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Phosphorus Shortage Induces an Increase in Root Exudation in Fifteen Eucalypts Species

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Abstract: A significant proportion of the carbon fixed by plants is transported to the roots and exuded to the rhizosphere. Exudates may have important roles in the rhizosphere, such as desorbing labile phosphorus (P) or mobilizing manganese (Mn) and other metal cations. This study evaluated the root exudation profiles of seedlings of 15 eucalypt species in response to a P shortage and if the ability to exude organic compounds was related to P and Mn accumulation in the shoots. The plants were grown on sand and were irrigated with nutrient solutions containing either sufficient P (500 μM) or low P (25 μM). Organic acids (OA), amino acids/polyamines, and phenolics were analyzed in the root exudates by UPLC-MS/MS. Plants with a low P level had low leaf P contents and growth reduction. A P shortage induced the exudation of the three groups of metabolites analyzed at higher levels than sufficient P availability. Despite that, the composition pattern of root exudates was similar among species under low or sufficient P concentrations. Citric and isocitric acids were the major OAs found in the exudates, followed by oxalic, malic, and succinic acids. Among the amino acids/polyamines identified, putrescine was the most abundant in all species, followed by glycine. Cinnamic acid was the predominant phenolic in the root exudates. Our results indicate that P limitation induces a conserved response genetic mechanism in eucalypts. Such results can be further investigated to adapt commercial clones to soils with low P availability.

Keywords: amino acids; Corymbia; Eucalyptus; manganese; organic acids; phenolic compounds; phosphorus; root exudation

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1. Introduction

In response to the low phosphorus (P) availability in soil, plants evolved adaptative strategies to increase P acquisition and improve P use efficiency [1]. These strategies include (i) root morphological changes, (ii) associations with mycorrhizal fungi, (iii) metabolic adaptations to increase the internal P economy, and iv) root physiological adaptations to increase P availability in the rhizosphere [1–3]. These physiological root responses to the P shortage comprise the secretion of enzymes such as phosphatases and the release of organic anions to the rhizosphere [1].

Plant roots constantly release different compounds into their surroundings by an excretion process known as exudation [4]. Root exudation encompasses carbon transport in the phloem to the roots and its release to the rhizosphere [5]. Exudates may act as microbial attractors or repellers and influence nutrient and water availability [6]. Roots can exude a wide variety of primary and secondary metabolites into the soil; organic acids (OA), sugars, mucilage, and amino acids are among the major primary metabolites exuded, and flavonoids, glucosinolates, or even hormones such as auxins or strigolactones are considered the most common secondary metabolites in root exudates [7,8]. The composition of root exudates varies according to the species, plant age, physiological and nutritional status, and environmental conditions [4]. Additionally, the pattern of root exudation can be quantitatively and qualitatively influenced by abiotic factors such as a water deficit,

flooding, salinity, temperature extremes, or nutritional limitation [4]. Under some of these stressing conditions, mainly nutritional and water limitations, root exudation can be a significant sink of the carbon fixed by photosynthesis [9].

The exudation of OA, di-, and tricarboxylic acids is specifically induced under a P deficiency. Carboxylic acids are important in the mobilization of inorganic P, solubilizing P bound to iron (Fe) and aluminum (Al) oxides/hydroxides in acidic soils [10], or by chelating the cations of the precipitated phosphates [11]. In general, organic anions at a low pH are fully dissociated and compete for sorption sites with inorganic and organic P, promoting mineral dissolution and stimulating the growth of microorganisms [1].

The exudation of phenolics by the roots occurs in several plant species, but information on their nature and amounts are still scarce, especially concerning the plant's response to nutritional stimuli [12]. Flavonoids, besides their role in plant–microbe communication, can be released by roots in response to the low availability of P in the soil, acting as metal chelators and with a possible role in the mobilization of poorly soluble forms of P in the soil [13]. Lupinus albus, a species that forms "cluster roots," exudes copious amounts of citrate and malate and a significant amount of isoflavonoids in response to low P in the soil [14]. The release of flavonoids together with OA has been associated with the protection of these carboxylates against microbial degradation in the rhizosphere [12]. In apple rootstocks (*Malus* × *domestica*), the P deficiency increased the root exudation of oxalate and flavonoids, in addition to the up-regulation of the expression of transporters mediating the exudation of coumarin and possibly other flavonoids [15]. Additionally, exudated OA and phenolics may contribute to the uptake of soil manganese (Mn) and iron (Fe) [16,17]. Some of the OAs exuded by roots as malic acid, for example, can reduce hydrous oxides of Mn, releasing Mn^{2+} ions that can be available for root absorption [18]. In this respect, the Mn concentration in leaves has been proposed as a proxy for root exudation, as organic acids mobilize metal cations such as Mn, Fe, and zinc [3], and possibly even silicon [19]. Besides the P shortage, the suboptimal Mn contents in plants have been related to the induction of OAs exudation to the rhizosphere [20].

Most works focused on the study of root exudation have been made with herbaceous species, and there is much less information regarding tree species [20]. It has been suggested that eucalypt species such as *Eucalyptus gummifera* can access inorganic P from insoluble forms of Fe and Al phosphates by releasing organic anions [21]. In *E. camaldulensis* and the hybrid *E. grandis* × *E. urophylla*, a relationship between the root exudation of OA and the tolerance of these species to Al was observed [22,23]. In recent studies by our group, 24 eucalypt species were evaluated for their ability to grow in soils with a low P availability and their responsiveness to the P supply [24], as well as the composition of the microbial communities on the rhizosphere being characterized [25]. The species most responsive to the addition of P were those with the lowest efficiency in using P and with the lowest efficiency in the P uptake. In contrast, species with high P-use efficiency were not responsive to the P supply [24]. The identity of the eucalypt species strongly influences the microbial communities in the rhizosphere, as the P availability modulated the microbiome in a species-specific manner [25]. It was suggested that root exudation might be involved in the eucalypt P uptake efficiency and the shaping of rhizosphere microbial communities [24,25]. In this study, we aimed to evaluate the response of root exudation to low P concentrations in eucalypt species that previously differed in their availability to grow under P-limited conditions [25]. We hypothesized that: (1) a low P concentration in the solution would increase root exudation, mainly OAs, which may desorb P from the soil particles; (2) eucalypts species would differ in the exudation profiles, which could be related to their ability to accumulate P or Mn in the shoots. In this study, we comprehensively present the root exudates profile of seedlings of 15 eucalypt species as challenged by the P shortage.

2. Material and Methods

2.1. Experimental Design, Biological Materials, and Pot Culture Experiment

The experiment was conducted in a 15 × 2 factorial completely randomized design, i.e., 15 eucalypt species and 2 P levels (low and sufficient P), with 11 replicates. The species were: *Corymbia citriodora*, *C. maculata*, *Eucalyptus acmenoides*, *E. brassiana*, *E. camaldulensis*, *E. deanei*, *E. globulus*, *E. grandis*, *E. microcorys*, *E. pellita*, *E. resinifera*, *E. robusta*, *E. saligna*, *E. tereticornis*, and *E. urophylla*. Seeds were obtained from Caiçara Sementes (Brejo Alegre, São Paulo, Brazil) and the Institute of Forestry Research and Studies (IPEF, Piracicaba, São Paulo, Brazil) produced from plants in clonal gardens.

The plants were kept in a greenhouse with natural light and temperature conditions. Seeds were germinated in trays with vermiculite, and when the seedlings had between 2 and 3 leaves, they were transplanted into 280 mL pots containing washed-sand and vermiculite mixture (4:1, v/v), with one seedling per pot. The seedlings received $\frac{1}{2}$ strength modified Hoagland solution [26], with a low (Low P—25 μM L^{-1}) and sufficient concentration (Suff P—500 μM L^{-1}) of P [27], according to the treatments. The nutrient solution was supplied twice a week, and the plants were watered when needed. The plants were maintained for 12 weeks under these conditions.

2.2. Collection of Root Exudates

At harvest, six plants per treatment were used for exudate collection, following modifications of previous methods [28,29]. Root collection was performed in blocks of one replicate (plant) per treatment between 9.30 and 12.30 h. For that, whole plants were carefully removed from the substrate, and the roots were immediately and gently washed in water and placed in 15 mL tubes with 8 mL of 0.2 mM L^{-1} of $CaCl_2$. The plants were maintained for 2 h under illumination and gentle agitation (130 rpm) on an orbital shaker. After this time, the roots were removed from the solution, which was immediately frozen. Roots were transferred to 50% ethanol and kept in the fridge until they were characterized as follows. The roots were scanned at a resolution of 600 dpi (Epson Expression 12000XL, Epson America Inc., Long Beach, CA, USA), and the images were analyzed with the software WinRhizo (Regent Instruments Inc., Montreal, QC, Canada). Total root length, root surface area, and root diameter were determined. Roots and shoots were air-dried at 60 °C for seven days for dry mass determination.

2.3. Determination of the Concentration of P and Mn

The dried shoots were ground and digested with nitric-perchloric acid (HNO_3–$HClO_4$) to determine the contents of P and Mn by plasma emission spectrophotometry (ICP-OES).

2.4. Determination of Organic Acids, Amino Acids, and Phenolics in Root Exudates

The solutions with root exudates were filtered in 0.22-mm membrane, equally divided into two tubes, and lyophilized. The content of one of the tubes was solubilized in 200 μL MilliQ water and used for the OA determination. The content of the other tube was solubilized in 200 μL of 80% methanol and used for amino acids, polyamines, and phenolics determination. In both cases, solubilization was made just before analysis. The qualitative determination was performed by ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS/MS, QTOF, quadrupole time-of-flight mass spectrometry, Micromass/Waters, Manchester, UK). The chromatographic separation was made using a Waters Acquity C18 BEH analytical column (150 × 2.1 mm id, 1.7 μm) at 30 °C. The mobile phases consisted of methanol (A) and aqueous 0.1% formic acid (B) at a flow rate of 0.2 mL min^{-1}. The gradient was 1% A for 2.5 min, increasing to 50% A in 5 min and then returning to 1% A in 8 min and kept on that until stabilization. Positive mode electrospray ionization-mass spectrometry (ESI-MS) and tandem ESI− MS/MS were done under the following conditions: capillary 3.5 KV, cone 30 V, and source and desolvation temperature were 150 and 300 °C, respectively. For ESI− MS/MS, the energy for the collision-induced dissociations was 15 eV. Data were acquired in the mass/charge (m/z) range

between 50 and 300. Ions were identified by comparing their m/z, retention time, and ESI−MS/MS dissociation patterns with pure standards [30,31]. The standards included were 10 OA (tartaric, malic, malonic, oxalic, citric, isocitric, maleic, fumaric, succinic, and lactic acids), 15 phenolics (quercetin, rutin, catechin, epicatechin, myricetin, vanillin, naringenin, *p*-cumaric acid, shikimic acid, ferulic acid, benzoic acid, chlorogenic acid, protocatechuic acid, gallic, and cinnamic acids), 21 amino acids (proline, lysine, histidine, glutamine, serine, alanine, glycine, asparagine, aspartic acid, glutamic acid, arginine, threonine, valine, leucine, isoleucine, tyrosine, tryptophan, cysteine, gamma-aminobutyric acid, ornithine, and citrulline), and four polyamines (spermine, spermidine, putrescine, and agmatine).

2.5. Data Analysis and Statistics

Data acquired from UPLC-MS/MS were processed with MassLynxV4.1 software (Waters Co., Milford, MA, USA). Data were analyzed by two-way ANOVA, and when possible, means were compared by the Scott–Knott at 5% significance using the software SISVAR [32]. Data related to counts and percentages were transformed into the log (x + 1) and arcsine $\sqrt{(x/100)}$, respectively, before statistical analysis. Pearson's correlation was used to check the correlation between the main variables and the concentration of P. Principal component analysis (PCA) was used to identify the variables that best explained the highest proportion of data set variance using Minitab 17 software (Minitab Inc., Shanghai, China).

3. Results

3.1. Plant Growth and P and Mn Concentrations

In general, shoots and roots of plants grown at low P produced a 42 and 52% lower biomass than plants under sufficient P (Figure 1a,b). However, some species showed similar shoot and root biomass production at both P concentrations. This was the case for the shoots of *C. citriodora*, *E. pellita*, and *E resinifera* (Figure 1a), and the roots of *E. brassiana*, *C. citriodora*, *E. pellita*, *E. resinifera*, and *E. tereticornis* (Figure 1b). The shoot-to-root (S:R) ratio was not significantly influenced by the P supply (Figure 1c), except for *E. globulus*, *E. robusta*, and *E. saligna*, which showed a higher S:R ratio when growing under a low P concentration (Figure 1c).

In all species, the shoot P concentrations were higher in plants grown on sufficient P than in low P (Figure 2a). The concentrations ranged from 0.34 to 0.65 g kg^{-1} in plants under low P and from 1.14 to 1.87 g kg^{-1} in plants under sufficient P (Figure 2a). At low P, the species with the highest shoot P concentrations were *E. grandis* and *C. maculata*, and at sufficient P, *C. maculata* was also the species with the highest P concentrations in the shoot (Figure 2a).

We observed a high interspecific variation for the shoot Mn concentration, especially at low P concentrations, ranging from 37 mg kg^{-1} in *E. camaldulensis* to 209 mg kg^{-1} in *C. maculata* (Figure 2b). In general, plants grown under sufficient P had more Mn than those under low P. Opposite to all other species, plants of *C. maculata* had significantly more Mn in the shoots at low P than at sufficient P (Figure 2b). *C. citriodora* showed a similar trend, but it was not statistically different. Under sufficient P, *C. maculata* also showed the highest value of Mn compared to all other species. Shoot P was positively correlated with shoot Mn concentrations under both low and sufficient P concentrations (Low P, r = 0.734 p = 0.002; Suff P, r = 0.664 p = 0.007) (Figure S1).

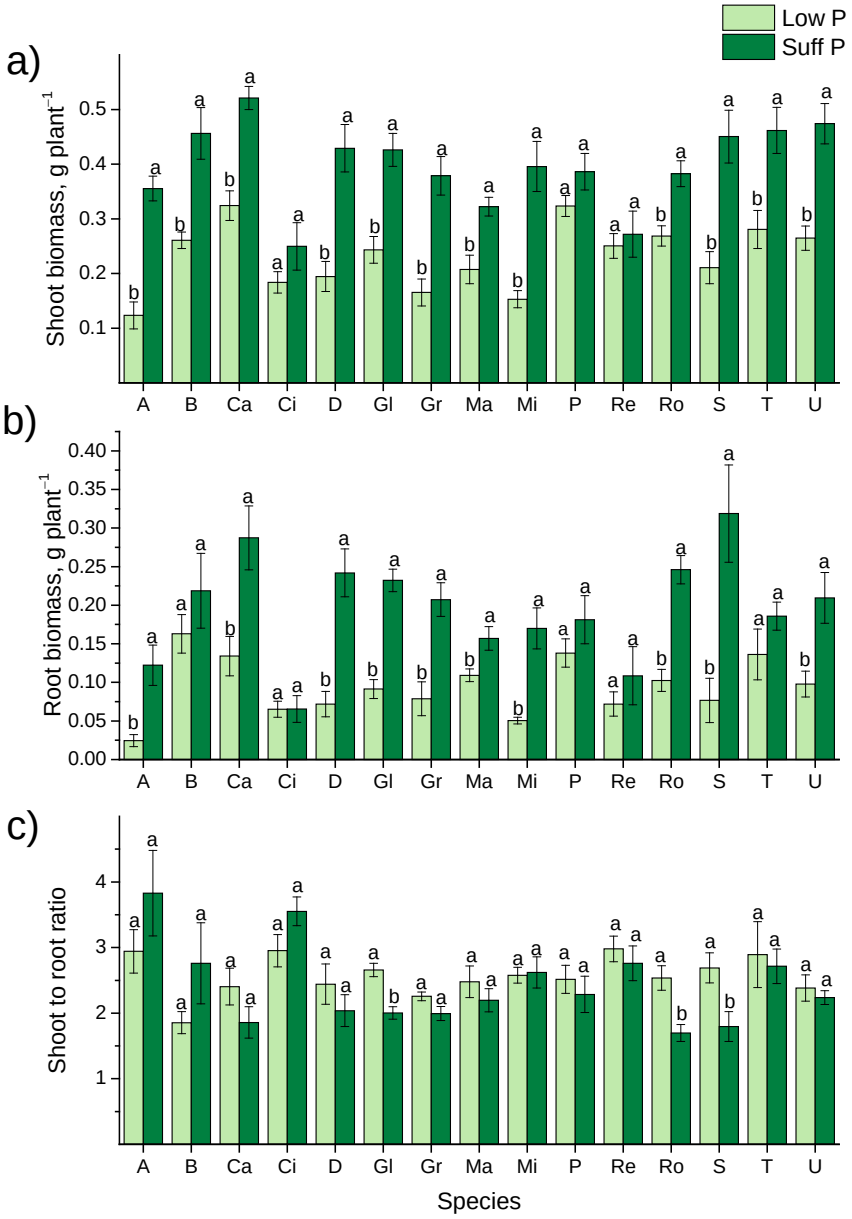


Figure 1. Shoot (**a**) and root (**b**) biomass production, and shoot-to-root ratio (**c**) of fifteen eucalypt species grown under low (Low P, clear color) and sufficient (Suff P, dark color) phosphorus concentrations. Different letters indicate significant differences between low and sufficient P concentrations within each species by the Tukey test ($p < 0.05$). Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

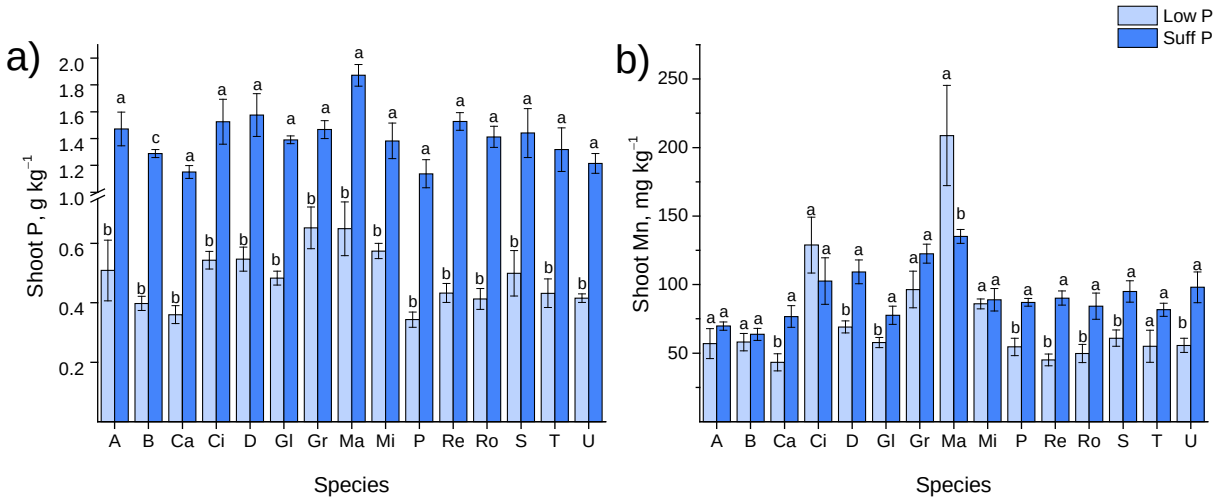


Figure 2. Concentration of phosphorus (**a**) and manganese (**b**) in shoots of fifteen eucalypt species grown under low (Low P, clear color) and sufficient (Suff P, dark color) phosphorus concentrations. Different letters indicate significant differences between low and sufficient P concentrations within each species by the Tukey test ($p < 0.05$). Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

3.2. Root Exudation: Organic Acids, Amino Acids/Polyamines, and Phenolics

The amount of total OA exuded by the roots of eucalypts was generally 30% higher under low P than under sufficient P (Figure 3 insert). However, the total amount of OA was significantly higher only in the low P plants of *E. brassiana*, *E. grandis*, *E. robusta*, *E. saligna*, and *E. urograndis* (Figure 3). Opposite to these species, *E. acmenoides*-exuded OA was highest in the plants grown at sufficient P. The total amount of OA released by the eucalypt roots was not significantly correlated with the shoot P concentrations ($r = -0.155$, $p = 0.413$) (Figure S1). However, although not significant, the correlation was positive when considering only the results for low P ($r = 0.444$, $p = 0.097$) (Figure S1). The OA profile, expressed as a percentage of the total OA exuded per total root area, showed that the main OA in eucalypt exudates were citric acid and its isomer isocitric acid, comprising, on average, 50 and 32% of the total OA composition, respectively (Figure 4). Oxalic acid and malic acid were also among the more common OA identified. We calculated the percentual change of each OA between the low and sufficient plants (Table 1). For most species, there was an increase in succinic, isocitric, lactic, maleic, citric, fumaric, oxalic, and tartaric acids. The largest increases were observed in isocitric and lactic acids. While isocitric acid was abundant in the exudates, lactic acid was not, representing less than 1% for most species (Figure 4). These OAs increased more than two thousand times in *E. urograndis*. Lactic acid increased more than one thousand times in *E. camaldulensis* and *E. robusta*. On the contrary, malic acid decreased in most of the species grown under low P. The same was observed for malonic acid, although it was detected only in four species. Interestingly, *E. acmenoides* was the only species presenting a decrease in the ratio of low to sufficient P plants, except for oxalic acid (Table 1).

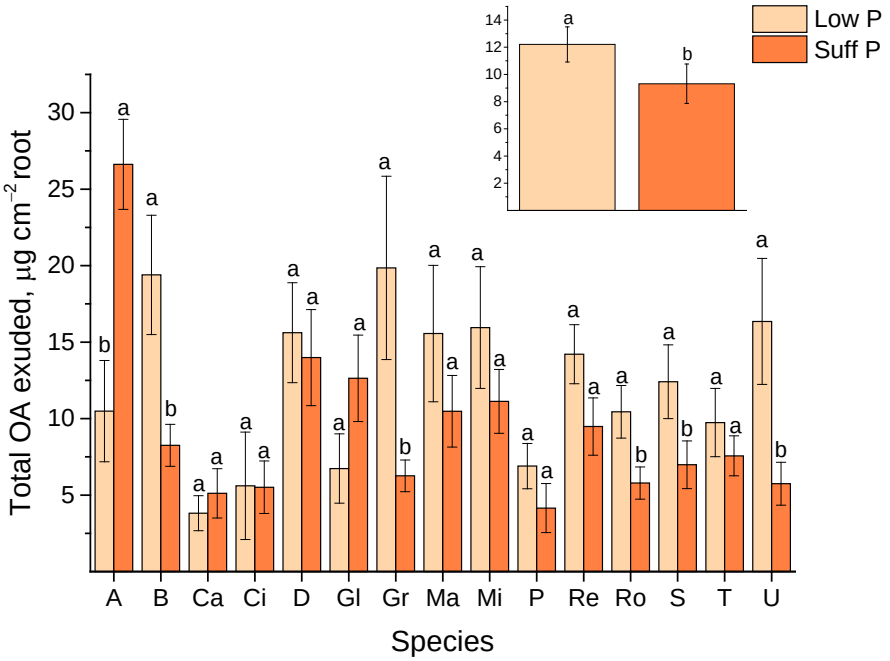


Figure 3. Total organic acid (OA) exuded by the roots of fifteen eucalypt species grown under low (Low P, clear color) and sufficient (Suff P, dark color) phosphorus concentrations. Different letters indicate significant differences between low and sufficient P concentrations within each species by the Tukey test ($p < 0.05$). Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

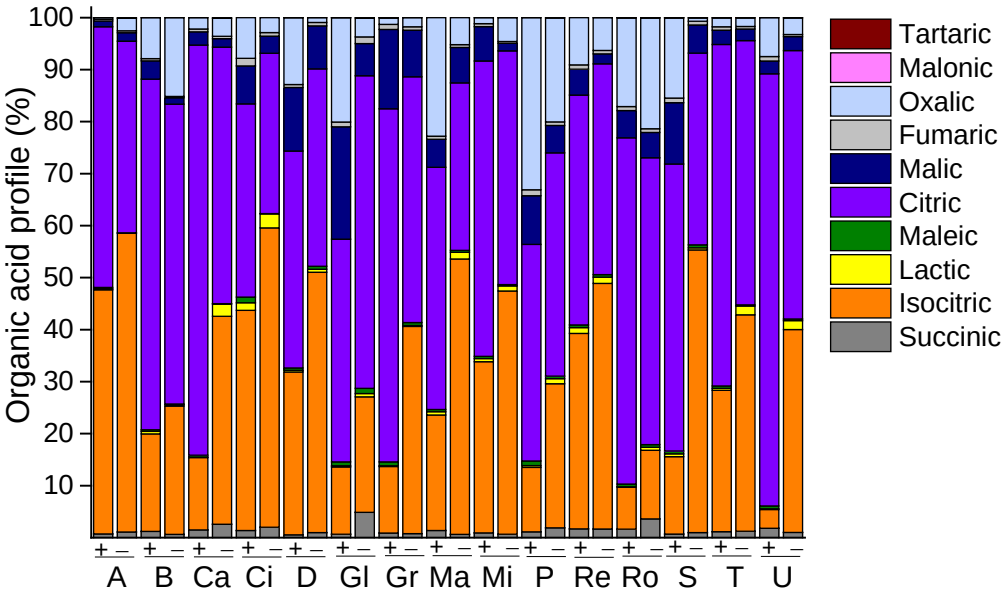


Figure 4. Organic acid profile in root exudates of fifteen eucalypt species grown under low (−) and sufficient (+) phosphorus concentrations. Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

Table 1. Percentage of change between the amount of individual organic acids exuded by roots under low and sufficient phosphorus concentrations in each eucalypt species. Blue-shaded cells indicate a decrease and orange-shaded cells indicate an increase in the percentage of change. nd = not found in the species.

Species	Succinic	Isocitric	Lactic	Maleic	Citric	Malic	Fumaric	Oxalic	Malonic	Tartaric
					Low P/Sufficient P (%)					
Eucalyptus acmenoides	−40.0	−52.2	−100.0	−67.3	−71.3	−59.2	−53.1	119.9	nd	−100.0
E. brassiana	18.8	189.7	17.4	8.2	88.3	−28.1	21.2	324.0	nd	450.7
E. camaldulensis	27.0	110.2	1394.6	−75.5	−54.3	−53.5	−35.6	16.9	nd	434.8
Corymbia citriodora	47.0	38.3	91.7	−67.6	−15.7	−55.9	−53.4	−62.6	nd	nd
E. deanei	94.1	69.1	102.0	26.3	−3.7	−28.4	18.1	−92.6	−100.0	186.9
E. globulus	301.9	−8.6	58.9	−27.9	−25.1	−84.7	−25.6	−90.3	−100.0	336.0
E. grandis	180.3	886.5	216.8	143.7	120.9	85.8	125.8	328.0	−100.0	119.5
C. maculata	−31.3	253.4	225.3	36.2	2.6	83.4	30.9	−66.2	nd	nd
E. microcorys	1.2	104.0	134.5	−20.8	13.6	−68.7	−14.0	476.9	nd	169.0
E. pellita	184.7	271.1	280.4	−3.6	71.3	−6.4	0.1	0.5	nd	434.3
E. resinifera	47.7	87.0	56.7	44.2	36.4	−22.2	18.8	3.3	nd	nd
E. robusta	303.4	197.8	1764.8	84.7	51.2	69.2	70.2	128.2	83.3	128.2
E. saligna	145.6	526.7	26.1	79.1	14.7	−14.4	35.8	−92.5	−100.0	282.1
E. tereticornis	39.1	98.6	435.7	−22.5	0.4	−11.2	−5.5	26.2	nd	1.6
E. urophylla	56.8	2945.4	2582.9	73.6	75.6	214.0	36.2	21.5	nd	371.8

Considering all species, the total amount of phenolics exuded by the roots of eucalypts was 2.3 times higher in plants under low than under sufficient P (Figure 5). Although there is a clear trend in all species, the total phenolic content increased significantly only in six species (*E. acmenoides*, *E. brassiana*, *E. camaldulensis*, *E. deanei*, *E. grandis*, and *E. microcorys*). The largest values were found in *E. acmenoides* and, although not statistically significant, in *C. citriodora*, which showed a considerable variation in the total amount of phenolics exuded by low P-treated plants (Figure 5). Cinnamic acid was the main phenolic in the root exudates in all species, comprising up to 99% of the 14 phenolics identified (Table S1). In exudates of *E. robusta* under sufficient P, around 11% and 7% of all the identified phenolics were naringenin and quercetin, respectively, whose amounts were non-representative in the exudates of this species under low P. In *E. acmenoides* and *E. urophylla* grown under sufficient P, quinic acid was represented in 3.2 and 3.9 wt% of the total phenolics in the root exudates (Table S1). In all these cases, the percentual contribution of these compounds to the total amount of phenolics in the root exudates practically disappeared.

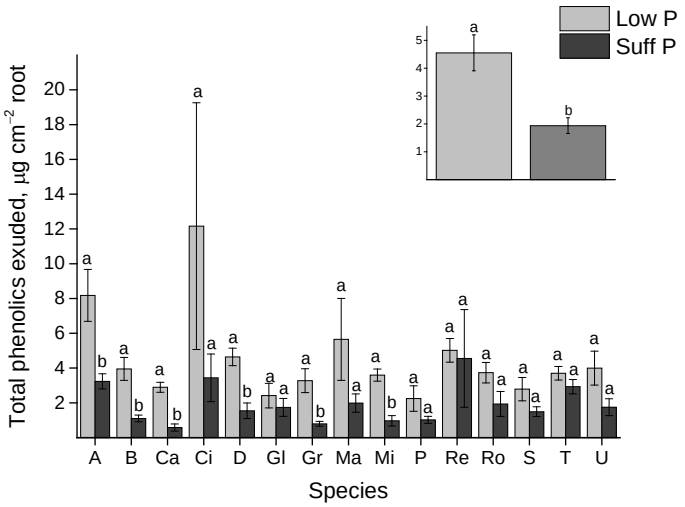


Figure 5. Total phenolics exuded by the roots of fifteen eucalypt species grown under low (Low P, clear color) and sufficient (Suff P, dark color) phosphorus concentrations. Different letters indicate significant differences between low and sufficient P concentrations within each species by the Tukey test ($p < 0.05$). Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

Amino acids and polyamines were also analyzed in root exudates. Under low P, the amount of total amino acids and polyamines in the root exudates was two times the amount found at sufficient P (Figure 6). Similar to phenolics, a clear trend was observed for total amino acids and polyamines in the exudate of all species, i.e., most had more amino acids in plants grown in low P. Still, only a few were statistically significant (*E. brassiana*, *E. deanei*, *E. microcorys*, *E. robusta*, *E. saligna*, and *E. tereticornis*). *E. tereticornis* showed the greatest differences between low and sufficient P (Figure 6). When looking at the amino acid/polyamine profiles of the root exudates, the major compound identified in all 15 species was putrescine, followed by glycine (Figure 7). Curiously, *E. urograndis* plants under sufficient P showed reasonable amounts of putrescine, glutamine, and lysine, and much less glycine. In addition, for amino acids, we calculated the ratio between the contents found in low P and sufficient P plants (Table 2). The amino acid/polyamine profile was generally little changed in response to the P concentration. In general, putrescine, agmatine, and glycine were the compounds with the highest increase in low P plants. On the contrary, lysine and glutamine were in a greater proportion in sufficient plants of *E. microcorys*, *E. resinifera*, and *E. urograndis* (Figure 7).

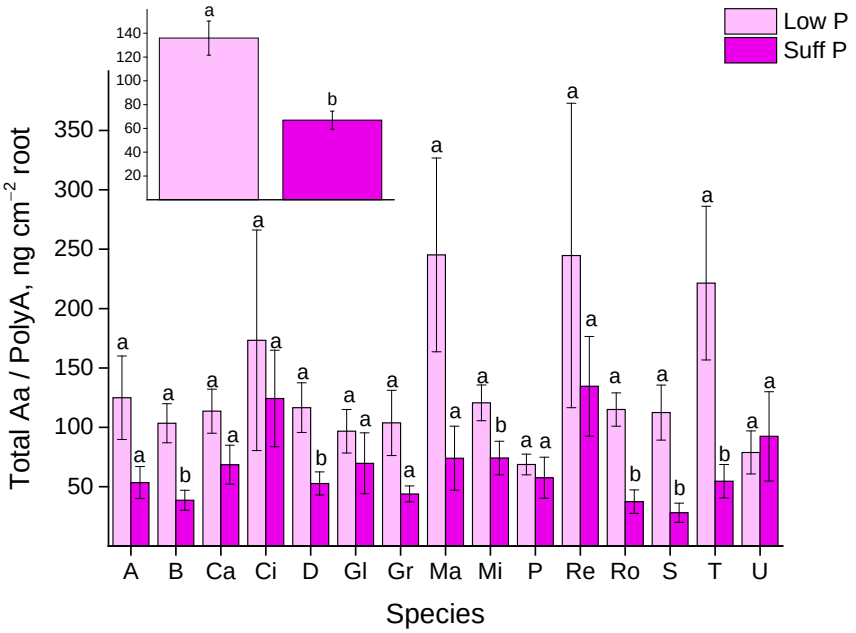


Figure 6. Total amino acid and polyamines (Aa/PolyA) exuded by the roots of fifteen eucalypt species grown under low (Low P, clear color) and sufficient (Suff P, dark color) phosphorus concentrations. Different letters indicate significant differences between low and sufficient P concentrations within each species by the Tukey test ($p < 0.05$). Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

The principal component analysis (PCA) for the different OA amounts present in the root exudates of the seedlings of the 15 eucalypt species is shown in Figure 8. PC1 and PC2 explained 33.1% and 18.9% of the total variance, respectively. The score plot (Figure 8a) shows the influence of the P concentration in the amounts of OA in the root exudates of the eucalypt species, clearly separating P treatments into two groups along the PC2 axis (Figure 8). In the loading plot (Figure 8b), malonic acid is isolated probably because of the low values found in most of the species (see Table 1). Still, in this plot, oxalic, malic, fumaric, and maleic acid are separated in the PC2, and they were the compounds in which the proportion between the low and sufficient P plants decreased (negative values in Table 1).

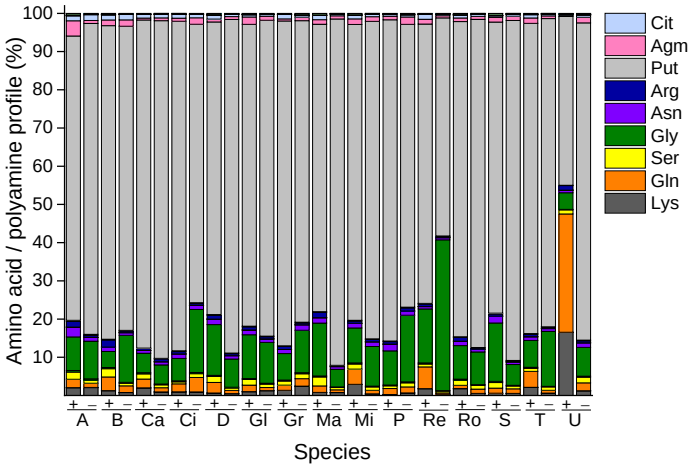
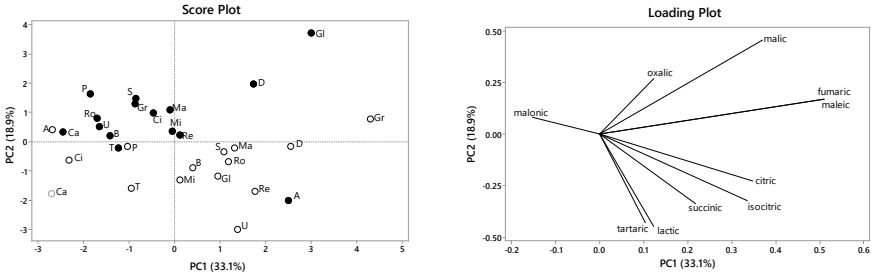


Figure 7. Amino acids and polyamines profile in root exudates of fifteen eucalypt species grown under low (−) and sufficient (+) phosphorus concentrations. Species abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*. Lys—lysine, Gln—glutamine, Ser—serine, Gly—glycine, Asn—asparagine, Arg—arginine, Put—putrescine, Agm—agmatine, Cit—citrulline.

Table 2. Percentage of change between the amount of individual amino acids/polyamines exuded by roots under low and sufficient phosphorus concentrations in each eucalypt species. Blue-shaded cells indicate a decrease and orange-shaded cells indicate an increase in the percentage of change.

Species	Lys	Gln	Ser	Gly	Asn	Arg	Put	Agm	Cit
					Low P/Sufficient P (%)				
Eucalyptus acmenoides	138.7	16.0	2.6	161.8	−8.8	−14.8	155.4	−54.0	167.2
E. brassiana	60.3	32.1	−17.6	685.2	63.8	−38.1	159.9	197.7	182.7
E. camaldulensis	−24.1	−21.2	−7.0	63.2	89.8	142.7	70.6	153.6	60.6
Corymbia citriodora	33.8	154.6	159.8	292.2	24.9	9.2	17.9	198.7	21.2
E. deanei	68.6	−33.9	−9.7	22.6	34.8	17.1	152.0	126.1	25.5
E. globulus	75.9	−34.5	−17.2	29.6	3.3	−6.2	45.2	−22.2	3.8
E. grandis	336.3	237.5	182.0	277.8	215.3	44.1	119.4	288.7	17.9
C. maculata	223.2	36.4	−12.5	10.2	64.9	−45.4	299.4	124.8	37.6
E. microcorys	−76.9	−62.0	7.7	84.2	66.3	75.6	74.5	34.9	3.4
E. pellita	212.5	20.4	79.1	133.2	−27.1	47.0	5.1	149.8	39.7
E. resinifera	−69.0	−88.2	8.8	407.8	68.5	−4.8	42.0	−17.0	−38.6
E. robusta	−7.2	310.1	130.1	199.1	91.8	0.5	219.8	156.6	130.6
Eucalyptus acmenoides	289.2	255.5	96.9	42.9	51.4	90.6	367.5	258.7	419.3
E. brassiana	16.2	−28.7	346.5	730.2	202.6	132.4	302.8	102.2	126.4
E. camaldulensis	−93.7	−94.3	16.8	48.0	54.1	−62.9	60.4	458.4	43.4



(**a**) (**b**)

Figure 8. Score (**a**) and loading (**b**) plots for PC1 vs. PC2 for organic acids amounts in the root exudates of seedlings of fifteen eucalypt species grown under low (white) and sufficient (black) P concentrations. Abbreviations: A—*E. acmenoides*, B—*E. brassiana*, Ca—*E. camaldulensis*, Ci—*C. citriodora*, D—*E. deanei*, Gl—*E. globulus*, Gr—*E. grandis*, Ma—*C. maculata*, Mi—*E. microcorys*, P—*E. pellita*, Re—*E. resinifera*, Ro—*E. robusta*, T—*E. tereticornis*, S—*E. saligna*, U—*E. urophylla*.

4. Discussion

Here, we show the effect of the P supply on the root exudate composition of 15 eucalyptus species. A clear trend observed for the compounds analyzed was that their amount on a root-area basis was the greatest when plants grew at a low P. The stimulation of root exudation has been suggested to constitute a strategy to mobilize P and other nutrients that are adsorbed to inorganic or organic surfaces [33]. Root exudates are also involved in the recruitment of microorganisms to the rhizosphere that may have P-solubilizing capabilities [34], the ability to produce siderophores or other mechanisms of plant growth promotion under nutrient limitation [35]. The reduced availability of P, confirmed by the low plant P contents, caused the growth reduction of eucalypt seedlings, which may have diverted photoassimilates towards the roots to be exuded as OA, amino acids/polyamines, phenolics, and other compounds not analyzed in this work [9]. OA exudation clearly showed a different pattern in eucalypts plants grown with low and sufficient soil P, as revealed by multivariate analysis (Figure 8), except for *E. acmenoides*, in which root exudation responded in quite a different form to the P concentration (Figure 8).

In this quantity, OAs were more exudated in the eucalypts than phenolics and amino acids/polyamines, which were in much lower amounts. These results are in line with other studies, which showed that OA was the most abundant metabolite class in root exudates of trees [36,37]. Surprisingly, the chemical profile of exudates in this study exhibited a high similarity among the eucalypt species. We expected a larger variation in the compounds exuded by the eucalypt species, as phylogenetic differences in the root chemical compounds were previously found, with more related species having more similar root chemistry [38].

The amount of OA in the exudates increased when seedlings were exposed to low P. With low P, roots exuded an average 30% higher amount of total OA than roots with a sufficient P concentration, but a great intraspecific variation was found. Although, in general, OA exudation was poorly correlated with the P and Mn concentration in the shoots, a closer positive correlation was found under low P, suggesting OA exudation was induced to mobilize P in plants that had lower P concentrations [33,39]. The P and Mn uptake capacity, as measured by the shoot P and Mn concentrations, is considered evidence of the nutrient demand for each species, and may contribute to triggering greater root exudation. Conversely, the Mn concentration in plants has been indicated to be a proxy of root exudation because of its desorption from the soil by OA [3]. Here, while the P and Mn concentrations in eucalypt shoots were positively correlated, the Mn concentration was not correlated with OA exudation since plants were grown with nutrient solution and not in soil. Mn can be present in the soil as insoluble hydrous oxides and can be reduced to soluble Mn^{2+}, a process that can be mediated by OAs [16]. However, Mn in our nutrient solution was supplied in a soluble form, which is the reason why there may have been a low effect of increased exudation on the Mn contents. In the yeast *Saccharomyces cerevisiae*, the high-affinity phosphate transporter PHO84 may function as a low-affinity Mn transporter [40]. In plants, several phosphate transporters have high similarity to PHO84. This is the case of two AtPH1 of *Arabidopsis thaliana* which are very similar to PHO4 [41,42], but they failed to complement the yeast *pho84* mutants. On the other hand, Muchhal et al. [43] could complement a yeast *pho84* mutant with two *A. thaliana* phosphate transporter cDNAs, AtPT1, or AtPT2. Despite that, there is no concrete evidence that the P transporters interact with Mn or that the Mn transporters interact with P. However, a high P concentration disturbs the acquisition of Mn in the barley plants [44].

In studies with rice, a low internal P concentration has been shown to trigger an increased flux of carbon toward OA synthesis by inducing the expression of enzymes of the tricarboxylic acid cycle [45,46]. This response may be related to the release of fixed carbon observed in P-deficient plants [16,47], as OA may accumulate in roots under nutrient deprivation [9].

We identified ten OAs in the root exudates of the studied eucalypt species. However, some were in trace amounts, such as tartaric and malonic acids. The tricarboxylic acids, citric and isocitric, were the major OA found in eucalypt root exudates, followed by

the dicarboxylic oxalic, malic, and succinic acids. Citric, malic, and oxalic have been reported as the most effective OA in mobilizing P [48,49]. However, the observed rapid degradation of di- and tricarboxylate exudates by microbes in the rhizosphere may reduce their effectiveness in mobilizing P for sorption sites [50,51].

In other studies, root exudates of *E. calophylla* and *E. marginata* showed similar OA compositions and quantities, with citric, malic, and succinic acids as the main OAs from those analyzed, while *E. marginata* produced greater amounts of exudates per unit of root weight than *E. calophylla* [52]. Clones of the hybrid *E. grandis* × *E. urophylla* showed the exudation of OA as citrate, oxalate, and malate by roots related to their higher Al tolerance and reduced after the P application [23]. In *E. camaldulensis*, the aluminum tolerance was attributed to the formation of Al^{3+}-citrate complexes within the roots and the low decomposition of citrate [22,53]. However, although citrate and maleic acid exudation was induced by Al in eucalyptus, the tolerance could not be related to the higher presence of these OAs [54].

Interestingly, even at low amounts, lactic acid was the only OA that showed the highest increase in all species, except for *E. saligna*. Such an increase in the exudate of low P plants suggests an effect on the anaerobic root respiration, where ethanol and lactic acid are produced. A P deficiency may decrease aerobic respiration in leaves [55,56] and roots [57]. It was suggested that a low P supply might suppress the cytochrome pathway in the roots of *Phaseolus vulgaris* grown under a P deficiency, restrict the phosphorylating capacity, or cause a partial inhibition of cytochrome oxidase activity [58]. Ethanol was not determined in the roots of the P-stressed eucalypt plants, but it is known that measurable amounts of ethanol may be produced in the roots due to the anaerobic respiratory pathway [59]. Additionally, regarding other nutrient stress, several sequences of the enzymes of anaerobic respiration were induced in roots of iron stress *Arabidopsis thaliana* plants [60]. Roots of *Beta vulgaris* stressed for iron increased the relative amounts of proteins and metabolites associated with anaerobic respiration [61]. Lactic acid is toxic to cellular metabolism, and in plants deprived of oxygen, when it is produced together with ethanol, it is released to the rhizosphere to avoid excessive accumulation [62].

Eucalypt seedlings also released higher amounts of amino acids/polyamines and phenolics when grown with low P. A higher concentrations of phenolics, flavonoids, and other secondary metabolites have been observed to be exuded by roots of nutrient-limited plants [4]. Amino acids, for example, can be found in the rhizosphere due to exudation or the proteolysis of existing peptides. The loss of nitrogen by amino acids exudation has been long established in several species, and although apparently in countersense due to the high energy costs of acquiring and assimilating nitrogen, several nitrogenous compounds play a role in microbes' recruitment or as chelating agents for metal ions in the soil [63,64]. The efflux of amino acids from the roots was initially thought to be by passive diffusion, however, other works suggest that the plants control the amino acid exudation, leading to characteristic distribution and amounts of amino acids [65,66]. Glycine was the most abundant amino acid in the root exudates of the eucalypt seedlings, and for some of the studied species, this amino acid increased under low P concentrations. Glycine was also one of the most exuded amino acids in herbaceous plants such as tomato, maize, white clover, and oilseed rape [65]. Additionally, in the Poaceae *Andropogon virginicus*, P-deficiency induced the exudation of higher levels of glutamine, glutamic acids, valine, and methionine [67]. Glutamine was identified in abundance in the root exudates of *E. urophylla*, mainly at low P. Glutamine and other amino acids can also be exuded by arbuscular mycorrhizal hyphae, suggesting that arbuscular mycorrhizal symbiosis may change root exudation, including in response to P availability [68]. However, putrescine was the most exuded nitrogenous compound by the eucalypt seedlings and among those we analyzed, followed by the amino acid glycine for most species. A high proportion of glutamine and lysine was found in *E. urophylla* under sufficient P conditions. Other polyamines such as agmatine and citrulline were also detected in the root exudates of eucalypts. Putrescine is increased by low-P stress in the roots of *Plantago lanceolata* [69] and

is suggested to be involved in phosphate starvation responses inhibiting root elongation [70]. Polyamines, particularly putrescine, have been related to abiotic stress responses in plants, including nutrient deficiency [71]. Thus, the high levels of polyamines and OA exudated indicate that a low P supply in eucalypts induces a stressing condition in roots.

Phenolics in eucalypt root exudates, in higher amounts in low than sufficient P concentrations, were dominated by cinnamic acid, at least relative to the 14 compounds analyzed here. *C. citriodora* was one of the species with higher amounts of total phenolics in its exudates when growing at low P concentrations and showing a high intraspecific variation. Root exudates and extracts of leaf litter of *C. citriodora* showed inhibitory effects on seed germination, suggesting allelochemical properties commonly attributed to some phenolic compounds [72]. Phenolics had been shown in root exudates of *E. camaldulensis* and suggested to be involved with OA in this species' Al tolerance [53]. In a study comparing root exudates of *E. grandis* monoculture plantations with those present in mixed plantations of *E. grandis* and *Alnus formosana*, metabolite profiling showed that secondary metabolites such as phenolic acids were present at the highest amounts in monocultures compared to mixed plantations that reduced the release of potential allelochemicals [73].

5. Conclusions

Our results showed that a P shortage reduced eucalypt growth and P contents while inducing root exudation, increasing the amounts of OA, amino acids/polyamines, and phenolics compounds in the exudates. Contrary to our expectations of greater differences in the composition profiles of root exudates according to the species identity, eucalypt species showed similar composition patterns of the exudates released by the roots, either under low or sufficient P concentrations. OA were in greater amounts in eucalypt exudates, followed by phenolics and nitrogenous compounds such as amino acids and polyamines in much smaller amounts. While citric/isocitric, oxalic, and malic acids were the main OAs in eucalypts exudates, cinnamic acid was the main phenolic, and putrescine was the main nitrogenous compound among those we analyzed here. Our results also suggest a typical stress condition induced by low P, leading to a diversion of photoassimilates to root exudation as a mechanism to improve phosphorus uptake. The increased exudation in plants of all studied species suggests that this is a conserved genetic mechanism in eucalypts, which should be better studied for the further adaptation of clones in soils with low P availability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092041/s1, Figure S1: Heatmap showing Pearson's correlation coefficients between total organic acid (total OA), shoot biomass (shoot mass), root diameter (root diam.), and P (shoot P) and Mn (shoot Mn) contents in the shoots, in 15 eucalypt species grown under low P and sufficient P conditions (a), only considering low P (b) or sufficient P (c) conditions, Table S1: Percentage of the total phenolics in the root exudates of seedlings of fifteen eucalypt species grown under low and sufficient P concentrations.

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