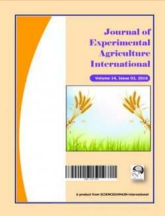

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Changes of Photosynthetic and Antioxidant Activity of *Phaseolus vulgaris* to Potassium

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Authors' contributions

This work was carried out in collaboration between all authors. Author VGU designed the experiment, evaluated, performed the data mining in R software and drafted the paper together with the last author. All other authors contributed equally in data collection, analysis and discussion.

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ABSTRACT

Greenhouse experiment was conducted with two cultivars of common beans and four concentrations of potassium (1, 2, 10 and 20 millimolar –mM) were supplied to the soil pots aiming to access variations in plant antioxidant defense systems (secondary metabolites and enzymatic mechanisms), growth parameters and leaf gas-exchange measurements. The total fresh leaf mass increased in both cultivars from 1 to 10 mM of potassium and the dry matter content decreased from 1 to 20 mM. Root volume significantly increased in Uirapuru cultivar. Potassium increased chlorophyll A in both in the range of 1 to 10 Mm. Catalase activity and carotenoids increased only in

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Dama cultivar. Potassium increased the photosynthetic activity in cultivar-dependent manner. Cultivar 'Dama' showed higher intrinsic water use efficiency, NDVI index, chlorophyll contents, total leaf mass and higher catalase activity. If the selection of cultivar under those traits is aimed, 'Dama' cultivar is a candidate and presented a linear stomatal conductance model.

Keywords: Common beans; potassium nutrition; photosynthesis; stomatal conductance models; growth parameters; antioxidant defense mechanisms; phenolics; normalized vegetative index; catalase.

1. INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume in human diets. It provides protein, complex carbohydrates, and valuable micronutrients for more than 300 million people in the tropics. Is the second most important source of calories after maize in Eastern and Southern Africa providing 32% of energy supply and the fourth in tropical America where it is also the third most important source of calories after maize and cassava and constitutes an important source of household income for small scale farmers [1,2]. The common beans are thought to provide up to 65% of protein [3] and currently estimated to be one of the most important legumes worldwide [4,5]. Beside the great importance, there are many factors that affect the high performance of the plant potential and photosynthesis.

Potassium (K^+) is the most abundant inorganic cation in plant cells. K^+ is vital for plant growth. Together with nitrogen (N) and phosphorous (P), K^+ belongs to the top three elements, the availability of which strongly determines crop yield. Undeniably, the application of mineral NPK-fertilizers was an essential cornerstone of the green revolution in the last century [6]. Limited resources [6] are now forcing us to investigate and better understand how the plants' K^+ demand can be satisfied with the minimum of fertilizer application [6]. The indispensable ground for providing answers to this complex question is the understanding of the exact roles of K^+ in plants [6]. Yao & Naeth [7] reported changes in physiological growth stages in barley from emergence and below ground biomass in response to potassium application.

In plant metabolic processes, potassium application has been reported to inhibit the uptake of cesium [8]. As reported by Rashid [9] potassium (K) has a critical role in plant physiology and comprises up to 10% of plants dry weight [10]. K provides regulatory control over different plant processes such as transpiration, starch synthesis, sucrose translocation, respiration and lipid synthesis and has a profound effect on the profile and distribution of the primary metabolites in plant tissues [9]. Changes in metabolite concentrations induced by K are multiple and include K dependence of metabolic enzymes, photosynthesis and long distance transport [9]. The primary metabolites such as soluble sugars particularly reducing sugars, organic acids and amino acids tend to increase in K deficient plants [9]. Experiment conducted by Rashid [10] found that K fertilization increased K content but reduced N, Si, free sugar and soluble protein contents in the plant tissues which resulted in the minimum reduction of relative water content (RWC) in rice plants.

Gautam and co-workers [11] also reported the beneficial effect of potassium application in improving the submergence tolerance of rice. All the cultivars evaluated showed inhibition of photosynthesis, and this was accompanied with decrease in stomatal conductance, chlorophyll and carbohydrate contents. The activity of antioxidants was found to be significantly higher. Potassium application improved the survival mainly because of maintenance of carbohydrates, chlorophyll and contributing to less lodging and leaf senescence. Furthermore, K application resulted in inhibition of lipid peroxidation and increase in catalase and peroxidase activities [11]. Potassium at higher levels was more beneficial in terms of improving survival, photosynthesis and plant growth [11]. Experiment conducted in peanut cultivars by Chakraborty [12] showed that external K application resulted in improved salinity tolerance in terms of plant water status, biomass produced under stress, osmotic adjustment and better ionic balance. A study conducted by Zain & Ismail [13] in rice aiming to understand the effects of potassium rates (80, 120 and 160 kg/ha) and types (KCl and K_2SO_4) on leaf gas exchange,

growth and biochemical changes under cyclic water stress concluded that increases in fertilization rates increased proline production, catalase, malondialdehyde (MDA) and transpiration rate. Higher potassium rates reduced water stress effects by inducing high transpiration which increased nutrient uptake to repair the damaged tissues and reduce oxidative stress. Potassium in KCl form increased net assimilation rate –NAR. Potassium has been also reported to play different roles such as biophysical and biochemical roles, improve tolerance to biotic and abiotic stress (salinity, drought, ammonium concentrations, cold, frost, light), reduce oxidative stress load of chilling stressed plants, role in photosynthesis, movement of plant organs, improve pathogen resistance, maintain osmotic potential of sieve tubes, cell turgor and phloem loading and transport [14-17].

K constitutes about 2.1–2.3% of the earth's crust and thus is the seventh or eighth most abundant element [14]. Therefore, soil K reserves are generally large [18]. However, large agricultural areas of the world are reported to be deficient in K availability. Soils inherently low in K are often sandy, waterlogged, saline, or acidic. Additionally, in intensive agricultural production systems, K has become a limiting element, in particular in coarse-textured or organic soils. In many cases, lower fertilizer K application in the context of unbalanced fertilization may result in a significant depletion of available soil K reserves, and thus in decreased soil fertility [14]. Beside extensive literature of potassium in other crops, a better understanding on how potassium affect the photosynthetic and antioxidant is of crucial importance to better manage the common beans.

Aiming to better understanding the role of potassium application in photosynthesis, agronomic attributes, antioxidant behavior and secondary metabolism of common beans, greenhouse experiment (pot experiment) was conducted with two contrasting cultivars (white-"carioca" and black colored tegument) .

2. MATERIALS AND METHODS

2.1 Plant Material and Soil Preparation

Two contrasting commercial common bean cultivars were selected for this experiment (white colored grains-carioca "TAA DAMA", hereinafter designed as Dama and black colored grains "IPR88 UIRAPURU", designed as Uirapuru, see Fig. 1). Dama cultivar is tolerant to rains even during harvesting time; it has growth cycle of 85 to 95 days, with plant height around 50 cm. Its growth habit is indeterminate (type III) and the shape of the grain is oblong. It has been argued to be moderately resistant to rust, angular stain, mildew and bacteriosis and resistant to common mosaic. Has long duration of light grain color for more than one year; higher productive ceiling, extensive adaptation and higher sieve yield [19]. Uirapuru was selected because presents excellent culinary qualities, is moderately tolerant to water stress [20], has a broad adaptation, with erect stems and branches which favors direct mechanical harvesting. Flowers and reaches maturity at 43 and 86 days after germination respectively [21]. Resistant to common mosaic virus, rust and powdery mildew and tolerant to high temperatures [21]. Sandy soil in the pots was firstly washed during03 times (24 hours each) with distilled-deionized water in other to remove any existence of minerals before seeding.

Fig. 1. (A) Cultivar TAA DAMA and (B)-IPR88 UIRAPURU

2.2 Greenhouse Experiment and Treatments

The experiment followed a randomized block design, with three blocks. Each block consisted of 10 treatments (2 genotypes –"Dama" and "Uirapuru", hereinafter designed as "UIRA" and four levels of potassium- 0, 1, 2, 10 and 20 mM applied in a form of potassium chloride (KCl), molecular weight of 74.548 g mol^{-1} and the KCl mass required for each concentration calculated according to the equation below –Eq. A). These 4 KCl concentration were chosen taking into account the previously published manuscript by Nieves-Cordones [15] which state levels of (>10mM) as higher concentration for uptake in non-selective channels (main pathways for K^+ uptake) while 1mM as intermediate level[10]. So according to previous reports [10], 2 high and 2 intermediate levels were chosen and considered adequate for pot experiments. Treatments in each block were represented by 3 pots (previously filled with soil) with 3 plants in each in a total of 9 plants per treatment/block. Bean seeds were surface sterilized by a commercial fungicide (carbendazim + tiram at a dose 300 mL/100 kg of seeds) before seeding in the pots and then 5 seeds were seeded and placed in the greenhouse under partially controlled conditions of light intensity, photoperiod, relative humidity and temperature. Seedlings were irrigated by basic salt of Murashige and Skoog nutrient solution (2mM potassium-total concentration and applied 1/4 per week) one time per week, during one month. Plants were watered regularly (one to two times per week) with distilled water and 30 days after potassium treatment, the evaluations were started.

$$\left[m = [\mathrm{C}] * \mathrm{Mw} * \mathrm{V} \right] \quad \text{(a)}$$

Where "m" is the required mass of KCl; [C] is the required concentration; Mw is the molecular weight of KCl and V is the total volume of water used (3.6 L).

2.3 Chlorophyll Content

Chlorophyll (Chla, Chlb and total) in the greenhouse was determined using chlorophyll electronic analyzer (CLOROFILOG CFL 1030, FALKER, serial 0520, Brazil). For that, 18 leaves in each treatment per block were measured in a total of 54 leaves per treatment in all experiment.

2.4 Normalized Difference Vegetative Index (NDVI)

As an important indicator of the status of chlorophylls, photosynthetic capacity and energy absorption by plant canopy, NDVI was measured using analyzer (PlantPen NDVI 300, Photon System Instruments, Brazil).

2.5 Leaf Gas Exchange Data

Photosynthetic parameters were measured using a portable photosynthesis system-IRGA (LI-6400XT-LI-COR, Biosciences Inc., Lincoln, NE, USA). Data of net photosyntensis (P_n), stomatal conductance (gs), intercellular carbon dioxide, transpiration, leaf to air temperature ratio, leaf saturation vapor, total conductance to carbon dioxide, intercellular to ambient carbon dioxide, intrinsic water use efficiency (WUE), stomatal limitation value (SL), carboxylation efficiency of Rubisco were accessed. Intrinsic water use efficiency, stomatal limitation value and carboxylation efficiency (CarbE) were calculated according to equations 1, 2 and 3 respectively.

$$\left[\mathrm{WUE} = \frac{P_n}{g_s} \right] \quad (1)$$

$$\left[\mathrm{SL} = 1 - \frac{C_i}{C_a} \right] \quad (2)$$

$$\left[\mathrm{CarbE} = \frac{P_n}{C_i} \right] \quad (3)$$

Where C_i and C_a means the intercellular and ambient carbon dioxide respectively, and g_s the stomatal conductance.

Using "plantecophys"-an R package for analysing and modeling leaf gas exchange data [22], photosynthesis and stomatal conductance models were fitted in other to better understand and quantify inter-specific differences in photosynthesis and model the rapid response to changes in environmental drivers such as light, humidity and temperature.

2.6 Dry Matter, Root Length, Root Volume and Total Leaf Mass

These parameters were evaluated at the final of the experiment, when plants reached the R5-R6

phenological stage according to Fernández [23]. Dry matter of leaves was measured by weighing the fresh and dry weight (Forced air oven, 72 hours, 60℃) of sample and represented as proportion of dry and wet sample weight (%), total fresh leaf mass by weighing all leaves collected per treatment and represented in grams, root length using a rule and root volume by the water volume dislocated by the roots.

2.7 Antioxidant Enzymatic System (Catalase-CAT and Ascorbate Peroxidase-APX)

Enzymes were extracted from 1 g of leaf tissue using a mortar and pestle with 5 ml extraction buffer containing 0.2 M K-phosphate buffer pH7, 20 mM DTT, 0.100 g PVP, 25 µL triton, 50 mM PMSF and 5 mM EDTA. The homogenate was centrifuged (3000 rpm/30 minutes) and the supernatant fraction was used to assay catalase, proteins and ascorbate peroxidase [24]. Protein content was determined using Bradford reagent [25] with bovine serum albumin as a standard. Catalase activity was assayed in a reaction mixture of 2 mL of 50 mM pH7 phosphate buffer containing hydrogen peroxide (25 µL) and 20 µL of enzyme extract. The decomposition of hydrogen peroxide was followed at 240 nm (ε=36 mM^{-1} cm^{-1}). Total APX activity was measured by monitoring the decline in absorbance at 290 nm, as ascorbate (ε= 2.8 $mM^{-1}cm^{-1}$) was oxidized, for 2 min using the method of Nakano and Asada [26]. The assay medium consisted of 600 µL of 50 mM K-PO_4 buffer (pH 7.0) with ascorbate, 600 µL of buffer with hydrogen peroxide and 300 µL of sample extract. APX activity was expressed in mM ascorbate.$min^{-1}mg^{-1}$ of proteins.

2.8 Non-enzymatic Antioxidant System (Carotenoids, Total Phenolics and Flavonoids) and UV-Visible Scanning of Phenolic Extracts

Carotenoids were determined by the method described by Lichtenthaler & Buschman [27] using cold acetone as a solvent and calculated by Lambert-Beer equations (4-6) respectively.

$$\left[C_{carot}\left(\mu g/ml\right) = (1000A_{470} - 1.90C_a - 63.14C_b)/214\right] \quad (4)$$

The total phenolic contents of were determined by Folin-Ciocalteau reagent (FCR) method. For a 2.0 mL total volume, 200 µL of extract were first mixed with 100 µL of FCR reagent after adding 1.40 mL distilled water and the contents were kept at room temperature for 10 minutes. Later, 300 µL of Na_2CO_3 aqueous solution (20%) were added and incubated for 1 hour. The absorbance was measured at 765 nm using a spectrophotometer. Total phenolics content was expressed as µg of gallic acid equivalents/g of dry extract (µg GAE/g) using a standard curve (0-1000 µg/mL) of gallic acid [28].

UV-visible (UV-vis) scanning (200-800 nm) using spectrophotometer of phenolic extracts was also performed. Spectra were normalized, baseline corrected, subjected to feature selection at the region of interest and non-supervised multivariate analysis (hierarchical cluster analysis-HCA and principal component analysis-PCA).

Total flavonoid content of plant extract was determined using aluminum chloride colorimetric method (Woisky & Salatino [29] and revised by Chang [30]) and standard solutions (0-1000 µg/mL of quercetin in 80% methanol). For that, 1 mL extract solution was mixed with 0.5 mL 95% ethanol (v/v), 0.1 mL 1 M potassium acetate, 0.1 mL aluminum chloride solution (10% $AlCl_3$), and 0.8 mL distilled water to a total volume of 2.5 mL. The mixture was well mixed and incubated at room temperature for 30 minutes versus reagent blank containing water instead of sample. Quercetin was used as the standard for the quantification of total flavonoid. Results were expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g).

2.9 Data Mining and Statistics

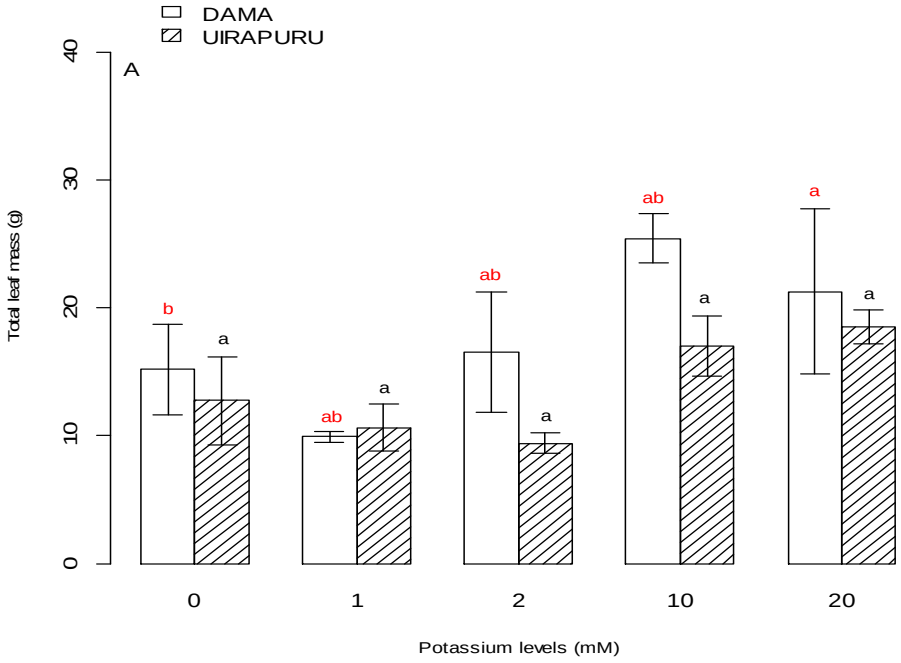
Data were summarized and subjected to normality (Shapiro-Wilk) and homogeneity (Levene) tests and analysis of variance (Two-way ANOVA). Tukey HSD ($p<0.05$) test was used where differences were found. Multivariate statistical techniques were also used in other to find important variables related to potassium in common beans. All analyses were performed in R software [31] using scripts produced by our group. A report in html format is provided as supplementary data for data analysis reproducibility. Leaf Gas exchange models were fitted using Plantecophys package [22]. Equations were made in Mathtype software.

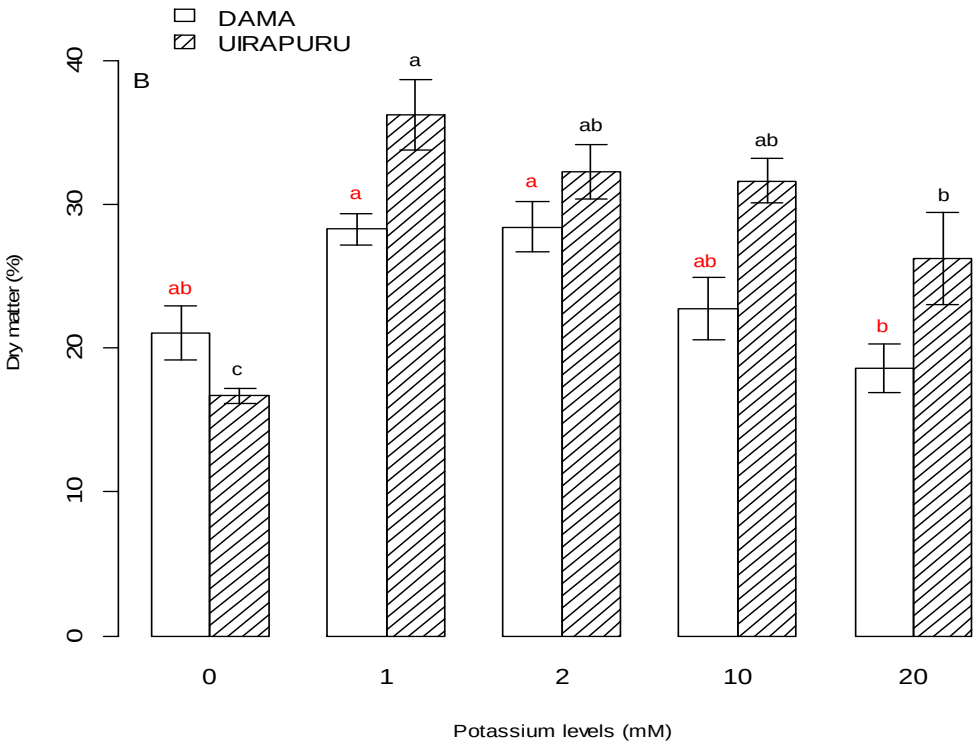
3. RESULTS AND DISCUSSION

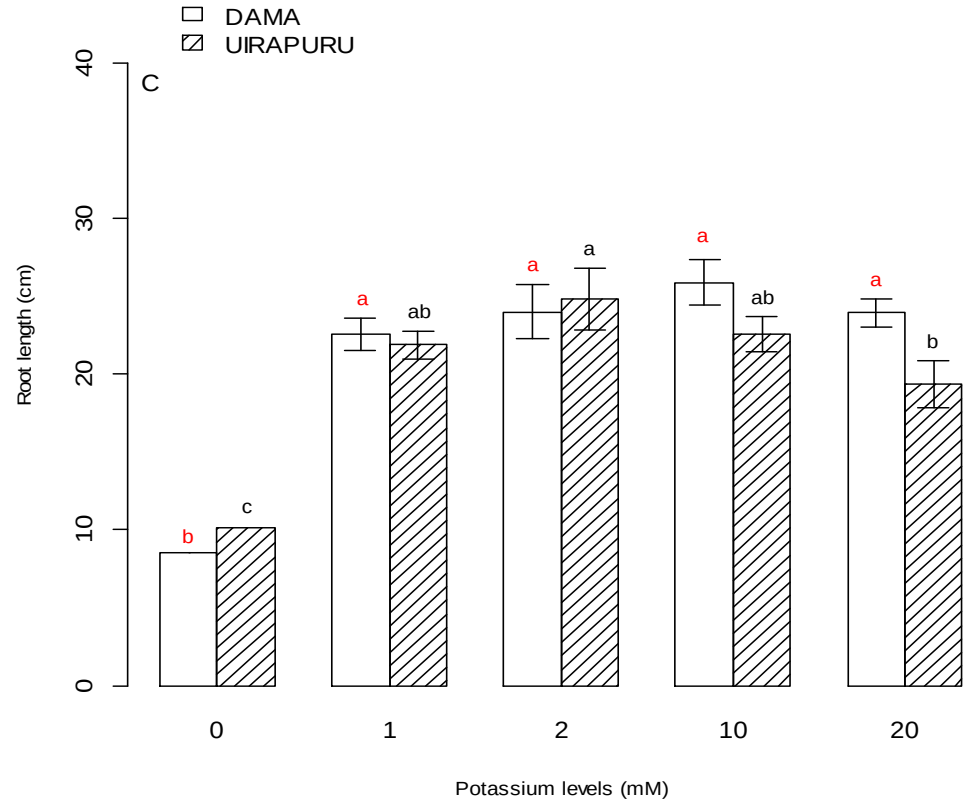
3.1 Growth Parameters (Total Fresh Leaf Mass, Dry Matter, Root Length and Root Volume)

Results of growth parameters are summarized in the Fig. 2(A-D). Two-way ANOVA of the total fresh leaf mass showed differences between potassium levels in Dama cultivar. 10mM of potassium nutrition provided major total fresh leaf mass ($p<0.05$) and 1mM the minor value of leaf mass. All other levels (0, 2, 20 mM) were statistically similar (Fig. 2A). Significant statistical differences were found between potassium at 10 Mm and other levels ($p<0.05$). Increases in potassium promoted the total leaf mass of "Dama" cultivar until level 10. For "Uirapuru" cultivar, non-significant differences were found (Fig. 2A). Previous results by Yaghub [32] also reported improvement of leaf mass by K application. Contrarily, K deficiency was found to decrease the leaf number, leaf area and reproductive dry weight [10]. These changes explain our results. Potassium deficiency affects the carbon-nitrogen balance. Improved plant survival and reduction of shoot elongation were also reported to be affected by K addition [11].

The dry matter of leaves (Fig 2B) differed between cultivars and potassium levels and was affected adversely by potassium. Independently of cultivar, the dry matter decreased gradually (1.5 fold and 1.4 fold) with increases of potassium levels in 'Dama' and 'Uirapuru' respectively from 1 to 20mM of potassium (Fig 2B). The 'Uirapuru' cultivar presented higher dry matter (16.7% fold) than 'Dama' ($P<0.05$). Tukey HSD test ($p<0.05$) showed significant differences between levels 1 and 2 with 10 and 20 in 'Dama' and between (2 and 10) with (1 and 20) in 'Uirapuru' (see supplementary data for details). Similar results were reported by Taibi [33] testing the effect of salt stress (NaCl) in Phaseoulus vulgaris. In their research shoot and root biomass decreased with increases of salinity. K induces phenotypic dry matter allocation plasticity and was reported to be responsible of carbon allocation to osmotic adjustment [34]. Root length and volume showed a direct relationship between potassium levels. When K concentration increased until 10 mM, the root length and volume (Figs. 2C and 2D) increased in cultivar dependent manner. In 'Dama' cultivar increases were observed until level 10 of potassium and in 'Uirapuru' until 2mM (Figs 2C-D). Besides, root length did not differed statistically in 'Dama' cultivar ($p<0.05$). Similar trends were also observed for root volume (see supplementary data). In Sulla carnosa, a forage legume the growth of vegetative organs was decreased by 50% by K deficiency being the stems more affected than roots and leaves [35].




DAMA
UIRAPURU
B
Dry matter (%)
0
10
20
30
40
ab
c
a
a
ab
a
ab
ab
b
b
0
1
2
10
20
Potassium levels (mM)


DAMA
UIRAPURU
C
Root length (cm)
0
10
20
30
40
b
c
a
ab
a
a
a
ab
a
b
0
1
2
10
20
Potassium levels (mM)

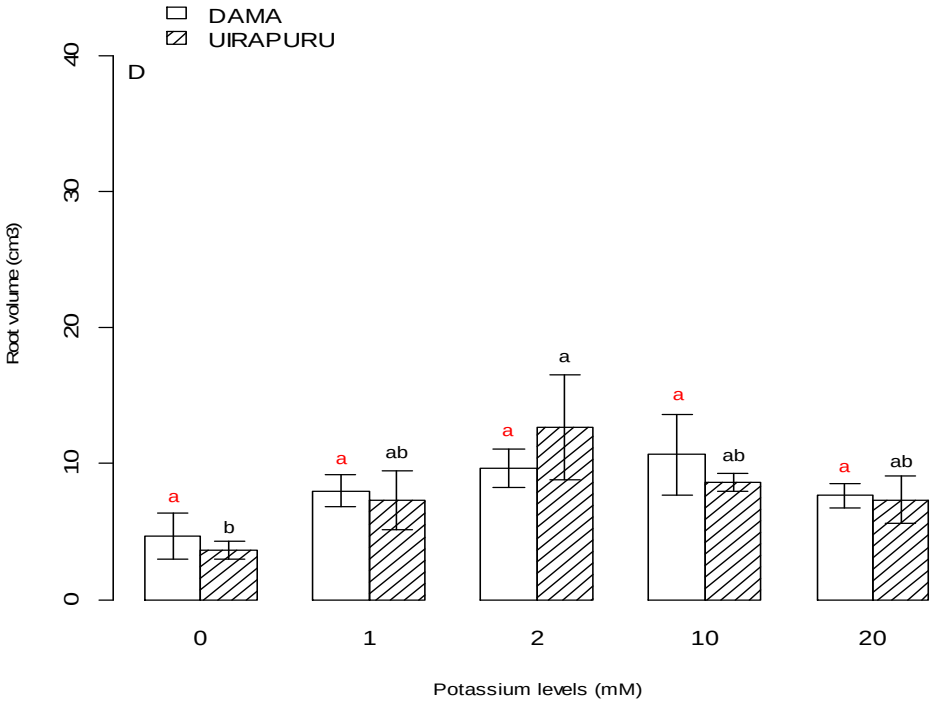


Fig. 2. Total leaf mass (A), dry matter (B), root length (C) and root volume (D) of the two cultivars subjected to potassium levels. Tukey HSD tests are presented in the supplementary data

When Pearson correlation of all variables studied ($p<0.05$) was done (Table 1), the total leaf mass (TLM), root volume (RV) and root length (RL) were found to be positively correlated with chlorophyll contents (Table 1) and these last two also correlated with catalase activity (Table 1). The dry matter content of leaves was negatively correlated with carotenoid content and TLM.

3.2 Photosynthetic Pigments, Enzymatic and Non-enzymatic Antioxidants

3.2.1 Chlorophyll A and B

Chlorophyll A and B increased with potassium application (Fig. 3A-B) in all cultivars. The accumulation of chlorophylls was higher in 'Dama' cultivar than 'Uirapuru' ($p<0.05$). Increases in chlorophyll contents may be attributed to the role of K in plant metabolism. Essentiality of K in plant life cycle is evidenced by its role in activating enzymes which are involved in various physiological processes such as energy metabolism, starch synthesis, nitrate reduction, photosynthesis and sugar degradation. As a component of plant cytoplasmatic solution, K reduces loss of water from leaf surfaces by regulating stomatal closure and increases water uptake efficiency of roots from soil mainly due to the role of K in regulating cellular osmotic potentials [36] which may explain our observed results. Cultivar 'Dama' was most efficient in activating chlorophyll pigments.

3.2.2 Catalase activity (CAT)

CAT in both genotypes increased until 2mM of potassium and then decreased gradually (Fig 3C). At higher levels of potassium, CAT was less important as enzymatic antioxidant system. The activation of CAT was markedly in 'Dama' cultivar than 'Uirapuru'. 'Dama' exhibited plants with better performance may be due to the activation of many antioxidant mechanisms. Contrarily, the level of ascorbate peroxidase (APX) was higher in 'Uirapuru', which decreased with potassium levels (Fig 3D). Trace amounts of APX were found in 'Dama'. Results indicated that these 2 enzymatic systems may be of lesser importance in all genotypes at higher rates of potassium.

3.2.3 Carotenoids

Carotenoids (Fig. 3E) increased with potassium levels. The rate was higher in 'Dama' than

Table 1. Pearson correlations of all variables. Chla, Chlb and Chlt mean chlorophyll a, b and total respectively. CAT-catalase activity; APX-ascorbate peroxidase activity; CAR- total carotenoids; FLA-total flavonoids; PHE-total phenolic compounds; TLM-total leaf mass; DM- dry matter; RL-root length; RV- root volume; TRA-transpiration rate; SVTleaf-leaf saturation vapor; Cond- stomatal conductance; CndTotal -total stomatal conductance; WUE-intrinsic water use efficiency; Ls-stomatal limitation value; Pn-net photosynthesis; CarbE- carboxylation efficiency of Rubisco; Ci and Ca-intercellular and ambient carbon dioxide respectively; Ti and Ta-intercellular and ambient temperature respectively and NDVI-normalized difference vegetative index

	Chla	Chlb	Chlt	CAT	APX	CAR	FLA	PHE	TLM	DM	RL	RV	Pn	Cond	Ci	TRA	Ti/Ta	SVTleaf	CndTotal	Ci/Ca	WUE	Ls	CarbE
Chla																							
Chlb	0.98* * * *																						
Chlt	1.00* * * *	0.99* * * *																					
CAT	0.43	0.32	0.41																				
APX	0.04	0.06	0.04	-0.34																			
CAR	-0.38	-0.31	-0.36	-0.26	0.10																		
FLA	-0.35	-0.30	-0.34	0.15	-0.54	-0.01																	
PHE	-0.52	-0.52	-0.52	-0.32	-0.13	-0.13	0.08																
TLM	0.61	0.63	0.61	0.19	-0.32	0.29	-0.26	-0.41															
DM	0.12	0.11	0.12	0.00	0.42	-0.62	-0.23	0.32	-0.46														
RL	0.85* *	0.81* *	0.84* *	0.58	0.16	-0.58	-0.21	-0.43	0.23	0.52													
RV	0.59	0.53	0.58	0.62	0.07	-0.46	-0.18	-0.35	0.11	0.53	0.86* *												
Pn	-0.09	0.02	-0.06	-0.46	-0.17	0.37	-0.13	0.31	0.41	0.02	-0.22	-0.08											
Cond	0.10	0.22	0.13	-0.33	-0.10	-0.04	0.00	0.17	0.08	0.27	0.05	0.04	0.64*										
Ci	-0.51	-0.59	-0.53	-0.10	0.21	-0.14	0.14	0.01	-0.62	-0.01	-0.30	-0.19	-0.62	-0.72*									
Tra	-0.17	-0.05	-0.14	-0.48	-0.18	0.20	0.03	0.22	0.08	0.07	-0.26	-0.15	0.76*	0.93* * * *	-0.59								
TI/Ta	0.27	0.38	0.30	-0.19	-0.07	-0.19	0.01	0.12	0.09	0.36	0.24	0.15	0.51	0.97* * * *	-0.76*	0.81* *							
SVTleaf	0.14	0.26	0.17	-0.28	-0.12	-0.05	0.03	0.15	0.11	0.24	0.08	0.03	0.60	0.99* * * *	-0.76*	0.90* * * *	0.98* * * *						
CndTotal	-0.27	-0.16	-0.24	-0.58	-0.13	0.23	-0.04	0.24	0.04	0.08	-0.34	-0.18	0.80* *	0.85* *	-0.46	0.98* * * *	0.70*	0.80* *					
Ci/Ca	-0.54	-0.62	-0.56	-0.11	0.17	-0.09	0.16	0.02	-0.58	-0.07	-0.35	-0.24	-0.59	-0.74*	1.00* * * *	-0.59	-0.79* *	-0.78* *	-0.46				
WUE	0.43	0.35	0.41	0.57	-0.04	0.19	-0.22	-0.26	0.55	-0.33	0.29	0.22	-0.23	-0.66*	-0.04	-0.69*	-0.58	-0.62	-0.71*	-0.01			
Ls	0.54	0.62	0.56	0.10	-0.18	0.06	-0.14	-0.01	0.58	0.08	0.35	0.24	0.59	0.74*	-1.00* * * *	0.59	0.79* *	0.78* *	0.45	-1.00* * * *	0.01		
CarbE	0.14	0.27	0.17	-0.34	-0.16	0.09	-0.04	0.22	0.30	0.19	0.03	0.04	0.83* *	0.95* * * *	-0.82* *	0.91* * * *	0.90* * * *	0.94* * * *	0.84* *	-0.83* *	-0.46	0.83* *	
NDVI	-0.52	-0.51	-0.52	0.08	-0.61	0.45	0.33	0.14	0.09	-0.39	-0.52	-0.19	0.40	0.24	-0.15	0.49	0.08	0.22	0.50	-0.11	-0.22	0.09	0.26

*Significance levels: p < .0001 '****'; p < .001 '***', p < .01 '**', p < .05 '*'*

***Chla, Chlb and Chlt mean chlorophyll a, b and total respectively. CAT-catalase activity; APX-ascorbate peroxidase activity; CAR- total carotenoids; FLA-total flavonoids; PHE-total phenolic compounds; TLM-total leaf mass; DM- dry matter; RL-root length; RV-root volume; TRA-transpiration rate; SVTleaf-leaf saturation vapor; Cond- stomatal conductance; CndTotal -total stomatal conductance; WUE-intrinsic water use efficiency; Ls-stomatal limitation value; Pn-net photosynthesis; CarbE- carboxylation efficiency of Rubisco; Ci and Ca-intercellular and ambient carbon dioxide respectively; Ti and Ta-intercellular and ambient temperature respectively and NDVI-normalized difference vegetative index.*

'Uirapuru'. Carotenoids, being antioxidants, have the potential to detoxify the plants from the effects of reactive oxygen species. Carotenoids are known to function as collectors of light energy for photosynthesis and as quenchers of triplet chlorophyll and O_2. Moreover, they dissipate excess energy via the xanthophyll cycle and can act as powerful chloroplast membrane stabilizers [33, 37, 38]. Increases in carotenoid contents indicate that those compounds play an important role under increases of potassium levels. As reported by Cazzonelli & Pogson [39], carotenoids play essential roles in development, photosynthesis, root mycorrhizal interactions and the production of phytohormones, such as abscisic acid and strigolactones.

3.2.4 Flavonoids

For flavonoid contents (Fig. 3F), increases were observed only for 'Dama' cultivar. Flavonoids protect the plant against ultraviolet radiations, have antimicrobial properties and act as a deterrent for herbivores by limiting assimilation of dietary proteins and inhibiting digestive enzymes [40]. Furthermore, flavonoids act as scavengers of free radicals such as reactive oxygen species (ROS), and also prevent their formation by chelating metals [41-44, 40]. Increases in flavonoids observed in our research may be related to antioxidant functions of those compounds mainly in 'Dama' cultivar which exhibited plants with better performance.

3.2.5 Total Phenolics

Regarding the phenolic content, decreases were observed until level 2 of potassium and then increased in Dama culttivar. Contrarily, for 'Uirapuru', an increase until 2mM and then decreased gradually (Fig. 3G). Phenolics were observed to play a role only for 'Dama' Cultivar at higher levels of potassium. This group of compounds is claimed to have the ability of free-radical scavenging and are essential to plant physiology and resistance to biotic and abiotic stresses. Besides removing free radicals, these compounds are capable of chelating metal, activating antioxidant enzymes, and inhibiting oxidases [41].

3.3 Normalized Difference Vegetation Index (NDVI)

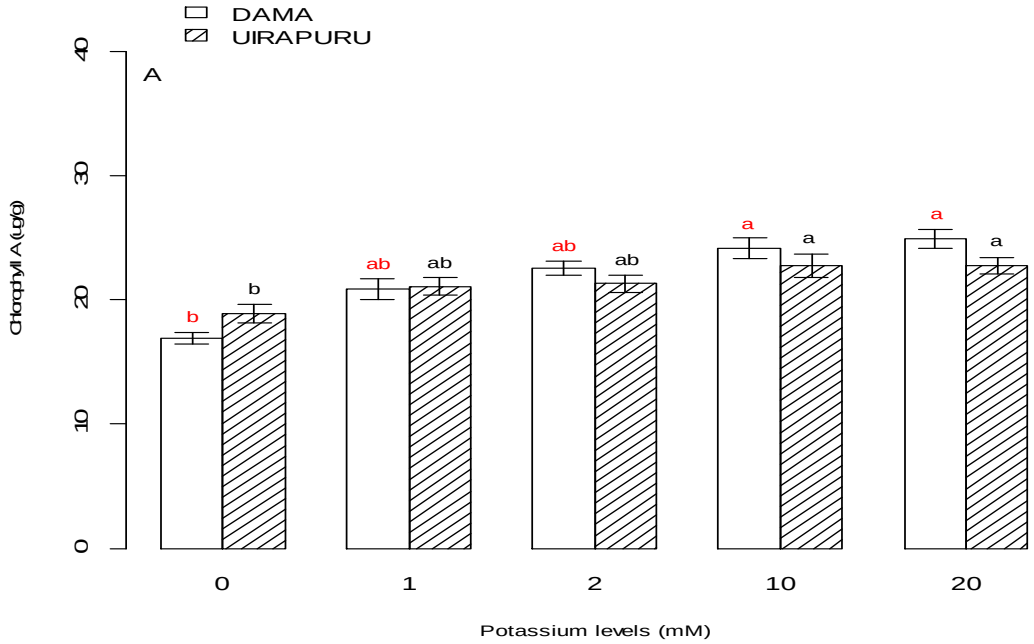
NDVI results are summarized in the Fig. 3H. As it can be observed, NDVI index increased gradually with potassium levels until 10mM. Live green vegetation was higher in 'Dama' cultivar than 'Uirapuru' but was statistically non significant ($p<0.05$, Tukey HSD test). The results were in accordance with the total biomass observed by that Cultivar. NDVI was higher in control plants than those at 20mM of potassium and was positively correlated with carotenoids, flavonoids, net photosynthesis, stomatal conductance, leaf saturation vapour and the carboxilation efficiency and negatively correlated with chlorophyll contents (See Table 1). Similar results were also reported by Penuelas [44] studying physiological changes in nitrogen and water limited sunflower leaves. Positive correlations with chlorophyll and secondary metabolites were also reported previously in aquatic vegetation [45-46]. NDVI was also positively correlated with leaf area index [47]. NDVI is a broadband index (i.e., normalized differences between the reflectance in the near infrared and the red regions of the light spectrum) that is related to green biomass and has been used to indirectly estimate photosynthetic capacity and net primary productivity [48] and assess whether the target being observed contains live green vegetation or not. NDVI correlates well with canopy features such as biomass, leaf area index (LAI), absorbed photosynthetically active radiation, and canopy photosynthetic capacity, but fails to capture dynamic physiological processes, which may occur on fine temporal and spectral scales [49,48].

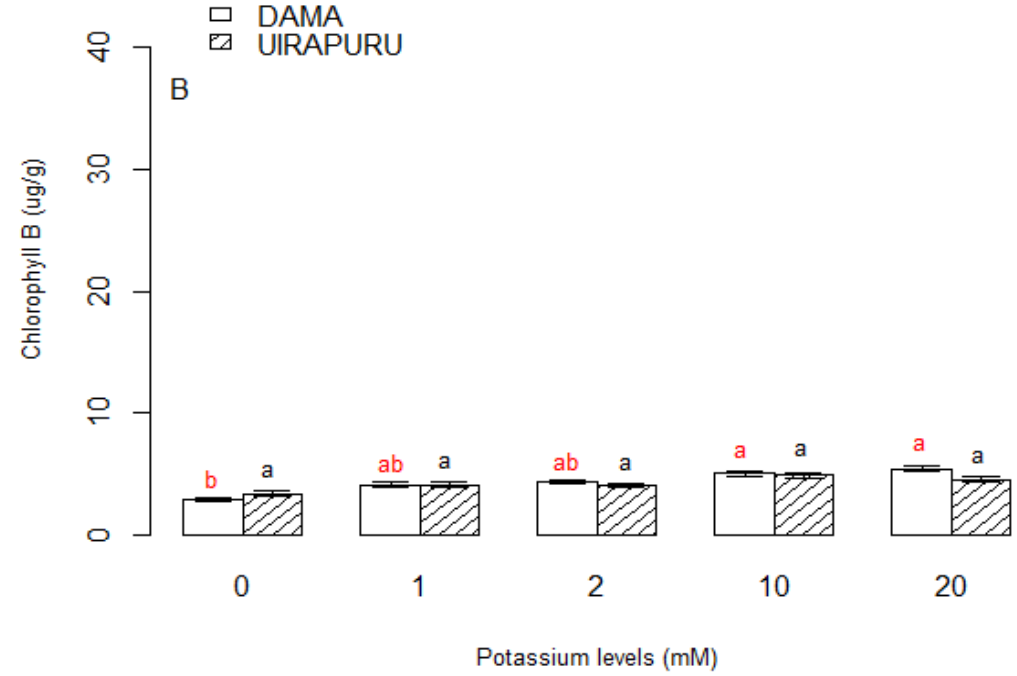
3.4 UV-visible Scanning of Phenolic Extracts

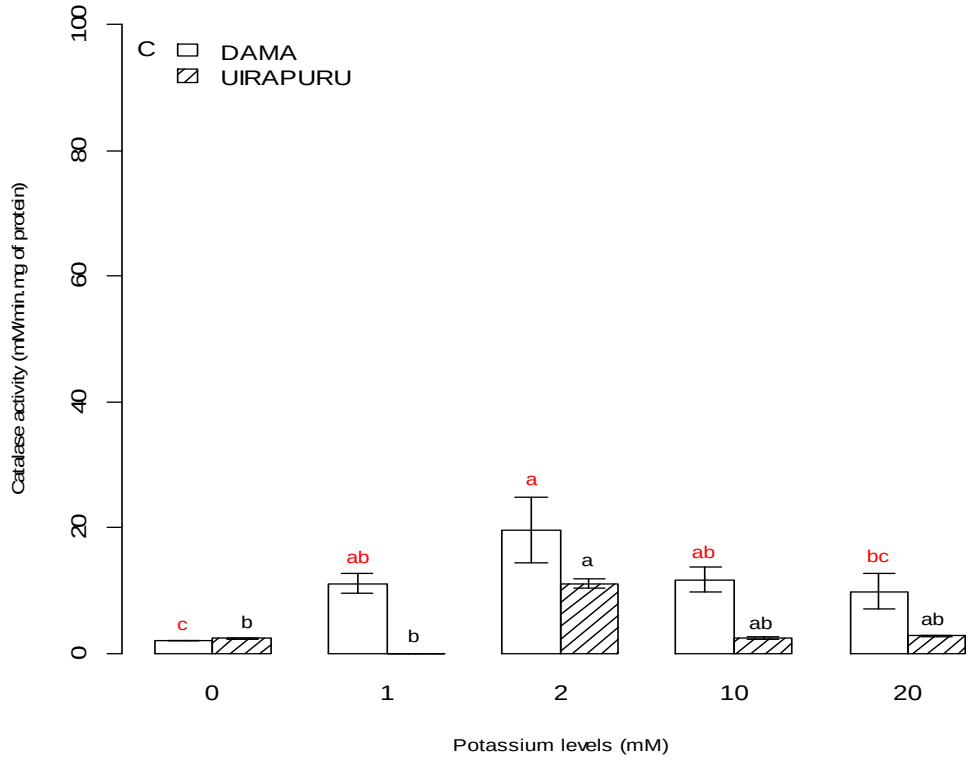
All sample spectra showed higher absorbance in the similar wavelength regions (300-500 nm - region of phenolics and carotenoids and 640-700nm (see supplementary Fig. 1). Those regions were selected for further multivariate analysis. HCA analysis showed four different groups. 'Dama' cultivar samples with 1 and 10 mM of potassium separated alone in distinct groups. The third group was composed mainly by 'Uirapuru' cultivar samples with 1, 2, 10, control and 'Dama' with 20 mM. The fourth group was composed by 'Dama' cultivar samples (control, 2 mM) and'Uirapuru'with 20 mM of potassium. The cophenetic correlation of HCA was 88% according to Euclidean distance (Fig. 4A).

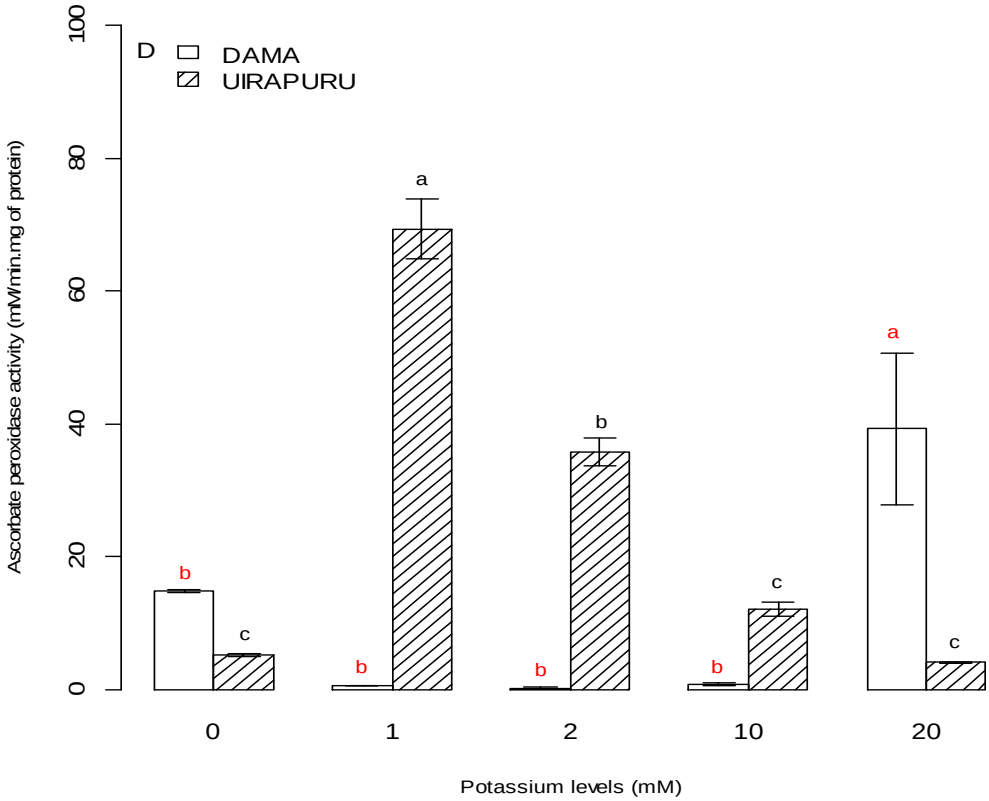
Principal component analysis showed that samples of cultivar 'Dama' with potassium levels of 1mM and 10mM separated alone duo to their

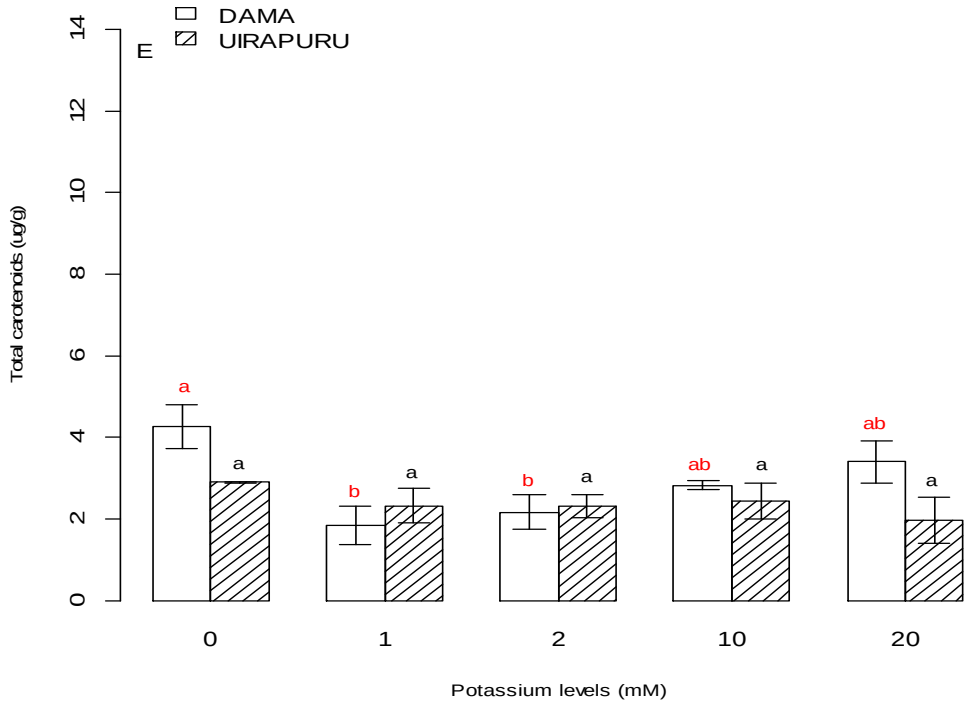
composition in chlorophyll contents as it can be observed by the loadings or Eigen values of PCA (Fig. 4B). Other samples were similar due their composition in phenolics and carotenoids. The total variance explained by PCA was 88.3%, being 48.6 and 33.7% for component 1 (PC1) and 2 (PC2) respectively.

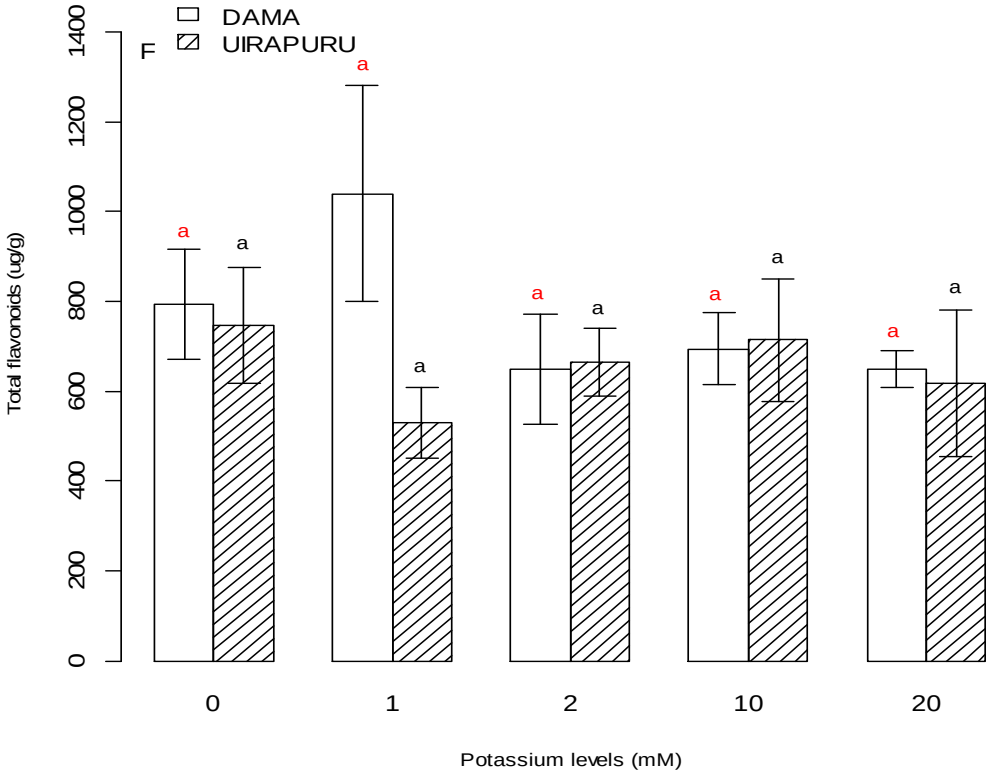





C
DAMA
UIRAPURU
Catalase activity (mM/min.mg of protein)
0
20
40
60
80
100
c
b
ab
b
a
a
ab
ab
bc
ab
0
1
2
10
20
Potassium levels (mM)


D
DAMA
UIRAPURU
Ascorbate peroxidase activity (mM/min.mg of protein)
0
20
40
60
80
100
b
c
b
a
b
b
b
c
a
c
0
1
2
10
20
Potassium levels (mM)


DAMA
UIRAPURU
E
Total carotenoids (ug/g)
0
2
4
6
8
10
12
14
a
a
b
a
b
a
ab
a
ab
a
0
1
2
10
20
Potassium levels (mM)


DAMA
UIRAPURU
F
Total flavonoids (ug/g)
0
200
400
600
800
1000
1200
1400
a
a
a
a
a
a
a
a
a
a
0
1
2
10
20
Potassium levels (mM)

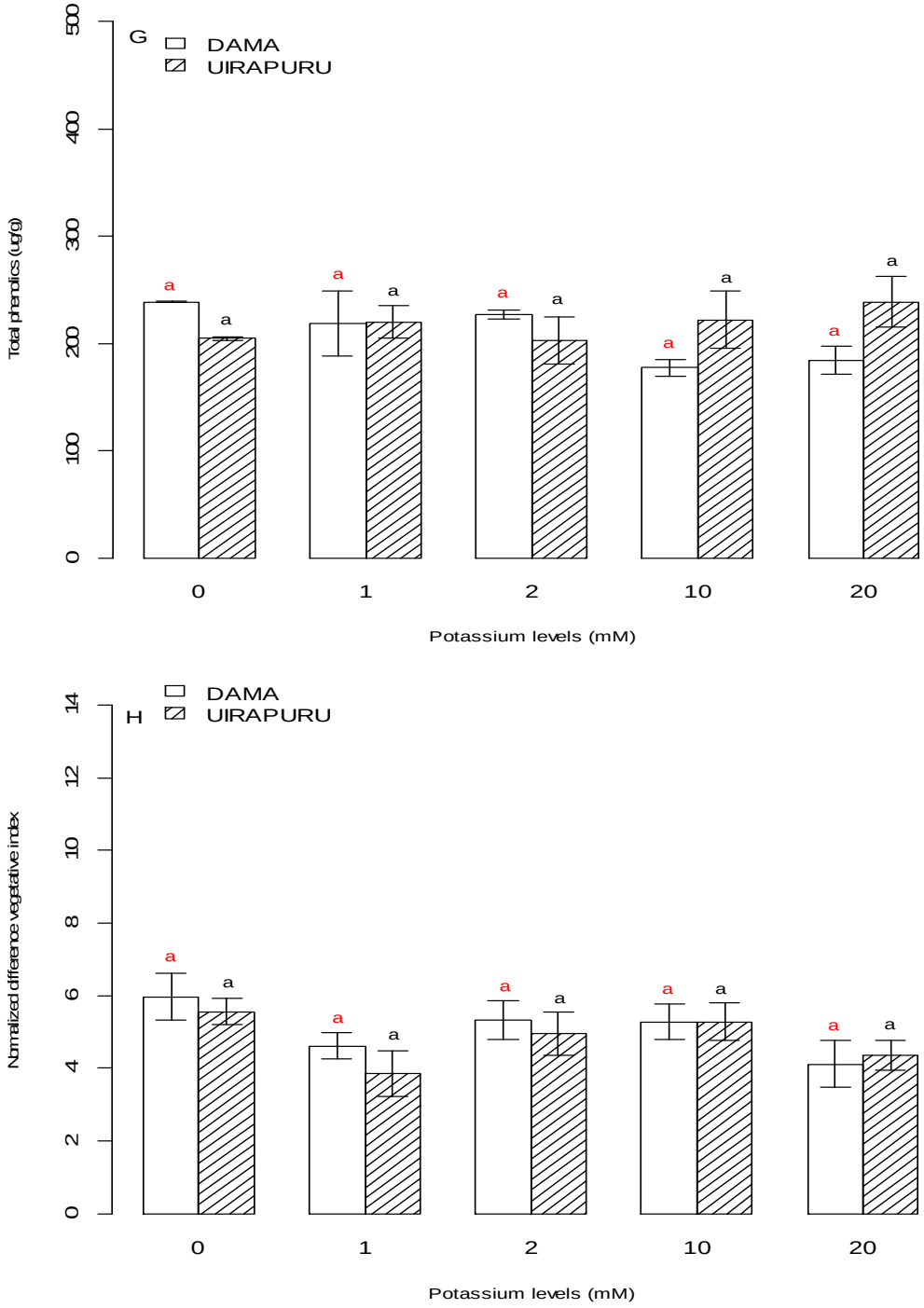


Fig. 3. Changes in chlorophyll A (A), chlorophyll B (B), catalase (C), ascorbate peroxidase (D), total carotenoid contents (E), total flavonoids (F), total phenolic contents (G) and NDVI index (H) with potassium levels for two common beans cultivars. Values are representative of mean and standard error of the mean

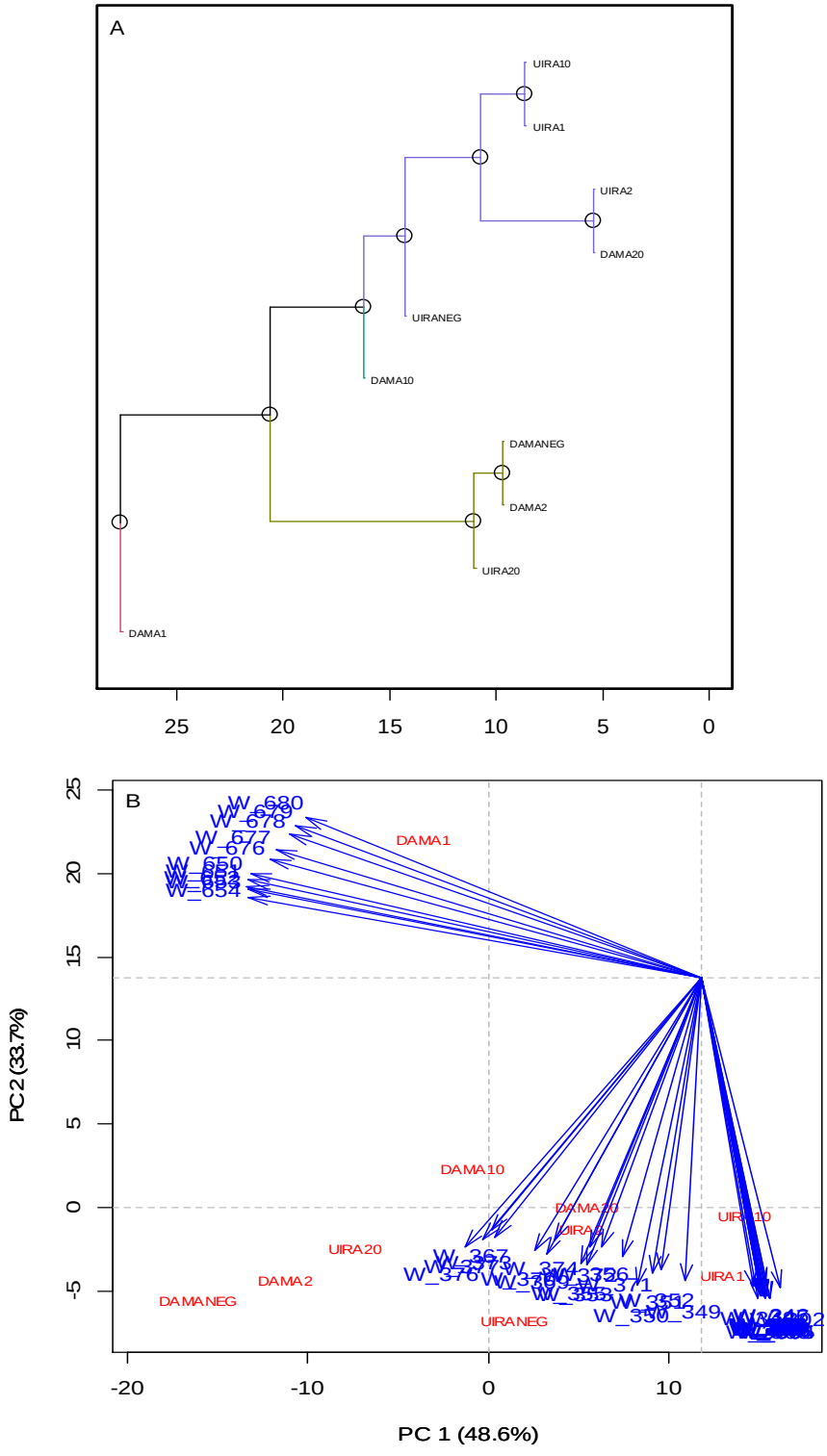


Fig. 4. (A) Hierarchical cluster analysis of UV-Visible scanning spectra of all samples (B) Score plot and loadings of principal component analysis (B)

3.5 Leaf Gas-exchange Data

Results of leaf gas exchange are summarized in the Table 2. The net photosynthesis of all cultivars increased gradually with potassium levels from 1 to 10mM. The rate of photosynthesis was higher in 'Uirapuru' cultivar except for control plants. A slight increase in stomatal conductance was observed for 'Dama' cultivar. Higher rate of stomatal conductance at 10mM of potassium was observed for 'Uirapuru'. Intercellular carbon dioxide decreased in all cultivars but the rate was higher for 'Uirapuru' at level 10 of potassium. The rate of transpiration in all cultivars increased with potassium until level of 10mM. The rate of transpiration was higher in 'Uirapuru'. The leaf to air temperature and leaf saturation vapor showed slight variation in plants treated with different potassium levels, except at level 10 where a maxima was observed in 'Uirapuru' cultivar. The total conductance to carbon dioxide showed similar trend as in stomatal conductance previously mentioned in the Fig. 5B. The rate of intercellular to ambient carbon dioxide decreased in all cultivars, but the decreasing rate was higher at 10mM of potassium for 'Uirapuru' cultivar. Cultivar 'Dama' showed water use efficiency and superior stomatal limitation value except at 10mM of potassium. Higher rubisco carboxilation was also observed at 10mM of potassium in 'Uirapuru'.

Table 2. Gas-exchange measurements of the two common bean cultivars. Net photosynthesis, stomatal conductance, intercellular carbon dioxide, Transpiration rate, Leaf to air temperature ratio, Leaf saturation vapor, Total conductance to carbon dioxide, Intercellular to ambient carbon dioxide, Intrinsic water use efficiency, Stomatal limitation value and Carboxylation efficiency of rubisco

Dama cultivar**										
Potassium amount	**0**		**1**		**2**		**10**		**20**	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Pn	6.31	1.6	2.82	2.4	3.32	0.9	5.19	2.6	3.50	2.1
Cond	0.10	0.0	0.04	0.0	0.04	0.0	0.08	0.0	0.04	0.0
Ci	274.05	2.0	299.69	64.4	258.32	25.3	261.44	16.8	249.16	29.6
Trmmol	2.60	0.6	1.04	0.5	1.15	0.2	1.77	1.0	1.07	0.5
Tl/Ta	0.57	0.3	1.29	0.2	1.28	0.1	0.97	0.4	1.24	0.2
SVTleaf	4.02	0.1	3.70	0.2	3.85	0.1	3.75	0.1	3.88	0.2
CndTotal	0.10	0.0	0.04	0.0	0.04	0.0	0.07	0.0	0.04	0.0
Ci/Ca	0.70	0.0	0.75	0.2	0.65	0.1	0.67	0.0	0.63	0.1
WUE	61.52	2.3	53.43	32.6	75.70	16.0	71.97	11.3	82.43	14.8
Ls	0.30	0.0	0.25	0.1	0.35	0.1	0.33	0.0	0.37	0.1
CarbE	0.02	0.0	0.01	0.0	0.01	0.0	0.02	0.0	0.01	0.0
Uirapuru cultivar										
Pn	3.25	0.8	3.77	3.1	3.76	2.6	6.67	3.2	5.29	3.0
Cond	0.09	0.0	0.07	0.0	0.08	0.1	0.70	0.7	0.07	0.0
Ci	311.20	26.1	302.73	21.2	301.69	6.1	205.81	129.2	275.08	55.6
Trmmol	2.31	1.0	1.33	0.4	1.66	1.3	4.79	0.9	1.66	0.7
Tl/Ta	0.73	0.4	1.15	0.2	0.97	0.6	6.18	8.8	1.01	0.4
SVTleaf	4.01	0.1	3.60	0.4	3.48	0.5	10.97	10.2	3.69	0.3
CndTotal	0.09	0.0	0.06	0.0	0.07	0.1	0.15	0.1	0.07	0.0
Ci/Ca	0.79	0.1	0.76	0.1	0.75	0.0	0.51	0.4	0.70	0.1
WUE	41.96	16.0	51.12	14.5	55.01	9.7	30.97	22.6	67.57	16.3
Ls	0.21	0.1	0.24	0.1	0.25	0.0	0.49	0.4	0.31	0.1
CarbE	0.01	0.0	0.01	0.0	0.01	0.0	0.05	0.0	0.02	0.0

*** Net photosynthesis (Pn). stomatal conductance (Cond). intercellular carbon dioxide (Ci). Transpiration rate (Trmmol). Leaf to air temperature ratio (Tl/Ta). Leaf saturation vapor (SVTleaf). Total conductance to carbon dioxide (CndTotal). Intercellular to ambient carbon dioxide (Ci/Ca). Intrinsic water use efficiency (WUE). Stomatal limitation value and Carboxylation efficiency of rubisco (CarbE)*

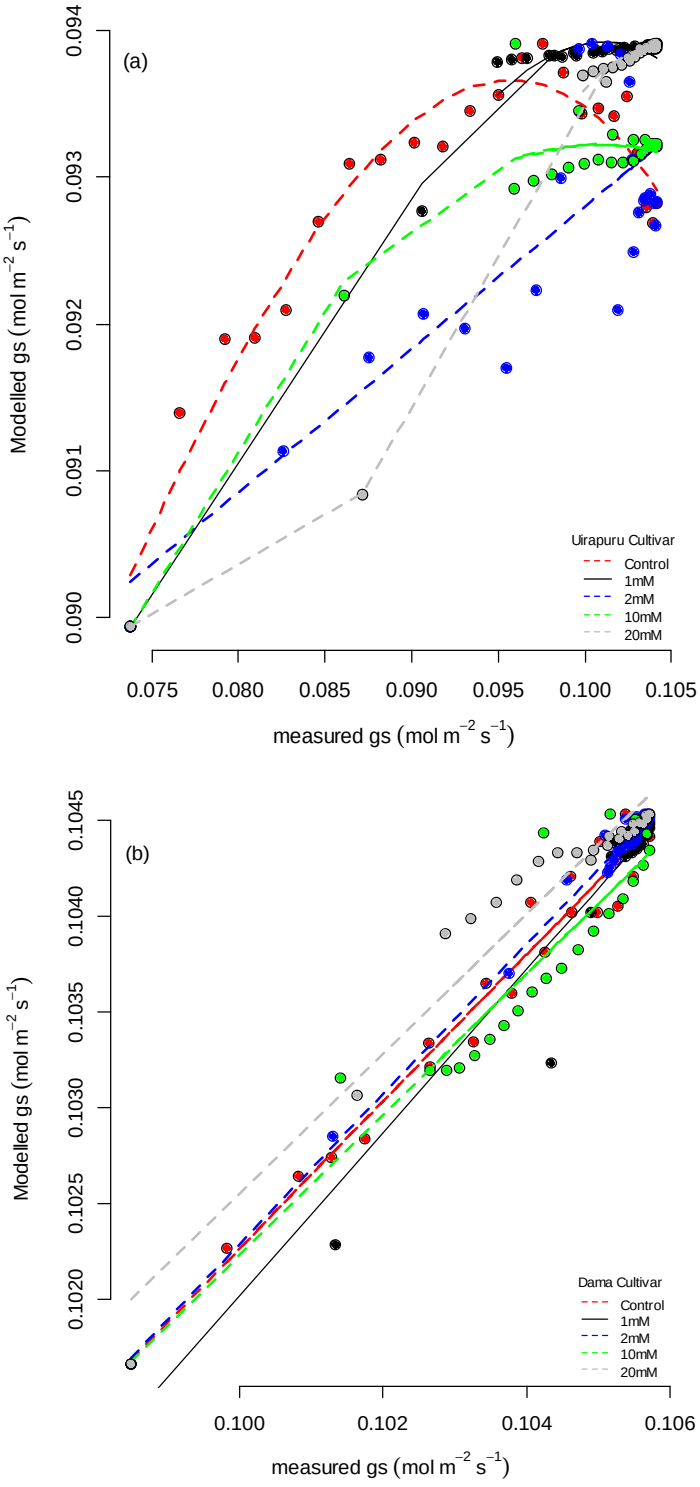


Fig. 5. Modeled (with the model of Medlyn et al., 2011) versus measured stomatal conductance (gs) for the two cultivars studied under different potassium levels. (A) for 'Uirapuru' and (B) for 'Dama' cultivars

Photosynthetic assimilation of carbon is a defining feature of plant kingdom. The fixation of large amount of carbon dioxide supports the synthesis of carbohydrates, which make up the bulk of plant biomass. Measurements of carbon assimilation rates are therefore crucial duo to their impact on the plant metabolism, growth and reproductive success [50]. As it can be observed in our results, potassium nutrition in the range of (1-10 mM) promoted higher carbon assimilation. The decrease of intercellular carbon dioxide may be attributed to the higher transpiration rate and assimilation activity observed. Cultivar 'Dama' was most efficient in water use and showed lower stomatal limitation value. Higher carbon assimilation rates in 'Uirapuru' cultivar may be attributed to the elevated stomatal limitation value. The carboxylation of rubisco was more pronounced at 10 mM of potassium in all cultivars. It is important to note that the presence of finite stomatal resistance to carbon dioxide cause carbon dioxide at sites of carboxylation to be less than that in the atmosphere, thereby reducing the rate of carbon dioxide assimilation somewhat below its potential. Nevertheless, stomata usually impose the largest resistance to diffusion [51]. Lower stomatal values in 'Dama' cultivar may have contributed to lower carbon dioxide assimilation rates observed in this research. Previous report has indicated that K deficiency promotes inhibition of enzymatic-photochemical processes [52]. Results indicate lower chlorophyll content and altered Rubisco activity as probable causes of photosynthetic impairment. Potassium deficiency was found to diminish photoprotection mechanisms due to reduced photosynthetic and photorespiratory capacity [52].

In the past, stomatal responses have generally been considered in relation to single environmental variables in part because the interactions between factors have appeared difficult to quantify in a simple way. A linear correlation between stomatal conductance (gs) and carbon dioxide assimilation rate has been reported when photon fluence was varied and when the photosynthetic capacity of leaves was altered by growth conditions, provided $C0_2$, air humidity and leaf temperature were constant [53]. In this research using the measured gs we fitted the stomatal conductance models of the two cultivars (Fig. 5A-B). A linear ('Dama' cultivar) and non-linear regression was found for 'Uirapuru' cultivar (Fig 5A-B). As we know, fundamental to any examination of stomatal functioning is the understanding of how rate of assimilation of carbon dioxide responds to potassium levels. Furthermore, stomatal movements provide the leaf with the opportunity to change both the partial pressure of carbon dioxide at sites of carboxylation and the rate of transpiration [51,54-56]. For 'Dama' cultivar (Fig. 5B) at all levels of potassium, the fitted models showed a linear relation, but such trend was not observed for 'Uirapuru' (Fig 5A).

Data of all variables studied were also correlated (Pearson correlation, $p<0.05$) aiming to find an association between them. As it can be observed in the Table 1, a positive correlation of root length with chlorophylls was found. Significant positive association was also observed in net photosynthesis with stomatal conductance and carboxylation efficiency (Table 1). The stomatal conductance was negatively correlated with the intercellular carbon dioxide and intrinsic water use efficiency. The transpiration rate was positively correlated with leaf temperature, leaf saturation vapor and stomatal conductance. Contrarily, a negative association of NDVI index with chlorophyll contents was observed (see Table 1). A positive correlation between the stomatal limitation value with the carboxylation efficiency of rubisco was also found.

When all dataset was subjected to principal component analysis (PCA) aiming to dimensionality reduction and find important variables, similarities and dissimilarities between the samples (Fig. 6), an interesting result was found, as it can be observed in the score plot and loadings of PCA. Control samples grouped together showing that there are similar and composed the first group in PCA. Such an observation was also found for those samples where 1mM of potassium was applied. Samples of 'Uirapuru' cultivar with 2 and 20 mM of potassium grouped together. A clear separation was observed from 'Uirapuru' at 10mM of potassium and the control samples. Samples of 'Dama' cultivar at 2, 10 and 20 grouped also together duo to their catalase activity and intrinsic water efficiency presented. Intercellular carbon dioxide and the ratio of intercellular to ambient carbon dioxide where the parameters that most contributed to sample clustering of control plants (see Fig 6). Contrary, 'Uirapuru' samples at 10mM stayed alone duo to phenolic contents, high level of NDVI index, stomatal conductance, net photosynthesis, transpiration and stomatal limitation value. The total variance explained by the model was 64.3%, being 36.6 and 27.7% for component 1 (PC1) and 2 (PC2)

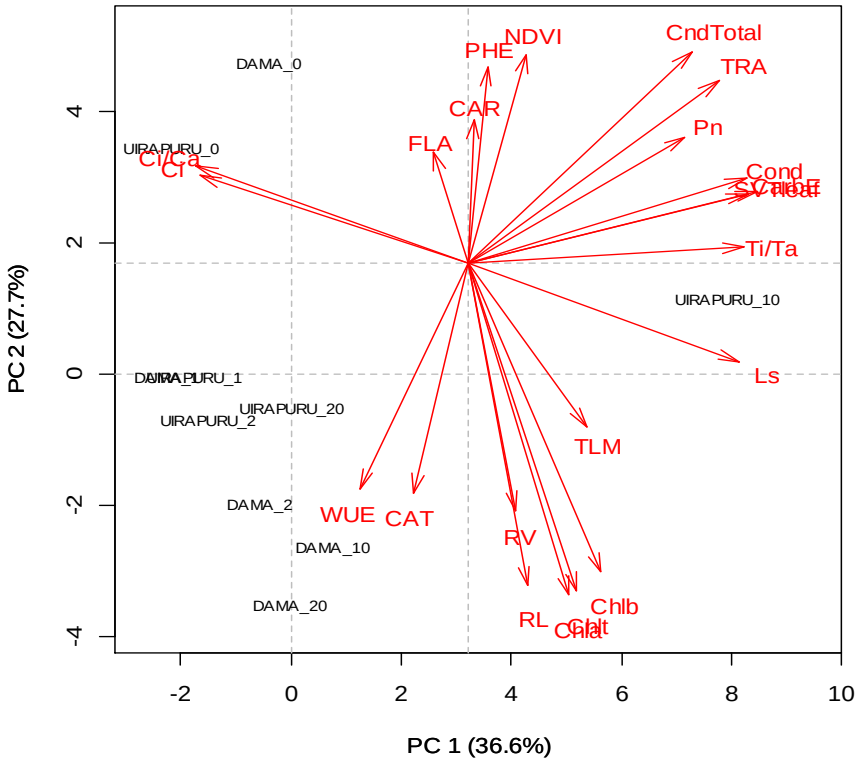


Fig. 6. Score and loading plot of Principal component analysis of all dataset showing sample clustering and the main variables correlated. The total variance explained by the two first components were 36.6% and 27.7% respectively

respectively. Most samples grouped in (PC1-/PC2-) axis. The control samples grouped in the (PC1-/PC2+) axis and 'Uirapuru' at 10 mM in (PC1+/PC2+) axis.

The loading values indicated that sample clustering in PC1 were highly influenced by the net photosynthesis, stomatal conductance, intercellular carbon dioxide, transpiration, intercellular to air temperature, carboxylation efficiency and stomatal limitation value. Those clustered in PC2 were most influenced firstly by the chlorophyll contents, followed by catalase activity, phenolic contents, root length, root volume, transpiration, water use efficiency and NDVI index.

4. CONCLUSIONS

The results of this study prompt us to conclude that there are considerable photosynthetic and biochemical differences between the cultivars studied in their response to potassium nutrition. Potassium range from 1 to 10mM promoted the plant biomass, root length and volume and chlorophyll contents. Increases in carotenoids, flavonoids and catalase activity may be correlated to their antioxidant activities and as scavengers of reactive oxygen species that can be produced during the high photosynthetic rate and transpiration. Cultivar 'Dama' was most tolerant to potassium levels and presented a linear relationship of stomatal conductance. Potassium levels also increased net photosynthesis, NDVI indices, carboxylation efficiency and transpiration, but the variation was in a cultivar-dependent manner. Intercellular carbon dioxide decreased with increases of potassium levels. Multivariate tools revealed that most samples of 'Dama' cultivar were most influenced by CAT and the intrinsic water use efficiency presented than 'Uirapuru'.

SUPPLEMENTARY DATA

A report in html format is provided for data reproducibility. Detailed statistical analysis not shown in the manuscript can be found here.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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