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Impact of phosphorus application on drought resistant responses of *Eucalyptus grandis* seedlings

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Eucalyptus grandis is the most widely planted tree species worldwide and can face severe drought during the initial months after planting because the root system is developing. A complete randomized design was used to study the effects of two water regimes (well-watered and water-stressed) and phosphorus (P) applications (with and without P) on the morphological and physio-biochemical responses of *E. grandis*. Drought had negative effects on the growth and metabolism of *E. grandis,* as indicated by changes in morphological traits, decreased net photosynthetic rates (P_n), pigment concentrations, leaf relative water contents (LRWCs), nitrogenous compounds, over-production of reactive oxygen species (ROS) and higher lipid peroxidation. However, *E. grandis* showed effective drought tolerance strategies, such as reduced leaf area and transpiration rate (E), higher accumulation of soluble sugars and proline and a strong antioxidative enzyme system. P fertilization had positive effects on well-watered seedlings due to improved growth and photosynthesis, which indicated the high P requirements during the initial *E. grandis* growth stage. In drought-stressed seedlings, P application had no effects on the morphological traits, but it significantly improved the LRWC, P_n, quantum efficiency of photosystem II (F_v/F_m), chlorophyll pigments, nitrogenous compounds and reduced lipid peroxidation. P fertilization improved *E. grandis* seedling growth under well-watered conditions but also ameliorated some leaf physiological traits under drought conditions. The effects of P fertilization are mainly due to the enhancement of plant N nutrition. Therefore, P can be used as a fertilizer to improve growth and production in the face of future climate change.

Abbreviations – Car, carotenoids; CAT, catalase; Chl *a*, chlorophyll a; Chl *b*, chlorophyll b; C_i, intercellular CO_2 concentration; E, transpiration rate; F_v/F_m, maximum quantum efficiency of photosystem II; G_s, stomatal conductance; H_2O_2, hydrogen peroxide; LRWC, leaf relative water content; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NH_4^+, ammonium; NO_3^-, nitrate; $O_2^{\bullet-}$, superoxide anion; P_n, net CO_2 assimilation rate; PCA, principal component analysis; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SP, soluble protein; SRWC, soil relative water content; SS, soluble sugar; TCA, trichloroacetic acid; TW, turgid weight.

Introduction

Altered temperature and precipitation regimes due to climate change are expected to increase the frequency and intensity of future drought, and consequently, the mortality of forests worldwide (Wu, Dijkstra, Koch, Peñuelas, & Hungate, 2011). Forest trees provide a variety of ecosystem services and help alleviate the impact of climate change (Harfouche, Meilan, & Altman, 2014; Neale & Kremer, 2011). The prediction of a global increase in drought stress would likely affect the growth and sustainability of forest trees by disturbing nutrients redistribution in soils (Schimel, Balser, & Wallenstein, 2007). Many studies have explored the effects of nutrients on forest tree species (He & Dijkstra, 2014), but very little attention has been paid to the role of nutrient fertilization in drought tolerance abilities of tree species.

Drought is a primary ecological factor that limits plant growth and development; however, the susceptibility of a plant to drought depends on the plant species, stress degree and growth stage (Demirevska et al., 2009). In plantations, drought is a frequent problem during seedling establishment because plants are exposed to high radiation and air movement when transplanted to the field; therefore, the evapotranspirative demand is high when the root system is small. Drought stress affects water flux and reduces carbon dioxide fixation via biochemical limitations (Flexas, Bota, Loreto, Cornic, & Sharkey, 2004). These changes lead to over-production of reactive oxygen species (ROS) and eventually photoinhibition (Parvaiz & Satyawati, 2008). These ROS, which include superoxide ions ($O_2^{\bullet-}$) and hydrogen peroxides (H_2O_2) under drought stress, result in considerable damage to proteins, DNA and lipids, thereby inhibiting plant growth (Tariq et al., 2017). In response to water deficit stress plants have evolved different protective mechanisms to reduce the damage to photosynthetic machinery from the over-generation of ROS and photoinhibition. These mechanisms include changes in the size and concentration of light-harvesting antennae, upregulation of antioxidant enzyme activities (superoxide dismutase [SOD], peroxidase [POD], catalase [CAT], etc.) and non-enzymatic antioxidant system (glutathione, ascorbate, carotenoids) (Apel & Hirt, 2004). The accumulation of compatible solutes such as soluble sugars (SSs) and proline has also been considered a defense mechanism in plants that utilizes osmotic adjustment to maintain cell turgor, gas exchange and growth under drought stress (Hessini et al., 2009). Moreover, these mechanisms also help protect cell membranes from rupturing and normalizing the functions and structures of enzymes and proteins (Villadsen, Rung, & Nielsen, 2005).

Phosphorus (P) is a major nutrient that is important for plant growth, but its low mobility in soil inhibits its uptake by plant roots. The availability of P has an important relationship with plant metabolism, and, in P-starved plants, photosynthesis is limited by the low concentration of P-containing compounds involved in primary metabolism (Warren, 2011). Moreover, P nutrition is closely related to nitrogen uptake: low availability of P can decrease nitrate uptake (Lambers et al., 2015). Drought affects plant growth by further decreasing P uptake, transport and distribution by decreasing mineral nutrient supply through mineralization, mass flow and diffusion (Rouphael, Cardarelli, Schwarz, Franken, & Colla, 2012) as indicated by the results of different studies (Cramer, Hawkins, & Verboom, 2009; Sardans & Penuelas, 2012). Several studies have suggested that P application enhances the response to drought by improving growth under conditions of water scarcity (Campbell & Sage, 2006; dos Santos, Ribeiro, Oliveira, & Pimentel, 2004; Garg, Burman, & Kathju, 2004). P application has shown significant positive effects on photosynthesis, stomatal conductance, membrane stability and water use efficiency (dos Santos et al., 2004; dos Santos, Ribeiro, de Oliveira, Machado, & Pimentel, 2006; Faustino, Bulfe, Pinazo, Monteoliva, & Graciano, 2013; Naeem & Khan, 2009).

Different mechanisms of drought tolerance have been reported in *Eucalyptus* species (Callister et al., 2008; Whitehead & Beadle, 2004). Morphological adaptations in response to drought stress vary in different *Eucalyptus* species and include limiting water loss by reducing leaf area and/or improving root surface area to enhance water uptake (Pita & Pardos, 2001). Moreover, *Eucalyptus* species reduce water loss through transpiration by closing stomata (Pita & Pardos, 2001; Warren, Bleby, & Adams, 2007), while in some species osmotic changes also contribute to balancing the cell water content under drought stress (Callister et al., 2008). *Eucalyptus grandis* is considered a highly valuable species of the *Eucalyptus* genus and is widely planted (Goncalves, Alvares, & Higa, 2013). *E. grandis* is evergreen, fast-growing, adaptable, commercially important (wood, paper, biofuel and firewood) and is being planted worldwide especially in China, India and Brazil (FAO, 2010, Ni, Zhang, Feng, & Liu, 2007). Due to the current anthropogenic disturbances, the plantation regions of this species are expected to face severe drought in the future (IPCC, 2013). It has already been found that plant species with fast growth rates are more prone to drought stress and nutrient limitations than slow-growing species (Weih, 2001). The adaptable mechanisms of *E. grandis* trees to overcome drought stress are still poorly understood and documented. For instance, research over a short

time-scale (18 days) conducted by Hu, Chen, and Hu (2012) suggested that *E. grandis* grows better under well-watered conditions, and under drought stress it shows tolerance strategies because of its sensitivity. However, it is difficult to clearly investigate the possible effects of drought and the tolerance and avoidance strategies of plants during short experiments. Moreover, the possible role of P fertilization in improving the drought tolerance mechanisms of *E. grandis* has not been studied. Drought and P interaction studies showed that P fertilization increased *E. grandis* growth under well-watered conditions, and the drought tolerance strategies were found to correspond to soil physical and chemical properties (Graciano, Goya, Frangi, & Guiamet, 2006; Graciano, Guiamet, & Goya, 2005). In addition to P application, Battie-Laclau, Laclau, and Domec (2014) revealed that potassium (K) and sodium (Na) application greatly improved the growth and metabolism of *E. grandis* under drought stress. However, the effects of P application on leaf photosynthetic apparatus, osmolyte accumulation, antioxidant system and nitrogenous compound accumulation under drought stress remain unclear. Different studies have suggested that P fertilization improves the drought tolerance of forest tree species by means of physiological and biochemical adjustments (Liu et al., 2015; Tariq et al., 2017, 2018). However, in any of those antecedents, plants responded to P fertilization by increasing growth. In this paper, we chose *E. grandis*, which is a species that has evolved in soils with low availability of P (Attiwill & Adams, 1996; Judd, Attiwill, & Adams, 1996), and is known to exhibit greater growth responses to P fertilization than N fertilization (Graciano et al., 2006). Therefore, the present research was designed to explore the answers to the following questions (1) how does drought stress affect the metabolism of *E. grandis* plants fertilized with P? (2) How does P application improve the drought tolerance ability of *E. grandis* by changes in dry mass partitioning? *E. grandis* is likely to play an emergent role in the future to meet social demands and improve ecosystem services, and its resistance to climate change deserves serious attention. Thus, there is dire need to further understand the tolerance potentials of valuable forest tree species and their responses to P fertilization under water deficit conditions to design proper management and conservation strategies in the face of future climate change.

Materials and methods

Plant collection and experimental design

The experiment was carried out at the Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan Province, southwest China. Uniform healthy seedlings (1-year-old) of *E. grandis* were acquired from Sichuan Agricultural University, Sichuan Province. Seedlings were transferred to plastic pots (10-l) filled with 4 kg of topsoil (pH 7.3; homogenized; 0.19% total nitrogen, 2.67% carbon). The pots were organized in a greenhouse (temperature range: 18–32°C; relative humidity range 50–85%) according to a complete randomized design, and the pots were watered regularly. After 2 months of growth, the seedlings were assigned to four treatments (three replicates/treatment) for 90 days: two water treatments (well-watered and water-stressed) and two P fertilization levels (with and without P fertilization, −P and +P respectively). Before application of treatments, we measured the total P (0.89 g kg^{-1}) and available P (27.6 mg kg^{-1}) in soil, according to the methods in Olsen and Sommers (1982). We extracted available P with 0.5 M of $NaHCO_3$ (pH 8.2) and molybdate-ascorbic acid was used for colorimetric measurement following the methods in Murphy and Riley (1962). The soil relative water content (SRWC) of the two water conditions (control: 80–85%; severe drought: 30–35%) was measured by the weight method (Xu, Zhou, & Shimizu, 2009). The pots were watered and weighed on a daily basis until they reached their corresponding target SRWC to replace the evaporated and transpired water. SRWC was calculated as follows:

$$\mathrm{SRWC} = \left[\left(W_{soil} - W_{pot} - DW_{soil}\right) / \left(W_{FC} - W_{pot} - DW_{soil}\right)\right]^{*} 100$$

where W_{soil} is the current soil weight (soil + pot + water), W_{pot} is the weight of the empty pot, DW_{soil} is the dry soil weight and W_{FC} is the soil weight at field capacity (soil + pot + water).

Sodium di-hydrogen phosphate (NaH_2PO_4, 25.5% P) was supplied as a P fertilizer, with the dose consisting of 129.3 mg P mixed in 200 ml of water per pot, and the fertilizer was applied every 30 days (i.e. three times for the entire experiment). The pots were switched every 5 days to avoid and reduce the systematic errors caused by fluctuations in local environmental conditions. All of the plants were harvested and sampled at the end of the experiment. The upper fully expanded leaves were collected from each plant for physiological and biochemical determination in the laboratory.

Analysis of growth and biomass

Growth was measured by quantifying plant height (cm), leaf area (cm^2) and stem diameter (mm) in a standard way using a measuring tape, leaf area meter and electronic calipers (CI 202 CID Bio Science, Inc., Camas, WA),

respectively. After digging the plants out of the soil, roots, shoots and leaves were detached and a subset was oven-dried at 70°C for 24 h to obtain their dry weights for biomass calculation. The shoot:root and leaf:root dry biomass ratios and Huber values were calculated. The Huber value is the ratio between the surface of the cross section of the active xylem stem and the total leaf area, i.e. the sapwood:leaf area ratio. As the seedlings were small, all of the stem xylems were active.

Determination of leaf relative water content

Fully-expanded leaves from each plant were collected as a single sample and their FW was measured. The samples were then immediately dipped into distilled water, in the dark at a 4°C temperature for 4 h. Turgid weight (TW) of the leaves was taken and then placed in an oven at 70°C for 24 h to obtain their dry weight (DW).

The following equation was used to calculate the leaf relative water content (LRWC) of the samples:

$$\mathrm{LRWC} = \left[(\mathrm{FW}-\mathrm{DW}) / (\mathrm{TW}-\mathrm{DW})\right] \times 100\%$$

Gas exchange and chlorophyll fluorescence measurements

The net CO_2 assimilation rate (P_n), stomatal conductance (G_s) and inter-cellular CO_2 concentration (C_i) and transpiration rate (E) were measured on fully expanded leaves at similar development stages with a portable open-flow gas exchange system (LI-6400, LI-COR Inc., Lincoln, NE). Measurements were performed in the late morning (9:00–11:00 h). The relative humidity of the air, concentration of CO_2 and photon flux density were maintained at 60–70%, 380 μmol mol^{-1} and 800 μmol m^{-2} s^{-1} in all measurements respectively. The same leaves were used to measure the maximum quantum efficiency of photosystem II (F_v/F_m) with a portable pulse amplitude modulated fluorometer (PAM-2100, Walz, Effeltrich, Germany), where the leaves were dark-adapted with clips for 20 min. After this time, minimal fluorescence (F_o) was measured under a weak pulse of modulating light over 0.8 s, and maximal fluorescence (F_m) was induced by a saturating pulse of light (8000 mmol m^{-2} s^{-1}) applied over 0.8 s. F_v/F_m was calculated, where F_v is the difference between F_m and F_o.

Determination of photosynthetic pigments

Two grams of fresh leaves were used to extract chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoids (Car) with 5 ml of 100% acetone. The samples were then kept in the dark at room temperature for 36 h and their respective spectrophotometric absorbance values were measured at 662, 645 and 470 nm (A662, A645 and A470, respectively). The following equations were used to determine the pigment concentrations: Chl $a = 11.75A662 - 2.35A645$; Chl $b = 18.61A645 - 3.96A662$; Car = (1000A470 − 2.27 Chl *a* − 81.40 Chl *b*)/227 (Liu et al., 2014).

Determination of osmolytes and nitrogenous compounds

Leaf extraction was performed in 0.2 g DW in three cycles using 80% ethanol (6 ml) at 80°C for 30 min. SSs of the resulting supernatant were measured according to the anthrone method (Zhang & Qu, 2003). Proline extraction was performed with 10% acetic acid (2 Ml) and 3% sulphosalicylic acid (5 ml). The resultants were analyzed following the methodology described by Liu et al. (2014). Soluble proteins (SPs) were measured with Bradford G-250 reagent. Frozen leaves (0.2 g) homogenized in deionized water (5 ml) were used for NO_3^- concentration, while frozen leaves (0.2 g) homogenized in 2 ml of 10% HCl were used for NH_4^+ concentration. The resulting supernatants were analyzed using the quantitative colorimetric method as described by Tang (1999).

Determination of ROS and lipid peroxidation

To determine the superoxide anion ($O_{2\bullet}^-$) production rate, fresh leaf samples (0.2 g) were taken and homogenized using 65 mM phosphate buffer (2 Ml; pH 7.8) and centrifuged at 5000*g* for 10 min (Elstner & Heupel, 1976). The production rate was monitored by the formation of nitrite from hydroxylamine in the presence of $O_{2\bullet}^-$. The incubation mixture was composed of supernatant (1 ml), 10 mM hydroxylammonium chloride (0.1 ml) and 65-mM phosphate buffer (0.9 ml; pH 7.8). After incubating for 20 min at 25°C, α-naphthylamine (7 mM) and sulfanilamide (17 mM) were supplemented to the incubation mixture and kept at 25°C for 20 min. After that, we added ethyl ether in the same volume and performed a centrifugation at 1500*g* for 5 min and the absorbance measurement was performed at 530 nm.

Determination of hydrogen peroxide (H_2O_2) was performed by observing titanium-peroxide complex absorbance at 410 nm (Patterson, MacRae, & Ferguson, 1984). We homogenized fresh leaves (0.2 g), in acetone (5 ml) and centrifuged them at 3000*g* for 10 min. The reaction mixture was composed of ammonia (0.2 ml), a titanium reagent (0.1 ml) and supernatant (1 ml). It was centrifuged at 3000*g* for 10 min. In the next step, the precipitate was washed five times with acetone and centrifuged for 5 min at 10 000*g*. After that, the precipitate was dissolved using 1-M H_2SO_4 (3 ml), and absorbance was measured at 410 nm.

Lipid peroxidation was determined by quantifying the malondialdehyde (MDA) concentration, following the thiobarbituric acid test, at three different wavelengths (450, 532 and 600 nm) (Zhou, Lam, & Zhang, 2007). Fresh leaves (0.25 g) were homogenized in 5 ml of 1% trichloroacetic acid (TCA) and centrifuged at 5000*g* for 10 min. In the next step, 1 ml supernatant was added to 20% TCA (4 ml; containing 0.5%-thiobarbituric acid) and heated at 95°C for 30 min. After heating, the mixture was immediately subjected to an ice bath and the absorbance was recorded at 450, 532 and 600 nm using a spectrophotometer (Zhou et al., 2007). The following equation was used to calculate MDA concentration: MDA (mol g^{-1} FW) = 6.45(OD532 − OD600) − 0.56OD450.

Determination of antioxidant enzyme activities

For antioxidant enzyme extraction, we took 0.1 g of fresh leaves and ground them with 5 ml of extraction buffer (50 mM Tris–HCl [pH 7.0], 0.1 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM AsA, 1 mM dithiothreitol and 5 mM $MgCl_2$) in a mortar. Then, the homogenates were centrifuged at 10 000*g* for 15 min at 4°C. The resulting supernatants were used to examine antioxidant enzyme activities. SOD activity was checked by examining the photochemical reduction inhibition of nitroblue tetrazolium (NBT) (Fu & Huang, 2001). The reaction mixture (3 ml) was composed of 0.1 mM NBT, 0.1 mM EDTA, 50 mM Tris–HCl (pH 7.8), 13.37 mM methionine, 0.1 mM riboflavin and 0.1 ml enzyme extract. The concentration of enzymes in response to 50% inhibition of NBT photochemical reduction at 560 nm wavelength was defined as one unit of enzyme activity. The Fu and Huang (2001) method was followed to determine CAT and POD activities. CAT activity was quantified by measuring the absorbance reduction in H_2O_2 at 240 nm. Three milliliters of the reaction mixture was composed of H_2O (12.5 mM), EDTA (0.1 mM), Tris–HCl (50 mM; pH 7.0) and enzyme extract (0.1 ml). A 0.01-change in absorbance per min at 240 nm was characterized as one unit of enzyme activity. The activity of POD was determined by examining the increase in absorbance due to the oxidation of guaiacol at a wavelength of 470 nm. The composition of the reaction mixture was guaiacol (10 mM), enzyme extract (0.1 ml), Tris–HCl (50 mM; pH 7.0) and H_2O_2 (5 mM). An absorbance change of 0.1 unit per min was characterized as one unit of POD activity.

Statistical analysis

All measurements were repeated three times, and the data organized in Microsoft Excel 2007 and presented as the mean values ± SE. We used SPSS v16.0 (SPSS Inc., Chicago, IL, 2007) to perform one-way analysis of variance (ANOVA) on the data. Duncan's multiple range test, with an alpha level of 0.05 for significance, was used to make pairwise comparisons of the mean values of a given response variable. Before fitting the ANOVAS, the data were checked for normality and homogeneity of variances. Origin pro v8.5 was used to draw the figure graphics, all of which show bars as the mean ± SE. To discuss all the traits measured in an integrative way, principal component analysis (PCA) was performed in two groups of variables, to simplify interpretation. First, a PCA with morphological variables was performed together with LRWC as a water-stress indicator. Then, a PCA with all physiological variables was performed. In both cases, water and P-fertilization treatments were added as sort criteria to help with the interpretation of graphs. Analysis was performed with InfoStat (Di Rienzo et al., 2015).

Results

Growth traits

Strong reductions were observed in dry mass accumulation and the size of drought-stressed seedlings compared with the well-watered seedlings; however, root biomass differences were not observed (Table 1). Regardless of P application, significant reductions were found in the leaf biomass (46.89%), shoot biomass (46.89%), leaf area (43.97%) and stem diameter (20.54%) of drought-stressed *E. grandis* compared with the well-watered plants. Phosphorus application significantly increased the leaf biomass (22.04%), shoot biomass (22.51%) and stem diameter (17.80%) under well-watered conditions. Moreover, P application slightly increased the height and leaf area, but the differences were not significant. As a consequence, the shoot:root and leaf:root biomass ratios were higher in the P-fertilized than those in the non-fertilized plants. In the case of drought-stressed plants, P application had no significant effects on the morphological traits of the *E. grandis* seedlings. However, the shoot:root and leaf:root biomass ratios were slightly higher in the +P than in those in the −P plants. The Huber values in the fertilized and non-fertilized plants were similar under both water-stressed conditions, but in P-fertilized plants the Huber value was higher in the well-watered plants than that in the drought-stressed plants.

Leaf relative water content, gas exchange, and chlorophyll fluorescence

Irrespective of P application, significant reductions in LRWC (46.40%), P_n (53.99%), C_i (30.52%), G_s

Table 1. Changes in growth and dry mass partitioning in response to P application under well-watered and drought conditions. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Values are means ± SE.

	Well-watered		Water-stressed	
Traits	− P	+ P	− P	+ P
Leaf biomass (g)	14.5 ± 1.5b	18.6 ± 1a	7.7 ± 1c	10.3 ± 1c
Shoot biomass (g)	38.2 ± 2.8b	46.8 ± 2.8a	21.4 ± 1.7c	26.7 ± 1.9c
Root biomass (g)	11.3 ± 0.7a	9.7 ± 0.7a	8.7 ± 0.9a	10.1 ± 0.9a
Leaf area (cm^2)	21.7 ± 1.8ab	23.2 ± 1.2a	13.5 ± 1.4c	17.3 ± 0.8bc
Height (cm)	66.0 ± 4.9ab	72.0 ± 4.8a	50.7 ± 4.3c	53.7 ± 4bc
Stem diameter (mm)	7.3 ± 0.1b	8.6 ± 0.2a	5.8 ± 0.3c	6.1 ± 0.4c
Shoot:root biomass	4.7 ± 0.3b	6.8 ± 0.6a	3.4 ± 0.3c	3.7 ± 0.2bc
Leaf:root biomass	1.3 ± 0.1b	1.9 ± 0.1a	0.9 ± 0.1c	1.0 ± 0.1bc
Huber value	2.0 ± 0.2ab	2.5 ± 0.2a	2.0 ± 0.2ab	1.7 ± 0.3b

Table 2. Changes in leaf relative water content, and the photosynthetic and chlorophyll fluorescence parameters in response to P application under well-watered and drought conditions. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Values are means ± SE.

	Well-watered		Water-stressed	
Traits	− P	+ P	− P	+ P
LRWC (%)	77.06 ± 5.4a	83.46 ± 4.05a	41.3 ± 2.63c	56.66 ± 5.68b
P_n ($\mu mol\ m^{-2}\ s^{-1}$)	8.39 ± 0.53b	11.19 ± 0.53a	3.86 ± 0.21d	5.33 ± 0.41c
C_i ($\mu mol\ mol^{-1}$)	285.7 ± 13.5a	292.6 ± 15.9a	198.3 ± 15.4b	234.8 ± 17.1b
G_s ($mol\ m^{-2}\ s^{-1}$)	0.31 ± 0.01a	0.36 ± 0.031a	0.02 ± 0.01b	0.03 ± 0.01b
E ($mmol\ m^{-2}\ s^{-1}$)	1.87 ± 0.3a	2.0 ± 0.1a	0.52 ± 0.1b	0.87 ± 0.2b
F_v/F_m	0.85 ± 0.02a	0.91 ± 0.03a	0.52 ± 0.02c	0.63 ± 0.01b

(50%), E (72.19%) and F_v/F_m (38.82%) in *E. grandis* were observed under drought stress compared with those in the well-watered conditions (Table 2). Phosphorus application under well-watered conditions had a significant positive effect on P_n (33.37%), while the other traits non-significantly improved. However, P fertilization under drought stress significantly enhanced LRWC (37.19%), P_n (38.08%) and F_v/F_m (21.15%) compared with its non-fertilized counterpart. Additionally, P fertilization slightly improved other physiological traits, but no significant differences were observed.

Biochemical changes

In well-watered conditions, the concentrations of NH_4^+ (90.5%), NO_3^- (68.75%) and SPs (133.9%) were significantly higher than those in drought-stressed seedlings regardless of P application. However, the opposite trend was found for SSs and proline concentrations as their concentrations were significantly higher under drought stress conditions than under well-watered conditions by 54 and 76.5%, irrespective of P application. Phosphorus fertilization significantly increased NO_3^- (14.18%) under well-watered conditions compared with its drought-stressed counterpart. In drought-stressed seedlings, P application significantly improved NH_4^+ (88.09%), NO_3^- (41.25%) and SP (142.12%) concentrations compared with their counterparts. However, there were no significant effects of P fertilization on SSs and proline concentrations under drought stress (Table 3).

Photosynthetic pigments

The photosynthetic pigment concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids (Car) were significantly lower in drought-stressed plants than those in well-watered plants by 46.05, 51.6 and 30% respectively regardless of the P application (Fig. 1). Phosphorus fertilization did not significantly increase the Chl *a* and Car concentrations under well-watered conditions, however, the Chl *b* (34.66%) concentrations were significantly increased in comparison with their counterparts. In drought-stressed plants, P application significantly enhanced Chl *a* (53.68%) and Chl *b* (80.44%) concentrations, but had no significant effect on Car concentration in the drought-stressed plants compared with their counterparts.

ROS production and lipid peroxidation

Lipid peroxidation was significantly higher in drought-stressed plants than that in well-watered

Table 3. Osmolytes accumulation and nitrogenous compounds reduction and assimilation in *E. grandis* for non-fertilized (−P) and fertilized (+P) treatments with and without water stress. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Values are means ± SE.

	Well-watered		Water-stressed	
Traits	− P	+ P	− P	+ P
Soluble sugar (mg g^{-1} DW)	0.50 ± 0.02b	0.52 ± 0.02b	0.77 ± 0.02a	0.91 ± 0.07a
NH_4^+ (mg g^{-1} DW)	0.80 ± 0.11a	0.94 ± 0.08a	0.42 ± 0.04b	0.79 ± 0.07a
NO_3^- (mg g^{-1} DW)	2.27 ± 0.08b	3.15 ± 0.14a	1.59 ± 0.15c	2.27 ± 0.14b
Soluble proteins (mg g^{-1} DW)	58.3 ± 7.5a	68.9 ± 4.3a	24.9 ± 8.4b	60.3 ± 3.8a
Proline (μg g^{-1} DW)	17 ± 1b	11 ± 1b	30 ± 3a	31 ± 3a
Leaf $NH_4^+ + NO_3^-$ content (mg $plant^{-1}$)	51.3 ± 7.6b	75.4 ± 0.9a	15.5 ± 2.9d	31.7 ± 3.2c
Leaf protein content (mg $plant^{-1}$)	874 ± 186b	1271 ± 65c	200 ± 73a	634 ± 108b

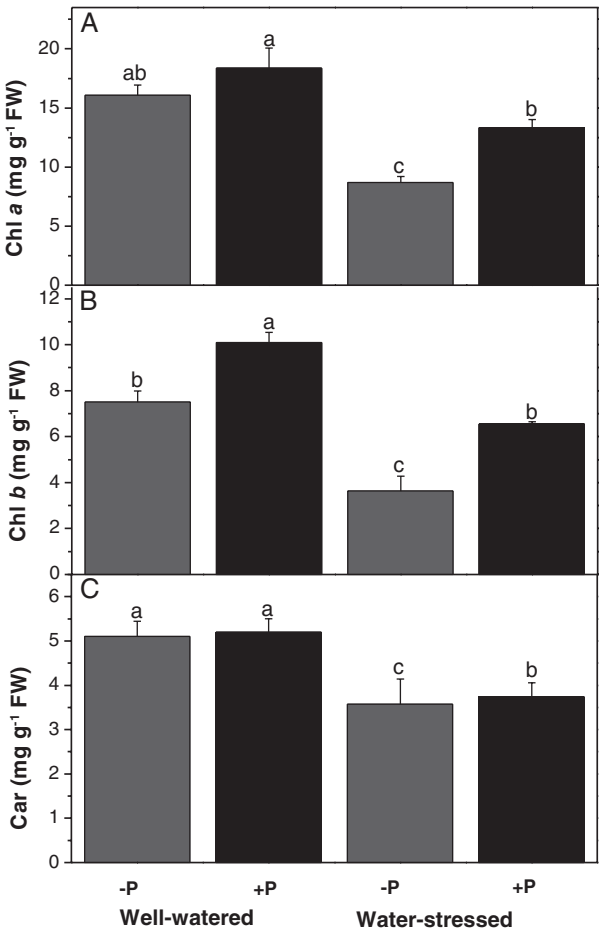

Fig. 1. Changes in chloroplast pigments (Chl *a* A, Chl *b* B and Car C) in response to P application under well-watered and drought conditions. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Vertical bars show ± SE.

plants, as indicated by higher MDA content (103.3%). However, P application significantly reduced the MDA contents (34.47%) in drought-stressed seedlings in comparison with their non-fertilized counterparts. Moreover, the H_2O_2 and $O_2^{\bullet-}$ production rates were significantly higher (24.75 and 50% respectively) in the drought-stressed plants than those in the well-watered, non-fertilized plants. However, the P application significantly decreased (50%) the $O_2^{\bullet-}$ level in plants under drought stress when compared with their non-fertilized counterparts, while it slightly decreased the H_2O_2 level (though not significantly) (Fig. 2).

Antioxidant system

Antioxidant enzyme (SOD, POD and CAT) activities were significantly higher (46.45, 228.57 and 60% respectively) in drought-stressed seedlings than those in well-watered seedlings regardless of P application. P fertilization had no significant effects on SOD, POD and CAT enzyme activities under well-watered conditions; however, it significantly reduced (28.28, 34.78 and 71.42% respectively) their activities in drought-stressed seedlings compared with their non-fertilized counterparts (Fig. 3).

Correlation between LRWC and morpho-physiological traits under drought

Plant growth was greatly improved with P fertilization, and a significant positive correlation was observed between all the morphological traits and LRWC (Fig. 4). However, there was no correlation between root biomass and Huber value with LRWC (Fig. 4A). A negative correlation was observed between SSs, proline and antioxidant system with LRWC (Fig. 4B).

Discussion

Morphological response of *E. grandis*

Drought is one of the major stresses that restrict the growth and productivity of forest trees around the world (Sankar et al., 2007). In the present

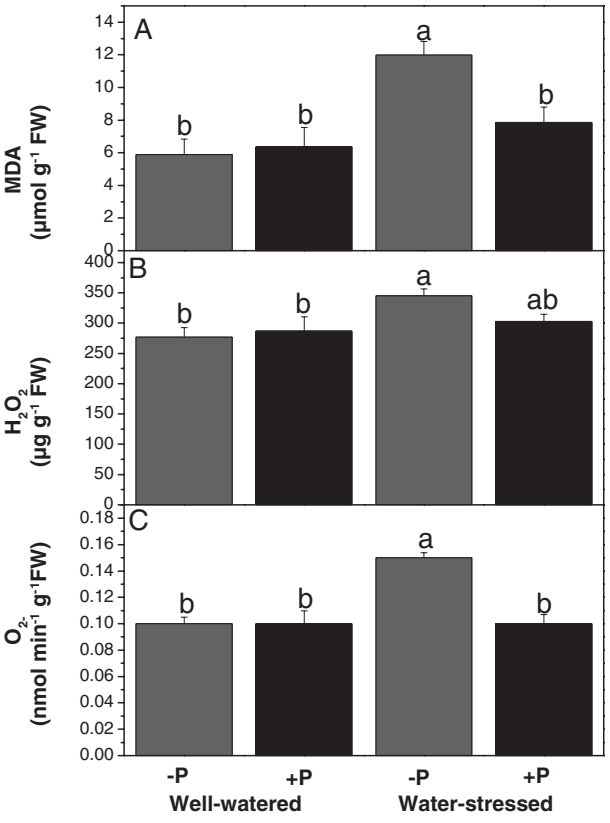

Fig. 2. Changes in lipid peroxidation (MDA, A), superoxide anion ($O_2^{\bullet-}$, A), and hydrogen peroxide (H_2O_2, C) in response to P application under well-watered and drought conditions. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Vertical bars show ± SE.

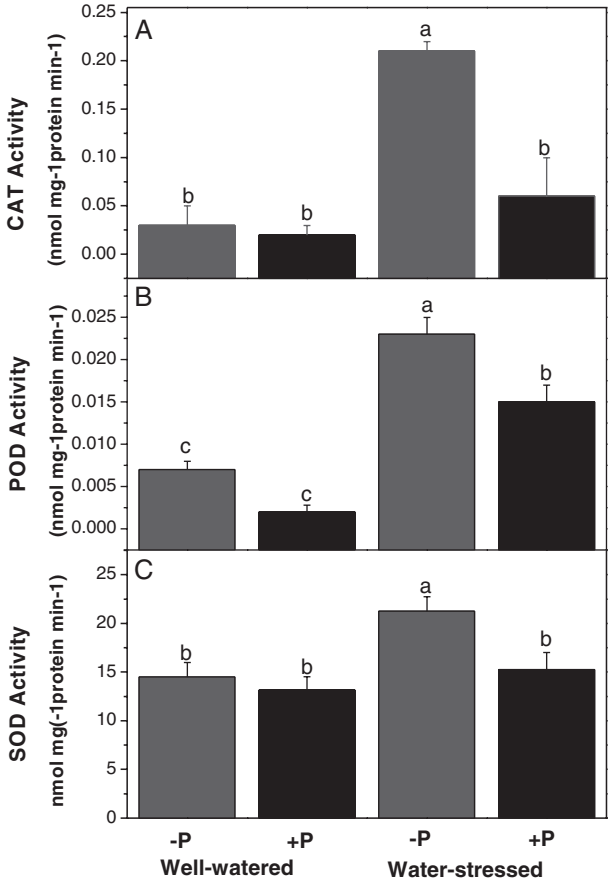

Fig. 3. Changes in catalase (CAT, A), peroxidase (POD, B), and superoxide dismutase (SOD, C) in response to P application under well-watered and drought conditions. The means followed by different letters indicate significant differences ($P \leq 0.05$) among the four treatments according to Duncan's test. Vertical bars show ± SE.

investigation, a strong reduction in the growth of drought-stressed seedlings of *E. grandis* was observed in comparison with well-watered plants, regardless of P application (Table 1). Water deficits in the soil adversely affect the availability and distribution of ions and nutrients, microbial activities and ultimately cell growth. Previous studies have suggested that P uptake by plant roots further decreases under water deficit conditions (Cramer et al., 2009; Sardans & Penuelas, 2012) and that plant growth is dependent on the availability of P. Our findings also revealed that height, stem diameter, leaf area, stem and leaf biomass and LRWC were significantly lower in drought-stressed seedlings than those in with well-watered plants which indicates strong sensitivity of *E. grandis* to water deficits in the soil (Tables 1 and 2). The present findings are in line with other studies that were conducted on different forest tree species (Liu et al., 2015; Oliveira, Medeiros, Frosi, & dos Santos, 2014; Tariq et al., 2017, 2018). However, root biomass was similar in the drought-stressed and well-watered plants, regardless of P application. As shoot dry matter was reduced by drought, the maintenance of root size implies lower shoot:root and leaf:root biomass ratios. The reductions in these ratios are an effective drought adaptive/tolerant strategy to alleviate the evaporative losses and water demands of the root system. The reduced leaf area in *E. grandis* seedlings is an effective drought tolerance strategy that is utilized to reduce the transpiration rate (Hu et al., 2012). Our findings clearly indicate that *E. grandis* is sensitive to drought stress, as indicated by the overall growth reduction; however, it has good drought tolerance strategies by maintaining high root biomass and reducing leaf area to cope with water deficit stress. Drought stress generally limits the acquisition of nutrients and their transportation to shoots (Garg, 2003). Phosphorus application had no significant effects on the morphological traits of *E. grandis* under drought stress. However, P application significantly

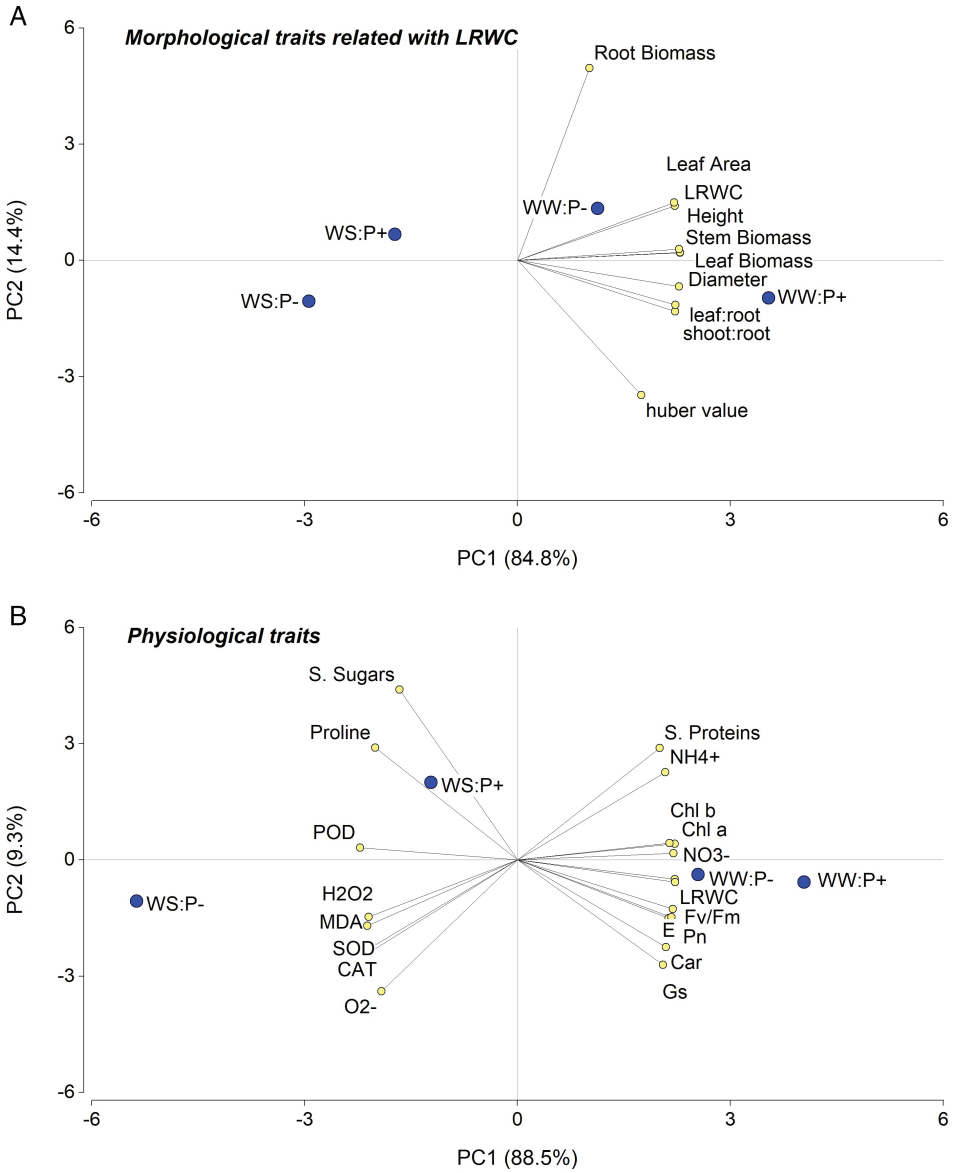

Fig. 4. Principal component analysis divided into (A) morphological traits related to LRWC and (B) physiological traits in *E. grandis* plants fertilized with P (P+) and non-fertilized (P–) exposed to well-watered (WW) or water-stress (WS) conditions. Vectors in the same sense and direction are positively correlated. Vectors in the opposite sense are negatively correlated. Orthogonal vectors are not correlated. Points indicate the position of the mean of each treatment with respect to the mean of all treatments (0,0).

increased the stem diameter and leaves and shoot biomasses under well-watered conditions indicating its high P requirements (Thomas, Montagu, & Conroy, 2006) and that P mobility is strongly reduced in dry soils (Singh & Singh, 2004). It has already previously been found that the demand for P is high during the first year of growth, while the demand is lower in later stages, because of internal recycling (Fernandez, Dias, Barros, Novais, & Moraes, 2000). The present findings are in line with the results of previous studies (Cicek, Yilmaz, Tilki, & Cicek, 2010), but contradicted the results of other studies conducted on other forest tree species (Liu et al., 2015; Tariq et al., 2017, 2018). The lack of effect of P fertilization under drought indicates that P uptake was more limited and difficult under low moisture content in the soil than in well-watered conditions and demonstrates that different species respond differently to P fertilization because they have varied physiological adjustments, nutrient availability and interaction and soil physical properties.

The Huber value reflects the capacity of the plant to adjust its biomass partitioning between xylem and leaves to maintain homeostasis in leaf water relations (Carter & White, 2009). We found that plants adjusted the proportion of xylem area to leaf area and maintained a nearly constant ratio. Compared with well-watered P-fertilized plants, the only significant decrease in Huber value was in the plants under drought conditions that were

fertilized with P (Table 2). This decrease could compromise LRWC, as a smaller xylem area should feed a higher proportion of leaves. However, under drought conditions, LRWC was higher in P-fertilized plants than that in non-fertilized plants. Therefore, despite the apparent negative effect of P fertilization on biomass partitioning between leaves and stems, P-fertilization enabled plants to maintain high LRWCs under drought. This result is possibly related to the higher accumulation of osmolytes in plants fertilized with P than in non-fertilized plants (Table 3), as will be discussed in the following sections.

LRWC, gas exchange, chlorophyll fluorescence and photosynthetic pigments

Maintaining high water potential is a great challenge to forest tree species because it is imperative to sustain normal growth and development. LRWC is considered an effective indicator of hydration capacity and tolerance of plant species (Parker, Pallardy, Hinckley, & Robert, 1982). As expected (Oliveira et al., 2014; Siddique, Hamid, & Islam, 2001), under drought stress, LRWC was significantly reduced in *E. grandis* seedlings compared with that in well-watered plants (Table 2). We found that P fertilization significantly improved the LRWC in drought-stressed plants with respect to non-fertilized plants in the same water condition, which is most likely due to the high concentrations of ions (such as NO_3^- and NH_4^+) and SP in leaves that contribute to diminishing the osmotic potential and enhancing water movement to leaves (Table 3). Various studies have shown that P fertilization can improve LRWC either improving the ability of roots to extract water, and other nutrients or by improving water conservation in plant tissues (Costa, Faustino, & Graciano, 2017, Graciano et al., 2005, Shubhra, Goswami, & Munjal, 2004). However, our findings are contradictory to several studies that suggested non-significant effect of P on the LRWC under drought stress (dos Santos et al., 2004; Liu et al., 2015). The differences in the responses are associated with different physiological, biochemical and molecular mechanisms against drought conditions. A significant reduction in the rate of CO_2 assimilation (P_n) was observed in drought-stressed seedlings compared with well-watered seedlings regardless of P application, which might be associated with a decline in LRWC and other factors. Stomatal closure is considered a key factor behind declined photosynthesis under drought stress conditions (Anjum et al., 2011). The reduction in G_s limits its gas exchange, results in a decline in the photosynthetic rate due to a significant drop in Rubisco activity (Reddy, Chaitanya, & Vivekanandan, 2004). Moreover, our findings also revealed significant decreases in G_s and C_i under water deficit conditions in non-fertilized plant seedlings as well as in fertilized seedlings. We found that the decline in P_n was related to not only a decrease in G_s but also an impaired photosynthetic apparatus, as indicated by the decline in F_v/F_m under water-stress conditions. Factors such as reduced LRWC, G_s, C_i and F_v/F_m might be responsible for reduced photosynthesis (Table 2) in drought-stressed seedlings and ultimately reduced plant growth (Table 1). Moreover, we found that P fertilization significantly improved the P_n rate in well-watered plants. In drought-stressed seedlings P application improved P_n and F_v/F_m but had no significant effects on stomatal conductance and transpiration rate. This result clarifies that P application can improve the drought tolerance potential of *E. grandis* by conserving water, as indicated by the high LRWC (Table 2) and the high uptake and assimilation of N (Table 3). Previous studies have reported increased P_n rates in different plant species under P fertilization, and these results are in line with our findings (Burman, Garg, & Kathju, 2009; Liu et al., 2015; Singh, Badgujar, Reddy, Fleisher, & Bunce, 2013). The possible reason behind the high P_n rate in response to P fertilization might be due to the improved LRWC, F_v/F_m and concentration of photosynthetic pigments (Fig. 1), which may be related to the higher concentrations and contents of inorganic N forms and SPs (Table 3). Our results showed that drought stress significantly diminished photosynthetic pigment concentrations (Chl *a*, Chl *b* and Car), which could be another reason for the low P_n rate under drought conditions (Frosi, Oliveira, Almeida-Cortez, & Santos, 2013; Rivas, Oliveira, & dos Santos, 2013) because leaf dehydration causes damage to lamellae vesiculation and chloroplast membranes (Anjum et al., 2011). Moreover, P application significantly improved Chl *a* and Chl *b* concentrations and resulted in a high P_n rate in fertilized drought-stressed plants because leaves with high chlorophyll concentrations can harvest a large amount of light over a short period of time. However, P application had no effects on Car concentration under any water treatment. Our results support those found in other studies (Sharma, 1995; Sinha, Sakal, & Kumar, 1995), but they are in contradiction with other results found with different severities and durations of water deficit conditions (Campbell & Sage, 2006; Singh et al., 2013; Zhang & Kirkham, 1996).

Osmolyte accumulation and nitrogenous compound assimilation

The accumulation of different types of solutes (organic and inorganic) helps plants to maintain the osmotic potential of the cell (Farooq, Wahid, Kobayashi, Fujita,

& Basra, 2009). Leaf turgor pressure can be maintained by accumulating proline, SSs and other solutes. Our findings showed that under drought stress conditions proline and SS concentrations were significantly higher than those in well-watered conditions (Table 3). These results are expected because increased SS concentrations can help plants preserve membrane integrity by maintaining the hydrophilic interaction between proteins and membranes (Hoekstra, Golovina, & Buitink, 2001). Similarly, a high accumulation of proline suggests osmotic adjustments under drought stress and may also help to detoxify ROS, resulting in membrane and macromolecule protection (Keunen, Peshev, Vangronsveld, Ende, & Cuypers, 2013). P application slightly improved these solute concentrations but had no significant effect on the drought-stressed seedlings. A constantly high level of these solutes with P fertilization could be a different strategy to maintain osmotic adjustments, membrane stabilization and protect macromolecules to cope with drought stress. Nitrogen is considered an essential nutrient for plant growth and metabolism since it is a part of the chlorophyll, amino acids, nucleic acid and proteins.

Drought stress is believed to decrease N uptake and the production of nitrogenous compounds (Garg et al., 2004; Lawlor & Cornic, 2002). Under drought stress, the concentrations of NH_4^+, NO_3^- and SPs significantly decreased compared with those in well-watered plants regardless of P application (Table 3) which might be related to the high activities of protease enzymes and the decline in the synthesis of proteins, as well as the lower P_n, i.e. less carbon to build metabolites. However, P application under drought conditions significantly improved NH_4^+, NO_3^- and SPs, which may be related to the up-regulation of associated enzymes activities (Burman et al., 2009; Burman, Garg, & Kathju, 2004). P application had a positive influence on the nitrogenous compounds under well-watered conditions due to a sufficient supply of nutrients and appropriate water level. Similarly, P fertilization enhances N uptake and accumulation even more than N fertilization in *E. grandis* plants growing on sandy and clay soils (Graciano et al., 2006). The increased assimilation of N can be related to small changes in the architecture and hydraulic conductivity of the roots (Costa et al., 2017). Our findings suggest that P application can maintain or enhance drought tolerance of *E. grandis* by maintaining high osmolyte concentrations and improving the reduction and assimilation of nitrogenous compounds. Additionally, our results highlight the interaction between mineral nutrition in plants, and the relationship between nutrient uptake and soil water availability.

Lipid peroxidation, ROS production and antioxidant system

The exposure of plants to drought stress often results in the generation of ROS such as anion radicals (O_2^-) and hydrogen peroxide (H_2O_2), which may react with lipids, proteins and DNA and cause oxidative damage and disrupt the normal functions of cells (Foyer & Fletcher, 2001). Our results showed that in drought-stressed seedlings, the levels of O_2^- and H_2O_2 were significantly higher than those in well-watered seedlings, irrespective of P application (Fig. 2), which might be due to a decline in the photosynthetic rate and other physiological impairments. Enhanced production of ROS caused damage to membrane lipid peroxidation as indicated by the significantly higher MDA content in the drought-stressed seedlings than that in well-watered seedlings. ROS not only cause lipid peroxidation, but also cause damage to proteins and inactivate enzymes involved in different physiological processes (Sairam, Rao, & Srivastava, 2002). Plants have a very specialized enzyme-catalyzed defensive system for ROS scavenging that is used to avoid injuries caused by ROS under drought stress. Antioxidative enzyme (SOD, POD and CAT) activities were significantly higher in drought-stressed seedlings than those in well-watered seedlings (Fig. 3). P fertilization had no effects on antioxidative enzyme activities under well-watered conditions but it significantly reduced their activities as well as the MDA level in drought-stressed conditions. The present findings suggest that *E. grandis* has a strong antioxidant defense mechanism to scavenge ROS and reduce damage caused by oxidative stress, while P application helps to reduce damage caused by ROS as indicated by reduced MDA and improvement to other physiological processes.

Relationship of LRWC with morpho-physiological adjustments under drought

As previously stated, LRWC is a trait that describes the water status of a plant. Application of P improved growth of plants might be due to a positive correlation between morphological traits and LRWC (Fig. 4). However, lack of correlation between root biomass and Huber value with LRWC indicates that there were physiological mechanisms that enabled plants to maintain their LRWC, without exploring constant volume and disrupting water conducting structures of xylem in P fertilized plants. As the shoot:root and leaf:root ratios were similar in fertilized and unfertilized drought-stressed plants, the effects of P in improving the LRWC must be due to physiological changes at the leaf level. The main physiological adjustment was the accumulation of osmolytes

(SSs and proline) and improved activities of antioxidative enzymes because a negative correlation was observed between these traits and LRWC. Active antioxidant system can avoid the possible damage caused by elevated level of ROS associated with decreased LRWC. The positive effect of P-fertilization was mainly due to improved N nutrition, as indicated by high concentrations and contents of inorganic N forms and leaf proteins. Subsequently, P fertilization enabled the reduction in the concentration of oxidative compounds in leaves than in non-fertilized plants, which is probably associated with high LRWCs under drought conditions. However, P-fertilization had an additional effect of accumulating proline and SSs under drought stress. In summary, P-fertilization improved plant growth and N nutrition while increased leaf:root ratio could have produced an imbalance in water transportation to leaves during dry days. As a consequence, if a shortage in soil water availability is imposed, the leaf water content can be maintained by better nutrition in leaves, which alleviates oxidative stress and improve photosynthesis than in non-fertilized plants.

Conclusions

Drought has negative effects on the growth and metabolism of *E. grandis* as indicated by reduced height, stem diameter, leaf and shoot biomass, photosynthesis, photosynthetic pigments (Chl *a*, Chl *b* and Car) and LRWC, upregulation of ROS and increased MDA levels. However, reduced leaf area and stomatal conductance, improved solutes (SS, proline) and nitrogenous compounds (SPs, NH_4^+ and NO_3^-) concentrations, and upregulation of antioxidant enzymes (SOD, POD and CAT) suggest that the effective drought tolerance strategies of *E. grandis* are effective. Moreover, P fertilization had positive effects on the growth and photosynthesis of *E. grandis* indicating its high P requirements during the seedling stage. In drought-stressed seedlings, P application had no significant effects on the morphological traits; however, it significantly improved LRWC, photosynthetic rate, maximum quantum efficiency of photosystem II, photosynthetic pigments and nitrogenous compounds and reduced lipid peroxidation. The present study suggests that P application plays an important role in growth and metabolism under well-watered conditions and helps to maintain the drought tolerance of *E. grandis* mostly through physiological rather than morphological adjustments. P fertilization is highly recommended for *E. grandis* plantations to improve their production and tolerance under well-watered and drought-stressed conditions.

Author contributions

A.T. and K.P. designed the study including experimental design. A.T., Z.L., M.A.D., O.A.O. and L.N. carried out the physiological studies. A.T. analyzed the data and drafted the manuscript. C.G., X.S., L.Z., F.S., K.P., O.A.O., X.W. helped in analyzing data and revising the draft. L.N., D.S., M.A.D., Z.L. contributed reagents, materials and analysis tools.

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