Daily balance of leaf sugars and amino acids as indicators of common bean (*Phaseolus vulgaris* L.) metabolic response and drought intensity

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ABSTRACT

Leaf soluble sugar, starch and free amino acid amounts were evaluated in two bean genotypes, Carioca and Ouro Negro, grown in 10 L pots in a greenhouse. This was realized during a single day for Carioca and during ten days of water deficit for both genotypes, at 06:00 and 18:00 h. During the day, an increase in all parameters occurred up to midday, while in the afternoon, carbohydrate amounts varied in opposition to amino acids amounts. Under water deficit, the leaf soluble sugars at 18:00 and their daily balance (amount at 18:00 minus amount at 06:00) increased on the 2nd day, with starch and amino acid amounts and their respective daily balances decreasing. On the 8th and 10th days, the soluble sugar daily balance was negative and the starch daily balance approached zero or was negative, while the amino acid daily balance was positive. During rehydration, soluble sugar daily balance was close to zero and starch daily balance increased, whereas amino acid daily balance was negative. The carbohydrate daily balance, strongly related to free amino acid daily balance, were good indicators for evaluating the stage of water deficit and metabolic capacity to respond to the stress in common bean. [*Physiol. Mol. Biol. Plants* 2009; 15(1) : 23-30] E-mail : greenman@amcham.com.br.; mauroguida@yahoo.com.br

Key words : Amino acid amount, Carbohydrate metabolism, Drought tolerance, Soluble sugars amount, Starch amount, Water deficit.

Abbreviations : DAE, Days after emergence, g_s , Stomatal conductance, VPD, Vapour pressure deficit, Ψ_l , Leaf water potential

INTRODUCTION

Drought is one of the most limiting factors for crop yield (Sinclair and Purcell, 2005), especially in the developing countries of Latin America, Africa and Asia, where water deficit sensitive crops, such as common bean (*Phaseolus vulgaris* L.), suffer from drought at some stage of growth, seriously reducing their yields even when irrigated with large intervals. Several different morphological and biochemical changes occur among species in response to water deficit and as such, drought tolerance is considered a multigenic trait (Pimentel, 2006). Among these responses, the inhibition of photosynthesis is central (Boyer, 1976; Lawlor, 1976) and this inhibition is accentuated when water deficit

occurs at the pollination stage, i.e. the preflowering stage for beans (Pimentel *et al.*, 1999a,b), inducing a low seed yield (Santos *et al.*, 2006). At this stage, the net CO₂ assimilation rate is increased (Pimentel *et al.*, 1999b), improving leaf carbohydrate reserves, which will insure embryo growth after pollination (Kramer and Boyer, 1995).

During drought, stomatal closure probably contributes to the limitation of CO₂ assimilation rate (*A*) in the early phases of leaf dehydration (Lawlor, 1976; 2002), especially for common bean (Pimentel *et al.*, 1999b), while large and direct metabolic damage can be considered as the main cause of photosynthesis inhibition with the decline of leaf water potential (Ψ_l), as indicated by Lauer and Boyer (1992) and Tang *et al.* (2002). The causes of this reduction in photosynthetic metabolism under drought are not yet clear, since the reduction not only varies among species, but also among

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varieties of the same species (Pimentel *et al.*, 1999b; Santos *et al.*, 2006; Pimentel, 2006).

Nowadays, it is easy to measure CO₂ assimilation, because there are a number of commercial portable gas-exchange systems, which allow the operator to obtain *A*, stomatal conductance (*g_s*), transpiration (*E*) and intercellular CO₂ concentration (*C_i*), at the push of a few buttons and without any prior experience, but with some pitfalls, as measuring photosynthesis under low CO₂ fluxes, when stomata close during water deficit, or at different air temperatures and water vapour pressure deficit (Long and Bernacchi, 2003). Also, a single instantaneous measurement of CO₂ assimilation rate or one sample of leaf for carbohydrate amount in a day can be poorly correlated with the daily plant response to environment, and several measurements are necessary to estimate the diurnal course of photosynthesis (Schulze, 1986; Long and Bernacchi, 2003).

The water deficit response of photosynthesis can also be evaluated by its final products, such as the variation in the shoot nonstructural carbohydrate content (Kramer and Boyer, 1995). Plant cells often accumulate sucrose in response to stress conditions (Farrar *et al.*, 2000), since it stabilizes proteins and membranes, contributes to osmotic adjustment and provides an energy reserve to accelerate and support metabolic recovery after rehydration (Hanson and Hitz, 1982; Kramer and Boyer, 1995). Actually, sugars are not only considered as fuel for cellular carbon and energy metabolism, but also play pivotal roles as signaling molecules for growth and stress metabolic response, and multiple sugar sensors, as hexokinases (HXP) and protein kinases (PK), and signaling pathways, as in antagonism with cytokinin for senescence, from transcription and translation to protein stability and activity (Rolland *et al.*, 2006).

Many researchers have investigated the relation between CO₂ assimilation and end-products in bean (Castrillo, 1992) and others species (Quick *et al.*, 1992; Farrar *et al.*, 2000; Wingler *et al.*, 2006), showing the important role of leaf soluble sugar amounts in the feedback regulatory mechanisms of photosynthesis. In addition, starch and amino acid biosynthesis, which are regulated by soluble sugar amounts, can also act as a secondary feedback regulatory mechanism of photosynthesis (Paul and Pellny, 2003). Therefore, the evaluation of the daily carbohydrate balance, i.e. the sunset minus sunrise sugar amounts, can bring more information about diurnal sugar metabolism under water deficit. In this study, the hypothesis that leaf

carbohydrate and free amino acid daily balances are another useful tool to evaluate the severity of drought, i.e. the stage of water deficit as stated by Sinclair and Ludlow (1986), and photosynthetic response of common bean genotypes was investigated.

MATERIALS AND METHODS

Plant material and growth conditions

Common bean (*Phaseolus vulgaris* L.) plants, genotypes Carioca and Ouro Negro were grown under greenhouse conditions in 10 L pots. Carioca is the genotype most commonly cultivated in Brazil and Ouro Negro is an important black seeded genotype, both showing drought tolerance when compared to other genotypes (Pimentel *et al.*, 1999b). For the first experiment, only Carioca was evaluated, while in the second, both genotypes were studied.

The first and second experiments were conducted at the Experimental Station of the Department of Crop Science at the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil (22°45'S, 43°41'W). The pots were arranged in a completely random block design, using 42 pots (1 genotype x 7 sample dates x 6 replications) in the first assay and 96 pots (2 genotypes x 8 sample dates x 6 replications) in the second assay. For the first and second experiments, each pot containing 10 kg of Ultisol was fertilized with an equivalent of 1.5 t CaCO₃ ha⁻¹ (CaCO₃), 20 kg N ha⁻¹ [(NH₄)₂HPO₃], 90 kg P₂O₅ ha⁻¹ (SPS) and 40 kg K₂O ha⁻¹ (KCl), according to the soil chemical analysis; similar to fertilization used in Brazilian crop fields. Additional fertilization was provided 25 days after seedling emergence (DAE), using soluble urea at a concentration of 40 kg N ha⁻¹, as proposed by Vieira (1998). In the first experiment, the air temperature during the plant cycle varied from 11 to 34 °C, with a mean temperature of 25 °C on the measurement days and maximal of 34 °C, while in the second, temperature varied from 10 to 34 °C. For the 12 measurement days, the mean temperatures and vapor pressure deficit (VPD) are shown in Fig. 3.

Plant water status evaluation

The predawn water tension in the xylem was measured daily, with a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA), on the fourth trifoliate leaf from the base of the plant, a mature but not senescent leaf. It was assumed that these measurements are a true estimate of the predawn leaf water potential (Ψ_l).

Leaf soluble sugar, starch and free amino acid content

For both experiments, the leaf soluble sugar, starch and free amino acid contents were measured in the same medium leaflet of the fifth trifoliolate leaf, which was the youngest fully expanded leaf. In the first experiment, six (replications) middle leaflets of the fifth leaf of plants from different pots were collected and immediately dried, at two-hourly intervals between 06:00 and 18:00 h, to determine the diurnal course of leaf metabolism. In the second experiment, the same procedure was adopted for sampling leaf tissues and soluble sugar, starch and free amino acid amounts were evaluated every two days from the onset of water withholding. The variation in soluble sugar, starch and free amino acid amounts from 06:00 (sunrise) to 18:00 h (sunset) was used to evaluate the leaf carbohydrate daily balance.

Samples of 100 mg of leaf dry matter were obtained after leaves were dried at 60 °C for 48 h. Leaf soluble sugar and starch contents were assayed as proposed by Farrar (1995), and leaf free amino acids were determined according to Yemm and Cocking (1955), using a spectrophotometer (Spectronic 20, Milton Roy, USA).

Yield components

At the end of the plant cycle in the second experiment, the effect of water deficit was evaluated on plant yield components: seed weight per plant, number of pods and seeds per plant and 100 grain weight.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the means were compared and segregated by the Student Newman Keuls test ($p < 0.05$) when significance was detected.

RESULTS

In the first experiment, with Carioca plants at the preflowering stage, carbohydrate amounts increased from morning to noon, oscillating during the afternoon, but no significant differences occurred (Fig. 1a, b). However, free amino acid amounts varied in opposition to carbohydrate amounts, from midday to the end of the day (Fig. 1c), but significant differences only occurred between amino acid amounts at 14:00 h and the amounts in the morning until noon and between afternoon amounts and the amount at 06:00 h.

In the second experiment, a severe water deficit was applied at the pollination stage of the two cultivars,

Fig. 1. Diurnal variation of leaf soluble sugar (a), leaf starch (b) and leaf free amino acid (c) amounts from sunrise to sunset, in well watered plants of the Carioca common bean genotype. Data are mean values $\pm$ SE ($n=6$). The letters indicate differences between hours of the day at $p < 0.05$.

Carioca and Ouro Negro. The predawn leaf water potential (Ψ_l) in both cultivars suffered no reduction until day six of water deficit (Fig. 2), dropping suddenly on day eight and more gradually during days nine and ten, when it approached -1.4 MPa. At the end of day ten, the water-stressed plants were rehydrated. Significantly higher values of Ψ_l were observed for Ouro Negro than for Carioca on stress days eight and nine (Fig. 2). Two days after rehydration, the Ψ_l was equivalent to day zero of water stress for both genotypes (Fig. 2).

The leaf soluble sugar daily balance for both genotypes was negative from day four to the end of water deficit (Fig. 4b, d). On day two of rehydration, Carioca showed a positive leaf soluble sugar daily

Fig. 2. Diurnal variation of leaf water potential of the common bean genotypes Carioca and Ouro Negro under severe drought, ten days of water deficit, and two days after rehydration. Data are mean values $\pm$ 0.02 ($n=6$). The arrow indicates the rehydration day and * the difference at $p<0.05$.

Fig. 3. Diurnal variation of mean air temperature ($^{\circ}\text{C}$) and vapor pressure deficit (KPa) during the experiment on severely drought-stressed plants of the common bean genotypes Carioca and Ouro Negro, ten days of water deficit and two days after rehydration. The arrow indicates the rehydration day.

Fig. 4. Leaf soluble sugar amounts at 06:00 and 18:00 h and leaf soluble sugar daily balance, given by the difference in soluble sugar amounts at 18:00 and 06:00 h of two common bean genotypes Carioca (a, b) and Ouro Negro (c, d) under severe drought, ten days of water deficit, and two days after rehydration. Data are mean values $\pm$ SE ($n=6$). The arrows indicate the rehydration day and * the difference at $p<0.05$.

Fig. 5. Leaf starch amounts at 06:00 and 18:00 h and leaf starch daily balance, given by the difference in starch content at 18:00 and 06:00 h of two common bean genotypes Carioca (a, b) and Ouro Negro (c, d) under severe drought, ten days of water deficit, and two days after rehydration. Data are mean values $\pm$ SE ($n=6$). The arrows indicate the rehydration day and * the difference at $p<0.05$.

balance (Fig. 4b), while for Ouro Negro the daily balance was still negative (Fig. 4d). With the severity of the stress, soluble sugar amounts at 18:00 h decreased.

At the onset of water deficit, starch amounts and daily balance (Fig. 5) presented behavior contrary to that of soluble sugar amounts (Fig. 4b, d). On day two of water shortage, the starch daily balance decreased to close to zero for Carioca (Fig. 5b), but it showed an increase for Ouro Negro (Fig. 5d). During the first four days of water deficit, the starch amounts at 06:00 h and 18:00 h (Fig. 5a, c) varied contrary to the variation of the air temperature and VPD (Fig. 3). A slight nonsignificant increase in starch amounts and daily balance (Fig. 5) occurred on day four, especially for Ouro Negro at 09:00 h, when a reduction in air temperature and VPD occurred on days three and four (Fig. 3). From day four to the end of the water stress, starch amounts were reduced for Carioca at 06:00 h and 18:00 h reaching the same values at the end of the

stress, while for Ouro Negro, they were reduced at 18:00 h, but varied at 06:00 h; however, the starch amounts were lower at 18:00 h than that at 06:00 h on days eight and ten (Fig. 5a, c). The starch daily balance during water deficit fell close to zero only on day ten for Carioca (Fig. 5b), though it was negative on days eight and ten for Ouro Negro (Fig. 5d). Two days after rehydration, the starch daily balance was positive for both genotypes (Fig. 5b, d).

During water deficit and rehydration, the amino acid daily balance (Fig. 6b, d) varied in contrast to the sugar daily balance (Fig. 4b, d). The amino acid daily balance was close to zero on day two and very negative on day six, but it was positive again on stress days eight and ten. On day six, the last day of high Ψ_l (Fig. 2), a sudden increase in the amino acid amounts occurred at 06:00 h for both genotypes (Figs. 6a, c), which was significantly higher than at 18:00 h, dropping again until the end of the stress, especially at 06:00 h. Rehydration

Fig. 6. Leaf free amino acid amounts at 06:00 and 18:00 h, and leaf free amino acid daily balance, given by the difference in free amino acid amounts at 18:00 and 06:00 h of two common bean genotypes Carioca (a, b) and Ouro Negro (c, d) under severe drought, ten days of water deficit, and two days after rehydration. Data are mean values $\pm$ SE ($n=6$). The arrows indicate the rehydration day and * the difference at $p < 0.05$.

induced a negative amino acid daily balance (Fig. 6b, d), because amino acid amounts at 06:00 h were significantly higher than at 18:00 h, for both genotypes (Figs. 6a, c).

At the end of the plant cycle, severe drought applied at the pollination stage caused a significant decrease in all component yields of both genotypes, except for the 100 grain weight of Carioca (data not shown).

DISCUSSION

In the first experiment investigating well-watered Carioca plants at the pollination stage, the leaf soluble sugar and starch amounts varied in the same way throughout the entire day (Fig. 1a, b). The lower carbohydrate content at 14:00 h was probably due to the higher maximal air temperature of the day (34 °C), with a consequent high VPD (2.1 KPa), probably inducing stomatal closure and reducing A , as stated by Schulze (1986). At the end of the day, the carbohydrate amounts

were close to the values at 06:00 h, but amino acid amounts were much higher than at 06:00 h (Fig. 1c). The free amino acid amounts varied in opposition to carbohydrate amounts, from midday to the end of the day (Fig. 1a, b, c), which could indicate the use of carbohydrates for amino acid synthesis. Amino acid amounts increased from 06:00 to 18:00 h, probably due to a reduction in growth and protein synthesis, especially during the afternoon, indicating a mild water deficit effect, due to atmospheric conditions (Schulze, 1986), to which both processes are considered to be very sensitive, as stated by several authors in the past (Hsiao, 1973; Boyer, 1976; Lawlor, 1976), while amino acids continued to be synthesized (Hsiao, 1973; Hanson and Hitz, 1982).

When a severe water deficit was imposed over ten days at the pollination stage of both genotypes, the Ψ response can be analyzed as proposed by Sinclair and Ludlow (1986), in three distinct stages of dehydration:

stage I, mild water deficit, where the rate of transpiration caused no reduction in Ψ_l until the day six of stress in this assay (Fig. 2); stage II, moderate water deficit, where Ψ_l dropped quickly from stress day six to day eight; and stage III, severe water deficit on days nine and ten (Fig. 2), where Ψ_l continued to decrease, though more gradually, until reaching a critical lethal Ψ_l value for the species (i.e. $\Psi_l = -1.5$ MPa for common bean, as stated by Boyer, 1976). Below this critical lethal Ψ_l value for the species, recovery is not possible, because alterations in cellular membrane composition occur, especially in the chloroplast and mitochondria, due to increased hydrolytic activity, which break down the membrane and are quickly followed by cell lysis (Vieira da Silva, 1976).

The soluble sugar daily balance (Fig. 4b, d) was negative for most of the water stress period. The accumulation of soluble sugars during day two of water shortage was probably due to the arrest in foliar growth, a process which is more sensitive with regard to water deficit than photosynthesis (Hsiao, 1973; Boyer, 1976) and sucrose synthase activity (Castrillo, 1992). From stress day four onward, the soluble sugar content at 18:00 h was significantly reduced, especially for Carioca, indicating a significant reduction in A during these days, reducing the soluble sugar daily balance to negative values (Fig. 4b, d). Therefore, the soluble sugar daily balance showed a sensitive response to mild water deficit and can be considered a good indicator for its effects in stage I of water deficit. However, starch amounts at 18:00 h and daily balance generally varied (Fig. 5) contrary to soluble sugars (Fig. 4) during water deficit. In general, up to day six for Ouro Negro and day eight for Carioca, the starch amounts tended to be higher at the end of the day (18:00 h) than in the morning (06:00 h), but these amounts were only significantly different for Ouro Negro on days two and four (Fig. 5a, c), indicating a certain accumulation of starch during these days or an increase in starch consumption by night respiration. During the first four days of water deficit, the starch amounts at 06:00 h and 18:00 h (Fig. 5a, c) varied contrary to the variation of the air temperature and VPD (Fig. 3), probably due to the water deficit effect of low air humidity on stomatal conductance (Schulze, 1986). However, a positive starch daily balance still occurred in leaves during mild water deficit, since starch hydrolysis only increases during severe water shortage (Hsiao, 1973; Vieira da Silva, 1976; Boyer, 1976). Soluble sugar and starch amounts acted contrary to one another, because starch synthesis occurs by combining

sugars, whereas starch hydrolysis forms sugars, which are not used for growth under water deficit conditions (Hsiao, 1973). The starch daily balance was close to zero for Carioca and negative for Ouro Negro only at stage III, severe water deficit, indicating the moment for rehydration to promote plant recovery. With rehydration, the starch amounts increased more at 18:00 than at 06:00 h (Fig. 5a, c), indicating starch accumulation during the day, while soluble sugar amounts increased minimally (Fig. 4a, c).

During water deficit, amino acid amounts showed two peaks at 06:00 h, on stress days two and six and upon rehydration (Fig. 6a, c), showing significantly higher amounts on day six at 06:00 than at 18:00 h, related to a variation in the free amino acid daily balance (Fig. 6b, d) contrary to the soluble sugar daily balance (Fig. 4b, d). The increase in amino acid amounts at 06:00 on day two (Fig. 6a, c) was probably due to a reduction in protein synthesis associated with growth arrest even at high Ψ_l (Fig. 2), as stated by Hsiao (1973), while the increase on day six could be due to proteolysis (Hanson and Hitz, 1982), with the beginning of the decline in Ψ_l (Fig. 2) indicating the onset of stage II of dehydration. On day ten of water shortage, a decrease in amino acid amounts occurred (Fig. 6a, c); however, the amino acid daily balance remained positive because its amounts at 18:00 h were a little higher than at 06:00 h (Fig. 6a, c). During rehydration, the amino acid daily balance was negative (Fig. 6b, d), probably due to the reprise in growth and improved protein synthesis upon the recovery of the plants. This occurred even after severe water deficit, which significantly reduced all yield components of both genotypes, except the 100 grain weight of Carioca (data not shown).

Thus, the daily balance of soluble sugars, starch, and amino acids were very useful for indicating bean plant response during the different stages of water deficit. Measurement of soluble sugar and amino acid amounts at the beginning and end of the day was a useful tool for sensing mild water deficit in stage I and the beginning of stage II, and starch amounts indicated the severity of stress during stage III, when rehydration is required for plant recovery and where these compounds are also indicative. The soluble sugar and amino acid amounts and daily balance acted contrary to one another, as stated by Wingler *et al.* (2006) for senescence, which is induced by drought (Hsiao, 1973), and can be viewed as metabolic signals for physiological responses to drought (Farrar *et al.*, 2000; Rolland *et al.*, 2006).

However, plant response to water deficit depends on an enormous number of plant traits at all levels of organization, from the molecular to the plant levels, and there is no universal trait for drought tolerance (Sinclair and Purcell, 2005), though depending on the species under study, a number of traits can be useful in the evaluation of drought tolerance. Therefore, the evaluation of carbohydrate balance associated with free amino acid balance appears to be another useful tool for the study of plant response to different stages of water deficit and drought tolerance mechanisms.

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