

Greenhouse experiment with soil shows that eucalyptus does not interfere allelopathically on grasses, maize and soybean aiming an integrated crop-livestock-forest system

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Abstract

Integrated, livestock, and forest systems represent sustainable agricultural models. A critical aspect of these systems is the strategic combination of plant species that do not inhibit one another's growth through allelopathy. This study assessed the effects of leaf extracts from various eucalyptus (*Corymbia henryi*, *Eucalyptus camaldulensis*, *E. dun nii*, *E. exserta*, *E. globulus*, *E. grandis*, *E. pellita*, *E. resinifera*, *E. saligna*, and *E. urophylla*) on the seed germination and growth of *Urochloa brizantha* cv. Marandú, *Urochloa ruziziensis*, *Panicum maximum* cv. Mombasa, *Zea mays*, and *Glycine max*. These species are commonly used in integrated crop-livestock-forest systems. The impact of eucalyptus leaf extracts on the target species was initially evaluated in the laboratory by measuring primary root protrusion and total seedling length. A subsequent greenhouse experiment assessed the influence of these extracts on the growth of the target species through biometric and biochemical analyses. Results showed that eucalyptus extracts had a more significant effect on seed germination (when applied to germination paper) than when incorporated into the soil. Moreover, the extracts exhibited minimal interference with the growth of the target species, suggesting their compatibility for use in integrated agricultural systems. Notably, *E. globulus* extract enhanced soil enzyme activity, indicating increased microbial activity.

1. Introduction

The search of high agricultural yields is closely linked to the adoption of innovative technologies and the development of sustainable practices. Various strategies can be implemented to enhance production while minimizing risks to human health and the environment. These include Integrated crop-livestock systems (ICL), which are well-established in numerous Brazilian regions, utilizing practices such as crop rotation and succession (de Moraes et al. 2014)). The inclusion of trees in this framework leads to the integrated crop-livestock-forest system (ICLF), which allows for diverse combinations like crop-livestock, crop-forest, forest-livestock, or crop-livestock-forest systems.

The ICLF system offers numerous advantages, including increased production and income diversity for producers, improved efficiency in natural resource use, enhanced soil nutrient cycling, thermal comfort for livestock, and greater animal productivity (Farias et al. 2020; Sekaran et al. 2021)). Additionally, it promotes environmental sustainability through conservation practices (dos Reis et al. 2021)). However, ICLF systems are naturally more complex than grain crops, with the duration of the cycle primarily dependent on the tree component, thus meaning that the use of inadequate trees may penalize the system in the long term. Therefore, it is essential to implement the system correctly to avoid irreparable future management issues (Serra et al. 2019)).

One of the main challenges of integrated crop-livestock-forest (ICLF) systems is the acceptance of the forestry component, which is the least adopted compared to the other three components included in the ICLF system (Skorupa et al. 2021). Additionally, the limited use of trees in the system is related to the lack of knowledge of the farmers regarding forestry and the timber market, the long-term returns in face of the high initial investments and potential losses in the crops composing the system due to the

shading produced by tree species (Skorupa and Manzatto 2019; Skorupa et al. 2021)). Additionally, biomass inputs from foliage may carry allelopathic substances, depending on the species, which could affect the growth of other species in the ICLF system (Glatzle et al. 2021). Eucalyptus trees contain allelopathic substances that can inhibit the germination of forage seeds (Carvalho et al. 2015), which emphasizes the need for future studies on seed germination and seedling emergence (Bieluczyk et al. 2021)).

Forages from the genus *Urochloa* (commonly referred to as brachiaria) and *Panicum* are extensively utilized in ICLF (Baptistella et al. 2020)). Alongside these species, grain crops such as maize and soybeans are also integral components of integrated crop-livestock-forest ICLF systems (Gontijo Neto et al. 2018; Behling et al. 2023)).

Among the tree species utilized in ICLF system, those of the genus *Eucalyptus*, native to Australia and belonging to the Myrtaceae family, are particularly noteworthy. This family includes over 800 species found across tropical and subtropical regions (Salehi et al. 2019) and countries like India, Brazil, China, and Australia (Li et al. 2014)). The wide variety of eucalyptus species enhances their adaptability to diverse environmental conditions (Moura et al. 2010), allowing their use for multiple purposes. These include the production of paper, cellulose, firewood, charcoal, timber, essential oils, ornamental applications, and windbreaks.

Despite the potential of eucalyptus in ICLF, there is still a debate on the allelopathic effect they can have on the other crops of the system (Espinosa-García et al. 2008)). Allelopathy is considered a significant factor contributing to the low biodiversity of plants under the canopy of eucalyptus plantations (Degefu et al. 2023). Allelopathy refers to the effects that one plant species can have on another, which can be either positive or negative (Cheng and Cheng 2015; Shan et al. 2023). The chemical compounds released by organisms, termed allelopathic substances, phytotoxins, or allelochemicals, are primarily non-nutritive and are produced mainly through the secondary metabolism of plants or as a result of microbial decomposition (Cheng and Cheng 2015). These allelochemicals can be released through various mechanisms, including the decomposition of leaves and plant materials, the exudation of metabolites from roots, leaching, and volatilization. They can significantly interfere with vital plant functions such as nutrient absorption, growth regulation, photosynthesis, respiration, membrane permeability, and enzymatic activity (Hoque et al. 2003; Inderjit 2005; Uddin et al. 2007; Abhilasha et al. 2008; Sun and He 2010; Yuan et al. 2013; Ahmed et al. 2018; Benchaa et al. 2018; Grichi et al. 2018; Puig et al. 2019). Among these, phenolic compounds - particularly phenolic acids and flavones - are significant allelochemicals that tend to leach into the soil due to their high-water solubility (Blum 2014; Segesso et al. 2019).

Previous studies have shown that eucalyptus leaf extracts may interfere in the germination of several species (Espinosa-García et al. 2008; Zhang and Fu 2009; Fikreyesus et al. 2011; Gurmu 2015)). Because of that there is still resistance by farmers to include eucalyptus in ICLF systems. Thus, understanding

dynamics of allelopathy is crucial for optimizing the integration of eucalyptus with forage and grain species in ICLF systems, as this can enhance biodiversity and promote agricultural sustainability.

This study aimed to evaluate the effects of leaf extracts from various eucalyptus species on the seed germination and growth of *Urochloa brizantha* cv. Marandú, *Urochloa ruziziensis*, *Panicum maximum* cv. Mombasa, *Zea mays*, and *Glycine max*, growing in soil. By examining these interactions, we obtained results that allowed us to conclude that eucalyptus does not interfere with maize, soybean and grasses in ICLF systems.

2. Material and methods

Leaves of the following eucalyptus were used to produce the extracts: *Corymbia henryi* (HEN – previously included in the *Eucalyptus* genus - (Parra-O. et al. 2009; Healey et al. 2021), *Eucalyptus camadulensis* (CAM), *E. dunnii* (DUN), *E. exserta* (EXS), *E. globulus* (GLO), *E. grandis* (GRA), *E. pellita* (PEL), *E. resiniferous* (RES), *E. saligna* (SAL) and *E. urophylla* (URO). The allelopathic effect was studied on the germination and growth of *Urochloa brizantha* cv. Marandu (Marandu grass), *Urochloa ruziziensis* (brachiaria ruziziensis), *Panicum maximum* cv. Mombaça (Mombaça grass), *Zea mays* L. (maize) and *Glycine Max* (L.) Merrill cv. 57H0123 IPRO (soybean). These species will be further referred to as target species.

2.1. Eucalyptus leaf collection and extracts preparation

Leaves of the ten eucalyptus species were collected at the Experimental Station of Forest Sciences of University of São Paulo in the municipality of Itatinga-SP (Lat.23°10' S Long. 48°40' W, altitude of 850 meters, Cwa (Koeppen) climate, average annual temperature of 20°C and average annual rainfall of 1350 mm. The age of the trees from which leaves were collected was 24 years (*E. camaldulensis*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. pellita*, *E. resinifera*, *E. saligna*, *E. urophylla*), 23 years (*E. exserta*) and eight years (*Corymbia henryi*). The leaves were collected in liquid nitrogen and transported to the laboratory, where they were freeze-dried, finely ground in a Wiley mill and then stored in a desiccator in the refrigerator until use. Ground dried eucalyptus leaves were used to prepare aqueous extracts at three concentrations: 0.5 g, 1.0 g and 2.0 g were added to 200 ml of water, resulting in solutions with concentrations of 0.25% (dose 1), 0.5% (dose 2) and 1% (dose 3). The mixtures were homogenized on an orbital shaker table at 100 rpm for 60 min, at 23°C, and filtered on ordinary filter paper. The extracts were prepared just before use.

2.2. Preliminary evaluations of forage seeds

The seed of the forages Marandu grass, *Brachiaria ruziziensis* and Mombaça grass may present dormancy or variation in germination capacity. Thus, a purity test was carried out on seed lots following the Rules for Seed Analysis (MAPA 2009), where the percentage composition by weight and, the identity of the different seed species and the inert material of the seed lot could be determined.

As these species can present dormancy, the following tests were carried out in transparent plastic boxes with blotting paper:

- a) Breaking dormancy of seeds with H_2SO_4 + 2% KNO_3 solution in an amount equivalent to 2.5 times the mass of the dry paper.
- b) Breaking dormancy of seeds with H_2SO_4 + water in an amount equivalent to 2.5 times the mass of dry paper.
- c) Seeds without dormancy break + water equal to 2.5 times the mass of dry paper.

In the three situations, the seeds were set to germinate in ideal conditions for the species, with a count of seven and 21 days for *Urochloa* and 10 and 28 days for *Panicum* (MAPA 2009). It was possible to observe dormancy only in the seeds of Marandu grass and *Brachiaria ruziziensis*, and the treatment of H_2SO_4 without the KNO_3 solution on paper promoted the same result compared to the treatment with the addition of KNO_3 . Thus, the dormancy breaking of Marandu grass and *Brachiaria ruziziensis* seeds was performed only with H_2SO_4 moments before they were used in the tests, whether in the laboratory or greenhouse. The germination was tested in the seeds of the five target species through germination and seedling emergence evaluations in sand.

2.3. Evaluation of the effect of eucalyptus leaf extracts on the seeds of target species

The allelopathic effects of eucalyptus leaf extracts were evaluated on primary root protrusion and seed vigour of the target plants in laboratory tests. The evaluations were carried out in transparent plastic boxes (11 x 11 x 3.5 cm) for the forage species (*Urochloa* and *Panicum*), with 50 seeds distributed on blotting paper. For maize and soybean, the evaluations were made on paper towel rolls previously moistened with a quantity of solutions equivalent to 2.5 times the weight of the dry paper. The boxes and rolls were wrapped in plastic bags to prevent water loss and kept at the ideal temperature for the germination of each species (MAPA 2009). After sowing, primary root protrusion evaluations were performed for three (maize and soybean) and seven days (*Urochloa* and *Panicum*).

A computerized analysis of seedlings was performed to evaluate the total length of seedlings using the Vigor-S (*soybean*) and SVIS® (maize, *Urochloa* and *Panicum*) softwares. Five replicates of 20 seeds per species were used, distributed in two rows in the upper third on two sheets of paper towels and covered with a third sheet. The substrate was previously moistened with water (control) or extract (doses 2 and 3), equivalent to 2.5 times its dry mass. The rolls containing the seeds were kept in a germination chamber at the ideal temperature for each species for three (maize and soybean) and six (*Urochloa* and *Panicum*) days. At the end of this period, the seedlings of each replication were transferred from the roll of paper towels to a sheet of blue E.V.A (ethyl vinyl acetate) with dimensions of 30 cm x 22 cm, corresponding to the size of the scanner's useful area, to provide the necessary contrast for analysis by the system. Next, the seedling images were digitized in the HP Scanjet 200 scanner, installed in an

inverted position inside an aluminum box (60 x 50 x 12 cm), adjusted at a resolution of 100 dpi (SVIS)® and 300 dpi (Vigor-S®) and coupled to a computer. Indices of vigour, uniformity of development and seedling length were obtained.

2.3. Evaluation of the effect of eucalyptus leaf extracts on the growth of target species in a greenhouse

Knowing the results of root protrusion and initial seedling growth obtained in the laboratory, some extracts were selected for further study in the greenhouse. Before sowing the seeds of the target species, the eucalyptus extracts were applied once a week and for seven weeks on the substrate in 8 L pots. The substrate was a mixture of soil, vermiculite and sand (1:1:2, v/v/v), which was kept moist using a drip system during this period. In each application, 50 ml of 1% extract was used. The irrigation drip system applied 100 ml of water per day, which was sufficient to keep the substrate moist and to prevent drainage, which could remove the applied extracts. The substrate used in the cultivation of the plants was sent for analysis in a private laboratory (Table S1).

Four seeds were sown per pot, but after thinning to keep two seedlings per pot. Soybean seeds were inoculated before sowing with Rizokop® (*Bradyrhizobium japonicum* strains SEMIA 5079 and 5080) at a dose of 300 ml 50 kg⁻¹ of seed. The plants were irrigated by drip, with 0.5 L/day divided into 5 irrigations of 0.1 L.

At 40 days after sowing (DAS) a fluorimeter (FluorPen, model P100) was used to evaluate the extracts' effect on the plant photosystems' quantum yield. Indirect chlorophyll concentration, epidermal flavonoid and anthocyanin index, and nitrogen balance index (NBI) were also measured using the DUALEX (Force-A) equipment.

The plants of Marandu grass, *Brachiaria ruziziensis*, Mombaça grass and maize were kept in the pots until 49 DAS, and the soybean plants were kept in the pots until they complete seed maturation (126 DAS) to evaluate the yield components (number of pods, mass and number of grains). In all target species data were obtained on plant emergence speed, tiller count in forages, plant height, root length, and dry matter mass (shoot and root).

The youngest fully expanded leaves of all target species were collected at 47 DAS for determination of peroxidase and polyphenol oxidase activities (Oliveira 1972; Draetta and Lima 1976), total proteins and soluble proteins (Bradford 1976), total soluble sugars (Dubois et al. 1956), starch (Yemm and Willis 1954), total amino acids (Yemm et al. 1955), total soluble phenols (Swain and Hillis 1959) and nitrate (Cataldo et al. 1975).

The determination and quantification of the nutrients of the leaves of the target species were performed by an energy-dispersive X-ray fluorescence spectrometer (EDX – 720) of the Shimadzu brand. The dried, ground and sieved samples in a 100-mesh stainless steel sieve (150 µm) were packed in cuvettes covered with a 5 µm thick PP film (No. 3520, Spex Ind. Inc.), and subsequently subjected to vacuum. The X-ray beam was generated by an Rh anode operating at 40 kV and 150 µA. The beam was focused using a 1 mm collimator, and the fluorescence photons were detected by an SDD detector with an acquisition

time of 200 seconds. The analyses were performed under vacuum, and the dead time of the detector was kept at less than 1% (Tezotto et al. 2013).

2.5. Soil analysis

At the end of the experiment in the greenhouse, soil samples were collected from the substrate and sent to a private laboratory for chemical analysis and to evaluate the activities of the enzymes β -glucosidase and acid phosphatase (Tabatabai 1994).

2.6. Statistical analysis

The experiments were conducted using a completely randomized design. In the laboratory phase, the doses of each of the ten eucalyptus extracts applied to each of the five target species were compared. Thus, four treatments (control and doses of 0.25%, 0.5%, 1.0% of extracts) and four replicates were established. In the greenhouse experiment, the comparison was carried out with some extracts and applied at 1.0% concentration in each target species. Thus, five treatments were defined (CONTROL, CAMALDULENSIS, GLOBULUS, HENRYI and UROPHYLLA), each with five replications. The data were submitted to the Shapiro-Wilk residual normality test and the homogeneity of variances test by the Bartlett test, and the data underwent transformation for the variables that did not meet the assumptions of the tests. Subsequently, an Analysis of Variance (ANOVA) was performed, and the means were compared by Tukey's test ($p \leq 0.05$). All analyses were performed with the R v.1.4.1106 software.

3. Results

3.1. Preliminary assessments of the seed species used

Laboratory tests were carried out to evaluate the quality of the seeds of the five target species. For the forage species, the purity test showed 73.5% of pure seeds in Marandu grass, 87.7% in Brachiaria ruziziensis and 82.4% in Mombaça grass, and in the three species, the inert material was composed of plant remains, straw, stone and empty seeds. In all subsequent tests, pure seeds were used.

Regarding seed viability, the germination percentage initially was 91% for Marandu grass, 92% for Brachiaria ruziziensis, 81% for Mombaça grass, 100% for maize and 97% for soybean. Regarding seed vigour, the percentage of seedling emergence of the seed lots used was 73% for Marandu grass, 78% for Brachiaria ruziziensis, 68% for Mombaça grass, 100% for maize and 96% for soybean.

3.2. Primary root protrusion and total seedling length

In laboratory tests it was observed a significant influence of some eucalyptus extracts on the protrusion of the primary root, of RESINIFERA in Marandu grass, PELLITA in Brachiaria ruziziensis, GRANDIS in maize and CAMALDULENSIS, DUNNII, GLOBULUS, HENRYI, PELLITA, SALIGNA and UROPHYLLA in soybean. For the total seedling length obtained by the Vigor-S and SVIS® programs, significant differences were observed for Marandu grass with the application of UROPHYLLA extract, for Mombaça grass with HENRYI extract, for maize with DUNNII, GLOBULUS and UROPHYLLA extracts, and soybean

with HENRYI extract. Table 1 presents the results of the two tests mentioned above, comparing each dose applied to the control treatment since this phase foresaw the selection of extracts and doses for the assay in the oven.

From these results, it was defined that a dose of 1% of the extracts of CAMALDULENSIS, GLOBULUS, HENRYI and UROPHYLLA would be used for the greenhouse assay.

Table 1. Primary root (A) and seedling (B) length protrusion tests, which compared the eucalyptus extracts treatment (dose) to control treatment, in each extract.

Extract	Dose (%)	Marandu grass		Brachiaria Ruziziensis		Mombaça grass		Maize		Soybean	
		A	B	A	B	A	B	A	B	A	B
UROPHYLLA	0		ab					ab		a	
	0.25		-		-		-	-		ab	-
	0.5		a					a		ab	
	1.0		b					b		b	
PELLITA	0			A						b	
	0.25		-	B			-	-		a	-
	0.5			B						ab	
	1.0			ab						b	
SALIGNA	0									ab	
	0.25		-		-		-	-		ab	-
	0.5									b	
	1.0									a	
GLOBULUS	0							ab		a	
	0.25		-		-		-	-		ab	-
	0.5							b		a	
	1.0							a		b	
HENRY	0					ab				b	a
	0.25		-		-	-		-		ab	-
	0.5					a				a	b
	1.0					b				a	b
CAMALDULENSIS	0									a	
	0.25		-		-		-	-		a	-
	0.5									a	
	1.0									b	
EXSERTA	0										
	0.25		-		-		-	-			-
	0.5										
	1.0										
DUNNII	0							ab		b	
	0.25		-		-		-	-		ab	-
	0.5							a		ab	
	1.0							b		a	
RESINÍFERA	0		a								
	0.25		b		-		-	-			-
	0.5		ab								
	1.0		a								
GRANDIS	0							ab			
	0.25		-		-		-	a			-
	0.5							b			
	1.0							ab			

Values followed by the same letter along the column are not significantly different ($p < 0.05$) from each other. (Red) smaller than the control; (Yellow) does not differ from control; (Green) greater than control; (-) has not been tested.

3.3. The effect of eucalyptus leaf extracts on the development of target species cultivated in a greenhouse

Marandu grass

Compared to the CONTROL treatment, the application of CAMALDULENSIS and GLOBULUS extracts increased the shoot dry matter of Marandu grass (Fig. 1). Nitrate concentration in the leaves was higher in the treatments with HENRYI and CAMALDULENSIS extracts (Fig. 1B). As for the enzymes peroxidase and polyphenol oxidase (Figs. 1C and 1D), lower activity was observed in the treatments CAMALDULENSIS, HENRYI and GLOBULUS extracts compared to CONTROL. Higher activity of acid phosphatase (Fig. 1E) was observed in the GLOBULUS and CAMALDULENSIS treatments, which differed from the CONTROL. Other analyses did not show statistical differences between the extract treatments and control plants (Supplementary Figs. S1, S2 and S3 and supplementary Tables S2, S3 and S4).

Brachiaria ruziziensis

Compared to the CONTROL treatment, extracts from HENRYI and CAMALDULENSIS applied to brachiaria ruziziensis led to a decrease in the leaf concentration of Ca and P, respectively (Figs. 2A and 2B). CAMALDULENSIS increased the activity of the leaf peroxidase (Fig. 2C) and GLOBULUS increased the activity of the soil acid phosphatase (Fig. 2D). The other biometric and biochemical evaluations did not present significant differences (Supplementary Tables S5, S6 and S7 and Supplementary Figs. S4, S5, S6 and S7).

Mombaça Grass

Compared to the CONTROL treatment, GLOBULUS increased chlorophyll (Fig. 3B) and nitrogen balance index (Fig. 3C) but decreased anthocyanin (Fig. 3C). α -glucosidase was increased by UROPHYLLA (Fig. 4E). The other results did not differ significantly (Supplementary Table S8 and Supplementary Figs. S8, S9 and S10).

Maize

Compared to the CONTROL plants, all four eucalyptus extracts increased flavonoids in maize (Fig. 4B) and the contrary was observed with nitrogen index balance (Fig. 4C). UROPHYLLA extract led to an increase in leaf peroxidase activity (Fig. 4D) and GLOBULUS an increase in soil α -glucosidase activity (Fig. 4F). The other analyses carried out did not show significant differences among treatments (Supplementary Tables S9 and S10 and Supplementary Figs. S11, S12 and S13).

Still in maize, compared to the CONTROL treatment, P leaf concentration decreased with GLOBULUS extract (Fig. 5A). Soluble protein was decreased with UROPHYLLA (Fig. 5C) and amino acids were increased with CAMALDULENSIS extract (Fig. 5F). Nitrate was also increased with UROPHYLLA (Fig. 5E).

Soybean

Compared to the CONTROL plants, CAMALDULENSIS extract induced an increase of leaf (Fig. 6A) and together HENRYI and GLOBULUS extracts a reduction leaf soluble phenolics (Fig. 6C). UROPHYLLA, HENRYI and GLOBULUS extracts increase total protein content in soybean leaves (Fig. 6D). While UROPHYLLA increased starch, HENRYI and GLOBULUS decreased soluble sugars (Fig. 6F). All

eucalyptus extracts increased soil acid phosphatase activity (Fig. 6G). Data without statistical differences can be found in Supplementary Table S11 and Supplementary Figs. S14, S15, S16 and S17).

4. Discussion

The germination experiment revealed varying responses to eucalyptus extracts applied to seeds. Notably, extracts from the leaves of *Eucalyptus camaldulensis*, *Eucalyptus globulus*, *Corymbia henryi*, and *Eucalyptus urophylla* exhibited negative effects on the rate of primary root protrusion. *E. camaldulensis*, *E. globulus*, and *E. urophylla* are among the species most extensively studied in relation to allelopathy (Zhang et al. 2022). Previous studies have also documented the negative impacts of eucalyptus extracts on the seed germination of various species (Yamagushi et al. 2011; Carvalho et al. 2015).

However, it is important to note that findings regarding seed germination may not fully reflect the dynamics occurring in soil environments. This prompted us to conduct a second experiment in a greenhouse setting, but using soil. Allelopathic substances tend to have a greater impact on seedling development than on seed germination, with root necrosis being a common symptom (Carvalho et al. 2015). It has been also noted that hormesis may be observed, characterized by toxic effects at high concentrations and stimulatory effects at lower doses (Carvalho et al. 2015). For instance, when evaluating the impact of *E. urograndis* extract on *Urochloa decumbens* and *Panicum maximum* seeds, it was found a decrease in the germination velocity index, an increase in the percentage of abnormal seedlings, and shorter shoot lengths at higher concentrations. Conversely, at lower concentrations, the shoot length was greater compared to the control, while all doses negatively affected root development.

Among the results obtained, the enzyme activity in the soil, specifically α -glucosidase and acid phosphatase, was particularly notable. Except for acid phosphatase activity in Mombaça grass, the other measurements indicated significantly higher enzyme activity in treatments with eucalyptus extracts, especially with GLOBULUS extract, which consistently differed from the control across all evaluated species. These two extracellular enzymes serve as bioindicators of soil quality: acid phosphatase is produced by plant roots, while α -glucosidase and acid phosphatase are produced by soil microorganisms. The activity of these enzymes, along with the chemical and physical properties of the soil, can influence the development of cultivated plants and the availability of nutrients (Neemisha and Sharma 2022)). α -glucosidase hydrolyzes low molecular weight carbohydrates from soil organic matter to produce glucose, an essential carbon source for soil microorganisms; meanwhile, acid phosphatase catalyzes the hydrolysis of phosphate esters in organic substrates containing phosphorus, thus making inorganic phosphorus available to plants and soil biota in the form of orthophosphates (Chen et al. 2013; Dotaniya et al. 2019)). The application of eucalyptus extracts in the soil could have influenced acid phosphatase activity through several mechanisms. Firstly, these extracts may have stimulated the growth and activity of specific soil microorganisms that produce these enzymes. Additionally, the presence of the extracts might have altered the pH of the soil, which can enhance microbial and,

subsequently, enzymatic activity. It is noteworthy that the initial pH of the substrates was around 4.8, a condition (Table S1) that could have favored acid phosphatase activity.

Moreover, eucalyptus extracts may facilitate beneficial interactions between plants and microorganisms, potentially increasing soil enzymatic activity as part of a plant defense response, or due to the release of root exudates that enhance microbial activity. The distinct enzyme activity observed with the GLOBULUS extract may be attributed to specific constituents or a synergistic effect of multiple compounds present in the extract. These interactions underscore the complexity of soil ecology and the role that allelopathic substances can play in regulating microbial dynamics and enzyme production in agricultural systems.

To a comprehensive understanding of enzymatic and microbial activity within integrated crop-livestock-forest (ICLF) systems, analyses should be conducted over various time periods, as numerous factors can influence soil-plant dynamics. For instance, it has been found that the allelopathic effect of eucalyptus on *Brassica chinensis* was more pronounced in sterile soils, suggesting that soil microorganisms mitigate the negative impacts of Eucalyptus on plant development (Lu et al. 2017).

The interactions among all components present in the ICLF system - including plant species, the types and quantities of substances released into the soil, the diversity and abundance of microorganisms, and the overall soil environment - create a complex web of allelopathic interactions that remain poorly understood. Given this complexity, integrating eucalyptus with other forages or crops may serve as a viable strategy for enhancing soil health and improving overall productivity. This approach can leverage the potential benefits of allelopathy while minimizing adverse effects, ultimately contributing to more sustainable agricultural practices.

The plant enzymes peroxidase and polyphenol oxidase are involved in induced defense mechanisms against pathogens, or in conditions of stress of plants, acting as enzymatic antioxidants (Duroux and Welinder 2003; Araji et al. 2014; Sullivan 2015; Pandey et al. 2017; Zhang 2023). Peroxidases also have other physiological functions such as lignification, suberization, auxin metabolism, protein assembly in the cell wall, oxidative stress response, and defence against pathogens (Pandey et al. 2017). Polyphenol oxidase is an oxide-reductase enzyme that participates in the production of phenolic compounds, which are precursors in the synthesis of lignin, strengthening plant walls and playing a crucial role in the defence response of plants (Araji et al. 2014).

The activity of peroxidase and polyphenol oxidase observed in Marandú grass treated with CAMALDULENSIS, HENRYI, and GLBULUS extracts, coupled with the observed increase in shoot dry mass, suggests that while the application of eucalyptus extracts may enhance the growth performance of the plants, it could potentially compromise their natural resistance to pathogens and environmental stresses. This phenomenon indicates a possible trade-off where enhanced growth may come at the expense of the plant's defense mechanisms. To validate these initial findings, further research is necessary, specifically designed to evaluate the plants' responses under various stress conditions and pathogen exposure. Such studies will provide deeper insights into the implications of eucalyptus extract

application on plant health and resilience, ensuring a comprehensive understanding of its effects within the integrated crop-livestock-forest systems.

In case of Mombaça grass, the observed lower quantum yield of photosystem II and higher concentration of soluble phenols in response to CAMALDULENSIS treatment suggest a potential stress response in the plants. A decreased quantum yield of photosystem II is indicative of photoinhibition, which can be a consequence of allelochemical influence (Zhou and Yu 2003)). Conversely, treatment with GLOBULUS was associated with improved photosynthetic efficiency, reflected by a higher chlorophyll index, which correlates with better energy absorption and emission, along with an elevated nitrogen balance index and enhanced quantum yield.

When examining treatments applied to maize, differences indicative of stress response or activation of resistance mechanisms were evident. Specifically, the activation of peroxidase and polyphenol oxidase enzymes in the Eucalyptus urophylla treatment coincided with lower levels of both total and soluble proteins, as well as amino acids, alongside a significant accumulation of flavonoids. This reduction in protein and amino acid concentrations aligns with the elevated levels of nitrate in the UROPHYLLA treatment, suggesting potential negative effects on nitrogen assimilation mechanisms. Under stress conditions, plants often exhibit a lower protein demand for growth, leading to greater allocation of phenylalanine for the synthesis of protective polyphenols, including flavonoids, which serve varied protective roles in plants (Mierziak et al. 2014; Ramarosan et al. 2022)).

It has been demonstrated a reduction in protein and chlorophyll concentrations in lettuce leaves treated with *E. globulus* extract (Puig et al. 2018). This suggests a possible early stress response to phytotoxicity that may not immediately affect overall plant development but inhibits the synthesis of these essential compounds in the leaves. Conversely, the authors noted that when the eucalyptus extract was applied via foliar spraying, there were no changes in protein and chlorophyll levels; however, significant inhibition of plant growth and root biomass was observed, indicating a systemic effect. The conclusion drawn by Puig et al. (Puig et al. 2018) highlighted that the modes of action of phytotoxins can vary based on the entry point into the plant, leading to different impacts on morphological and physiological parameters. This observation aligns with the results of the present study, where applying eucalyptus extracts to the soil resulted in more pronounced physiological interferences, while biometric evaluations yielded relatively few significant findings. This reinforces the idea that the method of application plays a critical role in determining the effects of allelochemicals on plant development and highlights the complexity of interactions within integrated crop-livestock-forest systems.

For soybeans, the application of GLOBULUS extract resulted in higher pod yield per plant compared to the CONTROL group, an outcome typically indicative of a healthy plant that effectively allocates resources for seed production. This finding aligns with biochemical observations suggesting that a well-nourished plant entering the reproductive phase may require fewer defense compounds, such as soluble phenols in the leaves. Consequently, this can lead to a redirection of resources towards protein synthesis and seed development, which may correlate with lower levels of starch and total soluble sugars.

However, it is crucial to acknowledge that multiple factors can influence these relationships. Consequently, while the benefits of eucalyptus extract in this context are promising, effective crop management practices remain essential to optimize soybean yield during the reproductive phase. This highlights the need for a holistic approach in agricultural practices to ensure the health and productivity of crops within integrated systems.

Although application of eucalyptus extracts significantly altered some physiological and morphological parameters in the target species, no symptoms of root necrosis or leaf chlorosis were observed in either the first or second experiments. This finding aligns with the results reported by Puig et al. (Puig et al. 2018) in adult lettuce plants, where the presence of various phenolic compounds and organic acids in the aqueous extract of *GLOBULUS* was identified.

Assessing the allelopathic effects of eucalyptus species presents challenges, particularly regarding the dosage of the extracts. As discussed previously (Nelson et al. 2021)), many studies involving eucalyptus species employ artificial doses that may not accurately reflect natural concentrations, potentially skewing the results. Additionally, many studies use extracts obtained with organic solvents or boiling water, which are far from the real situation seen in the field. This is the reason we chose to extract ground leaves with water at room temperature. Furthermore, the complexity of these systems - including variations in plant species and age, the type and quantity of allelochemicals released into the soil, and the climate as well as the chemical, physical, and biological aspects of the soil - makes it difficult to draw definitive conclusions about integrated agricultural systems.

Additionally, soil microorganisms can modify the effects of these extracts, either mitigating or enhancing the allelopathic impacts, which further complicates the landscape of current understanding. Given the limited knowledge about microbial diversity in ecosystems, delineating the interactions within these systems remains a significant challenge for researchers and practitioners alike. This emphasizes the need for comprehensive studies that consider the interplay of various factors influencing the outcomes of integrated crop-livestock-forest systems.

5. Conclusions

The results obtained allow us to conclude that (1) Eucalyptus extracts interfere more in seed germination when in direct contact (germination paper) than in the presence of substrate (soil); (2) within the conditions established in the study, eucalyptus extracts have little interference with the species *U. brizantha*, *U. ruziziensis*, *P. maximum*, *Zea mays* L. and *Glycine max*, indicating the allelopathy is not a major issue in ICLF system composed by these species; (3) *E. globulus* extract promotes greater activity of soil enzymes; (4) new studies on allelopathy should be carried out focusing on soil microorganisms.

Declarations

Data Availability Statement: All data related to this work was included in the figures and tables, including supplementary material.

Conflict of Interest: The authors declare no conflict of interest

Author contribution: M.R., D.C.H.E., and J.L.C.B. executed the experiment and conducted data

analyses and wrote the first draft of the manuscript. P.M. planned the experiment, helped with data analyses, helped to write the first draft and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Supplementary material: It contains the chemical soil analysis of the substrate before experiment installation and information on several analysis (ecophysiological, biochemical and nutritional) carried out with the target plants exposed to the eucalyptus extracts, which did not differ statistically.

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Figures

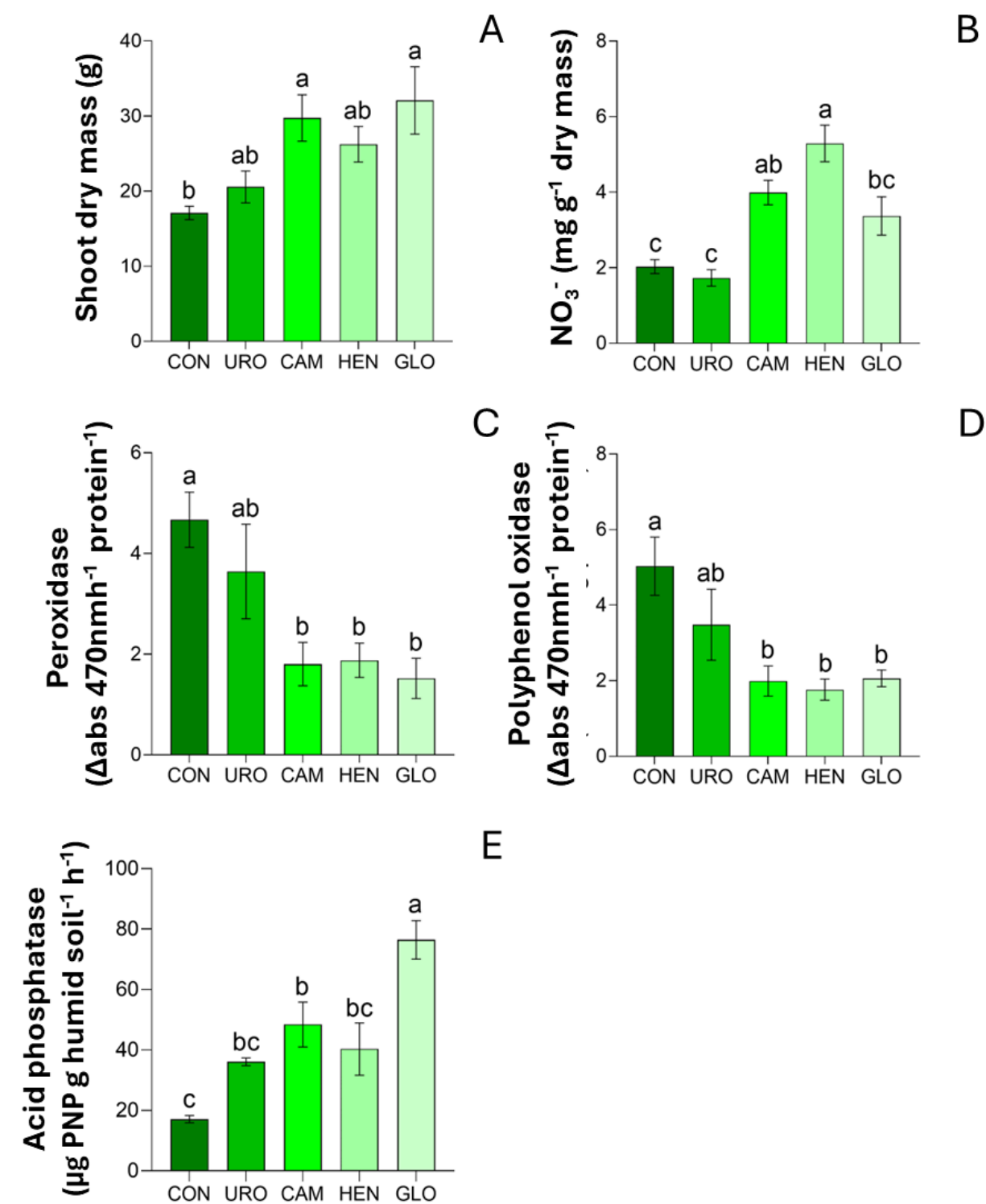


Figure 1

Effect of eucalyptus extracts on shoot dry matter (A), NO₃⁻ leaf concentration (B), peroxidase (C) and polyphenol oxidase (D) leaf activities and soil acid phosphatase activities (Fig. 1E) in the Marandu grass greenhouse experiment. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p<0.05$).

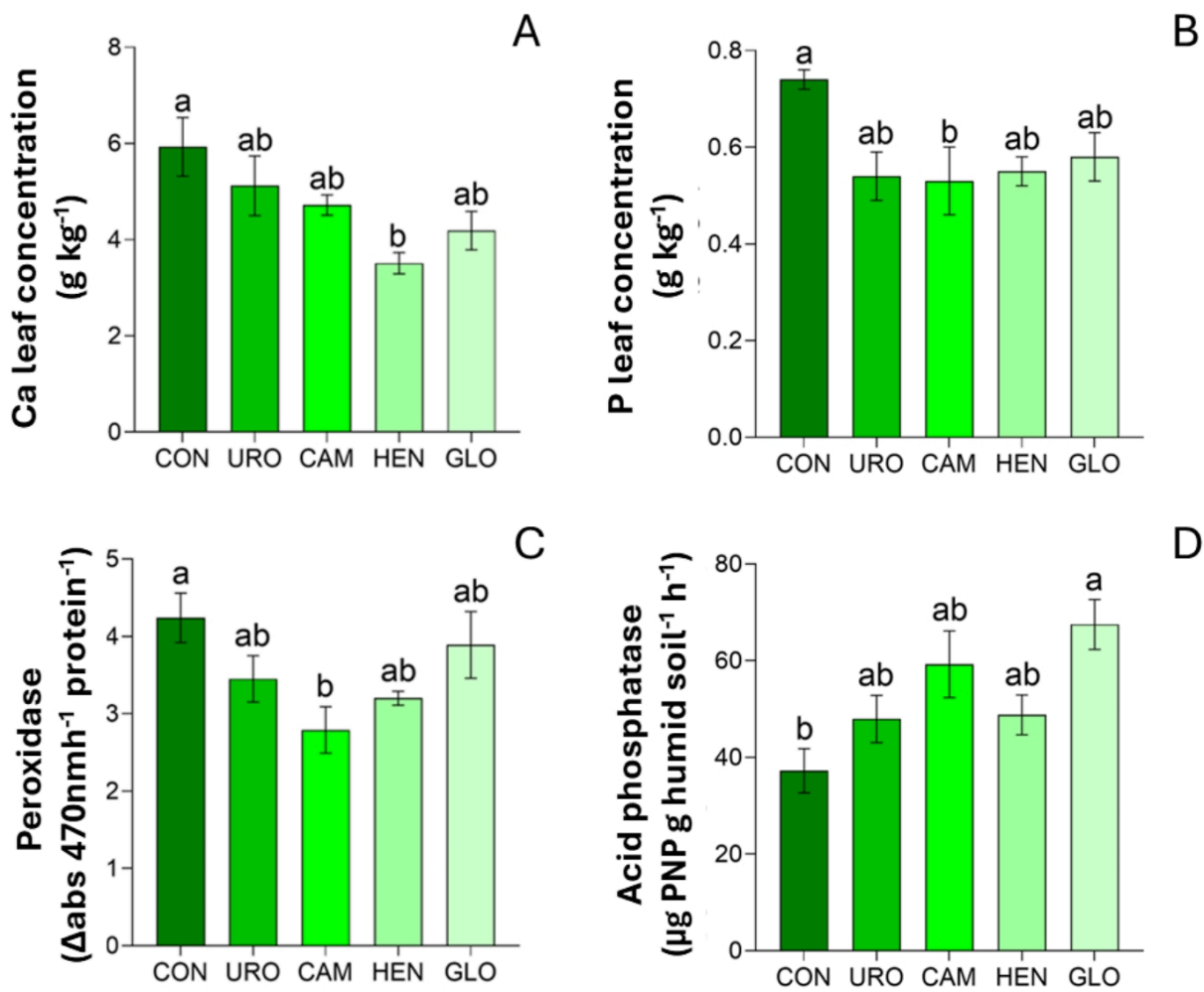


Figure 2

Effect of eucalyptus extracts on leaf concentration of Ca (A) and P (B), leaf peroxidase (C) activity and soil acid phosphatase activity in the brachiaria ruziziensis greenhouse experiment. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p<0.05$).

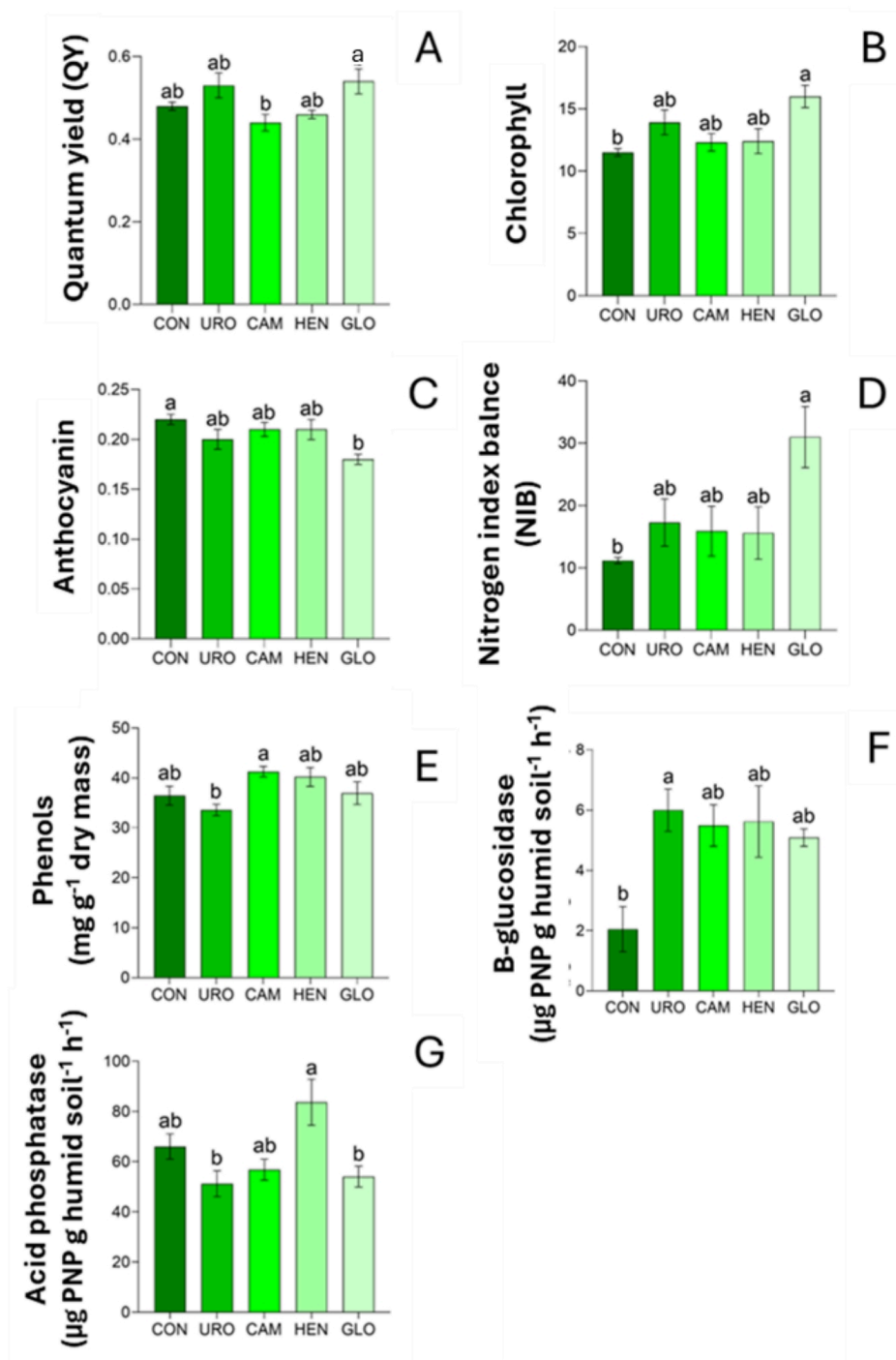


Figure 3

Effect of eucalyptus extracts on quantum yield (A), chlorophyll (B), anthocyanins (C), and nitrogen index balance (D), leaf concentration of total soluble phenols (E), soil activities of β -glucosidase (F) and acid phosphatase (G) in the Mombaça grass greenhouse experiment. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p < 0.05$).

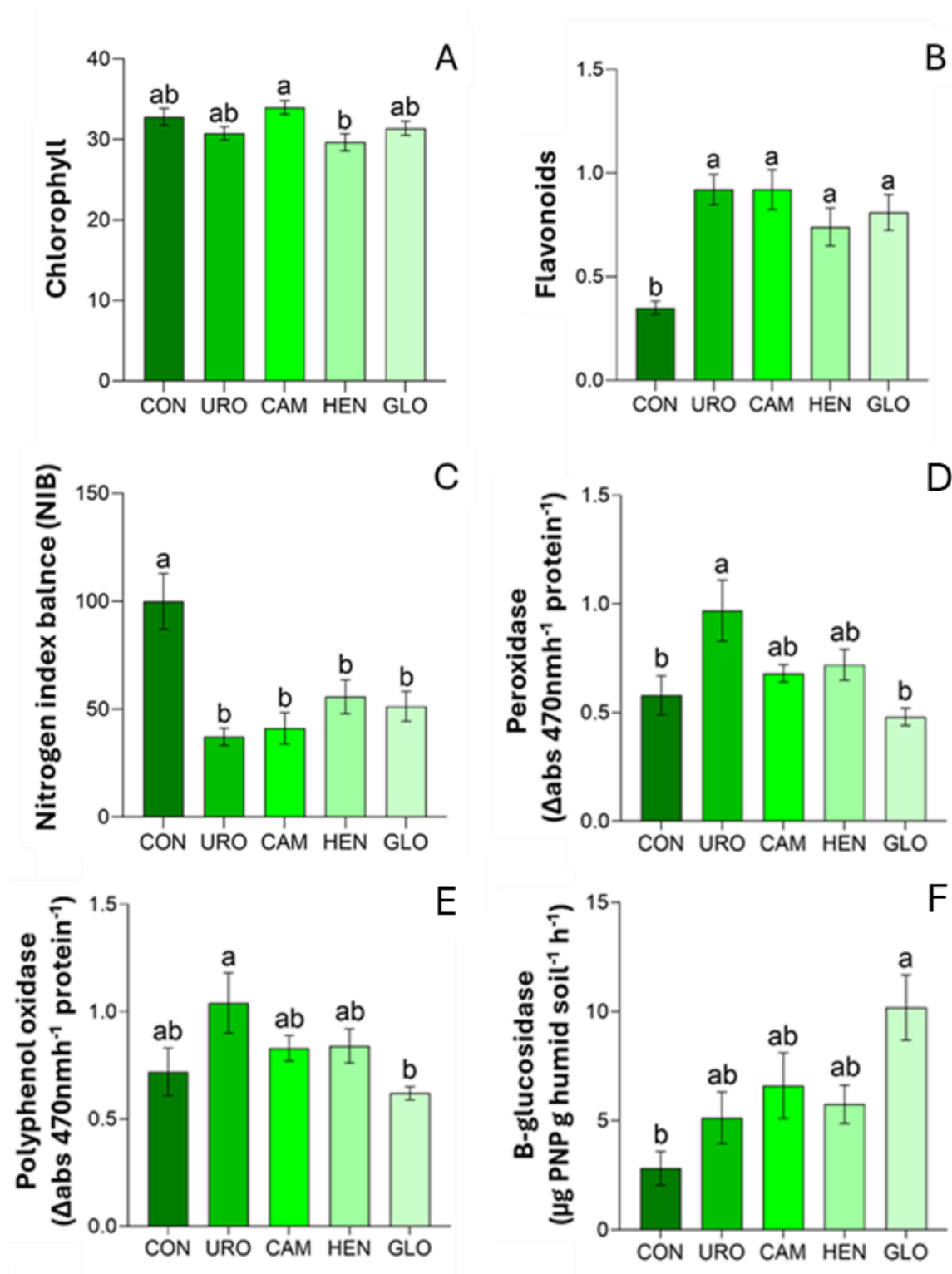


Figure 4

Effect of eucalyptus extracts on the chlorophyll (A), flavonoids (B) and nitrogen index balance (C), leaf peroxidase (D) and polyphenol oxidase activities (E) and soil β -glucosidase activity (F) in the maize greenhouse experiment. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p < 0.05$).

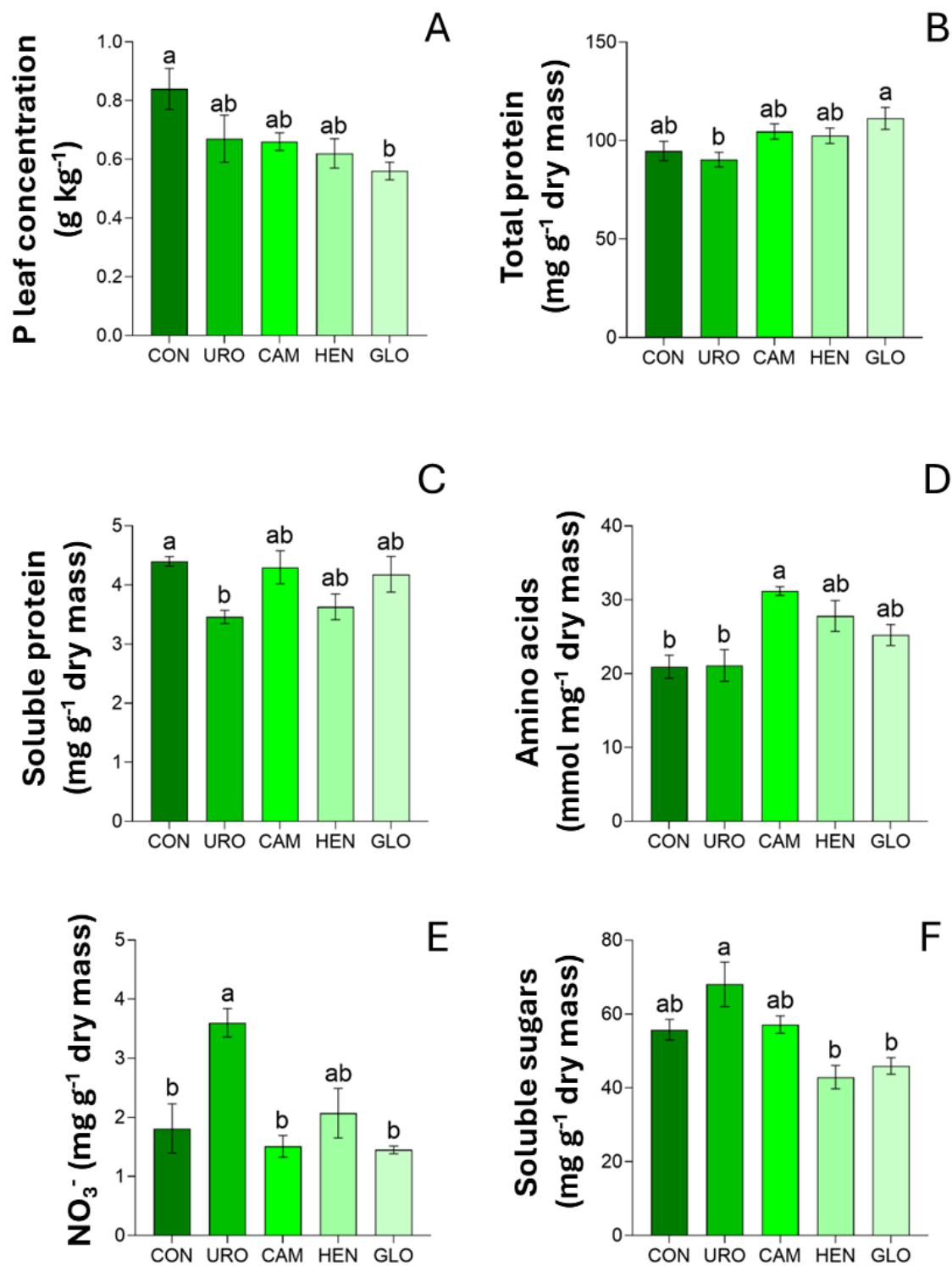


Figure 5

Effect of eucalyptus extracts on the P (A), total protein (B), soluble protein (C), amino acid (D), nitrate (E) and total soluble sugars (F) concentrations in leaves of the maize greenhouse experiment. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p < 0.05$).

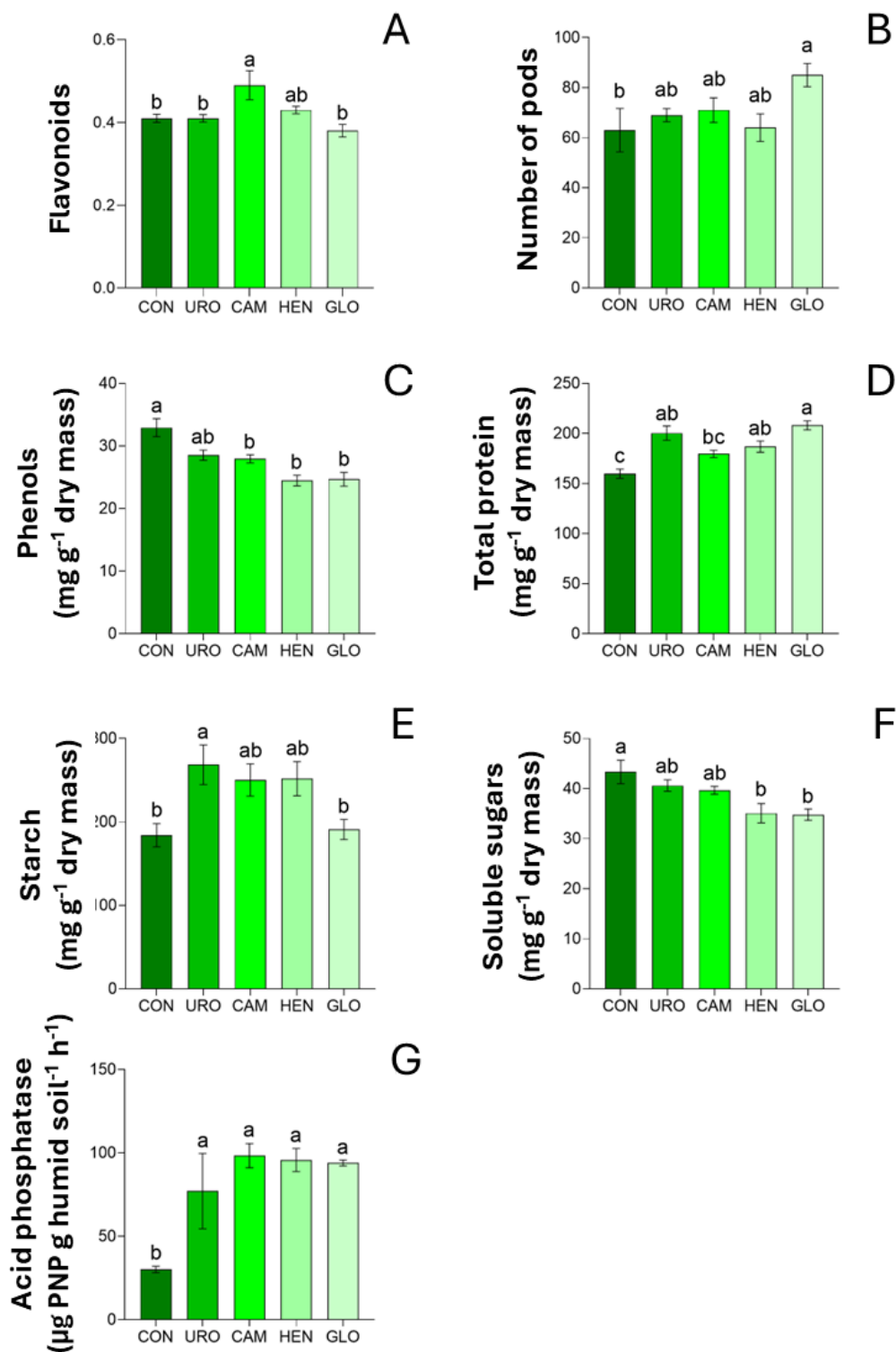


Figure 6

Effect of eucalyptus extracts on the flavonoids index (A), number of pods (B), concentration of total soluble phenols (C), total protein (D), starch (E) and total soluble sugars (F), and acid phosphatase activity (G) in soil collected from the soybean greenhouse experiment pots. Bars indicate standard errors. Equal letters did not differ statistically by Tukey's test ($p < 0.05$).

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