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Genotypic variation in response to drought stress is associated with biochemical and transcriptional regulation of ureides metabolism in common bean (*Phaseolus vulgaris* L.)

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Genotypic variation in response to drought stress is associated with biochemical and transcriptional regulation of ureides metabolism in common bean (*Phaseolus vulgaris* L.)

Abstract: Ureidic legumes such as common bean (*Phaseoulus valgaris* L.) plants export nitrogen from the nodules to shoots and leaves as ureides during symbiotic biological nitrogen fixation. Common bean gene encoding allantoinase (allantoin amidohydrolase, EC 3.5.2.5), is a key enzyme that catalyses the hydrolysis of allantoin to allantoic acid. It plays a role in ureide generation for export and ureide catabolism to generate a nitrogen source in sinks tissues. As such, one of the adaptive mechanisms of plants to drought stress, is associated with ureides accumulation. To identify genetic variation of common bean in response to drought stress, changes in the expression of *ALLANTONAISE* (*PvALN*) gene and ureides content were examined in the leaf tissues of the three common bean genotypes (CAL96, DAB514 and DAB541) and one tepary bean genotype (*Phaseolus acutifolius* A.Gray). Amongst all the genotypes, the suggested drought susceptibility in DAB514 common bean genotype, was probably attributed to a repressed *PvALN* expression rate which were corroborated by an impaired ureides levels, and reduced plant growth. On contrary, drought stress induced an upregulated relative expression of *PvALN* coupled with an increase in allantoin and allantoate in DAB541 common bean genotype. In addition, the sustained plant growth in CAL96 was probably attributed to a steady amount of allantoin synthesized under drought stress. Taken together, DAB541 and CAL96 common bean genotypes are the promising genotypes with an induced upregulated transcriptional control of catabolism and/or biosynthesis of ureides, hence potential genotypes for selection and introduction under Botswana semi-arid conditions.

Key words: common bean; drought stress; ureides: allantonaise; allantoin; allantoate

Genetska spremenljivost odziva navadnega fižola (*Phaseolus vulgaris* L.) na sušni stres je povezana z biokemičnim in transkripcijskim uravnavanjem presnove ureidov

Izvleček: Ureidne stročnice kot je navadni fižol (*Phaseoulus valgaris* L.) transportirajo med simbiontsko vezavo dušik iz nodulov v liste kot ureide. Pri navadnem fižolu je pomemben gen, ki kodira alantoinazo (alantoin amidohidrolaza, EC 3.5.2.5), ključni encim, ki katalizira hidrolizo alantoina v alantoinsko kislino. Ta ima pomembno vlogo pri tvorbi ureidov za njihov eksport in razgradnjo kot vir dušika v tkivih ponora. Pri rastlinah je eden izmed prilagoditvenih mehanizmov na sušni stres povezan s kopičenjem ureidov. Za določitev genetske variabilnosti navadnega fižola na sušni stres so bile analizirane spremembe v izražanju gena za alantoinazo, *ALLANTONAISE* (*PvALN*) in vsebnosti ureidov v listnih tkivih pri treh genotipih navadnega (CAL96, DAB514 and DAB541) in enem genotipu ostrega fižola, *Phaseolus acutifolius* A.Gray. Med vsemi genotipi bi občutljivost genotipa DAB514 navadnega fižola verejtno lahko pripisali zavrtju izražanja gena *PvALN*, kar je bilo povezano z zmanjšano tvorbo ureidov in slabšo rastjo. V nasprotju je sušni stres vzpodbudil povečano izražanje tega gena, kar je bilo povezano s povečanjem vsebnosti alantoina in alantoata pri genotipu DAB541. Dodatno bi ohranjeno rast genotipa CAL96 lahko pripisali stalni količini allantoina, ki se sintetizira med sušnim stresom. Zaključimo lahko, da sta genotipa navadnega fižola DAB541 in CAL96 obetajoča, z vzpodbujeno povečano transkripcijsko kontrolo katabolizma in/ali biosinteze ureidov, ki bi lahko služila kot potencial za izbor in uvajanje ustreznih genotipov v sušnih razmerah Botswane.

Ključne besede: navadni fižol; sušni stres; ureidi; alantonaza; allantoin; alantoat

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1 INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important grain legume in the human diet due to its high nutritional properties, such as proteins, vitamins and minerals (Broughton et al., 2003). One of the major benefits of common beans in agriculture is their capacity to symbiotically fix atmospheric nitrogen through associations with soil nitrogen-fixing rhizobia, thus reducing the need to use nitrogen fertilizers (Coleto et al., 2014). As such, common bean plants are not dependent on nitrogen fertilization for growth due to their ability to form symbioses with atmospheric di-nitrogen fixing bacteroid located in root nodules. Plant growth and productivity is dependent on the accessibility of the newly available fixed nitrogen from the root to the vegetative and reproductive plant tissues.

Ureide allantoin and its degradation derivate allantoate are a group of soil heterocyclic nitrogen compounds that play an essential role in the assimilation, metabolism, transport, and storage of nitrogen in higher plants (Smith & Atkins, 2002). They serve as the vehicle for storage and xylem transport of symbiotically fixed nitrogen from root to the shoot, and as such play a key role in nitrogen utilization in ureide-type legumes (Kohl et al., 1990; Smith & Atkins, 2002; Zrenner et al., 2006). Once delivered to sink tissues, allantoin is converted to allantoate, which in-turn can be broken down completely to glyoxylate, releasing four molecules of ammonia and two molecules of CO_2. Genes encoding allantoinase (allantoin amidohydrolase, EC 3.5.2.5), catalysis the first step in the degradation of the ureide allantoin and the synthesis of allantoate, the second most prominent ureide. It is therefore unique in this pathway such that it plays a role in ureide generation for export from the nodules as well as ureide catabolism to generate a nitrogen source in leaves and other nitrogen sinks (Muñoz et al., 2001; Watanabe et al., 2014; Werner et al., 2013).

Adaptive mechanisms of plants to abiotic stresses such as drought, include changes in the expression of genes involved, biosynthesis of compatible osmolytes and scavenging systems for reactive oxygen species (Han et al., 2014; Hasegawa et al., 2000). The inhibition of nitrogen utilization under drought stress, is proposed to be attributed to N-feedback regulation, in which ureides would be among the signaling molecules triggering the inhibition (Charlson et al., 2009; King & Purcell, 2005; Rachid Serraj, Vadez et al., 1999). The induction and activation of enzymes with a subsequent increased levels of intermediary metabolites, particularly ureides allantoin and allantoate play a vital role in plant responses and adaptation to abiotic stresses (Alamillo et al., 2010; Smith & Atkins, 2002) . In soybean, high ureides levels in shoots and leaves correlated with nitrogen fixation inhibition (Rachid Serraj, Vadez, et al., 1999). In *Arabidopsis thaliana* (L.) Heynh. mutant lacking *ALLANTONAISE (ALN)*, high levels of allantoin metabolites were reported due to an activated allantoin biosynthetic genes and/or repression of *ALN* expression rate. The response suggested that ureide metabolism and accumulation contribute to the abiotic stress response, which is regulated, at least in part, at the transcriptional level. In addition, this implied a possible elevated drought stress tolerance, possibly by reducing oxidative damage. (Irani & Todd, 2016).

The symbiotic nitrogen fixation showed to be extremely sensitive to drought stress and this effect could result in decreasing N accumulation and yield of legume crops (Serraj, 1999; Rachid Serraj, 2003). However, ureide-exporting legumes, such as common beans are more sensitive to drought stress due to rapid decline in nitrogen fixation compared to amidic ones (Purcell et al., 2004; R Serraj, 1999; Rachid Serraj, Vadez et al., 1999). On contrary, a variable degree of nitrogen fixation inhibition due to drought stress was found among the bean genotypes. An increase in both mRNA levels and *ALN* activity with a concomitant increase in roots, shoots and leaves ureide levels in common bean in response to drought was attributed to an elevated synthesis of allantoate (Alamillo et al., 2010). Remarkably, other studies demonstrated a positive correlation between suppressed nitrogen fixation and accumulation of ureides in stems and leaves of both sensitive and tolerant genotypes. Further variability was associated with the rise in allantoate level coupled with an increase in *ALLANTOINASE* gene expression and enzyme activity in the most sensitive genotype, which increased after inhibition of nitrogen fixation, suggesting that ureides originate in vegetative tissues as a response to water stress, probably mediated by the induction of allantoinase (Coleto et al., 2014).

The overreliance on erratic rain coupled with relatively poor soil quality has resulted in poor productivity of crops in Botswana, making the agricultural sector most vulnerable to climate change (FANRPAN, 2017). Crop diversification such as the use of drought-tolerant legumes with enhanced nitrogen fixation ability and improved utilization of the newly fixed nitrogen to enhance crop productivity crops has been hailed as one of the potential adaptive measures to mitigate climate change. As such, Botswana has considered the introduction of common bean into the cropping system as one of the climate smart agriculture approaches, combating poverty, environmental degradation, and improving soil health. This was further justified by its high nutri-

tive value and commercial benefits such as source of income for many rural household (Beebe et al., 2013; Molosiwa et al., 2019). However, information on the performance of the potential common bean genotypes for introduction, particularly nitrogen fixation and utilization capability and crop productivity under Botswana conditions remain elusive. Therefore, this study was conducted to identify the growth and genetic response of common bean genotypes under drought stress. Biochemical analysis of ureides-derived metabolites and transcriptional analysis of *Phaseoulus vulguris ALLANTOINASE* relative gene expression was conducted for the identification and selection of the best and promising common bean genotypes in terms of nitrogen fixation and utilization under drought stress.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND GROWTH CONDITIONS

Common bean genotypes were selected based on their superior stability, adaptability and yield performance in the previous studies conducted at Sebele and Pandamatenga respectively (Molosiwa et al., 2019). These includes three common bean (*Phaseolus vulgaris* L.) genotypes (DAB541; DAB514; CAL96) and GK011 tepary bean (*Phaseolus acutifolius* A.Gray.; GK011), the latter being reported in previous studies as a drought tolerant bean. The experiment was conducted in a growth cabinet, in a randomized block design, with six replications, under a 16 h light/8 h dark photoperiod at 25 °C and a light intensity of 100-150 µmol photons $m^{-2}s^{-1}$. Plants were exposed to water holding treatment three (3) weeks after emergence. Drought stress experiment consisted of two treatments, namely drought stress treatment by withholding water application with a serious drought stress (35-45 % water holding capacity) and the control by ensuring maximum water holding capacity by watering (70-80 %).

2.2 UREIDES ACCUMULATION: ALLANTOIN AND ALLANTOATE

The determination of ureides allantoin and allantoate were performed by differential analyses of glyoxylate derivative according to published protocol (Lescano, 2020). Ureides were extracted from leaf 15 mg liquid nitrogen grounded leaf tissue samples by boiling it in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 18,000 xg for 25 min at 4 °C to ensure the absence of debris and a clear supernatant containing ureides. Six biological replicates of 100 µl aliquots of each sample were collected in three separate tubes for the measurement of endogenous glyoxylate, allantoic acid-derived glyoxylate and allantoin-derived glyoxylate. Glyoxylate is converted into glycolic acid-phenylhydrazone and then oxidized by ferricyanide in the presence of concentrated acid and phenylhydrazine to give red-colored 1,5-diphenylformazan. The absorbance of supernatants was measured using a spectrophotometer at 535 nm.

2.3 TRANSCRIPTOMIC ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES

Total RNA was extracted using a Quick-RNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, United States) as per the manufacturer's protocol and treated with *DNase1* (Zymo Research Corporation, Irvine, CA, United States). The quantity and quality of the isolated RNA were evaluated, respectively, using a NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific) and by 1 % electrophoresis agarose gels according to manufacturer's instructions. The quality of each cDNA and the RT-qPCR were checked per by using standard PCR reaction and the housekeeping gene *PvACTIN-2* primers (Díaz-Leal et al., 2012) and *PvALN* (Table 1). These primer pairs were designed using GeneScript qPCR primer design (https://www.genscript.com/tools/pcr-primers-designer/advanced). Luna Universal qPCR Master Mix (New England Biolab Inc., MA, USA) and primers were used to determine RNA expression. The qPCR reactions were performed using triple replicates of cDNA samples in 96-well plates and performed on the LineGene 9600 (Hangzhou Bioer Techonology), following SYBR Green/FAM detection. Reactions were prepared in a total volume of 20 µl according to Luna® Universal qPCR Master Mix Protocol (M3003; New England Biolab Inc., MA, USA) containing: 1x Luna Universal qPCR Master Mix, 10 µM of forward and reverse primer, 100 ng cDNA template and nuclease-free water. The PCR cycles consisted of 1 cycle of initial denaturing at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s. The melting curve was obtained by applying increasing temperature from 60 to 90 °C. The relative fold change for *Phaseoulus vulgaris* ALLANTOINASE *(PvALN)* was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and normalized against the housekeeping *PvACTIN-2* gene (Díaz-Leal et al., 2012).

Table 1: Primer pairs used to determine expression of genes

Gene	Forward Primers	Reverse Primer
ACTIN-2 (PvActin-2)	5'-TTGCTTTCAAGGAGGGGGTATGC-3'	5'-GGAGCTTGGAACCTTTCGGTGC-3'
ALLANOTONAISE (PvALN)	5'-ACAAGCATGATGCAGGTGCTGTGA-3'	5'-TGCCTCCACGACATCGCACA-3'

2.4 DATA ANALYSIS

The data collected were subjected to analysis of variance (ANOVA) using MINITAB computer software program, significant means were separated using pairwise Tukey comparison at $p < 0.05$.

3 RESULTS

3.1 PHYSIOLOGICAL BIOMASS RESPONSE

To determine the response of common bean to drought stress, fresh biomass was determined from above ground plant tissues after watering was withheld for 10 consecutive days. Biomass for the three genotypes including GK011, CAL96 and DAB541 did not differ under normal water growth conditions. However, a significant reduced biomass was observed in DAB514 common bean genotypes compared to GK011 tepary bean as well as compared to the two common bean genotypes, namely, CAL96 and DAB541. Comparing drought stressed plants to their relative control plants demonstrated no significant variation in biomass in GK011 tepary bean and DAB541 common bean genotype. However, drought stress induced a 38.6 % and 38.7 % significant reduction in biomass in CAL96 and DAB514 respectively. Further biomass comparisons were made between drought stressed plants in all the genotypes. The result indicated a 42.6 % reduction in

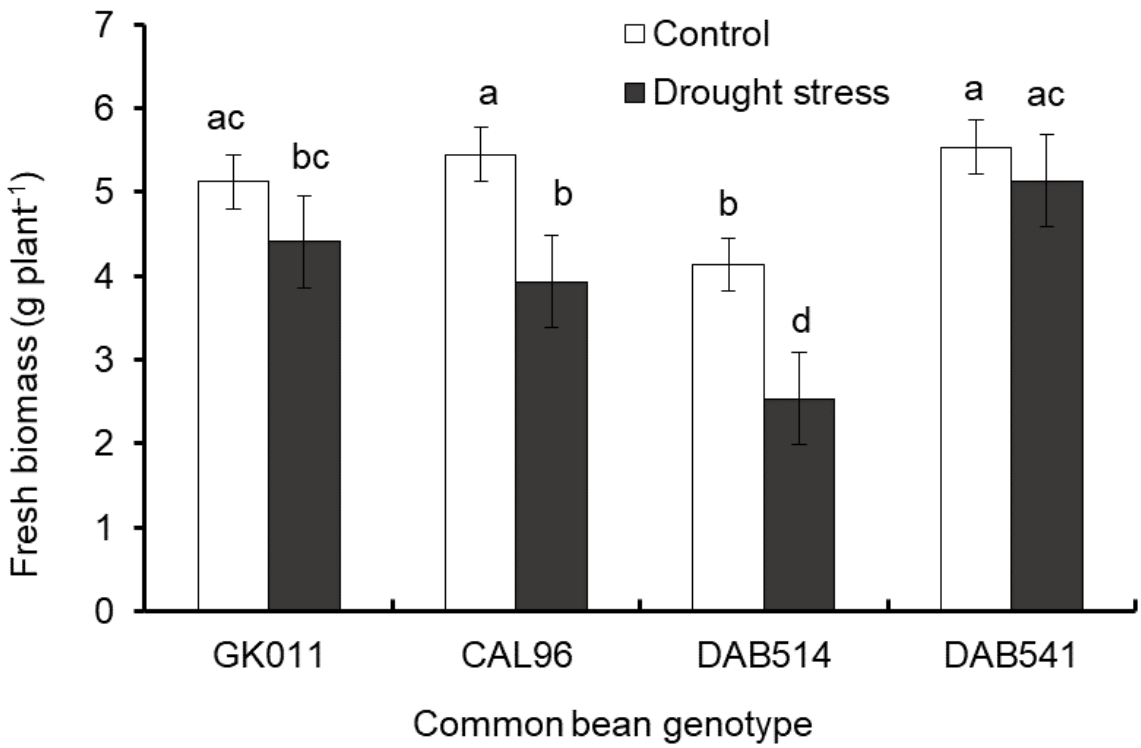

Figure 1: Biomass of common bean genotypes (*Phaseolus vulgaris* L.) in response to drought stress. Biomass was measured unbve ground plat tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. The standard error of mean of three independent flow cells is indicated by the error bars (n = 6). Bars with different lowercase letters indicate significant differences ($p < 0.05$)

biomass in drought stressed DAB514 plants relative to drought stressed GK011 tepary bean. In addition, a 35.6 % and a 50.6 % reduction in biomass in drought stressed DAB514 plants were observed in comparison to drought stressed CAL96 and DAB541 common bean plants respectively. Taken together, all comparisons demonstrated the existence of clear variation between DAB514 and DAB541 common bean genotypes in terms of biomass (Figure 1).

3.2 UREIDES ACCUMULATION IN RESPONSE TO DROUGHT STRESS

To investigate the production of ureides-derived metabolites in response to drought stress, levels of allantoin and allantoate were measured in the leaves of drought stressed plants and control plants (Figure 2). The results were visualized using heat map (Figure 3), generated with MINITAB analytical software (version 21.1). The result demonstrated a significant increase in allantoin metabolite in CAL96 (50.1 %), DAB514 (45.5 %) and DAB541 (47.1 %) common bean genotypes compared to the GK011 tepary bean under normal water conditions. A significant 60.0 % and 23.8 % increase in allantoin accumulation between the plants under normal condition and drought stress was detected for GK011 tepary bean and DAB541common bean genotypes respectively. In contrast, allantoin content was not significantly affected between the control plants and the drought stressed DAB514 and CAL96 common bean genotypes.

In respect to allantoate metabolite, the levels of allantoate were not significantly affected in CAL96 and DAB514 common bean genotypes compared to GK011 tepary bean under normal water growth conditions. The study further compared variation in allantoate levels for the drought stressed plants compared to their relative control plants. The result exhibited 24.6 %, 26.5 % and 47.8 % of reduced allantoate levels for GK011 tepary bean and two common bean genotypes, namely, CAL96 and DAB514 in drought stressed plants compared to the control plants. In contrast, DAB541 common bean genotype elicited a 31.0 % significantly increased levels of allantoate in the drought stressed plants relative to their control plants. Taken together, the response of DAB541 common bean genotype under water stress showed a similar trend for both allantoin and allantoate. Thus, water stress induced a significant increase in both allantoin and allantoate metabolite levels (Figure 2).

3.3 RELATIVE GENE EXPRESSION OF UREIDE METABOLISM

To assess the correlation between allantonaise ureide and changes in *ALLANTONAISE (PvALN)* relative gene expression, both metabolic accumulation of allantonaise and relative *PvALN* gene expression was performed on the leaves of genotypes. To assess whether the accumulation of ureides results from changes in the transcription of genes related to ureide metabolism, quantitative real time PCR was performed to determine the mRNA levels of genes coding for key enzymes in the synthesis of ureides, *ALLANTONAISE (ALN)*. Expression level of *PvALN* gene in the three replicates samples were normalized against the expression of *ACTIN-2* as the internal control. According to the pairwise Tukey comparison, the relative expression of *PvALN gene* in water-deficit plants compared to the control plants was significantly depressed for all the common beans genotypes, except for DAB541. GK011 tepary bean showed the highest 7.7-folds reduction in the relative expression of *PvALN*. This decrease was however insignificantly different from DAB514 and CAL96 common bean genotypes, which also showed a decreased *PvALN* expression rate by 3.2 and 5.4-folds respectively. Intriguingly, only DAB541 common bean genotype, showed an increase in the expression rate *PvALN* mRNA (1.2-folds) in the leaves of drought stressed plants relative to the control plants (Figure 4).

4 DISCUSSIONS

Legumes are agronomically and economically important in many cropping systems because of their ability to assimilate atmospheric nitrogen and maintaining soil fertility. These are highly desirable traits to consider in the improvement of legume productivity for sustainable agricultural practices (Serraj, 1999; Rachid Serraj, 2003, 2003; Rachid Serraj et al., 1999). Drought stress is one of the most important environmental factors that regulate plant growth and development and limit its production. Legumes exhibit reduction in nodulation and biological nitrogen fixation in response to drought stress (Pimratch et al., 2008). Accumulation of ureide compounds has been reported in several plant species under stress conditions, and a considerable number of research articles argue for a hindered rather than active ureide catabolism as the survival trait for plants subjected to periods of mild drought or salinity due to the

alternative prime stress signaling function of uric acid and allantoin.

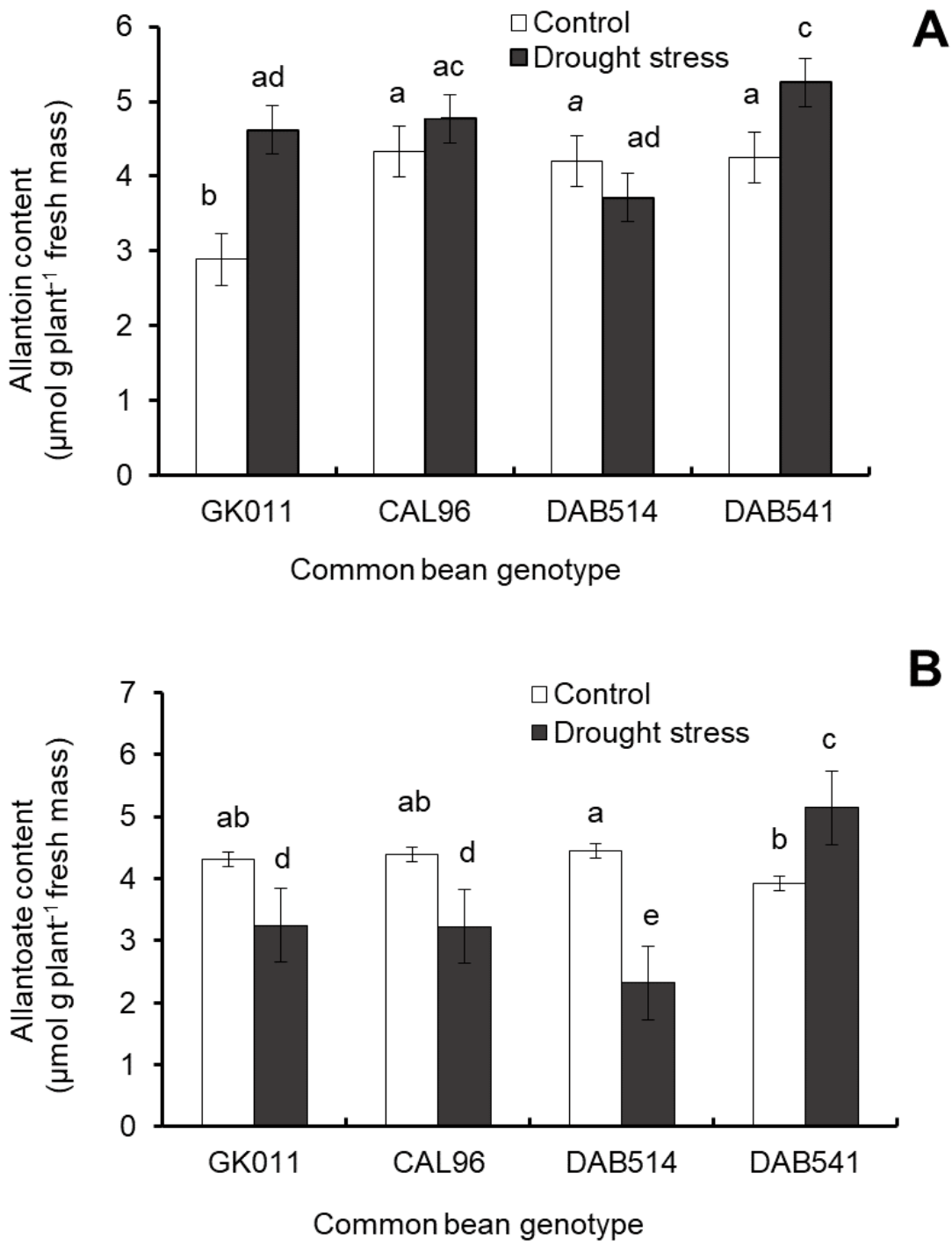

Figure 2: Ureides accumulation in response to drought stress. Ureides measurement consisted of allantoin (A) and allantoate (B) accumulation for pants under control (T0) and drought stress (T1). Ureides accumulation was measured on leaf tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. Bars with different letters are statistically different according to $p < 0.05$. The standard of mean of three independent flow cells is indicated by the error bars error (n = 6)

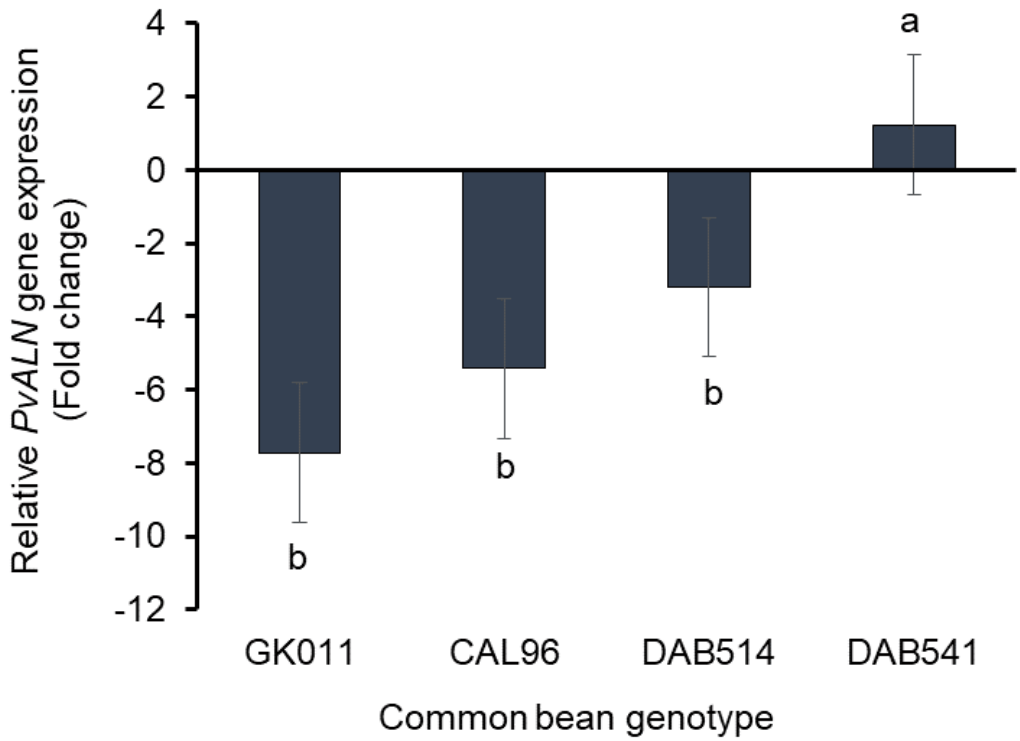

Figure 4: Relative *Phaseolus vulgaris ALLANTONAISE* (*PvALN*) gene expression in common beans in response to drought stress. Relative gene expression was measured on leaf tissues of three common bean genotypes (CAL97; DAB514; DAB541) and GK011 tepary bean. Bars with different letters are statistically different according to $p < 0.05$. The standard of mean of three independent flow cells is indicated by the error bars error (n = 3)

The current study evaluated the response of three common bean genotypes to drought stress at both biochemical and transcriptional level. Firstly, the response of common bean genotypes under normal growth conditions were tested against tepary bean (*Phaseolus acutifolius* A. Gray), a relatively higher drought-tolerant crop than common bean (*Phaseoulus vulgaris*) and serving as genetic resource for food and genetic enhancement of related legumes (Mwale et al., 2020). The insignificant growth rate in terms of biomass under normal growth condition was accompanied by a significant increase in ureide allantoin levels in CAL96, DAB514 and DAB541 common bean genotypes relative to GK011 tepary bean. On contrary, the allantoate content was not affected in CAL96 and DAB514 when compared to GK011 tepary bean. Taken together, the normal growth rate of common bean genotypes compared to GK011 tepary bean might have been sustained by an enhanced assimilation and metabolism of nitrogen, which is attributed increased levels of allantoin and a sustained level of allantoate. Taking into consideration the 16 hours day growth period in the current study, this results are consistent with *Arabidopsis thaliana* studies, which indicated that allantoin ureide degradation is important for the growth and development during vegetative growth under long-day conditions (Takagi et al., 2018).

The response of bean genotypes was further evaluated under drought stress by comparing plants under drought stress against their relative control ones. Intriguingly, all the common bean genotypes, including tepary bean revealed a similar trend of induced inhibited plant growth under drought stress. However, only DAB514 common bean genotype showed a significant reduced plant growth in drought stressed plants compared to their relative control plants. The impaired plant growth rate in DAB514 was positively associated with the reduction in both allantoin and allantoate levels, with a concomitant induced down-regulated *PvALN* relative gene expression. This response proposed an impaired ureides degradation at transcriptional level, which inevitably negatively affected assimilation and use of fixed N and eventually plant growth in DAB514 common bean genotype under drought stress. This finding is contrary to reports that indicated that DAB514 common bean genotype as a stable and high

yielding genotype under drought stress (Molosiwa et al., 2019). Our results implies that DAB514 common bean genotype is a drought-sensitive genotype possibly due to an impaired ureides metabolism at both chemical and transcriptional level with a substantial reduced plant growth. Though similar results were observed in terms of a suppressed expression of *PvALN* coupled with low levels of allantoate, the plant growth rate was not affected in water stressed CAL96 common bean genotype. The suppressed expression of *PvALN* in CAL96 common bean genotype might be responsible for an impaired rate of degradation of allantoin and the synthesis of allantoate, subsequently owing to a steady amount of allantoin synthesized under drought stress.

Water deficit also resulted in another notable increase in ureides allantoin and allantoate levels coupled with an induced upregulated relative expression of *PvALN* in DAB541 common bean genotype. This is in concert with studies on *Arabidopsis*, *Phaseolus vulgaris*, and Soybean which demonstrated an increase in shoot ureides under drought stress (Alamillo et al., 2010; Ladrera et al., 2007; Rachid Serraj, 2003; Vadez & Sinclair, 2001). This advocated for an increased transcriptional regulation of purine metabolism by *PvALN*, which in turn resulted in enhancing both the degradation of the ureide allantoin and the synthesis of allantoate (Alamillo et al., 2010; Coleto et al., 2014). This response suggested that ureide accumulation is a general response to drought stress and is regulated at the transcriptional level mainly through the induction of allantonaise degradation and the subsequent allantoate synthesis in DAB541 common bean genotype leaf tissues.

5 CONCLUSIONS

The current study evaluated the response of common bean genotypes to drought stress by assessing ureides metabolism at biochemical and transcriptional level coupled with the ultimate plant growth in terms of biomass. Overall results suggested a degree of genetical variation among common bean genotypes. The enhanced plant growth or maintained growth rate under drought stress in DAB541 and CAL96 common bean genotypes was probably due to an enhanced degradation of the ureide allantoin and the synthesis of allantoate metabolites. These findings suggested an enhanced ureide generation for export and ureide catabolism to generate a nitrogen source in leaves under drought. Therefore, the study concludes that DAB541 and CAL96 common bean genotype are potential genotypes for selection and introduction under Botswana semi-arid condition. Molecular reverse genetic studies can further be conducted to confirm ureides metabolism and crop performance of DAB541 and CAL96 common bean genotypes under drought stress.

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