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Research article

Manganese toxicity amelioration by phosphorus supply in contrasting Mn resistant genotypes of ryegrass

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ABSTRACT

We evaluated whether phosphorus (P) ameliorates manganese (Mn) excess harmful effects on photosynthetic performance, growth, oxidative stress, and antioxidants in ryegrass. Two perennial ryegrass genotypes, Banquet-II as Mn-resistant and One-50 as Mn-sensitive genotype, were growth under hydroponic conditions subjected to increased P (25, 50, 100, 200 and 400 μM), excess (750 μM) and sufficient Mn (2.4 μM) for 15 days. Growth rate, lipid peroxidation (LP), enzymatic and non-enzymatic antioxidants, photosynthetic parameters, and pigments were determined. Significant reduction of photosynthesis and growth in One-50 was observed under Mn-excess combined with low and adequate P, recovering under greater P-doses. The P concentration of both genotypes was enhanced towards increased P-supply, regardless of Mn treatments. Shoots Mn-concentration remained constant in both genotypes under Mn-excess, independently of P-levels; meanwhile, Banquet-II roots Mn-concentration increased 23% by P-supply. Furthermore, Banquet-II roots showed higher superoxide dismutase (SOD) activity than One-50, which increased towards the highest P dose under sufficient and excess of Mn. A high dose of phosphorus amendment alleviated Mn-toxicity in Mn-sensitive genotype (One-50). Besides, in the Mn-resistant genotype, enhanced plant performance is highlighted, explained by a high Mn-accumulation in roots and increased SOD activity, decreasing Mn translocation to shoots and therefore protecting the photosynthetic apparatus.

1. Introduction

Phosphorus (P) and manganese (Mn) are essential nutrients for plant growth and development (Millaleo et al., 2010), participating both in important metabolic processes. As an essential micronutrient, Mn takes place actively in photosynthesis by forming part of the structure of proteins in the oxygen-evolving complex, participating in the H_2O photolysis, electron transport and also as an enzyme antioxidant-cofactor (Goussias et al., 2002; Millaleo et al., 2010). Deficiency of Mn in plants affects the water-splitting systems of photosystem II (PSII), whereas Mn excess can lead to damages of the photosynthetic machinery, specifically PSII subunits (Millaleo et al., 2010). Both excess and deficiency of Mn result in alterations of the photosynthetic rate and enzyme activities, as well as absorption, translocation, and utilization of other mineral elements, such as Ca, Mg, Fe and P (Huang et al., 2016).

In volcanic ash-derived soils, the concentration of Mn^{2+} (the available form of Mn) increases under low pH and reduced conditions in the soil solution, becoming potentially toxic for plants (George et al., 2012; Mora et al., 2006). The excess of Mn may increase the production of oxygen reactive species (ROS) (Boojar and Goodarzi, 2008), provoking oxidative stress, which leads to damage to macromolecules and

Abbreviations: P, phosphorus; Mn, Manganese; SOD, superoxide dismutase; LP, lipid peroxidation; ROS, oxygen reactive species; RGR, relative growth rate; TBARS, thiobarbituric acid reacting substances; MDA, Malondialdehyde; DPPH, 2.2-diphenyl-1-picrylhydrazyl; NBT, nitro blue tetrazolium; FDR, False discovery rate; PCA, Principal Component Analysis; A, photosynthetic rate; g_s, stomatal conductance; T, transpiration; ΦPSII, effective photochemical efficiency; ETR, electron transport rate; NPQ, non-photochemical quenching; Chl $a+b$, total chlorophylls; Chl a/b, chlorophyll a/b ratio; β-car, β-carotene; Vio, violaxanthin; Neo, neoxanthin; Lut, lutein; RSA, radical scavenging activity; TP, total phenols

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disturbance of metabolic pathways, disruption of cellular homeostasis and physiological processes (Hegedus et al., 2001). As a consequence, oxidative stress is generated and lipid peroxidation (LP) of biomembranes occurs in the cells, leading to disorganization of membrane structure and loss of photochemical activities (Inostroza-Blancheteau et al., 2017; Mora et al., 2009). To alleviate oxidative stress induced by Mn toxicity, plants have developed antioxidant defense systems with the relevant capacity to scavenge ROS and chelate metals, which involves secondary metabolites such as phenolic acids and flavonoids and antioxidant enzymes such as superoxide dismutase (SOD) (Michalak, 2006). The contribution of phenolic acids in minimizing Mn toxicity in *Lolium perenne* L. was recently reported (Inostroza-Blancheteau et al., 2017). Likewise, in roots from soybean plants cultivated under the toxic concentration of Mn, an increase in SOD and peroxidase (POD) activities were proposed as the main physiological responses at high Mn uptake (Santos et al., 2017).

Phosphorus, another key nutrient for plants, is often considered as a limiting nutrient for plant growth and development (Marschner, 2012). In Andisols, the amount of total P is generally high, but due to the high adsorption of the available P-forms (HPO_4^{2-} and $H_2PO_4^{-4}$) to Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+} cations in soil, its availability for plants is considerably reduced (Shen et al., 2011). It has been shown that high P concentration in the soil increases the Mn uptake by roots of *Solanum tuberosum* under Mn deficiency conditions (Marsh et al., 1987). On the other hand, in *Hordeum vulgare* cultivated under elevated P condition, the Mn uptake by roots is impaired (Pedas et al., 2011). Similarly, other studies indicated that P could reduce Mn toxicity in *Glycine* max (Nogueira et al., 2004), *S. tuberosum* (Sarkar et al., 2004), *L. perenne* and *Trifolium repens* (Rosas et al., 2011). In agreement, in *Pseudotsuga menziesii* low P supply partly alleviated the negative Mn effects on biomass production (Ducic and Polle, 2007). However, despite the numbers of evidence supporting the positive effects of P supply on Mn plant toxicity, the interactions between Mn and P are still not well understood. Our current knowledge about the mechanisms of Mn and P interaction and the role of P in regulating Mn accumulation and toxicity in forage species is limited.

In the Andisols of southern Chile, the perennial ryegrass (*L. perenne*) is a major cultivated forage grass species. This species is very appreciated because of its high yield potential (Rosas et al., 2011). Recently, new genotypes were introduced in southern Chile, and the toxic effects of Mn on physiological, biochemical, and molecular attributes have been studied (Inostroza-Blancheteau et al., 2017; Reyes-Díaz et al., 2017). These studies revealed a differential degree of Mn-resistance between genotypes, appearing Banquet-II as the most resistant and One-50 the most sensitive genotype. Despite the great interest in P as a factor that influences Mn-absorption and toxicity on plant metabolism and functionality, little is known about the mechanism involved in the resistance capacity of the new Mn-contrasting cultivars of ryegrass. Moreover, there are no reports regarding the P influence on Mn excess in Banquet-II (Mn-resistant) and One-50 (Mn-sensitive) genotypes. Thus, the following hypothesis was stated: a high P nutrition ameliorates Mn-toxicity effects, decreasing oxidative stress and improving plant performance, mainly in the Mn-sensitive ryegrass genotype. Therefore, this study aimed to evaluate whether P ameliorates the harmful effect of Mn-excess on growth, photosynthetic performance, oxidative stress and antioxidants in contrasting Mn-excess resistant ryegrass genotypes and understand the mechanisms of Mn toxicity resistance mediated by P, displayed by the same genotypes.

2. Materials and methods

2.1. Plant material and growth conditions

For this study, two perennial ryegrass genotypes with contrasting resistance to Mn excess were selected and used (Banquet-II as Mn-resistant and One-50 as Mn-sensitive genotype) based on previous studies (Inostroza-Blancheteau et al., 2017). The seeds were surface sterilized and germinated on paper moistened with deionized water in a growth chamber with 16 h light at 20 °C. Seven-day-old seedlings were transferred to 7 L pots (112 seedlings per pot) containing aerated nutrient solution (Taylor and Foy, 1985) for two weeks. After that, the nutrient solution was supplied with five P treatments (25, 50, 100, 200, and 400 μM P), supplied as K_2HPO_4, in combination with two Mn concentrations, a sufficient Mn (2.4 μM) and an excess (toxic) Mn (750 μM). Concentrations of 2.4 μM of Mn and 100 μM P were considered as control. The first concentration is described as an optimal Mn supply for ryegrass (Rosas et al., 2007) and the second as an adequate P supply for the Taylor and Foy (1985) solution. To balance the increment of K with the increased treatments of P, KCl was added to the solution (Rosas et al., 2011). The excess or toxic Mn concentration was previously determined by our research group (Inostroza-Blancheteau et al., 2017).

The nutrient solution was aerated and renovated every three days, and the pH was adjusted daily to 4.8 (Rosas et al., 2011). The experiment was performed for 15 days, and after this period, photosynthetic related parameters were evaluated. Besides, shoots and roots were harvested from the same plants in the middle of the light period, snap-frozen in liquid nitrogen and stored at −80 °C for biochemical analyses.

2.2. Plant biometric growth parameters

Growth parameters were analyzed according to Hoffmann and Poorter (2002) and expressed as main relative growth rate (RGR) = (lnW2)-(lnW1)/(*t2*–*t1*) being *t1* and *t2*, 0 and 15 days, respectively. Shoots and roots of 20 seedlings were weighed (W1) and dried before the beginning of treatments. At the end of the experiment (15 days) samples from each pot were weighed (W2) and dried in a forced-air oven for 2 day at 65 °C.

2.3. Determination of Mn and P concentration

Dry samples of shoot and roots were burned to ashes for 8 h at 500 °C and later digested with 2 M HCl (Sadzawka et al., 2007). The Mn concentration was determined using a simultaneous multi-element atomic absorption spectrophotometer (Model 969 atomic absorption spectrometer, Unicam, Cambridge, UK). Phosphorus concentration was determined using the molybdenum blue assay as previously described (He and Honeycutt, 2005).

2.4. Measurements of photosynthetic performance

The photosynthetic performance was determined through the CO_2 assimilation and the photochemical efficiency of PSII. The net CO_2 assimilation (*A*) was measured on attached leaves using a portable CO_2 infrared gas analyzer (Licor 6400) equipped with a cuvette, which controlled the light source (300 μmol photons $m^{-2}s^{-1}$), temperature, humidity and CO_2 itself (400 ppm), with a flow rate of 200 mL min^{-1} and 80% external relative humidity. The temperature inside the leaf chamber was maintained at 20 °C. The photochemical efficiency of PSII was determined by the *in vivo* fluorescence emission of chlorophyll *a* on intact leaves with a portable modulated fluorescence system FMS 2 (Hansatech Instruments, King's Lynn, UK), according to the methodology described earlier (Reyes-Díaz et al., 2009).

2.5. Pigments analyses

Chlorophylls, carotenoids and determinations were performed in phase reversed, solvent gradient, high performance liquid chromatography (HPLC, Agilent Technologies Inc., San Jose, California, USA) following the protocol described by (García-Plazaola and Becerril, 1999).

2.6. Antioxidant related parameters

As oxidative stress indicator, the lipid peroxidation (LP) of membranes was determined in fresh material using thiobarbituric acid reacting substances (TBARS) assay, according to Du and Bramlage (1992). Malondialdehyde (MDA) product was measured at 532, 600, and 440 nm to correct the interference generated by TBARS-sugar complexes.

The radical scavenging activity (RSA) of shoots and roots were tested in methanolic extracts using the free radical 2.2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method as described by Chinnici et al. (2004). The absorbance was measured at 515 nm in a spectrophotometer (UNICOR 2800 UV/VIS, Spain) using Trolox as the standard. Total phenols (TP) concentration was determined following the previously described procedure (Slinkard and Singleton, 1977). The total SOD was extracted and assayed according to Giannopolitis and Ries (1977) by monitoring the superoxide radical-induced nitro blue tetrazolium (NBT) reduction at 560 nm.

2.7. Statistical analyses

The experimental design was a complete randomized block with two genotypes, ten treatments, and three replicates. Each replicate consisted in one pot containing 112 plants. The effects of different Mn and P supplies were assessed by two ways ANOVA, where factors were genotypes and treatments. Significance was assigned at $P < 0.05$ with Tukey's test. All the analyses were performed using Sigma Stat 4.0 software (SPSS, Chicago).

The relationships among variables were examined using Pearson correlation analysis at a significance level of $P < 0.05$. The resulting P-values were corrected using False discovery rate (FDR) script displayed by the Rbio software (www.biometria.ufv.br). Moreover, a Principal Component Analysis (PCA) was performed to reduce the dimensionality of the data set and identify the variables that explained a higher proportion of the total variance (Minitab® 18.1, Minitab Inc., Philadelphia).

3. Results

3.1. Effect of P and Mn treatments on plant relative growth rates

Significant interactions between genotype and treatment for shoots ($P < 0.001$) and roots ($P = 0.016$) were found for the RGR. In both genotypes, tissues subjected to sufficient Mn and increased P-doses did not change their shoot RGR compared to control the control treatments (Fig. 1). However, a significant reduction of shoots RGR in One-50 (Mn-sensitive genotype) were observed under Mn excess combined with low P (25–50 μM) and adequate P concentrations (100 μM), relative to control values ($P < 0.05$) (Fig. 1). Banquet II showed to decrease its RGR under Mn excess only when P was low. Despite this growth reduction in both genotypes, One-50 exhibited higher RGR under a combination of Mn excess with greater P-doses (200 and 400 μM), recovering values similar to the control.

Roots RGR of the Mn-resistant genotype (Banquet-II) exceeded those of the Mn-sensitive One-50 genotype in all treatments ($P < 0.05$). Compared with the control, RGR of One-50 roots was steadily reduced under sufficient Mn and different P treatments, being the lowest RGR under Mn excess combined with low and adequate P (Fig. 1).

3.2. P and Mn concentration in shoots and roots

Regarding P and Mn concentration in roots and shoots tissues, there was a significant interaction between genotype and treatment ($P < 0.001$). In both genotypes, roots and shoots exhibited reduced P concentration under the lowest P supply, enhancing their P concentration towards increased P-supply, regardless of Mn treatments (Fig. 2).

Under sufficient Mn and increased P-doses, Mn concentrations in shoots and roots were low in both genotypes, when compared with those obtained under an excess of Mn and increasing P (Fig. 2). Under Mn excess, the Mn concentration in shoots remained constant in both genotypes, independently of the variation of P-levels. In roots under Mn excess condition, the Mn concentration of Banquet-II increased by about 23% by P-supply. Likewise, as in control treatments, the Mn concentration of Banquet-II shoots and roots were significantly higher than One-50 under sufficient Mn, similarly as occurred in Banquet-II roots under Mn excess ($P < 0.001$) (Fig. 2). Analyzing the percentage of total Mn concentration in tissue, One-50, and Banquet-II control plants showed a similar proportion of shoot and root Mn (~50% of Mn). Under Mn excess with P-supply, the Mn shoot and root proportion increased 38 and 61% in One-50 and, ~24 and 75% in Banquet-II (Supplementary Table S1). Both increments were independent of P-supply.

3.3. Photosynthetic performance

An interaction between genotype and treatment for photosynthetic rate (A), stomatal conductance (g_s) and transpiration (T) ($P < 0.001$) was found. Under sufficient Mn condition, Banquet-II genotype increased A under enhanced P (35%), whereas in One-50 remained constant (Table 1). The photosynthetic rate decreased strongly under Mn excess and lower and sufficient P condition in One-50 (~40%) compared to the control, while in Banquet-II was observed a lower decrease (24%) at 25 μM P combined with Mn-excess (Table 1). Both genotypes reached similar A values as controls with high P supply, augmenting afterward at the highest P-supply, being A 14% and 36% higher in One-50 and Banquet-II, respectively. Stomatal conductance exhibited similar behavior to A in both genotypes with sufficient Mn but was reduced under Mn excess and the lowest P treatment, incrementing afterward with higher P doses (200 or 400 μM). In both genotypes, transpiration did not change at different treatments under sufficient Mn (Table 1).

Concerning the chlorophyll fluorescence parameters, it was observed that interaction between genotype and treatment was significant for effective photochemical efficiency (ΦPSII), electron transport rate (ETR) and non-photochemical quenching (NPQ) ($P \leq 0.001$). The ΦPSII and ETR of One-50 decreased in plants exposed to an excess of Mn regardless of the amount of P added compared to control (Table 2). In Banquet-II, these parameters were not affected. Conversely, NPQ increased in One-50 plants grown under Mn excess, while in Banquet-II, the NPQ diminished under this condition (Table 2).

3.4. Pigments analyses

Interactions between genotype and treatment were found for all analyzed pigments ($P \leq 0.001$). Total chlorophylls (Chl $a+b$), Chl a/b ratio, β-carotene (β-car), violaxanthin (Vio), neoxanthin (Neo) and lutein (Lut) concentrations did not change under different treatments in Banquet-II (Tables 1 and 3). In contrast, One-50 increased two-fold β-car under Mn-excess and different P-doses in relation to the control (Table 3). Violaxanthin and Neo concentrations of One-50 were reduced by Mn excess and P-supplies, while anteraxanthin (Ant) was augmented by an excess of Mn and 50 μM of P, afterward, the same pigment was reduced at the highest P-doses. Concerning the proportion between Vio and Ant, there was a reduction in the Vio/Ant ratio under Mn excess treatment, augmenting at high P-doses. Differently, in Banquet-II, Ant decreased under Mn-excess and low (25 and 50 μM P) and sufficient P-treatments compared to control, recovering its value with the higher doses of P. The Vio/Ant ratio was augmented by an excess of Mn and subsequently reduced with higher P supply. Lutein was decreased by Mn-excess and 50 μM of P in One-50, compared to control, with a slightly recovering by a higher P-supply (Table 3).

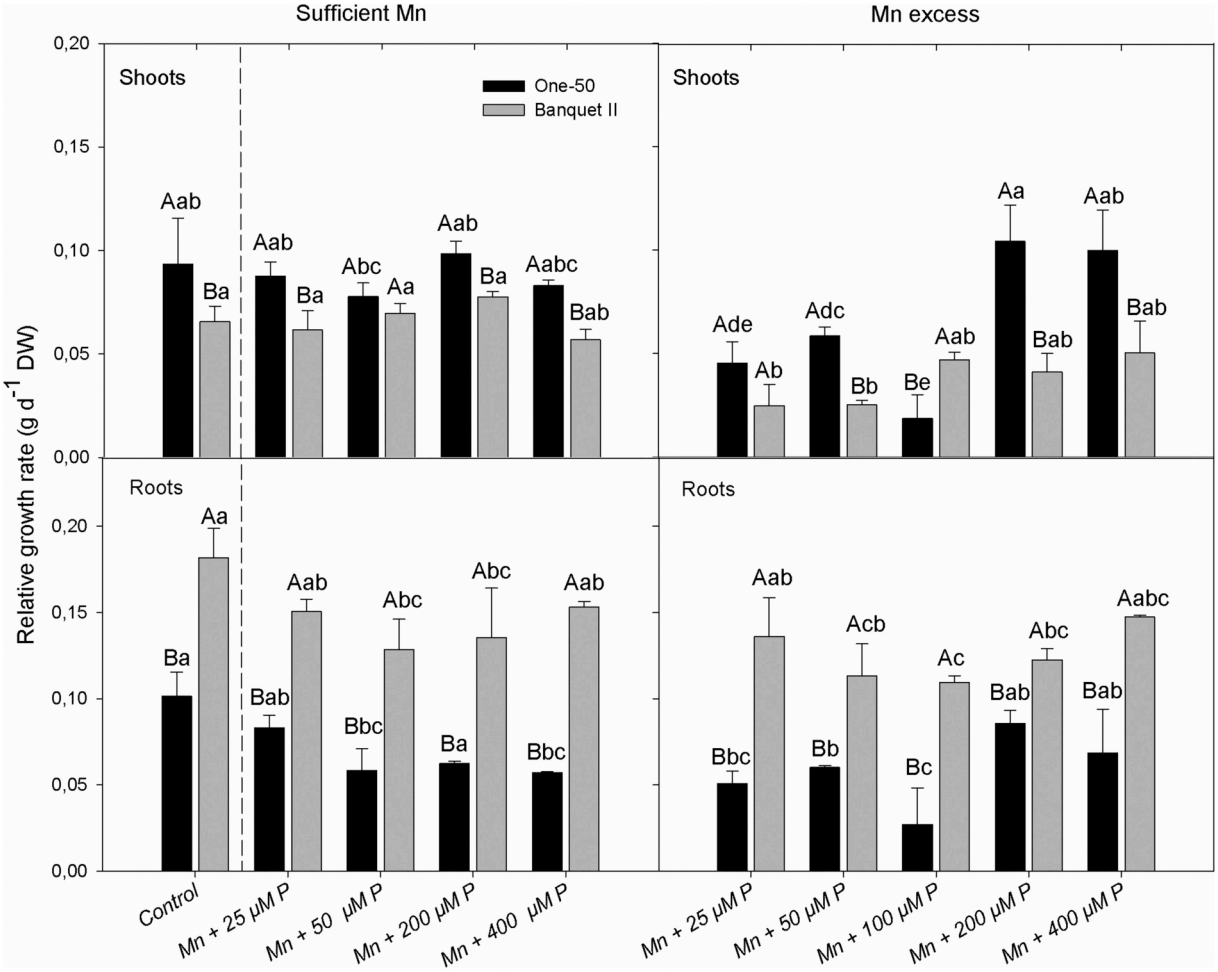

Fig. 1. Relative growth rate of shoots and roots in two ryegrass genotypes subjected to increasing P doses with a sufficient (2.4 μM) and excess (750 μM) of Mn. Dotted lines indicate the control treatment (2.4 μM Mn + 100 μM P). Different lowercase letters indicate statistically significant differences between treatments for the same genotype and different uppercase letters show differences between genotypes for the same treatments (Tuckey's test, $P \leq 0.05$).

3.5. Lipid peroxidation and radical scavenging activity

For lipid peroxidation (LP) in shoots and roots in both genotypes, a significant interaction was found ($P \leq 0.001$). The most evident changes in LP were observed in One-50, where shoots exhibited highest LP (63%) under sufficient Mn treatment and maximum P-supply, or by an excess of Mn accompanied by less or highest P (~2 fold), in comparison to control (Fig. 3). In roots with sufficient Mn and lower P, LP of both genotypes was higher in comparison to control (76%) (Fig. 3).

For radical scavenging activity (RSA) in shoots and roots, a significant interaction between genotypes and treatments ($P \leq 0.001$) was observed. In shoots, evident decreases of RSA, compared with the control, were verified in Banquet-II in the treatment with sufficient Mn and increasing P-supplies, while in One-50, this decrease only occurred under the highest P-supply (Fig. 3). However, in the treatments with Mn excess and increasing doses of P, shoots RSA of both genotypes went down compared to those treated with sufficient Mn (Fig. 3).

Under sufficient Mn, roots of Banquet-II reduced their RSA significantly with increasing P-levels (74%), while One-50 increased RSA under high P-levels compared to low P-level (25 and 50 μM P). Under excess of Mn, the Mn-sensitive One-50 decreased RSA at 50 μM P, remaining RSA levels without variations by the enhanced P-supplies (Fig. 3).

3.6. Total phenols (TP) and superoxide dismutase (SOD) activity

A significant interaction between genotype and treatment for TP in roots and shoots ($P < 0.001$) was found. Higher and maintained TP concentrations were observed in shoots of One-50 under sufficient Mn and increased doses of P compared to Banquet-II (Fig. 4). Instead, in plants subjected to an excess of Mn and increased P dose, generally, no significant differences were observed in the TP concentration of Banquet-II shoots (Fig. 4). Under Mn excess and different P dose, the TP concentration in shoots of One-50 decreased around 70% compared to control. On the other hand, in the roots of Banquet-II, a decrease in TP was observed at the highest Mn plus the lowest and the greatest P supply. Interaction between genotype and treatments were observed for SOD in roots and shoots ($P < 0.001$). It appears clearly that the roots of the investigated genotypes exhibit higher SOD activities than shoots (Fig. 5). Roots of Banquet-II showed greater SOD activities than One-50, increasing towards the highest P dose under sufficient and excess of Mn. In shoots, minor differences were observed between treatments for Banquet-II genotype in comparison with roots (Fig. 5).

3.7. Correlation between variables and principal components analyses

The association between the evaluated traits were calculated by Pearson correlation coefficients for all pairs of physiological and biochemical features at low and high P, sufficient- and Mn-excess in roots and shoots of Banquet-II and One-50 (Fig. 6). In the correlation matrix

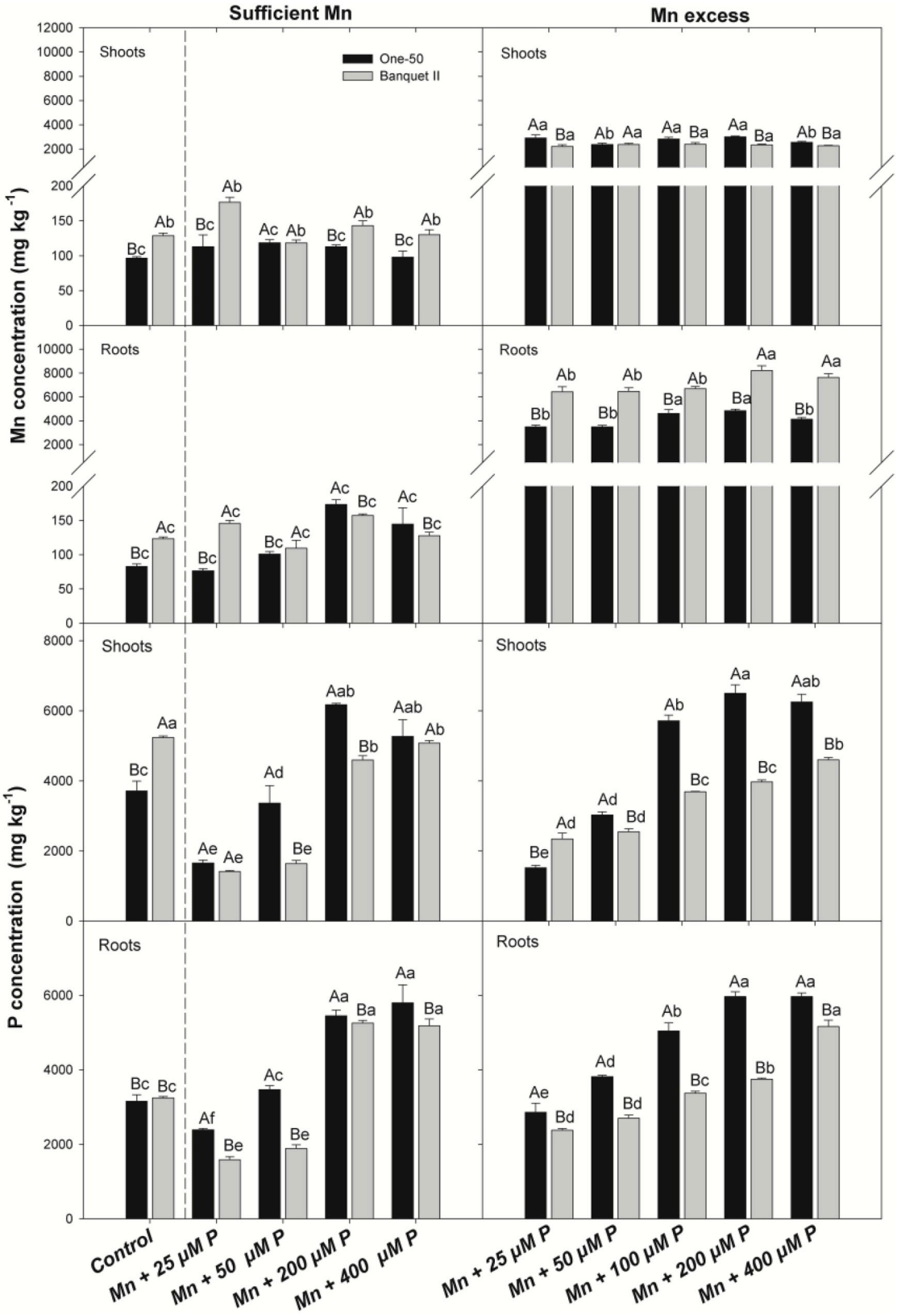

Fig. 2. Mn and P concentrations of shoots and roots in ryegrass genotypes subjected to increasing P doses with a sufficient (2.4 μM) and excess (750 μM) of Mn. Dotted lines indicate the control treatment (2.4 μM Mn + 100 μM P). Different lowercase letters indicate statistically significant differences between treatments for the same genotype and different uppercase letters show differences between genotypes for the same treatments (Tuckey's test, $P \leq 0.05$).

data set for roots, any relevant correlations were observed (Supplementary Fig. S1); in contrast, data set obtained for shoots of Banquet-II and One-50 under sufficient Mn exhibited 47 and 16 significant correlations ($P < 0.05$), respectively (Fig. 6a and c). In Banquet-II, 32 significant correlations were positive and 15 negatives (Fig. 6c); whereas, in One-50, 12 correlations were positive and four

Table 1
Gas-exchange parameters and chlorophylls concentration in *Lolium perenne* genotypes under different Mn and P supplies. Different lowercase letters indicate statistically significant differences among treatments for the same genotypes and different uppercase letters show differences among genotypes for the same treatments. Tuckey's test, $P \leq 0.05$. *A*: Net CO_2 assimilation, g_s: Stomatal conductance, T: Transpiration.

Treatments	One-50					Banquet-II				
	A (μmol CO_2 m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	T (mol H_2O $m^{-2}s^{-1}$)	Chl*a* + *b* (mg g^{-1} FW)	Chl*a*/*b*	*A* (μmol CO_2 m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	T (mol H_2O $m^{-2}s^{-1}$)	Chl*a* + *b* (mg g^{-1} FW)	Chl*a*/*b*
Sufficient Mn (2.4 μM)										
Control	5.21 ± 0.22 Ab	0.25 ± 0.03 Aa	2.52 ± 0.06 Aab	1.12 ± 0.03 Ba	1.77 ± 0.10 Bab	5.17 ± 0.10 Ac	0.26 ± 0.02 Ab	2.94 ± 0.21 Aa	2.59 ± 0.10 Aa	3.08 ± 0.07 Aab
25 μM P	5.14 ± 0.09 Ab	0.22 ± 0.01 Aab	2.58 ± 0.19Aa	0.87 ± 0.02 Bab	2.10 ± 0.32 Ba	5.29 ± 0.16 Ac	0.24 ± 0.02 Ab	3.01 ± 0.10 Aa	1.89 ± 0.18 Ab	3.10 ± 0.16 Aab
50 μM P	5.17 ± 0.10 Ab	0.24 ± 0.03 Aa	2.55 ± 0.07 Aab	0.90 ± 0.00 Bab	2.05 ± 0.17 Ba	5.17 ± 0.13 Ac	0.25 ± 0.01 Ab	2.94 ± 0.05 Aa	2.59 ± 0.20 Aa	3.07 ± 0.17 Aab
200 μM P	5.22 ± 0.07 Bb	0.23 ± 0.03 Bab	2.47 ± 0.25 Ba	1.09 ± 0.04 Ba	1.78 ± 0.05 Bab	7.03 ± 0.27 Aa	0.40 ± 0.04 Aa	3.55 ± 0.21 Aa	2.54 ± 0.16 Aa	3.03 ± 0.03 Aab
400 μM P	5.35 ± 0.10 Bb	0.24 ± 0.03 Ba	2.45 ± 0.22 Ba	1.09 ± 0.02 Ba	1.74 ± 0.08 Bab	6.95 ± 0.19 Aa	0.38 ± 0.04 Aa	3.38 ± 0.38 Aa	2.53 ± 0.18 Aa	3.02 ± 0.09 Aab
Mn excess (750 μM)										
25 μM P	3.31 ± 0.10 Bc	0.15 ± 0.01 Abc	1.97 ± 0.07 Abc	0.55 ± 0.04 Bb	1.65 ± 0.05 Bb	3.94 ± 0.33 Ad	0.14 ± 0.03 Ac	2.08 ± 0.11 Ab	2.08 ± 0.14 Ab	2.98 ± 0.12 Ab
50 μM P	3.10 ± 0.23 Bc	0.14 ± 0.04 Bc	1.83 ± 0.20 Bc	0.53 ± 0.03 Bb	1.71 ± 0.16 Bb	5.19 ± 0.23 Ac	0.25 ± 0.04 Ab	2.96 ± 0.05 Aa	2.42 ± 0.18 Aa	3.05 ± 0.16 Aab
100 μM P	3.25 ± 0.26 Bc	0.16 ± 0.02 Bbc	1.92 ± 0.16 Bbc	0.52 ± 0.02 Bb	1.76 ± 0.12 Bab	5.31 ± 0.10 Ac	0.25 ± 0.01 Ab	2.94 ± 0.10 Aa	2.52 ± 0.12 Aa	3.36 ± 0.14 Aa
200 μM P	5.16 ± 0.19 Bb	0.24 ± 0.01 Ba	2.39 ± 0.15 Ba	0.73 ± 0.04 Bb	1.78 ± 0.04 Bab	6.25 ± 0.30 Ab	0.38 ± 0.04 Aa	3.21 ± 0.21 Aa	2.56 ± 0.14 Aa	2.92 ± 0.22 Ab
400 μM P	5.94 ± 0.10 Ba	0.24 ± 0.02 Ba	2.45 ± 0.11Ba	0.72 ± 0.04 Bb	1.84 ± 0.17 Bab	7.04 ± 0.18 Aa	0.35 ± 0.03 Aa	3.21 ± 0.27 Aa	2.68 ± 0.10 Aa	3.04 ± 0.12 Aab

Table 2
Fluorescence related parameters in *Lolium perenne* genotypes under different Mn and P supplies. Different lowercase letters indicate statistically significant differences among treatments for the same genotype and different uppercase letters show differences among genotypes for the same treatments. Tuckey's test, $P \leq 0.05$

Treatments	One-50					Banquet-II				
	Fv/Fm	ΦPSII	ETR	NPQ	Fv/Fm	Fv/Fm	ΦPSII	ETR	NPQ	Fv/Fm
Sufficient Mn (2.4 μM)										
Control	0.82 ± 0.01 Aa	0.28 ± 0.02 Ba	35.05 ± 2.66 Ba	0.97 ± 0.03 Bc	0.82 ± 0.01 Aa	0.83 ± 0.01 Aa	0.34 ± 0.02 Aa	43.37 ± 2.17 Aa	1.39 ± 0.09 Aa	0.83 ± 0.01 Aa
25 μM P	0.82 ± 0.01 Aa	0.28 ± 0.02 Aa	35.55 ± 2.24 Aa	0.99 ± 0.11 Bc	0.82 ± 0.01 Aa	0.83 ± 0.01 Aa	0.29 ± 0.01 Ab	36.54 ± 1.23 Ab	1.51 ± 0.04 Aa	0.83 ± 0.01 Aa
50 μM P	0.82 ± 0.01 Aa	0.28 ± 0.00 Ba	35.58 ± 0.24 Ba	1.03 ± 0.12 Bc	0.82 ± 0.01 Aa	0.83 ± 0.00 Aa	0.34 ± 0.02 Aa	42.47 ± 2.74 Aa	1.48 ± 0.02 Aa	0.83 ± 0.00 Aa
200 μM P	0.82 ± 0.01 Aa	0.28 ± 0.01 Ba	36.18 ± 1.33 Ba	1.06 ± 0.02 Bb	0.82 ± 0.01 Aa	0.82 ± 0.00 Aa	0.33 ± 0.01Aab	41.75 ± 1.09 Aab	1.36 ± 0.05 Aab	0.82 ± 0.00 Aa
400 μM P	0.82 ± 0.01 Aa	0.28 ± 0.02 Ba	35.99 ± 2.20 Ba	1.14 ± 0.05 Bb	0.82 ± 0.01 Aa	0.83 ± 0.00 Aa	0.33 ± 0.02 Aa	41.92 ± 2.08 Aa	1.43 ± 0.07 Aab	0.83 ± 0.00 Aa
Mn excess (750 μM)										
25 μM P	0.82 ± 0.01 Aa	0.20 ± 0.02 Bb	25.25 ± 1.90 Bb	1.30 ± 0.03 Aab	0.82 ± 0.01 Aa	0.83 ± 0.00 Aa	0.33 ± 0.02 Aab	41.75 ± 2.40 Aab	1.25 ± 0.07 Abc	0.83 ± 0.00 Aa
50 μM P	0.82 ± 0.01 Aa	0.19 ± 0.01 Bb	24.70 ± 0.99 Bb	1.31 ± 0.11 Aab	0.82 ± 0.01 Aa	0.83 ± 0.00 Aa	0.33 ± 0.02 Aa	42.14 ± 2.64 Aa	1.03 ± 0.11 Bc	0.83 ± 0.00 Aa
100 μM P	0.82 ± 0.01 Aa	0.19 ± 0.02 Bb	24.49 ± 2.19 Bb	1.44 ± 0.02 Aa	0.82 ± 0.01 Aa	0.83 ± 0.01 Aa	0.33 ± 0.02 Aa	42.19 ± 2.73 Aa	1.18 ± 0.11 Bbc	0.83 ± 0.01 Aa
200 μM P	0.82 ± 0.01 Aa	0.18 ± 0.02 Bb	23.58 ± 1.89 Bb	1.30 ± 0.12 Aab	0.82 ± 0.01 Aa	0.83 ± 0.00 Aa	0.33 ± 0.01 Aa	41.85 ± 1.44 Aa	1.25 ± 0.14 Abc	0.83 ± 0.00 Aa
400 μM P	0.82 ± 0.01 Aa	0.21 ± 0.01 Bb	26.83 ± 0.67 Bb	1.48 ± 0.05 Aa	0.82 ± 0.01 Aa	0.83 ± 0.01 Aa	0.33 ± 0.01 Aab	41.59 ± 0.72 Aab	1.17 ± 0.04 Bbc	0.83 ± 0.01 Aa

Table 3
Pigments on *Lolium perenne* genotypes under different Mn and P treatments. Different lowercase letters indicate statistically significant differences among treatments for the same genotypes and different uppercase letters show differences among genotypes for the same treatments. Tuckey's test, $P \leq 0.05$. β-car: β-carotene, Lut: Lutein, Neo: Neoxanthin, Vio: Violaxanthin, Ant: Antheraxanthin, Vio/Ant: Violaxanthin/Antheraxanthin ratio.

Treatments	One-50						Banquet-II					
	β-car (μg g^{-1} FW)	Lut (μg g^{-1} FW)	Neo (μg g^{-1} FW)	Vio (μg g^{-1} FW)	Ant (μg g^{-1} FW)	Vio/Ant	β-car (μg g^{-1} FW)	Lut (μg g^{-1} FW)	Neo (μg g^{-1} FW)	Vio (μg g^{-1} FW)	Ant (μg g^{-1} FW)	Vio/Ant
Sufficient Mn (2.4 μM)												
Control	81.2 ± 6.1 Bc	371.2 ± 22.5 Aa	103.4 ± 11.0 Aa	154.1 ± 9.3 Aa	6.2 ± 0.7 Ab	24.9 ± 1.2 Aab	95.6 ± 6.4 Aab	261.0 ± 16.3 Bab	112.8 ± 6.5 Aa	83.2 ± 9.3 Ba	5.2 ± 0.6 Ba	16.3 ± 2.5 Bb
25 μM P	95.6 ± 4.6 Abc	336.3 ± 26.0 Ab	96.6 ± 5.5 Aab	133.2 ± 5.4 Ab	6.1 ± 0.6 Ab	21.6 ± 1.7 Aabc	76.8 ± 6.7 Bb	198.0 ± 17.5 Bb	75.1 ± 6.7 Bb	62.8 ± 1.1 Ba	3.1 ± 0.3 Bbc	20.4 ± 2.2 Bb
50 μM P	89.1 ± 2.5 Bc	337.3 ± 54.4 Ab	91.9 ± 8.3 Aab	127.8 ± 8.7 Abc	5.4 ± 0,6 Ab	24.0 ± 4.3 Aab	99.0 ± 9.5 Aab	253.9 ± 12.1 Bab	91.2 ± 10.9 Aab	88.6 ± 3.4 Ba	4.3 ± 0.2 Bb	20.6 ± 1.1 Ab
200 μM P	104.5 ± 7.6 Ab	378.6 ± 26.1 Aa	101.8 ± 10.4 Aab	169.3 ± 9.2 Aa	5.7 ± 0.4 Ab	29.9 ± 2.4 Aa	99.6 ± 5.8 Aab	251.4 ± 16.5 Bab	106.2 ± 6.3 Aa	85.6 ± 5.6 Ba	5.5 ± 0.4 Aa	15.7 ± 1.1 Bb
400 μM P	123.4 ± 15.7Ab	387.1 ± 37.0 Aa	113.0 ± 4.3 Aa	160.1 ± 13.6 Aa	5.7 ± 0.5 Ab	28.1 ± 1.4 Aa	97.6 ± 7.4 Bab	257.8 ± 18.9 Bab	108.9 ± 12.4 Aa	83.4 ± 5.7 Ba	5.1 ± 0.4 Aa	16.5 ± 0.4 Bb
Mn excess (750 μM)												
25 μM P	175.7 ± 14.3 Aa	328.2 ± 13.3 Ab	84.6 ± 1.8 Ab	110.4 ± 7.0 Acd	6.3 ± 0.3 Ab	17.7 ± 1.8 Bcd	81.1 ± 4.3 Bb	238.9 ± 5.4 Bab	87.3 ± 5.5Aab	79.1 ± 6.3 Ba	2.3 ± 0.3 Bc	34.8 ± 2.0 Aa
50 μM P	175.5 ± 4.2 Aa	242.4 ± 13.4 Ad	79.1 ± 5.8 Bb	105.6 ± 12.0 Acd	7.9 ± 0.6 Aa	13.4 ± 0.7 Bd	89.4 ± 6.0 Bab	240.8 ± 11.5 Aab	105.1 ± 9.2 Aa	84.7 ± 3.4 Ba	2.7 ± 0.2 Bc	32.1 ± 3.5 Aa
100 μM P	112.4 ± 9.1 Ab	224.7 ± 8.9 Ad	75.5 ± 6.5 Bb	106.1 ± 3.7 Acd	5.5 ± 0.2 Ab	19.4 ± 1 Bb	85.2 ± 5.3 Bb	247.2 ± 12.1 Aab	98.2 ± 9.2 Aab	81.7 ± 2.2 Ba	2.2 ± 0.2 Bc	37.7 ± 3.1 Aa
200 μM P	116.9 ± 12.8 Ab	273.5 ± 28.7 Acd	77.8 ± 1.9 Bb	105.7 ± 6.7 Acd	5.1 ± 0.5 Ab	21.1 ± 2.4 Ab	85.6 ± 7.3 Bab	250.3 ± 11.2 Aab	101.9 ± 15.9 Aa	80.1 ± 5.1 Ba	4.1 ± 0.1 Bb	19.3 ± 0.7 Ab
400 μM P	112.0 ± 6.49 Ab	296.3 ± 6.93 Abc	77.9 ± 4.4 Bb	97.9 ± 5.6 Ad	3.6 ± 0.3 Bc	27.6 ± 3.9 Aa	108.6 ± 8.6 Aa	274.8 ± 10.6 Aa	100.1 ± 22.1 Aab	86.8 ± 6.3 Aa	6.1 ± 0.4 Aa	14.4 ± 1.6 Bb

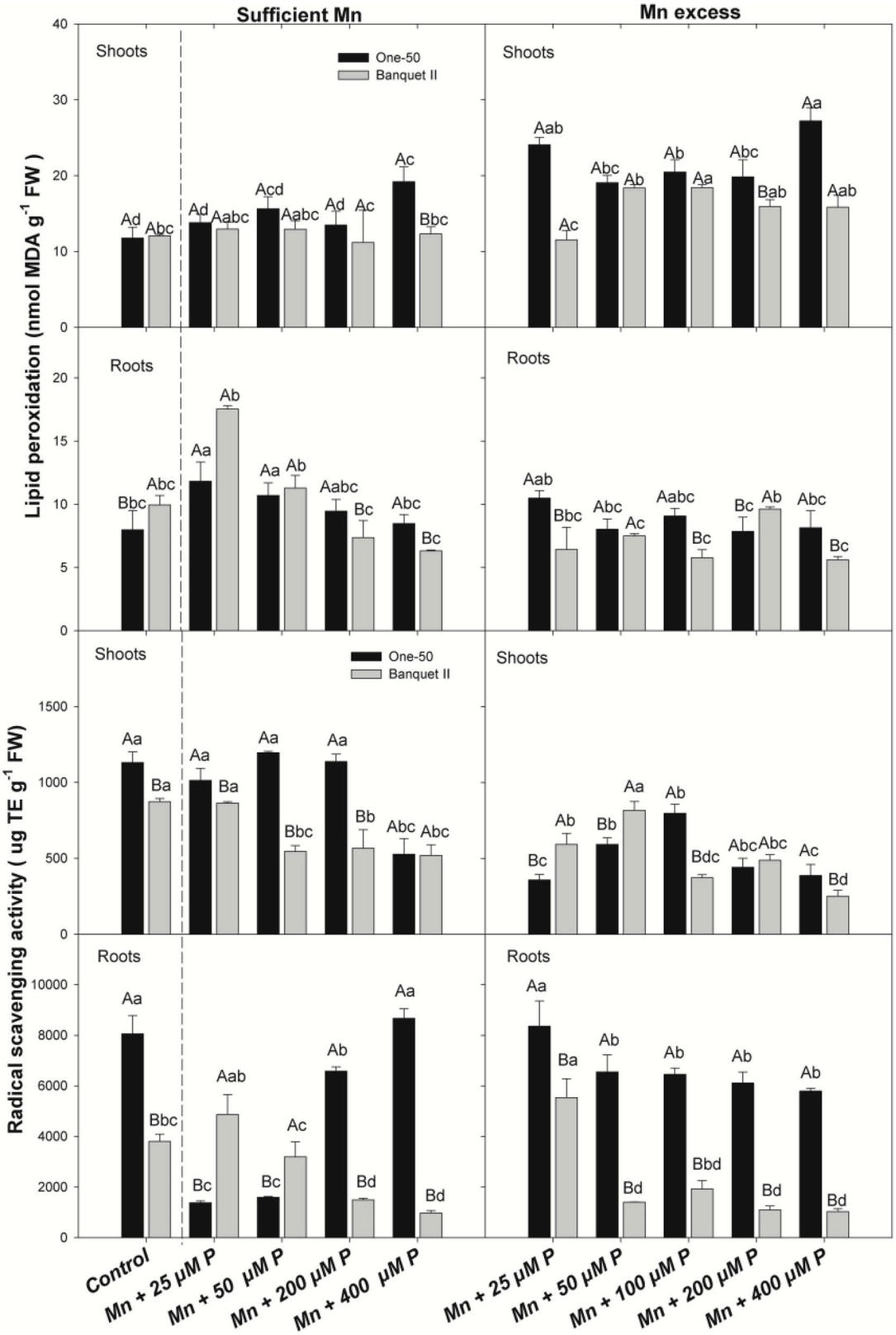

Fig. 3. Lipid peroxidation and radical scavenging activity of shoots and roots in ryegrass genotypes under increasing P doses being sufficient 2.4 μM and excess 750 μM of Mn. Dotted lines indicate the control treatment (2.4 μM Mn + 100 μM P). Different lowercase letters indicate statistically significant differences between treatments for the same genotype and different uppercase letters show differences between genotypes for the same treatments (Tuckey's test, $P \leq 0.05$).

negatives (Fig. 6a). Under Mn excess, shoots of One-50 exhibited more significant correlations compared with sufficient Mn condition (Fig. 6b), being Chl $a+b$ levels highly correlated with A, g_s and T (r = ~0.9). In Banquet-II, the amount of P in shoots was positively correlated with A (r = 0.90), g_S (r = 0.83) and T (r = 0.73). In contrast, the amount of Mn in the shoot of Banquet-II and One-50 did not

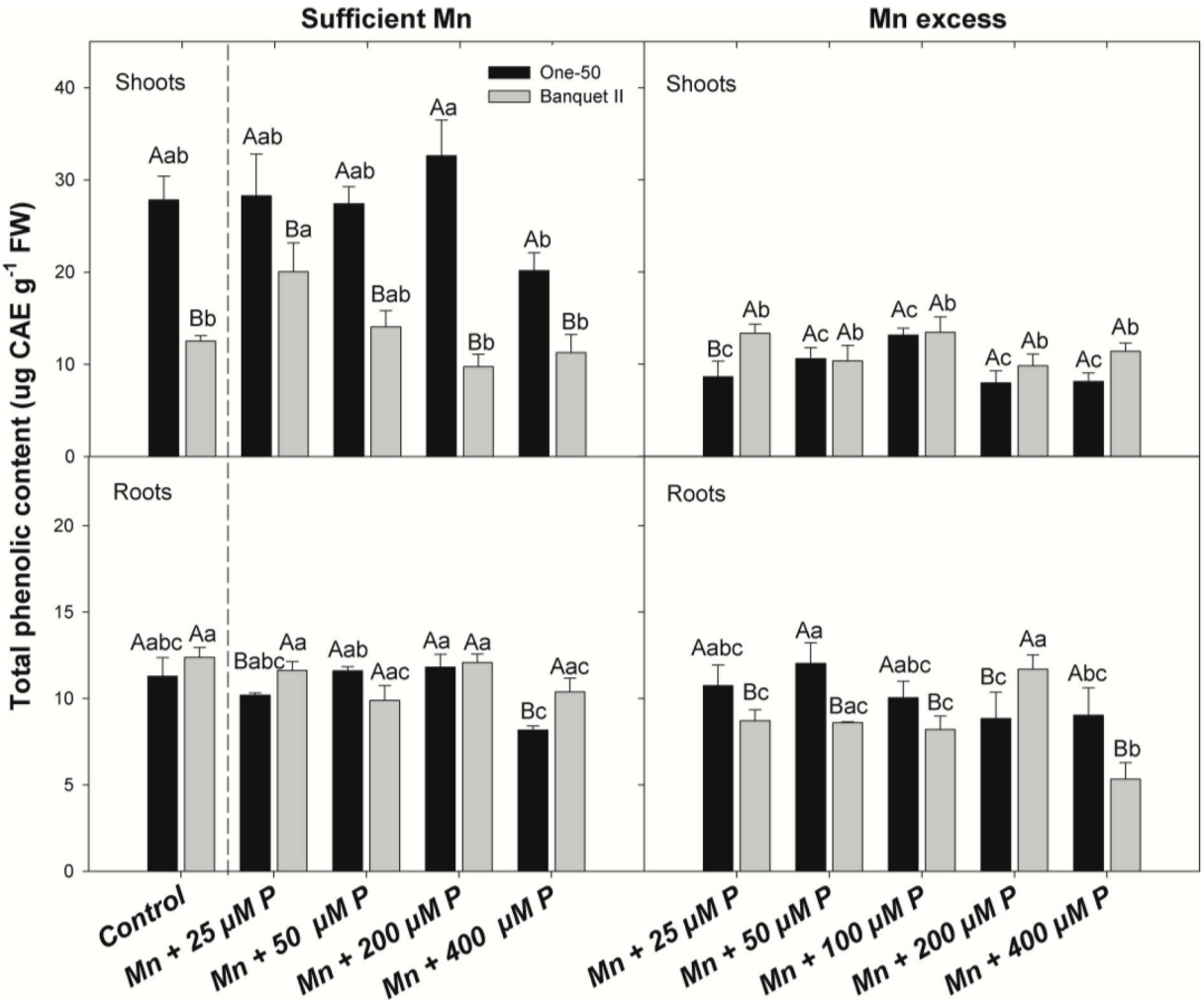

Fig. 4. Shoots and roots total phenolic content in ryegrass genotypes subjected to increasing P doses with a sufficient (2.4 μM) and excess (750 μM) of Mn. Dotted lines indicate the control treatment (2.4 μM Mn + 100 μM P). Different lowercase letters indicate statistically significant differences between treatments for the same genotype and different uppercase letters show differences between genotypes for the same treatments (Tuckey's test, $P \leq 0.05$).

correlate with any of the evaluated parameters.

Data sets obtained for all traits were averaged and normalized (min, max normalization) to perform a principal component analysis (PCA) for each tissue and Mn treatments, where the effect of P supply was observed (Fig. 7). In shoots subjected to sufficient Mn, PCA separated into two groups (Fig. 7a). The component 1 (PC1) explained 53.7% and component 2 (PC2) explained 21.5% of the total variation separating One-50 from Banquet-II genotype. Photosynthetic pigments, RGR, and TP contributing highly to the separation of the Mn-sensitive (One-50) genotype (Fig. 7b). Moreover, photosynthetic parameters and Mn concentration were responsible for the group separation of the Mn-resistant genotype (Banquet-II) (Fig. 7b). On the other hand, in roots under sufficient Mn, PCA separated the variables into four groups by high and low P in both genotypes, indicating the effect of P treatments on responses to toxic Mn (Fig. 7c). Under low P, the variables that highly contributed to PC1 were mainly LP in One-50 genotypes. Under high P, the variables that highly contributed to PC2 were SOD in Banquet-II and RSA and P concentration in One-50 (Fig. 7d). For shoots under excess of Mn, PC1 and PC2 explained 52.9% and 18.8%, respectively, and clearly separated the treatments into four groups by high and low P in both genotypes (Fig. 7e). The variables that highly contribute to PC1 were related to the RGR, damage, and Mn concentration of One-50, which was mostly affected than Banquet-II genotype (Fig. 7f). Under high P, SOD activity and photosynthetic parameters were improved in Banquet-II (Fig. 7f). For roots, under Mn excess, PCA formed two groups independently of P-treatments, where PC1 explained 59.3% of the total variation and PC2 explained 18.7% (Fig. 7g). This separation was according to Mn concentration, RGR, and SOD (Fig. 7h). Moreover, biochemical traits (TP, LP, and RSA) were the variable that better explained the separation of One-50.

4. Discussion

The Mn toxicity affects plant productivity, and its negative effects can be alleviated by P in some plants (Fernando and Lynch, 2015; Rosas et al., 2011). Thus, the mechanisms by which P interacts with Mn, and reduce the negative effects of Mn-toxicity displayed by some plant species and specific genotypes are of great interest in plant nutrition. Nonetheless, this interaction is still not well understood in forage plants (Rosas et al., 2011) because it is a genotype and environmental dependent response. As previously demonstrated, the ryegrass genotype Banquet-II exhibits Mn-resistance, accumulating higher amounts of Mn in roots in comparison to the Mn-sensitive genotype One-50 (Inostroza-Blancheteau et al., 2017; Reyes-Díaz et al., 2017). In the present study, we observed a decrease in the Mn shoot/root proportion under Mn excess; where Banquet-II accumulated 23% more Mn than One-50 in roots, suggesting that the uptake of Mn is genotype-dependent in ryegrass. We also verified that the excess of Mn reduces shoot and root growth of both genotypes, which was also observed under lower and adequate P doses, especially in the sensitive genotype (One-50) when

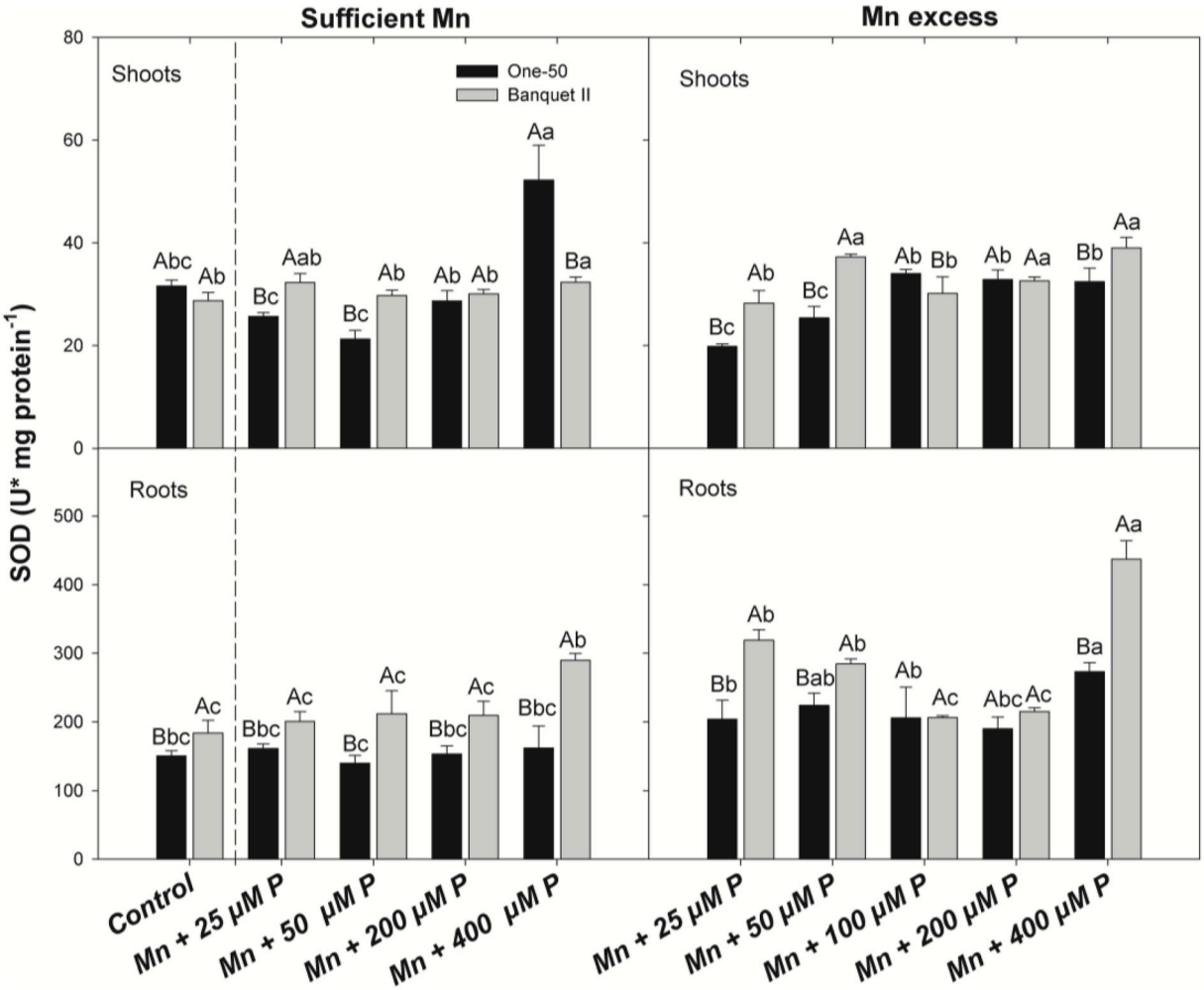

Fig. 5. SOD activity of shoots and roots in ryegrass genotypes subjected to increasing P doses with a sufficient (2.4 μM) and excess (750 μM) of Mn. Dotted lines indicate the control treatment (2.4 μM Mn + 100 μM P). Different lowercase letters indicate statistically significant differences between treatments for the same genotype and different uppercase letters show differences between genotypes for the same treatments (Tuckey's test, $P \leq 0.05$).

compared to the control treatment. Furthermore, we verified that this behavior is reverted by the addition of P (Fig. 1), suggesting that higher P-doses can ameliorate the negative effects of toxic Mn on the growth of both sensitive and tolerant genotypes. These results are in close agreement with those reported in *L. perenne and Trifolium repens* (Rosas et al., 2011), where both species displayed inhibition of plant growth by excess of Mn, counteracting this effect by supplying P. Despite the Mn resistance exhibited by Banquet II cultivar, it behaved as sensitive to low P supply. In addition, we verified that RGR correlates with P concentration in shoots only in the Mn-resistant Banquet-II (r = 0.62) (Fig. 6d). This result suggests that P addition might reduce the negative Mn effect via precipitation of Mn in low soluble complexes, which accumulate in vacuoles and/or in other organelles (Hall, 2002; Sarkar et al., 2004).

In the present study, the concentration of Mn in shoots was not affected by P-nutrition in the Mn-resistant Banquet-II. Differently, in *L. perenne* cv. Nui and *T. repens* plants under Mn toxicity conditions; the Mn concentration was associated with an increase in P-concentration in roots and shoots of both species (Rosas et al., 2011). However, in barley, increases in P-supply directly interfere with Mn-uptake in roots leading to Mn deficiency in shoots (Pedas et al., 2011). Together with our findings, these studies indicate that the benefits of P supply in plants under toxic levels of Mn are species-specific and cultivar-specific responses.

4.1. The effects of Mn toxicity and P on antioxidant defense mechanisms

It is known that Mn toxicity induces oxidative stress in plants, developing enzymatic and non-enzymatic antioxidant defense mechanisms depending on plant species (Michalak, 2006). The results presented here are in agreement with those previously reported (Inostroza-Blancheteau et al., 2017) and suggest that RSA and TP are involved in mechanisms against toxic Mn levels in One-50 cultivar. Interestingly, our results also indicated that SOD activity, enzyme that constitute the first line of the enzymatic antioxidative response (Yu and Rengel, 1999), exhibited higher activity in roots than in shoots when subjected to sufficient and excess of Mn in presence of high doses of P. This was clearly observed in the Mn-resistant genotype, which exhibited the highest SOD activity in roots by the highest P dose. In roots of One-50 plants subjected to an excess of Mn showed increased activity of this enzyme with the addition of the highest P concentration (Fig. 5). Generally, SOD is reported to increase under toxicity of metals such as Al (Parra-Almuna et al., 2018), Cd (Arshad et al., 2015) and Cr (Sayantan, 2013). Also, SOD is reported to increase its activity under P-deficiency in *Oriza sativa* (Poli et al., 2018) and *L. perenne* cv. Expo (Parra-Almuna et al., 2018). However, to our knowledge, this is the first study that indicates the activation of SOD enzyme by high P addition under Mn-excess. In this sense, our results suggest that activation of SOD activity displayed by P application might play an important role in Mn excess tolerance mechanism in ryegrass, by which ROS production

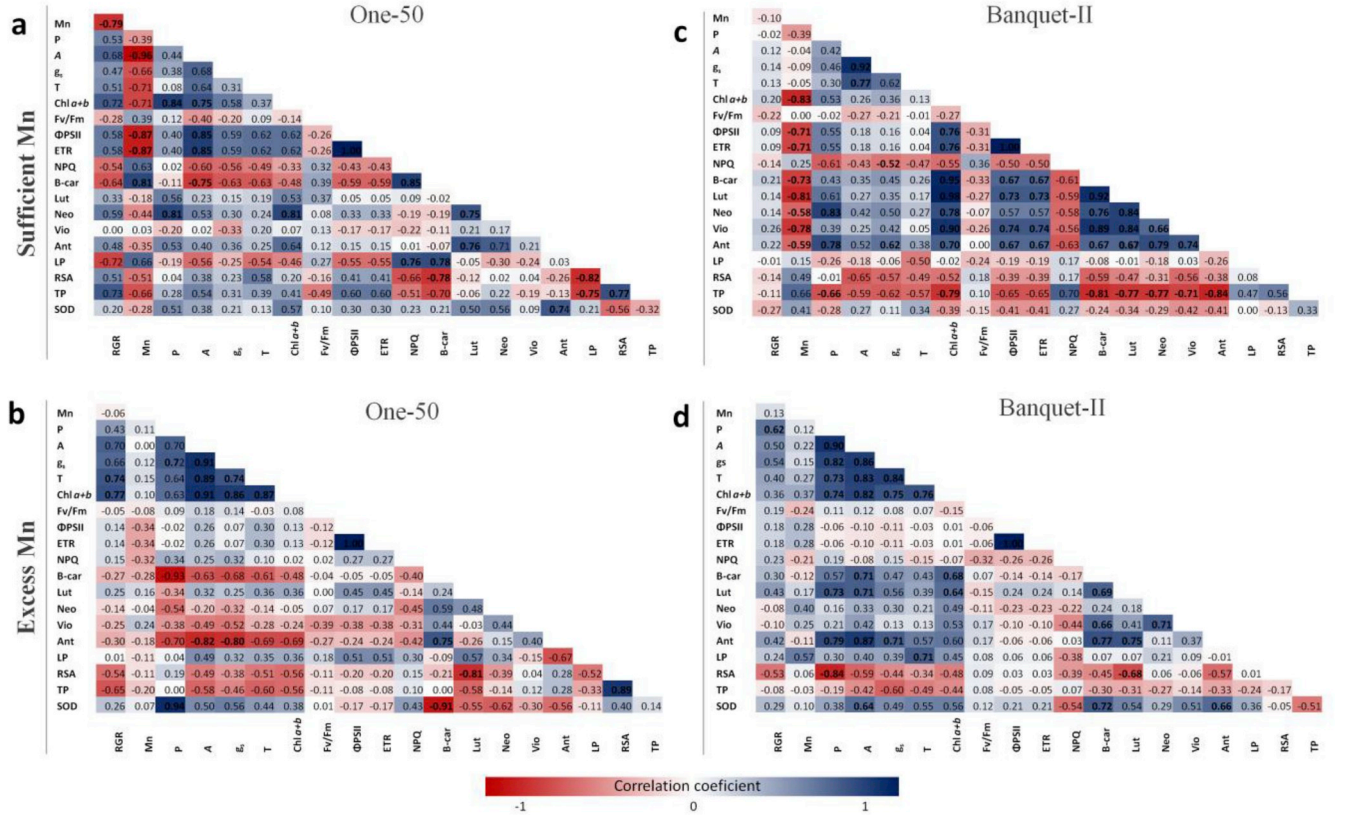

Fig. 6. Pearson correlation coefficients matrices derived from physiological and metabolic data from shoots of One 50 and Banquet-II under sufficient and excess of manganese supply. Negative and positive correlation coefficients are presented in red (ranging from 0 to −1) and blue (ranging from 0 to 1) colors, while the numbers within each colored box give the correlation coefficients (R value). The significant R values are indicated in bold ($P < 0.05$). *P*-values were corrected by False discovery rate (FDR) test in Rbio software to remove false positives correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

is reduced, decreasing oxidative stress generated by Mn-excess. However, the mechanism by which P activates SOD enzyme remains unknown.

4.2. The effects of Mn toxicity and P on photosynthetic performance

It has been documented that P-supplies counteracts the negative effects caused by Al and Cd on photosynthesis in *Citrus grandis* and *Triticum aestivum* (Arshad et al., 2015; Jiang et al., 2009). Concerning the Mn effects, it has been shown that Mn induces stomatal closure in plants (Misra et al., 2018). In agreement, we observed that the effect of Mn on g_s is reverted by P-supply, where the Mn-resistant Banquet-II exhibited enhanced *A* (36%) and g_s, overpassing the control (Table 1). Likewise, shoots P concentration of this genotype correlated with *A*, g_s, T and total chlorophylls (Fig. 6d), suggesting that higher P concentration enhances these parameters under Mn-excess condition. These studies stand out the importance of P participation in the regulation of Calvin-Benson cycle (Anwaruzzaman et al., 1995). Also, under higher P-supplies and excess of Mn, we verified that the Mn-sensitive One-50 genotype was able to recover *A* with respect to the control (14%).

Furthermore, we demonstrated that photochemical parameters are generally affected by the excess of Mn in the Mn-sensitive genotype, but P-supply does not ameliorate these parameters under Mn-toxicity (Table 2). Among the photochemical parameters, NPQ, an indicator of thermal energy dissipation in plants, avoiding detrimental effects of stresses (Adams et al., 2004), decreased under toxic Mn in the Mn-resistant Banquet-II and increased in the Mn-sensitive (One-50) independent of P-supply (Table 2). This result indicates that the Mn-sensitive genotype is more affected by the Mn-excess than Banquet-II. Interestingly, Banquet-II exhibited the lowest NPQ values under high Mn and high P-treatments in parallel with a higher concentration of antheraxanthin (Table 3), an intermediate pigment of the xanthophylls cycle involved in the thermal energy dissipation in plants (Adams and Barker, 1998). Besides, the violaxanthin/antheraxanthin ratio was shown to be increased in One-50 with P supply, while in Banquet-II, the same ratio was reduced (Table 3). These results suggest that a high P nutrition could favor energy dissipation through an increase of antheraxanthin in the xanthophylls cycle pool, without a direct relation with NPQ, under Mn excess. Furthermore, in Banquet-II, the up-regulation of xanthophyll cycle activity could avoid the excess of energy passing to O_2 via chlorophyll and thus protecting D1 protein of PSII of damage. Thus, in Banquet-II, the results indicate that under excess of Mn, P could lead to an activation of the xanthophyll cycle, allowing photosynthesis to increase in the response of P-supply. However, further studies are necessary to solve the mechanism by which a high P supply could activate this cycle.

5. Conclusions

A high dose of phosphorus amendment alleviated Mn-toxicity in Mn-sensitive genotype (One-50). Besides, in the Mn-resistant genotype, enhanced plant performance is highlighted, explained by a high Mn-accumulation in roots and increased SOD activity, decreasing Mn translocation to shoots and therefore protecting the photosynthetic apparatus. It is important to point out that the physiological and biochemical mechanism to Mn-excess and P was different in each tissue of the Mn-resistant Banquet-II. In roots, higher Mn accumulation under Mn excess and high P was observed, enhancing SOD activity, contributing to the decrease of LP and RSA levels, allowing root growth. Besides, in shoots, it is also important to note that in the resistant

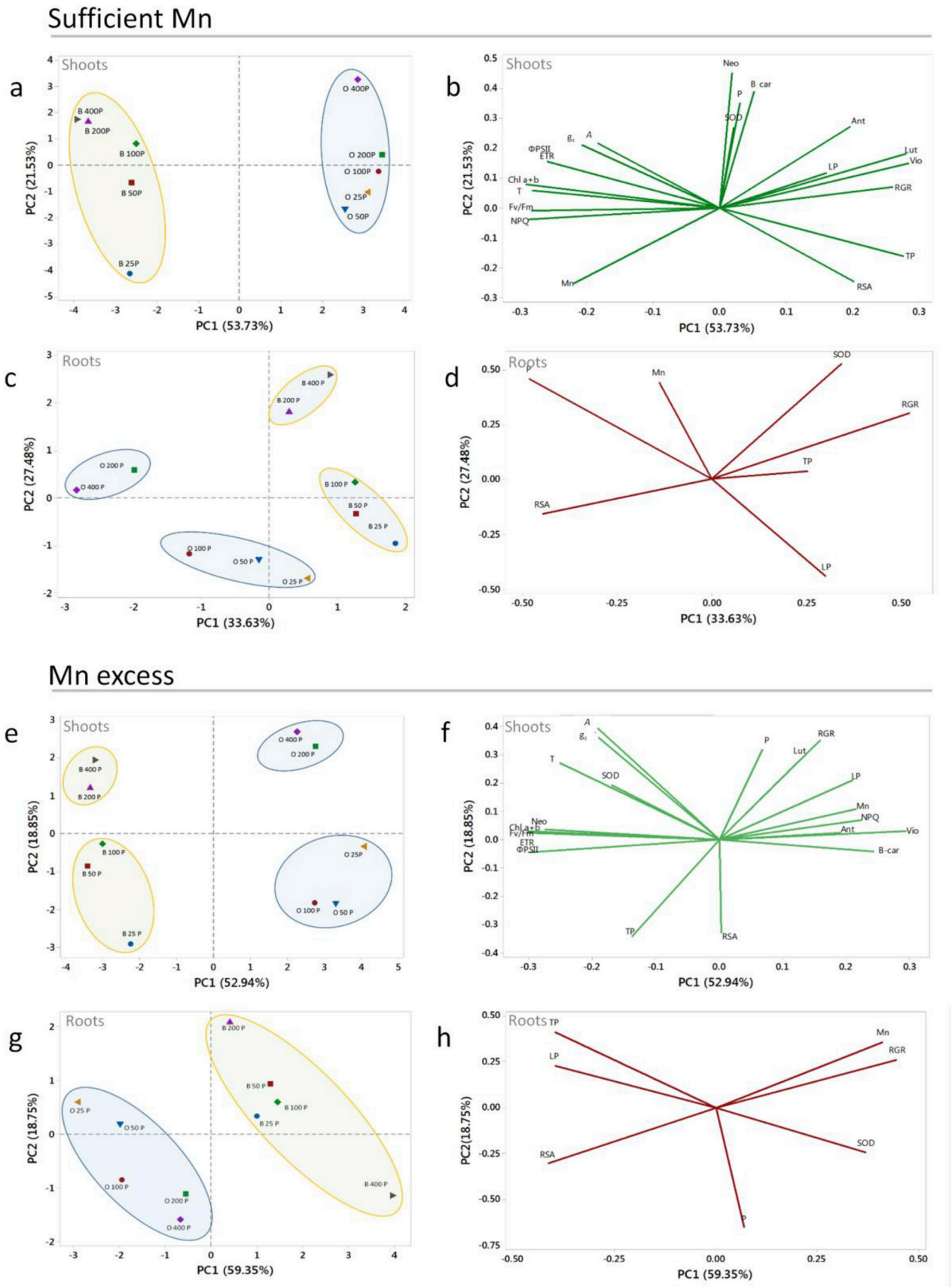

Fig. 7. PCA analysis score and loading plot per each analysis are shown. a-b, Mn excess in leaves, c-d, Mn excess in roots, e-f Sufficient Mn in leaves, g-h Sufficient Mn in roots. Genotypes One-50 and Banquet-II are distinguished by a yellow and blue circle, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genotype, the xanthophyll cycle is activated under P-supply, which could protect the photosynthetic apparatus under Mn-excess. These mechanisms together could allow coping Mn toxicity in the Mn-resistant Banquet-II under Mn-excess condition. Also, our findings are the first approach to understand the possible role of P nutrition in regulating Mn accumulation and toxicity in the new ryegrass genotypes.

Author's contribution

GB and MR-D designed and coordinated the experiment. GB and MR-D carried out the physiological and biochemical analyzes. GB, MR-D and ALE performed statistical analysis. GB and MA formulated the manuscript and MR-D, ALE and AN-N revised and corrected it. GB, MR-D, MA, ALE and AN-N revised and improved the current version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.034.

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