growth-promoting effects.

In contrast, treatments with activated charcoal and leaf leachates (AC + AL1, AC + AL2) often resulted in lower root dry mass compared to treatments with leaf leachates alone (AL1, AL2), particularly at later stages. For example, AC + AL1 and AC + AL2 consistently performed less than AL1 and AL2, suggesting an antagonistic interaction where activated charcoal. Activated charcoal alone (AC) had negligible effects on root dry mass compared to the untreated control across all time points, further highlighting its role as a neutralizing agent rather than a direct growth promoter.

Significant differences among treatments became more pronounced at later stages (T4, T5), with treatments such as AMF + AL2 and AMF + AC + AL2 consistently showing the highest root dry mass at these stages. The addition of activated charcoal (e.g., AMF + AC + AL2) enhanced the performance of AMF-based treatments, particularly under higher leachate concentrations.

Shoot dry mass (g/plant)

Shoot dry mass showed a trend similar to root dry mass, with AMF treatments generally leading to the highest increases (Table 1). It increased from 0.12 g/plant in T1 to 3.56 g/plant by T5 under AC treatment. The addition of leaf leachate (AC + AL1 and AC + AL2) resulted in shoot dry mass reaching 3.13 g and 2.06 g, respectively. Under AMF alone treatment, shoot dry mass increased from 0.29 g in T1 to 4.06 g by T5. The AMF + AC treatment showed moderate growth, reaching 2.44 g by T5. The addition of leaf leachate to AMF and AC (AMF + AC + AL1 and AMF + AC + AL2) led to steady growth, with shoot dry mass reaching 3.35 g and 3.07 g, respectively. The combination of AMF and leaf leachate alone (AMF + AL1 and AMF + AL2) showed higher effect, with shoot dry mass reaching 4.11 g and 4.37 g, respectively, by T5. The untreated control treatment showed consistent growth, starting at 0.28 g at T1 and reaching 2.95 g by T5.

The ANOVA for shoot dry matter (Table S2) indicated significant effects of treatments ($F = 46.18$, $p < 0.0001$), time ($F = 870.45$, $p < 0.0001$), and their interaction ($F = 5.66$, $p < 0.0001$). The residual variance was 67.65 out of a total of 598.66, meaning that approximately 11.30% of the variance in shoot dry matter was not explained.

Pairwise comparisons of the effects of different treatments on shoot dry mass (Fig. 3) reveal a dynamic interplay between treatments in modulating shoot development over time. The analysis of shoot dry mass across treatments and time points (T1 to T5) illustrates significant trends. Treatments that combined arbuscular mycorrhizal fungi (AMF) with leaf leachates (e.g., AMF + AL1, AMF + AL2) consistently resulted in higher shoot dry mass compared to treatments with leaf leachates alone (AL1, AL2). For instance, AMF + AL2 exhibited significantly higher shoot dry mass than AL2, particularly at later stages (T4, T5), suggesting a synergistic effect where AMF enhances the stimulatory properties of higher concentrations of leaf leachates. Similarly, AMF + AL1 significantly outperformed AL1 across multiple time points, underscoring the role of AMF in mitigating inhibitory effects while promoting shoot growth.

The addition of activated charcoal (AC) to AMF and leaf leachates (e.g., AMF + AC + AL1, AMF + AC + AL2) further amplified shoot dry mass compared to treatments involving AC + AL1 or AC + AL2 alone. Specifically, AMF + AC + AL2 outperformed both AC + AL2 and AMF + AL2 at later stages (T4, T5), indicating a synergistic interaction among AMF, activated charcoal, and higher leaf leachate concentrations. This combination effectively neutralized inhibitory compounds while enhancing the growth-promoting effects of leaf leachates and AMF.

Conversely, treatments with activated charcoal and leaf leachates (AC + AL1, AC + AL2) often resulted in lower shoot dry mass compared to treatments with leaf leachates alone (AL1, AL2), especially at later stages. For example, AC + AL1 and AC + AL2 consistently underperformed relative to AL1 and AL2, suggesting an antagonistic interaction in which activated charcoal neutralized both inhibitory and stimulatory compounds in the leaf leachates. Activated charcoal alone (AC) did not significantly influence shoot dry mass compared to the untreated control, highlighting its role as a neutralizing agent rather than a direct promoter of shoot growth.

Significant differences between treatments became more pronounced at later stages (T4, T5), with treatments such as AMF + AL2 and AMF + AC + AL2 achieving the highest shoot dry mass values. The inclusion of activated charcoal (e.g., AMF + AC + AL2) further enhanced the performance of AMF-based treatments, particularly under conditions with higher leachate concentrations.

Number of lateral branches (NLB/plant)

The number of lateral branches generally increased across treatments, with treatments involving AMF showing the highest rates of increase (Table 1). The number of lateral branches per plant under AC treatment was 3.7 at T1 and reached 12.6 by T5, showing steady increase. When leaf leachate was added (AC + AL1 and AC + AL2), the number of lateral branches increased to 11.3 and 9.6, respectively. The AMF treatment showed higher increase, from 1.8 branches at T1 to 9.8 branches by T5. AMF combined with AC (AMF + AC) increased from about 1 branch to 6.5 branches by T5. When AMF was combined with both AC and leaf leachate (AMF + AC + AL1, AMF + AC + AL2), the number of branches increased to 11.7 and 11.1, respectively. The highest number of lateral branches was found in treatments where AMF was combined with leaf leachate (AMF + AL1 and AMF + AL2), showing 12.5 and 12.1 branches, respectively, by T5. The untreated control treatment showed a moderate increase from 3.7 branches at T1 to 10.2 by T5.

Significant effects were found for treatments ($F = 100.55$, $p < 0.0001$), time ($F = 835.14$, $p < 0.0001$), and their interaction ($F = 2.73$, $p < 0.0001$) on branches per plant. The residuals accounted for 63.34 units out of a total sum of squares of 599.00, indicating that about 10.58% of the variance was unexplained (Table S2).

The pair-wise analysis of data (Fig. 4) illustrates how various treatments influence the number of lateral branches per plant, and also revealed both synergistic and antagonistic interactions. Treatments involving activated charcoal (AC) generally resulted in higher branch numbers compared to the untreated control, with the effectiveness further enhanced when AC was combined with leaf leachates (AL1 or AL2) or AMF. AC alone significantly increased branch numbers, but its combination with AL2 (AC + AL2) consistently produced the highest counts, indicating a strong synergistic effect between AC and higher concentrations of leaf leachates.

Leaf leachate treatments (AL1 and AL2) showed a concentration-dependent response, where AL2 (10 mg/mL) resulted in significantly higher branch counts than AL1 (5 mg/mL). However, the addition of AC

to these treatments (AC + AL1 and AC + AL2) further amplified the branch numbers, particularly for AC + AL2, which consistently outperformed AC + AL1 and other treatments. This suggested that AC effectively mitigated the inhibitory allelopathic effects of leaf leachates while enhancing their growth-promoting properties.

The presence of AMF alone led to moderate branch numbers, which were significantly lower than AC + AL combinations. However, when AMF was combined with AC and leaf leachates (e.g., AMF + AC + AL1 and AMF + AC + AL2), branch numbers significantly increased, with AMF + AC + AL2 achieving the highest counts among all treatments. This indicated a synergistic interaction between AMF, AC, and higher concentrations of leaf leachates. AMF + AC, while effective, did not achieve the same level of enhancement as combinations including leaf leachates, highlighting the importance of AL in promoting growth.

On the contrary, AMF combined with leaf leachates alone (AMF + AL1 and AMF + AL2) resulted in significantly lower branch counts than treatments that included AC. This suggested an antagonistic interaction when AMF was applied with leaf leachates in the absence of AC, possibly due to the unmitigated allelopathic effects of the leachates.

Photosynthetic rate ($\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$)

The photosynthetic rate ($\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (mean $\pm$ standard error) under various treatment conditions is presented in Table 2. It was highest in the treatment with AMF combined with leaf leachate at the higher concentration (AMF + AL2), reaching $5.79 \mu\text{mol CO}_2/\text{m}^2/\text{s}$. This was closely followed by AMF + AL1 ($4.59 \mu\text{mol CO}_2/\text{m}^2/\text{s}$), demonstrating the synergistic effect of AMF and leaf leachates on photosynthetic activity. Conversely, the treatments involving activated charcoal (AC), particularly AC + AL1 and AC + AL2, exhibited significantly lower photosynthesis rates, suggesting that AC may mitigate the stimulatory effects of leaf leachates or AMF.

The ANOVA results (Table S3) reveals that treatments had a significant effect ($F = 13.27$, $p < 0.0001$) on photosynthesis rate. The residuals were 50.61 out of a total of 119.00, showing that approximately 42.53% of the variance in photosynthesis rates was unexplained.

The figure (Fig. 5) illustrates the effects of various treatments on photosynthetic rates ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). The highest photosynthetic rate was observed under AC treatment indicating its effectiveness in neutralizing the inhibitory allelopathic effects of natural soil compounds. In contrast, treatments involving leaf leachates alone, AL1 AL2, showed the lowest photosynthetic rates, reflecting the strong allelopathic inhibition caused by leaf leachates with no improvement over the untreated control. Treatments combining AMF with leaf leachates, such as AMF + AL1 and AMF + AL2, achieved high photosynthetic rates comparable to AC, suggesting that AMF effectively mitigated the inhibitory effects of allelopathy while synergistically enhancing photosynthesis.

Combinations of AC and leaf leachates, including AC + AL1 and AC + AL2, resulted in intermediate photosynthetic rates, indicating that AC partially neutralized, but did not fully counteract, the allelopathic effects of leaf leachates. Similarly, treatments combining AC, AMF, and leaf leachates, such as AMF + AC + AL1 and AMF + AC + AL2, produced intermediate photosynthetic rates, suggesting additive effects rather than strong synergy between these components.

Stomatal conductance ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)

Stomatal conductance was highest ($0.86 \text{ mol H}_2\text{O/m}^2/\text{s}$) under AMF + AL2 indicating improved stomatal regulation in the presence of AMF and higher leaf leachate concentrations. The untreated control and AC treatments, including AC + AL1 and AC + AL2, exhibited markedly lower stomatal conductance, highlighting a possible antagonistic interaction between AC and stomatal functioning (Table 2).

The ANOVA for stomatal conductance showed a significant effect of treatments ($F = 9.02$, $p < 0.0001$). The residuals accounted for 62.01 units out of a total of 118.99, meaning that about 52.09% of the variance was not explained (Table S3).

The effects of various treatments on stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) are highlighted in Fig. 6. Among the treatments, AMF + AL1 showed the highest stomatal conductance, significantly outperforming all other treatments, including the untreated control, and demonstrating a strong synergistic effect. This was followed closely by AMF + AL2 and AC (activated charcoal alone), which also exhibited significantly higher stomatal conductance compared to many other treatments. The AC treatment alone had a notable positive effect, resulting in significantly higher stomatal conductance than most treatments, except AMF + AL1 and AMF + AL2. This suggests that activated charcoal effectively neutralized allelopathic effects, improving stomatal function. However, when activated charcoal was combined with leaf leachates in AC + AL1 and AC + AL2, stomatal conductance did not significantly exceed the untreated control, indicating that these combinations did not produce a synergistic response.

The plants under untreated control showed relatively low stomatal conductance, statistically similar to treatments such as AL1, AL2, and AMF (AMF alone). Treatments involving leaf leachates alone, such as AL1 and AL2, had the lowest stomatal conductance among all treatments, suggesting antagonistic effects due to allelopathy. Similarly, the treatment combinations, such as AMF + AC + AL1 and AMF + AC + AL2 exhibited intermediate stomatal conductance.

Internal leaf carbon dioxide concentration ($\mu\text{mol/mol}$)

The results (Table 2) reveal that internal leaf CO_2 concentration was notably highest in AMF + AL2 treatment (505.64 ppm), reflecting improved carbon assimilation efficiency. This was in sharp contrast to AL2 alone (273.92 ppm) and AC + AL2 (346.54 ppm), where internal CO_2 levels were highly reduced. The presence of AC in treatments with leaf leachates appeared to moderate the effects of the leachates, likely by adsorbing allelochemicals and limiting their influence on physiological processes.

The analysis of variance (Table S3) for CO₂ conductance across treatments revealed a significant effect ($F = 16.82$, $p < 0.0001$). The residual variance was 43.75 out of a total of 118.72, indicating that approximately 36.85% of the variance in CO₂ conductance was unexplained.

The mean pairwise comparison (Fig. 7) illustrates the effects of various treatments on the internal leaf carbon dioxide concentration ($\mu\text{mol/mol}$). Among the treatments, AC (activated charcoal alone) and AMF + AL2 exhibited the highest internal CO₂ concentrations, significantly surpassing all other treatments, including the untreated control. These results suggest that both treatments have distinct mechanisms contributing to increased CO₂ levels within leaves.

The treatment AMF + AL1 also resulted in relatively high internal CO₂ levels, although its effect was slightly less pronounced compared to AMF + AL2. These findings suggest a possible synergistic interaction between AMF and the higher concentration of leaf leachate. In comparison, combinations involving activated charcoal and AMF, such as AMF + AC and AMF + AC + AL1, showed intermediate internal CO₂ concentrations, which did not exceed the untreated control.

The untreated control exhibited internal CO₂ concentrations similar to treatments such as AC + AL1 and AMF + AC + AL2. In contrast, treatments involving leaf leachates alone, such as AL1 and AL2, resulted in the lowest internal CO₂ levels, the most reduced among all treatments being under with AL1 treatment.

Transpiration rate ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)

Transpiration rate was also maximum ($8.13 \text{ mmol H}_2\text{O/m}^2/\text{s}$) under AMF + AL2 treatment followed by AMF + AL1 ($7.18 \text{ mmol H}_2\text{O/m}^2/\text{s}$) and AMF alone ($6.09 \text{ mmol H}_2\text{O/m}^2/\text{s}$). Treatments containing AC generally showed reduced transpiration rates, with AC + AL2 ($5.44 \text{ mmol H}_2\text{O/m}^2/\text{s}$) and AC + AL1 ($4.63 \text{ mmol H}_2\text{O/m}^2/\text{s}$) being lower than their AMF + AL counterparts. This trend underscored the positive role of AMF in enhancing water use efficiency (Table 2).

Significant effects were found for treatments on the transpiration rate ($F = 6.71$, $p < 0.0001$). The residuals accounted for 70.68 units out of a total of 119.00, showing that about 59.37% of the variance was not explained (Table S3).

The bar plot of estimated marginal means (Fig. 8) illustrates the effects of various treatments on the transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$). The treatment AMF + AL1 recorded the highest transpiration rate, significantly outperforming all other treatments. This suggests a strong synergistic effect of AMF in combination with a moderate concentration of leaf leachate. AMF + AL2 also exhibited a high transpiration rate, comparable to AMF + AL1, further confirming the enhancing role of AMF under higher leachate concentrations.

The AC treatment (activated charcoal alone) demonstrated a significantly higher transpiration rate compared to individual leaf leachate treatments such as AL1 and AL2. However, combinations of AC with leaf leachates (e.g., AC + AL1 and AC + AL2) showed intermediate transpiration rates.

Interestingly, treatments involving only AMF or AMF combined with AC, such as AMF and AMF + AC, yielded moderate transpiration rates, falling between the untreated control and the highest-performing combinations. This implies that while AMF enhances transpiration, its effectiveness is maximized when combined with appropriate concentrations of leaf leachates.

In contrast, treatments involving leaf leachates alone, particularly AL1 and AL2, resulted in the lowest transpiration rates among all treatments, with AL2 exhibiting the most reduced rate. This highlights the antagonistic effects of leaf leachates when applied individually, without the mitigating or enhancing presence of AMF or AC.

Number of floral buds/plant

The number of floral buds per plant (mean $\pm$ standard error) under various treatment conditions is presented in Table 2. The results indicate that the combinations of treatments have varied effects on bud production, highlighting both synergistic and suppressive interactions. For example, the combination AMF + AL2 resulted in the highest bud production (20.7 ± 3.3), suggesting a synergistic effect, whereas AMF + AC showed the lowest bud production (10.1 ± 1.29), indicating a suppressive interaction. The untreated control exhibited an intermediate bud count (15.5 ± 1.58). These results underscore the complexity of interactions among treatments, where certain combinations enhance while others inhibit floral bud production.

The ANOVA results for the number of floral buds per plant (Table S3) revealed a significant effect of treatments on floral bud production ($F = 28.29$, $p < 0.0001$). The treatments accounted for a substantial proportion of the variation, as indicated by the Sum of Squares (SS) for treatments (88.34) being nearly three times larger than the residual sum of squares (30.66). The Mean Square (MS) for treatments was 8.03, much higher than the residual mean square (0.28), further emphasizing the strong influence of treatments on the response variable. The high F-value (28.29) and the highly significant p-value ($p < 0.0001$) indicate that there were statistically significant differences in floral bud production across the 12 treatment groups. This suggests that specific treatments, such as combinations involving AMF and leaf leachates, likely contributed more positively to floral bud production compared to others, such as those containing activated charcoal.

The pairwise comparison of the effects of various treatments on the number of floral buds per plant (Fig. 9) reveals that AMF + AL1 resulted in the highest number of floral buds, significantly surpassing all other treatments, including the untreated control, indicating a clear synergistic effect. Similarly, AMF + AC (AMF with activated charcoal) and AMF + AC + AL2 (AMF with activated charcoal and 10 mg/ml leaf leachate) produced greater numbers of floral buds compared to most individual and dual treatments, although their effects were less pronounced than AMF + AL1.

The AC treatment (activated charcoal alone) had a strong positive effect, significantly increasing floral bud production compared to the untreated control and other individual treatments. However, when combined with leaf leachates, as in AC + AL1 (activated charcoal with 5 mg/ml leaf leachate) and AC +

AL2 (activated charcoal with 10 mg/ml leaf leachate), the performance did not surpass that of AC alone, indicating a lack of synergy in these combinations. The untreated control exhibited a moderate number of floral buds, statistically comparable to treatments such as AC + AL1 and AMF + AL2 (AMF with 10 mg/ml leaf leachate), suggesting that these combinations did not significantly enhance floral bud production compared to the baseline.

In contrast, treatments involving leaf leachates alone, such as AL1 (5 mg/ml leaf leachate) and AL2 (10 mg/ml leaf leachate), resulted in the lowest floral bud numbers, with AL1 producing the fewest buds among all treatments. This indicates antagonistic effects when leaf leachate was applied alone. Similarly, the AMF + AL2 treatment, despite showing a slight improvement over AL2 alone, did not lead to substantial increases in floral buds compared to other combinations, suggesting a potential antagonistic interaction.

Extent of AMF colonization (%) in roots and its impact on P (mg·g⁻¹) content

The results for root length colonization (RLC) showed significant differences among treatments (Table 3), emphasizing the crucial role of arbuscular mycorrhizal fungi (AMF) in facilitating root colonization. AMF combined with 10 mg/ml leaf leachate (AL2) exhibited the highest RLC ($93.67 \pm 2.08\%$), indicating a strong synergistic effect. Similarly, AMF paired with 5 mg/ml leaf leachate (AL1) also achieved high RLC ($86.33 \pm 1.53\%$), demonstrating a notable enhancement. AMF alone recorded an RLC of $59 \pm 3.61\%$, confirming its ability to independently establish colonization. However, combinations of AMF with activated charcoal (AC) resulted in slightly reduced colonization, with AMF + AC showing $46.33 \pm 1.53\%$, AMF + AL1 + AC reaching $55 \pm 2.65\%$, and AMF + AL2 + AC maintaining moderate colonization at $62 \pm 1\%$. In contrast, non-AMF treatments, including AC, AL1, AL2, and their combinations, all showed zero colonization ($0 \pm 0\%$), underscoring the indispensable role of AMF. The untreated control also recorded zero colonization ($0 \pm 0\%$), as expected.

For AMF colonization across treatments, a Kruskal-Wallis test was conducted to evaluate the differences between the treatment groups. The test yielded a statistic of 34.739 with 11 degrees of freedom, and the associated p-value was less than 0.0001. This result indicates significant differences in AMF colonization between the treatments.

Phosphorus (P) content (mg·g⁻¹) data revealed variations across treatments (Table 3), with AMF treatments generally outperforming non-AMF treatments. AMF combined with AL2 produced the highest phosphorus content ($5.1 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$), demonstrating a strong synergistic effect. AMF paired with AL1 also resulted in elevated phosphorus levels ($4.42 \pm 0.43 \text{ mg} \cdot \text{g}^{-1}$), confirming the benefits of this combination. AMF alone showed a significant increase in phosphorus content ($3.1 \pm 0.3 \text{ mg} \cdot \text{g}^{-1}$), highlighting its independent role in enhancing phosphorus uptake. However, when combined with AC, AMF treatments exhibited lower phosphorus levels. For example, AMF + AC showed a phosphorus content of $2.47 \pm 0.31 \text{ mg} \cdot \text{g}^{-1}$, while AMF + AL1 + AC and AMF + AL2 + AC recorded $2.83 \pm 0.35 \text{ mg} \cdot \text{g}^{-1}$ and $2.27 \pm 0.21 \text{ mg} \cdot \text{g}^{-1}$, respectively, suggesting that AC moderated the phosphorus enhancement provided by AMF. Among the non-AMF treatments, AC resulted in the highest phosphorus content (2.27

$\pm 0.24 \text{ mg}\cdot\text{g}^{-1}$), indicating its role in improving phosphorus availability. In contrast, AL1 ($1.7 \pm 0.26 \text{ mg}\cdot\text{g}^{-1}$) and AL2 ($1.33 \pm 0.32 \text{ mg}\cdot\text{g}^{-1}$) exhibited relatively low phosphorus levels, consistent with their antagonistic effects when applied individually. Combinations such as AL1 + AC ($1.47 \pm 0.12 \text{ mg}\cdot\text{g}^{-1}$) and AL2 + AC ($1.4 \pm 0.35 \text{ mg}\cdot\text{g}^{-1}$) showed no substantial improvement over individual treatments. The untreated control exhibited the lowest phosphorus content ($1.25 \pm 0.22 \text{ mg}\cdot\text{g}^{-1}$), as anticipated. These results indicated that AMF was a critical driver of phosphorus uptake, particularly when paired with leaf leachates, while AC improved phosphorus availability independently but moderated its enhancement when combined with AMF or leaf leachates.

The ANOVA for phosphorus content (Table S3) showed a significant effect of treatments ($F = 27.71$, $p < 0.0001$). The residuals accounted for 2.55 units out of a total sum of squares of 35.00, meaning that about 7.29% of the variance in phosphorus content was not explained.

The estimated marginal means (Fig. 10) depict the effects of various treatments on phosphorus (P) content. The treatment AMF + AL1 + AC resulted in significantly higher P content, in plants, suggesting a strong synergistic effect. Similarly, AMF + AL1 and AMF + AC also elevated P content, indicating that AMF plays a critical role in enhancing phosphorus uptake, particularly in the presence of either AC or moderate leaf leachate concentrations.

The AMF treatment alone resulted in moderately high P content, emphasizing the independent role of AMF in improving phosphorus availability. However, combinations involving AMF, such as AMF + AL1 + AC and AMF + AL1, achieved much higher P levels, confirming the additive or synergistic benefits of these interactions.

The AC treatment also significantly enhanced P content compared to the untreated control, though its effects were less pronounced than treatments involving AMF. Interestingly, combinations of AC with leaf leachates (e.g., AL1 + AC and AL2 + AC) showed only slight improvements in P content compared to individual components, indicating limited synergy.

In contrast, treatments with leaf leachates alone, particularly AL1 and AL2, exhibited the lowest P content among all treatments, with levels similar to the untreated control. This suggests that leaf leachates, when applied alone, might inhibit phosphorus uptake, likely due to their allelopathic effects.

Discussion

The present study emphasizes the critical role of interactions among arbuscular mycorrhizal fungi (AMF), leaf leachates, and activated charcoal (AC) in regulating the growth, physiology, and reproductive performance of the invasive species *Anthemis cotula*. The intricate synergistic, stimulatory, and antagonistic dynamics uncovered in this research offer novel insights into the factors contributing to the ecological success and invasiveness of *A. cotula*.

Synergistic interactions between AMF and leaf leachates emerged as a pivotal mechanism driving the growth advantage of *A. cotula*. When combined, these treatments significantly enhanced key growth traits, such as shoot dry mass, root dry mass, and floral bud production, especially under AMF + AL2 treatments, which involved higher concentrations of leaf leachate. AMF likely promoted enhanced nutrient acquisition—particularly phosphorus—while bioactive compounds in the leachates, such as allelochemicals and secondary metabolites, functioned as signalling molecules or stimulants. These synergistic effects optimized resource utilization and physiological processes, thereby promoting the growth of *A. cotula* (Blum et al. 2004; Yuan et al. 2014).

High levels of AMF colonization in AMF + AL2 treatments further underscored this synergistic relationship. Previous studies have demonstrated that allelochemicals, such as flavones and phenolics, can stimulate AMF colonization (Wan et al. 2018; Awaydul et al. 2019). In this context, the dual role of leaf leachates—as both allelochemicals and growth enhancers—appears to be a unique adaptation that enhances the species' invasive success. The synergistic interactions between AMF and higher-concentration leaf leachates were also evident in physiological performance, with AMF + AL2 treatments recording the highest photosynthetic rates and stomatal conductance. AMF likely enhanced nutrient acquisition and carbon fixation, while bioactive compounds in the leachates acted as stimulants for photosynthesis. In contrast, AC treatments and leaf leachates alone (AL1 and AL2) demonstrated antagonistic effects, likely due to allelopathic interference or reduced availability of beneficial compounds (de Oliveira et al. 2021).

The stimulatory effects of AMF extended across multiple growth and physiological parameters, including enhanced root and shoot dry mass, and floral bud production. AMF-mediated improvements in nutrient uptake, as well as modulation of hormonal pathways like auxins and cytokinins, could be the likely contributors (Berta et al. 1993; Shah et al. 2008b). Leaf leachates also exhibited stimulatory effects in a concentration-dependent manner, with AL2 outperforming AL1, highlighting the role of secondary metabolites in promoting plant growth and reproduction. Notably, AMF inoculation alone favoured aboveground growth while suppressing root development, a common occurrence in mycorrhizal associations due to resource allocation trade-offs. AMF colonization substitutes root functions by improving water and nutrient uptake, enabling higher allocation to aboveground structures (Shah et al. 2008b; Berta et al. 1993).

The inclusion of AC (activated charcoal) introduced antagonistic dynamics by adsorbing bioactive compounds in leaf leachates, thereby neutralizing their growth-promoting effects. This was particularly evident in AC + AL1 and AC + AL2 treatments, where plant height and reproductive traits were reduced compared to AMF + leaf leachate combinations. However, AC also mitigated the auto-toxic effects of certain allelochemicals, as reflected in moderate improvements in some growth traits. This dual effect highlights the nuanced role of AC in shaping plant-soil interactions by modulating the availability of signalling and inhibitory compounds (Inderjit et al. 2005; Li et al. 2010).

Reproductive success, as indicated by floral bud production, was maximized under AMF + AL2 treatments. AMF facilitated resource allocation toward reproduction, while bioactive compounds in the leachates likely stimulated floral-inducing chemicals. The reduced reproductive performance in AC + AL2 treatments further emphasized the importance of bioactive compounds in driving the reproductive allocation of *A. cotula*.

This study presents a novel understanding of how AMF and leaf leachates interact to mediate the invasive success of *A. cotula*. While previous research has largely focused on root exudates in influencing AMF symbiosis (Cantor et al. 2011; Zhu et al. 2020), the findings here highlight the previously unexplored role of leaf leachates in enhancing AMF colonization and overall plant performance. These results suggest that *A. cotula* may exploit its own allelochemicals to promote AMF symbiosis, further driving its invasiveness.

At the same time, the observed auto-toxic effects of leaf leachates in the absence of AMF underscore the complexity of these interactions. Activated charcoal confirmed the role of allelochemicals as both growth stimulants and inhibitors, depending on their environmental context. This dual functionality may represent a unique adaptation that enables *A. cotula* to thrive in diverse ecosystems while suppressing potential competitors.

Conclusion

In conclusion, the study underscores the importance of synergistic, stimulatory, and antagonistic interactions among AMF and leaf leachates in shaping the invasive traits of *A. cotula*. The findings provide valuable insights into the complex interplay of biotic and abiotic factors that mediate plant-soil interactions, with significant implications for managing invasive species and promoting sustainable ecosystem management. Further research into the chemical composition of leaf leachates and their signalling pathways is warranted to deepen our understanding of these mechanisms.

Declarations

Supplementary information

Supplementary Tables (DOCX 46kb)

Conflict of interest

The authors declare that they do not have conflict of interest.

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Author Contribution

Afshana and ZAR conceived the idea and designed the study together with MAS and IR. Afshana did greenhouse trials, collected the data. Afshana and ZAR analysed the data. Afshana wrote the first draft of the manuscript. ZAR revised and corrected the manuscript.

Acknowledgement

Afshana and ZAR conceived the idea and designed the study together with MAS and IR. Afshana did greenhouse trials, collected the data. Afshana and ZAR analyzed the data. Afshana wrote the first draft of the manuscript. ZAR revised and corrected the manuscript.

Data availability

All data is provided in the form of tables or figures.

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Figures

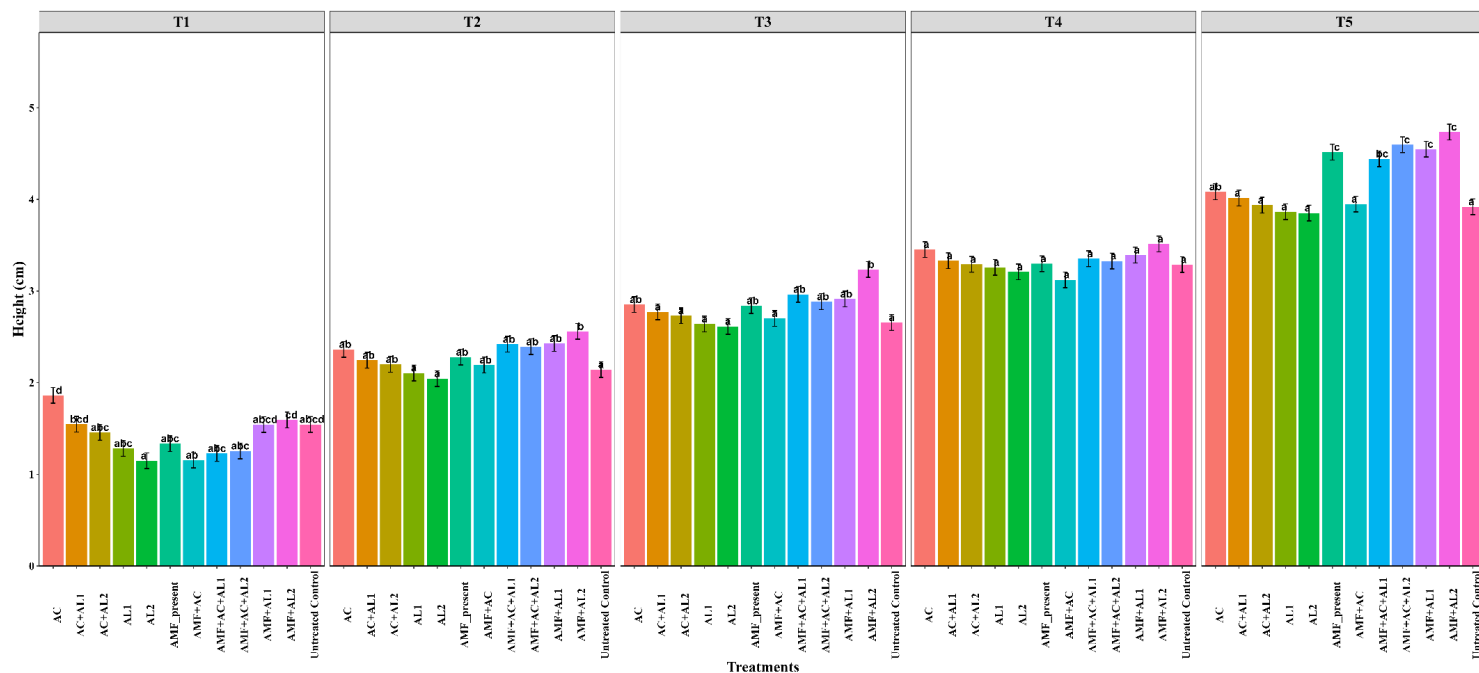


Figure 1

Estimated marginal means of height (cm) on the transformed scale for each treatment and time combination, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

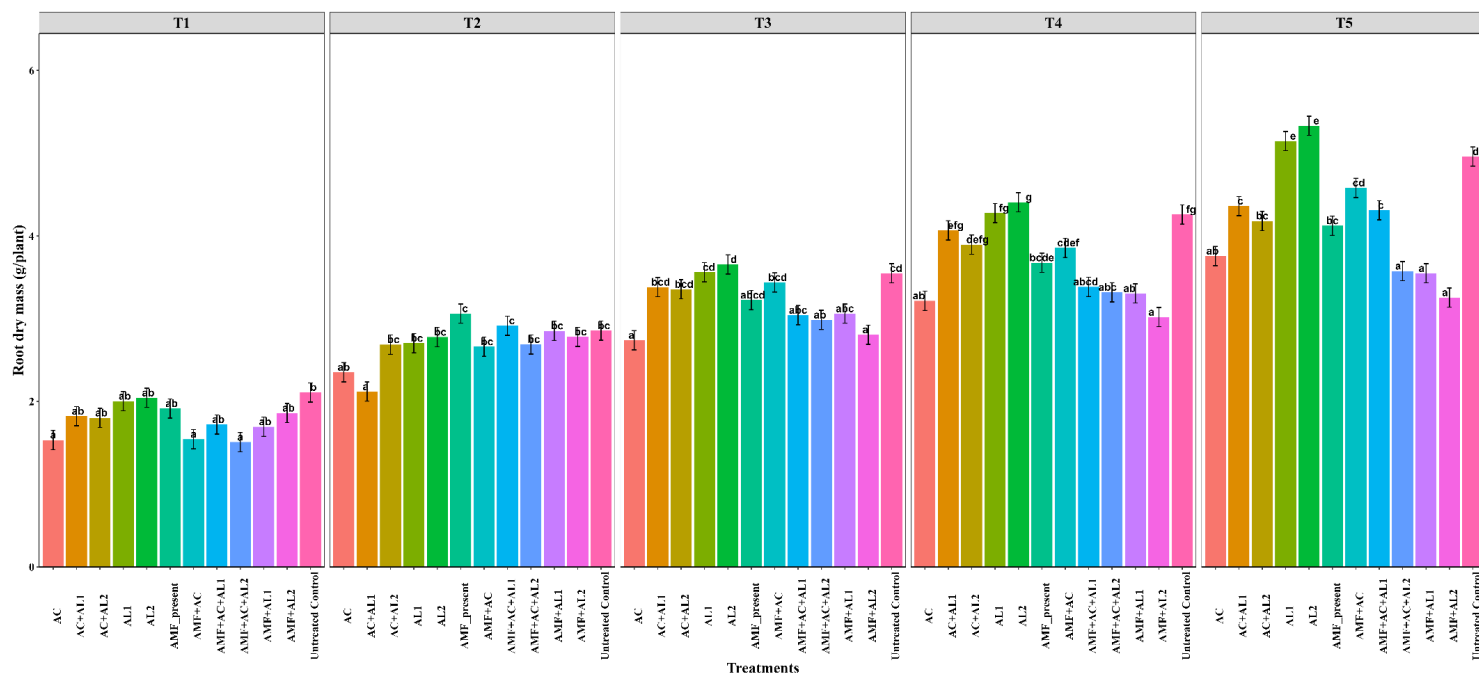


Figure 2

Estimated marginal means of root dry mass (g/plant) on the transformed scale for each treatment and time combination, based on a linear model. Error bars represent $\pm$ standard errors on the transformed

scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

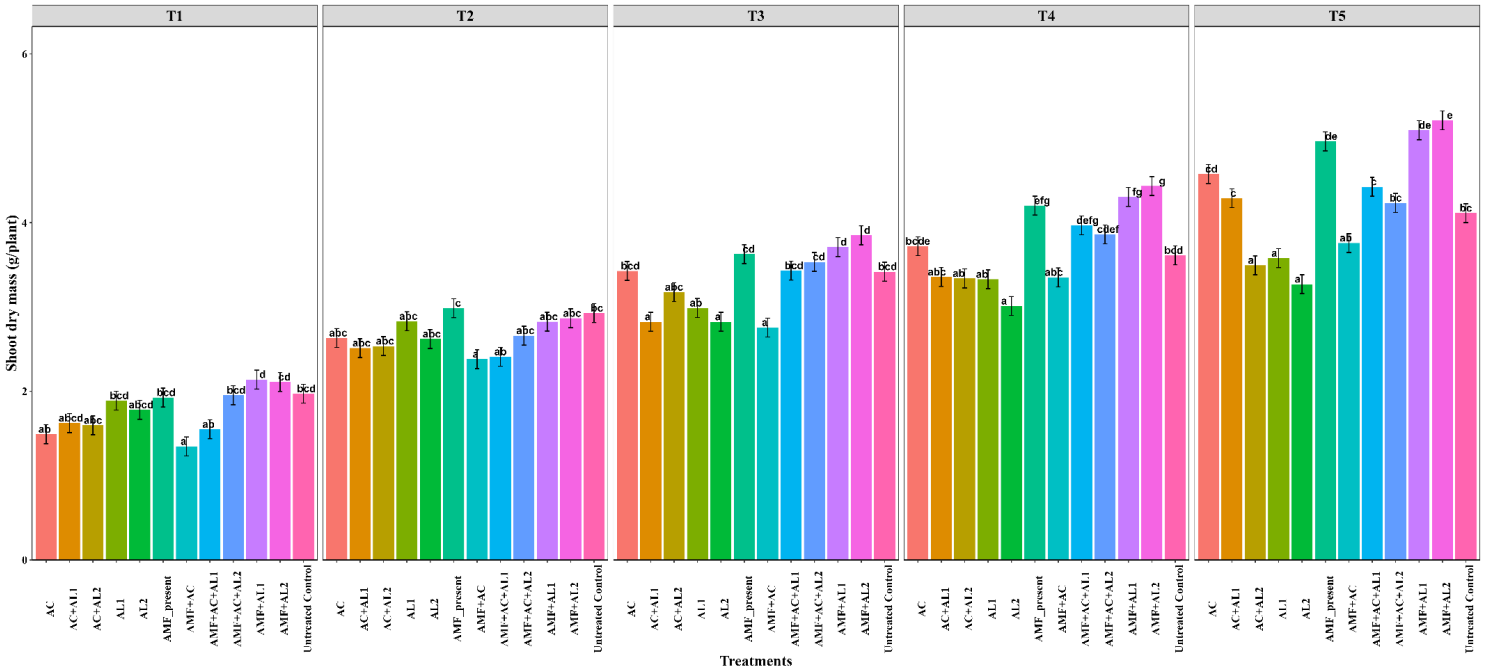


Figure 3

Estimated marginal means of shoot dry mass (g/plant) on the transformed scale for each treatment and time combination, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

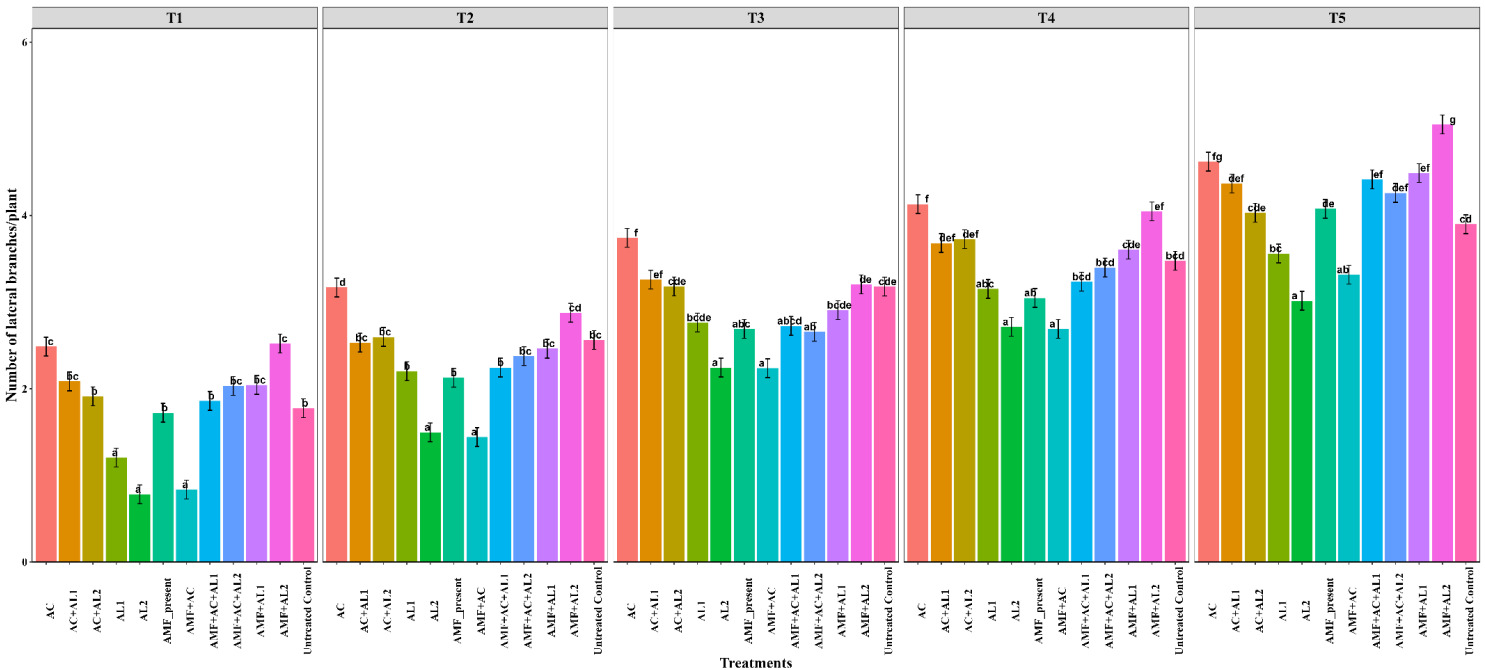


Figure 4

Estimated marginal means of number of lateral branches/plant on the transformed scale for each treatment and time combination, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

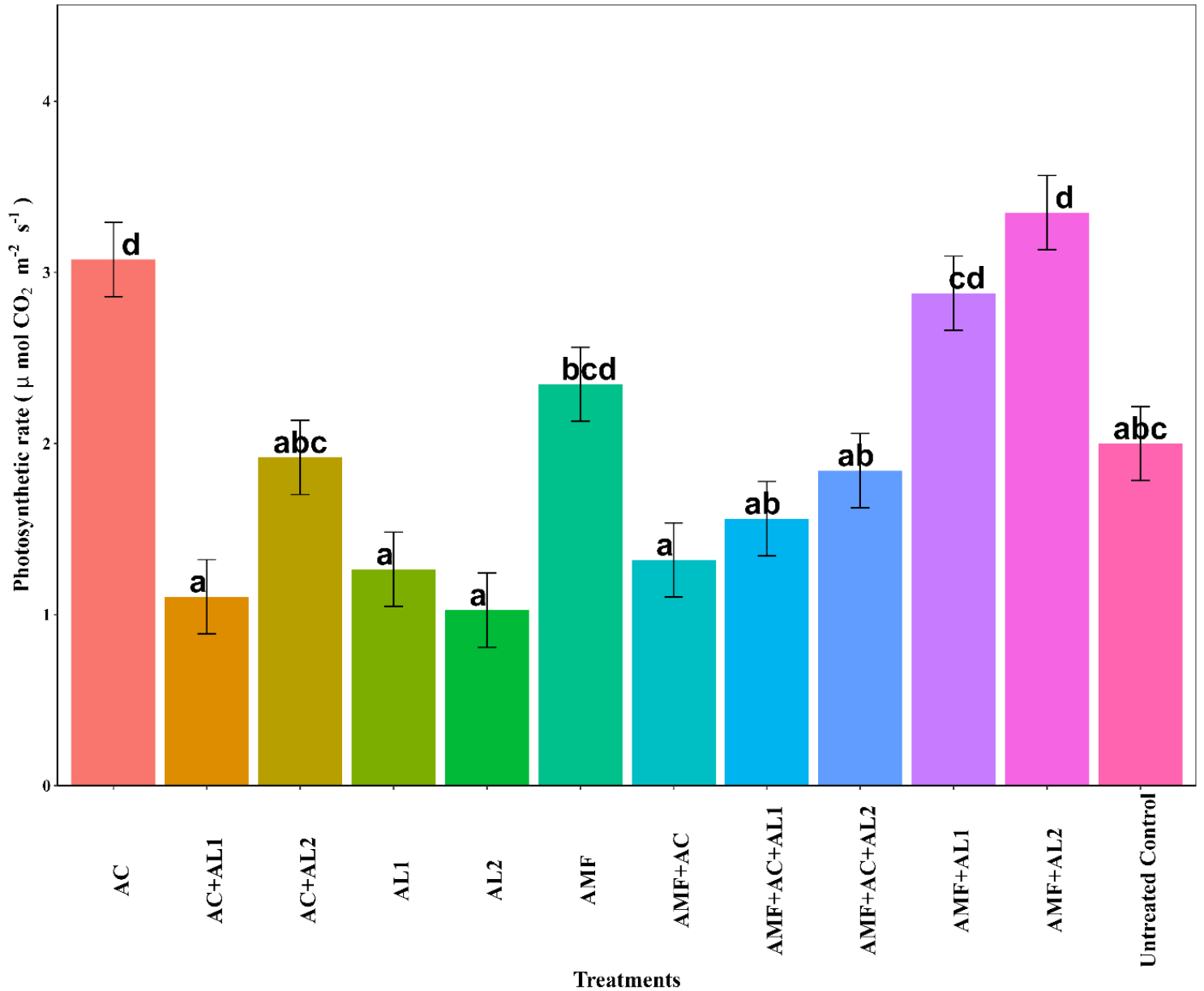


Figure 5

Estimated marginal means of photosynthesis rate ($\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$) on the transformed scale for each treatment, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

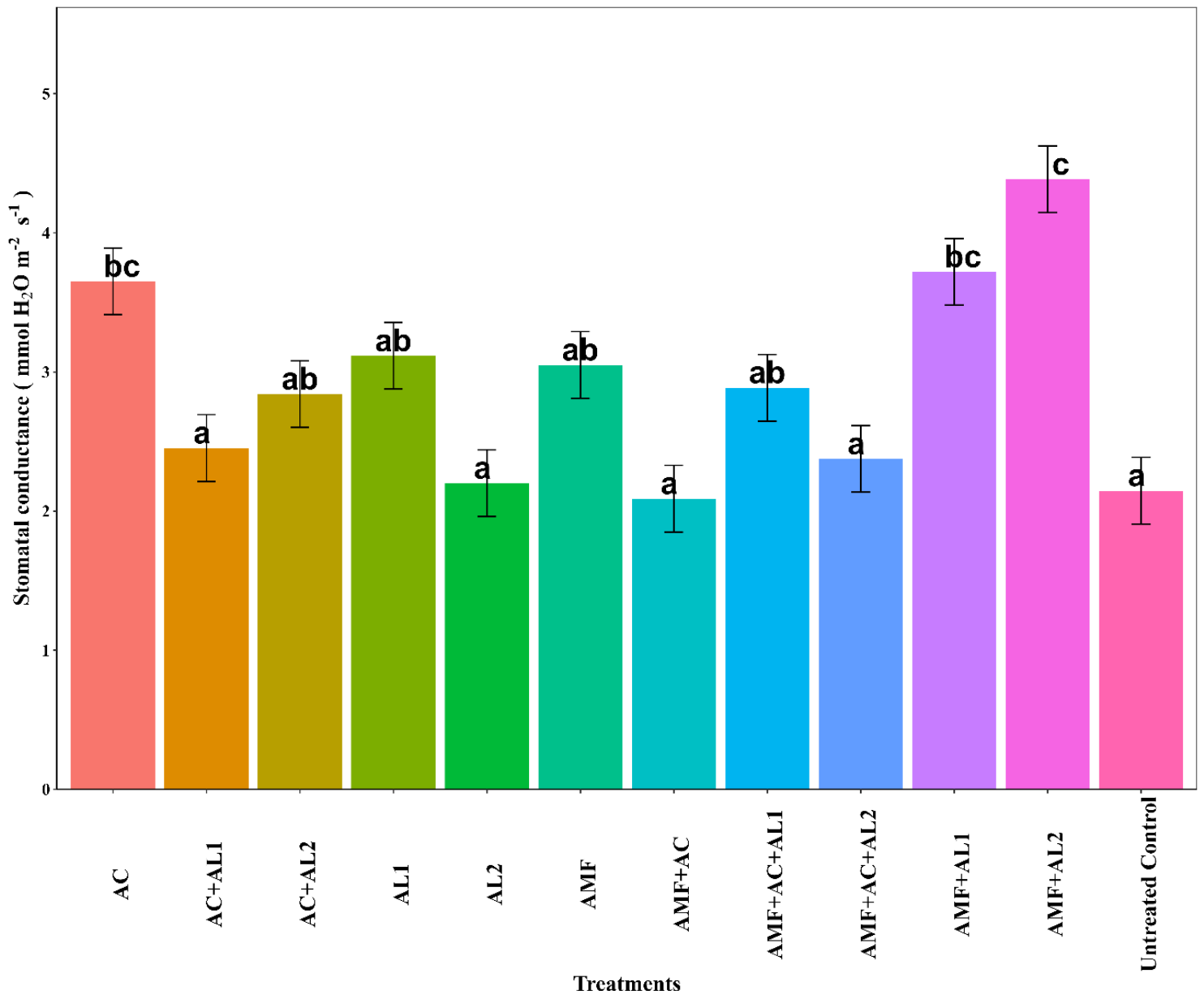


Figure 6

Estimated marginal means of stomatal conductance (mmol H₂O m⁻²s⁻¹) on the transformed scale for each treatment, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

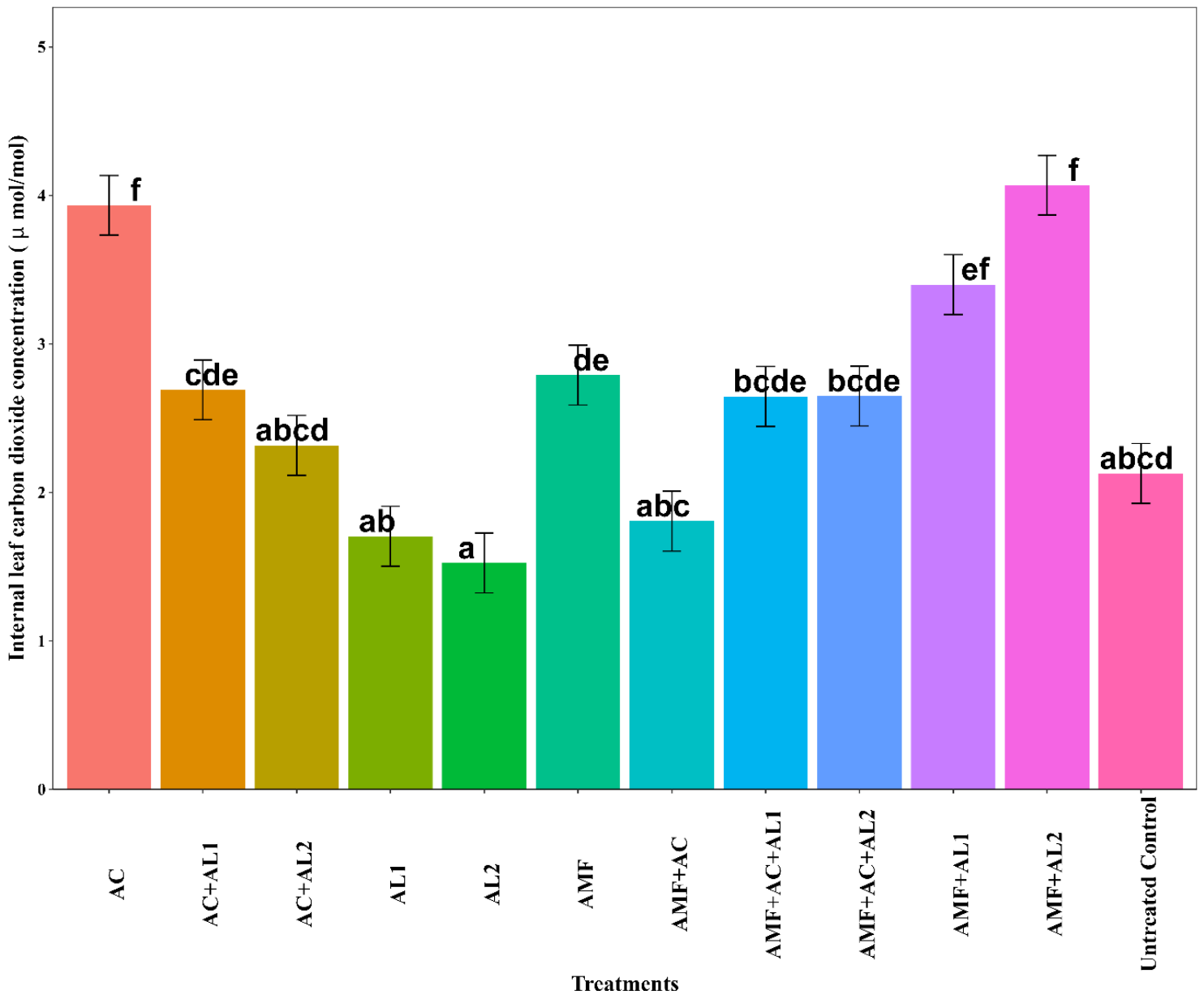


Figure 7

Estimated marginal means of Internal CO₂ concentration (μmol/mol) on the transformed scale for each treatment, based on a linear model. Error bars represent ± standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

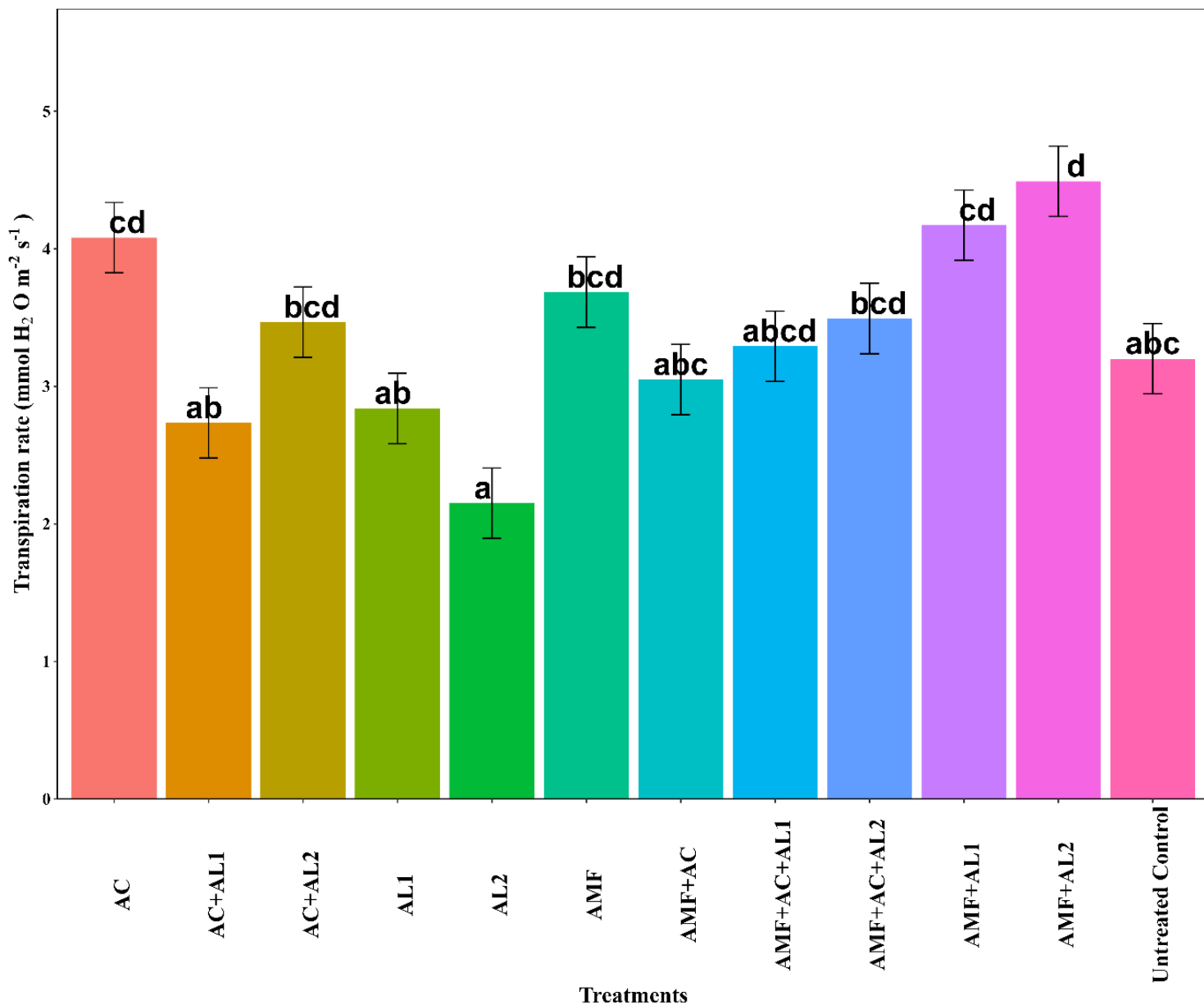


Figure 8

Estimated marginal means of Internal CO₂ concentration (μmol/mol) on the transformed scale for each treatment, based on a linear model. Error bars represent ± standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

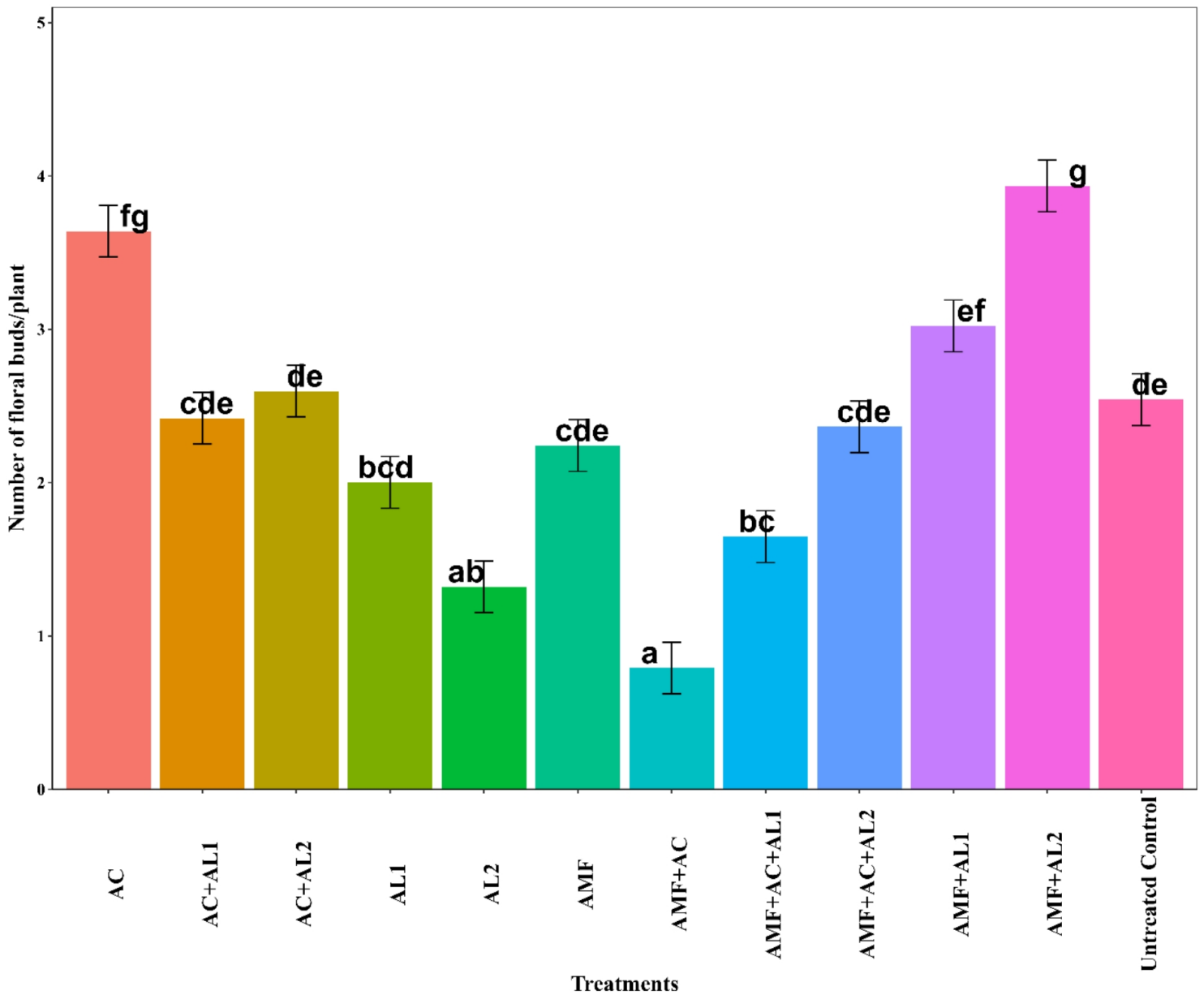


Figure 9

Estimated marginal means of number of floral buds/plant on the transformed scale for each treatment, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

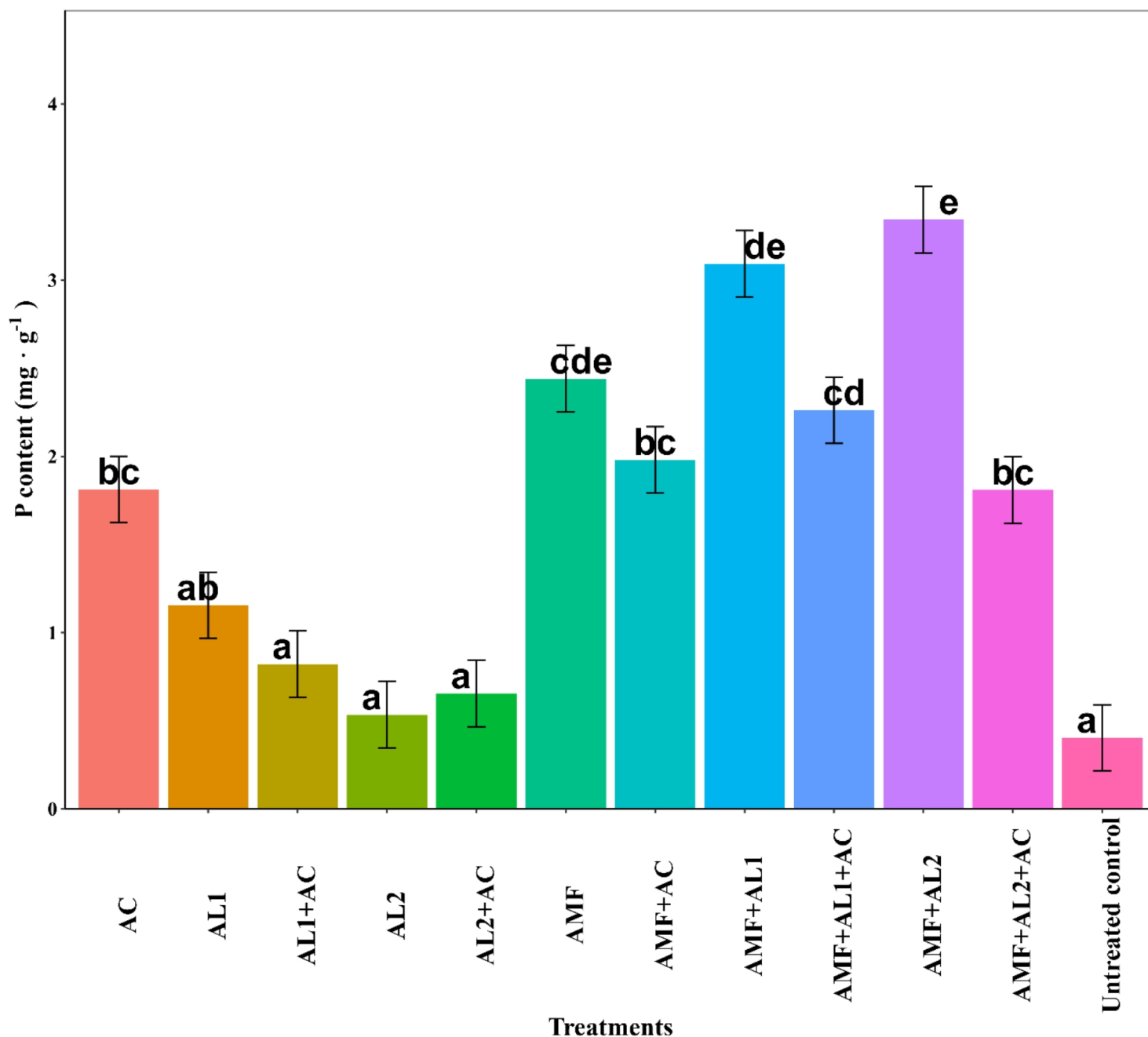


Figure 10

Estimated marginal means of P content (mg·g⁻¹) on the transformed scale for each treatment, based on a linear model. Error bars represent $\pm$ standard errors on the transformed scale. Compact letter display (CLD) letters indicate statistically significant differences between groups (Sidak-adjusted pairwise comparisons, $p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTableS1S3.docx](#)