Carbon partitioning and assimilation as affected by nitrogen deficiency in cassava

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Abstract

Plants of cassava (*Manihot esculenta* Crantz) were raised in a sand root medium watered with nutrient solutions, under greenhouse conditions. As the N-supply increased, shoot dry mass was enhanced to a greater extent than root dry mass, thus leading to an increased shoot to root ratio. In leaves, contents of total soluble saccharides, non-reducing saccharides, and inorganic phosphate increased linearly with increasing N-supply. An opposite response was found for reducing saccharides and starch. In general, content of non-reducing saccharides was considerably greater than starch content. Activity of sucrose synthase was not detected, regardless of the N-treatments; by contrast, activity of neutral and acid invertases increased with increasing N-availability. Roots accumulated more total soluble saccharides, but less reducing saccharides and starch, as the N-supply increased. Photosynthetic rates decreased with increasing N-deficiency. Such a decrease was circumstantially associated to reducing saccharide, but not starch, accumulation. Results suggest a limited capacity for carbon export from source leaves under N-limitation.

Additional key words: invertase; *Manihot esculenta*; nitrate; photosynthesis; saccharide; sucrose synthase.

Introduction

The rate of N-assimilation is an important determinant of plant growth and development, and a primary mean by which N influences growth is alteration of plant acquisition and internal distribution of carbon (Rufty *et al.* 1984). Under N-limitation, shoot growth is often inhibited to a greater extent than root growth, resulting in a decrease in the shoot to root ratio. The restricted plant development seems to be due chiefly to a decreased leaf area rather than to a declined rate of net carbon assimilation (P_N) per unit leaf area (Sage *et al.* 1987, Lima *et al.* 2000).

As long as sink capacity is decreased with low-N supply, carbon partitioning among different plant organs as well as carbon allocation among metabolic pathways such as starch formation, sucrose formation, and glycolysis may be profoundly altered (Rao and Terry 2000). In general, saccharides accumulate under N-limitation, which may lead to a feedback inhibition of P_N, although such a feedback inhibition has not always been found (Sage *et al.* 1989). Different experimental approaches have demonstrated that saccharides play a key role in this regulatory mechanism by repressing the expression of photosynthetic genes (Stitt *et al.* 1995, Roitsch 1999). At biochemical level, feedback inhibition of P_N could arise from a limited sucrose synthesis with the consequent restriction of inorganic phosphate (P_i) recycling to chloroplasts (Goldschmidt and Huber 1992). Also, mechanical distortion of the chloroplast by enlargement of starch grains has been suggested to decrease ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) catalysis (DeLucia *et al.* 1985, Grub and Mächler 1990), though this is a matter under debate (Goldschmidt and Huber 1992). Moreover, N-limitation may lower P_N simply by decreasing the content of photosynthetic pigments and electron carriers, as well as the synthesis of several enzymes involved in the Calvin cycle, particularly RuBPCO (Terashima and Evans 1988).

To some Latin American, Asian, and particularly African countries cassava is a major staple crop, and one of the most important primary sources of food energy in the diet of more than 600 million people. In 2001, cassava plantation totalled about 17 million hectares world wide yielding almost 180 million tons of tuberous roots. Smallholders chiefly cultivate cassava in sandy impoverished,

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soils, adopting low-input systems in which little or no application of fertiliser is the norm. Thus nutrient deficiencies, particularly of N, are common. Even so, cassava produces reasonably well under these circumstances (El-Sharkawy *et al.* 1993). However, little is known about how cassava acclimates to N-starvation. The aim of this work was, then, to examine the effects of N on growth, photosynthesis, as well as carbon partitioning and allocation in cassava plants grown in a range of N-supplies.

Materials and methods

General: Two experiments were conducted independently, each lasting 90 d, using plants of cassava (*Manihot esculenta* Crantz cv. Cigana Preta) grown under greenhouse conditions with midday irradiance about 1 000 μmol(photon) m^{-2} s^{-1}, in Viçosa (20°45'S, 42°15'W), south-eastern Brazil. The first experiment was installed on 15 December 1998, and the second one on 20 September 2000. Growth conditions, as described below, were essentially the same for both experiments.

Two stem cuttings of cassava were planted on each of 11 000 cm^3 pots filled with washed river sand. After 10 d, plants were thinned to one per pot (five pots per treatment). Plants were then watered daily and fertilised twice weekly with nutrient solutions modified from Hoagland and Arnon (1950) in order to contain four contents of NO_3^- (0.5, 3, 6, and 12 mM; Experiment 1) or two contents of NO_3^- (3 and 12 mM; Experiment 2). Original Hoagland's solution was modified in such a way as to vary N-contents whilst keeping an optimal content of the other nutrients. Solutions were adjusted to pH 5.7–6.0. Weekly, pots were thoroughly washed with tap water and subsequently with distilled water in order to avoid salinisation of the substrate. Sampling and measurements were performed 90 d after planting. All leaf samplings were made using central leaf lobes from the youngest, fully expanded leaves. Roots were thoroughly washed with tap water and subsequently with distilled water, after which sections from their middle parts were collected for analyses.

Experiment 1: Plants were harvested and separated into above-ground parts, absorbing roots, and tuberous roots (diameter above 5 mm; Tankou *et al.* 1990). These were oven-dried at 75 °C for 96 h, and subsequently weighted.

A portable, open-system infrared gas analyser (*LCA-4, ADC*, Hoddesdon, UK) fitted to a Parkinson leaf chamber operating at 5 cm^3 s^{-1} was used to measure the net CO_2 assimilation rate (P_N). During measurements (09:00–11:00), made under ambient CO_2 with artificial irradiance (1 100 μmol m^{-2} s^{-1}), air temperature inside the greenhouse varied from 31 to 33 °C, and relative humidity was about 60 %. Further technical details are described in DaMatta *et al.* (1997).

For saccharide analyses, fresh plant tissues of approximately 0.5 g were immersed in boiling 80 % ethanol and then stored at –20 °C. An aqueous extract from this material, obtained as described in DaMatta *et al.* (1999), was used for determination of reducing saccharides following Nelson (1944) and total soluble saccharides according to McCready *et al.* (1950), with modifications given in Amaral *et al.* (2001). Non-reducing saccharides were estimated as the difference between total soluble saccharides and reducing saccharides. The pellet remaining from the extraction of soluble saccharides was digested with $HClO_4$ according to McCready *et al.* (1950), for starch analysis. Starch content was assessed following the same procedure as used for total soluble saccharides. Starch was expressed as glucose equivalents multiplied by 0.9.

Experiment 2: This experiment was conducted to evaluate the effect of N-supply on the activity of sucrose-catabolising enzymes, namely neutral and acid invertases (EC 3.2.1.26) and sucrose synthase (SuSy; EC 2.4.1.13). Based on the results of Experiment 1, two NO_3^- contents (3 and 12 mM) were chosen in order to impose either a moderate deficiency or an adequate supply of N.

Leaf tissues were plunged into liquid nitrogen and then stored at –80 °C until analyses. Frozen tissues were ground (0–4 °C) in 5 cm^3 of medium containing 50 mM HEPES buffer (pH 7.0), 10 mM 2-mercaptoethanol, 2 % polyvinylpolypyrrolidone, 1 % polyvinylpyrrolidone, 1 mM EDTA, and 10 mM $MgCl_2$. The resulting slurry was centrifuged at 18 000×*g* for 20 min at 2–4 °C. An aliquot of the supernatant was desalted through filtration chromatography on a *Sephadex G-25* column, and the eluted constituted the extract for enzyme assays.

For acid invertase assay, 50 mm^3 of extract were added to 950 mm^3 of reaction medium containing 125 mM Na-acetate buffer (pH 4.5), 50 mM sucrose, and 15 mM $MgCl_2$. For neutral invertase, Na-acetate buffer was replaced by 125 mM K-phosphate buffer (pH 7.5). For SuSy assay, performed in the hydrolysis direction, 100 mm^3 of desalted extract were added to 900 mm^3 reaction medium composed by 25 mM HEPES-NaOH buffer (pH 6.5), 125 mM sucrose, 15 mM $MgCl_2$, and 2 mM UDP. For all assays, adding enzyme extract started the reactions followed by incubation at 28 °C for 15 (SuSy) or 30 (invertases) min. Reactions were stopped by the addition of 1 cm^3 copper alkaline reagent (Nelson 1944). The mixture was centrifuged at 1 000×*g* for 5 min, after which enzyme activities were determined through formation of reducing saccharides (Nelson 1944). For blanks, sucrose (invertases) or UDP (SuSy) was omitted from the reaction medium.

Statistics: The plants were distributed over a randomised block design with four and two treatments (NO_3^-

applications) in the first and second experiment, respectively. Each experimental plot was constituted by one plant per pot. For Experiment 1, best-fit regression equations were adjusted to determine relationships between dependent variables as a function of applications of NO_3^-. Correlation analysis was also performed. When applicable, differences among means were tested by the Duncan test. For Experiment 2, differences between means were examined by the F test.

Results and discussion

Carbon allocation and partitioning: Dry mass of the whole-plant, shoots, and tuberous roots increased curvilinearly, while dry mass of absorbing roots did it linearly, with increasing N-supply (Fig. 1). Changes in dry mass of shoots were much more pronounced than that of roots and, as a consequence, shoot to root ratio increased with increasing N-availability (Fig. 1). This indicates that adjustments in carbon partitioning between the shoot and the root system occurred in response to N-supply.

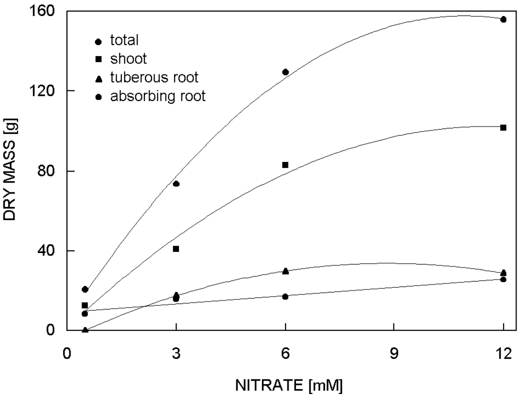

Fig. 1. Dry mass of cassava plants as a function of NO_3^- applications. Means of five replicates. Equations from regression analyses are: total ($y = -1.28\,x^2 + 27.95\,x + 5.20$; $r^2 = 0.99^{**}$); shoot ($y = -0.75\,x^2 + 17.42\,x + 1.24$; $r^2 = 0.99^{**}$); absorbing roots ($y = 1.38\,x + 9.09$; $r^2 = 0.94^{**}$); and tuberous roots ($y = -0.49\,x^2 + 8.59\,x - 4.06$; $r^2 = 0.99^{**}$). $^{**}p < 0.01$.

In leaves, while contents of reducing saccharides (assumed to be mostly glucose and fructose) decreased, those of non-reducing saccharides (assumed to be mostly sucrose) increased with increasing N-applications (Fig. 2*A,B*). Since non-reducing saccharides predominated over reducing saccharides, content of total soluble saccharides therefore increased in response to N-supply (Fig. 2*C*). Except for plants grown under severe N-deficiency, non-reducing saccharide content, and likely sucrose, was significantly higher than starch content. In plants grown at the highest N-application, for example, content of non-reducing saccharides was more than 3-fold larger than starch content (compare Fig. 2*B* with Fig. 2*D*), so cassava is a high leaf-sucrose former. These results support the assumption of Angelov *et al.* (1993) that cassava shows a marked ability to divert the newly photosynthetically fixed carbon to form sucrose.

Table 1. Activities of sucrose synthase and neutral and acid invertases [mg(glucose) kg^{-1}(FM) s^{-1}] in leaves of cassava plants grown with two NO_3^- applications. Means of five replicates. $^{*}p < 0.05$; $^{**}p < 0.01$; coefficient of variation (CV) is also presented. – = not detectable.

Enzyme	NO_3^- applications [mM]		CV [%]
	3	12	
Sucrose synthase	–	–	–
Neutral invertase	0.595	0.987^{*}	25.2
Acid invertase	2.243	4.435^{**}	12.5

There was no detectable activity of SuSy in leaves, regardless of the N-treatments (Table 1). According to Heldt (1997), source leaves of sucrose-accumulating species have little, if any, activity of SuSy, which is much more expressive in storage tissues accumulating starch or in growing tissues in which sucrose is associated with synthesis of cell wall components. On the other hand, activity of invertases, particularly that of acid invertase, decreased considerably in the low-N *versus* high-N plants (Table 1), suggesting that ability for sucrose degradation was less in the former. Also, export capacity was likely to be depressed with decreasing N, as will be discussed below. Assuming that the sucrose pool represents the balance between synthesis, export, and degradation, a decreased sucrose content in N-deficient plants should have mostly resulted from a depression in its synthesis. In fact, decreases in the activity of sucrose-phosphate synthase (SPS), the key enzyme in sucrose biosynthetic pathway, occurs in N-starved plants (Makino *et al.* 1994, Isopp *et al.* 2000).

The content of leaf P_i increased linearly as a function of N-supply (Fig. 2*F*). Because NO_3^- would impair P_i accumulation in the vacuoles of root cells, an increased P_i loading into xylem was expected (Lamaze *et al.* 1984), which would explain the observed relationship between leaf P_i and NO_3^- supply.

In N-deficient leaves much starch is accumulated (Oparka *et al.* 1987, Rufty *et al.* 1988, Wang and Tillberg 1996, Guidi *et al.* 1998) and our results confirm this (Fig. 2*D*). Such accumulation indicates a carbon source-sink imbalance (Stitt 1991), *i.e.* carbon assimilation exceeded carbon use. Indeed, P_N per unit leaf area decreased remarkably less than total biomass (compare Fig. 5*A* with Fig. 1). Moreover, the ratio of starch to non-reducing saccharides in leaves, which provides an indicator of carbon partitioning (Galtier *et al.* 1993), increased with decreasing N (Fig. 2*E*), suggesting impairments in export capacity paralleling a stimulation of starch formation.

The restricted capacity for sucrose synthesis, and the associated restricted generation of cytosolic P_i required for transport of triose-phosphate out of chloroplasts, would account for the diversion of photosynthetically fixed carbon into starch. This suggestion was reinforced, even though circumstantially, by the negative correlations between non-reducing saccharides with starch (Fig. 3*A*) and P_i with starch (Fig. 3*C*). Furthermore, lowering in demand for amino acids and proteins, as growth was depressed with declining N-supply (Fig. 1), should have increased the availability of hexoses, also leading to starch synthesis (Robinson 1996). This might be supported, though circumstantially, by the positive correlation between starch and reducing saccharides (Fig. 3*B*). In addition, NO_3^- *per se* may directly affect starch synthesis: activity of ADP-glucose pyrophosphorylase, a key enzyme in the starch biosynthetic pathway, but not SPS activity, is repressed by NO_3^-, suggesting that starch synthesis would be stimulated under low NO_3^- (Stitt 1999).

Table 2. Contents [g kg^{-1}(DM)] of total soluble saccharides (TSS), reducing saccharides (RS), non-reducing saccharides (NRS), starch, non-structural saccharides (NSS), and starch to NRS ratio in tuberous roots of cassava plants grown with four NO_3^- applications (plants grown with 0.5 mM NO_3^- did not produce tuberous roots). Means of five replicates. Different letters represent statistically significant difference among means for each parameter (p < 0.05; Duncan test). Coefficient of variation (CV) is also presented.

Parameter	NO_3^- application [mM]			CV [%]
	3	6	12	
TSS	85.3 b	119.7 ab	141.5 a	19.2
RS	6.1 c	12.3 b	17.8 a	26.1
NRS	79.7 b	107.4 ab	123.7 a	18.4
Starch	821.3 a	648.2 b	693.1 b	13.2
NSS	906.7 a	767.9 a	834.6 a	12.7
Starch/NRS	10.6 a	6.1 b	5.6 b	22.8

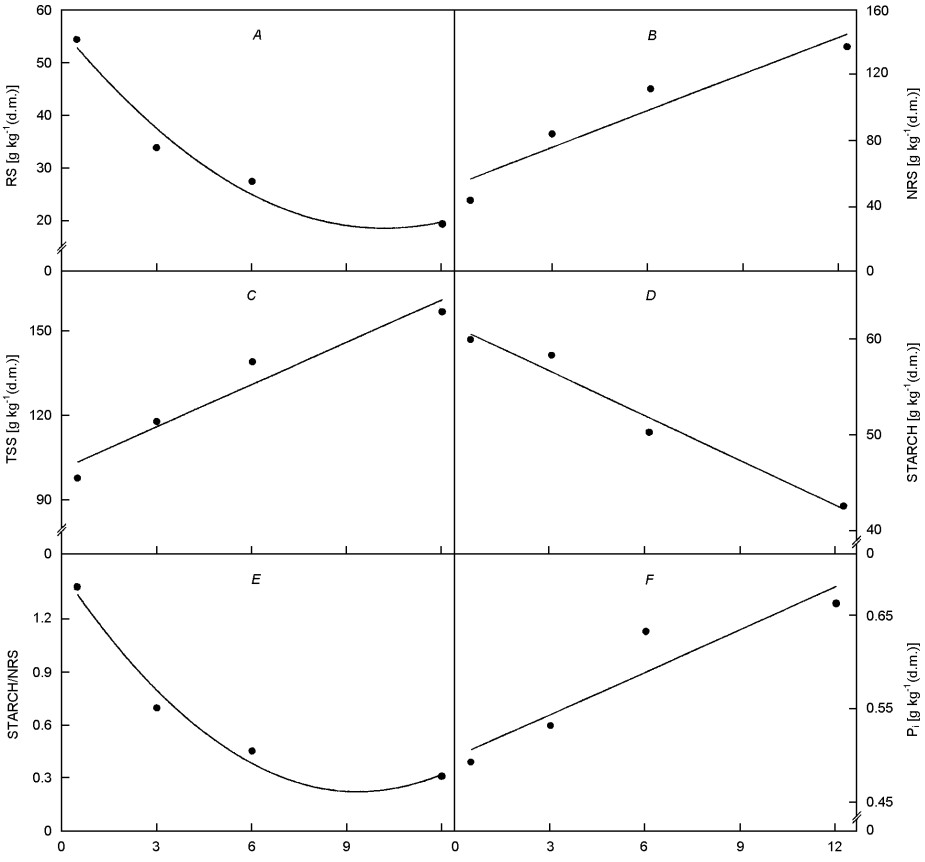

Fig. 2. Leaf contents of reducing saccharides (*A*), non-reducing saccharides (NRS) (*B*), total soluble saccharides (TSS) (*C*), starch (*D*), starch to NRS ratio (*E*), and inorganic phosphate (*F*) as a function of NO_3^- applications in cassava plants (note differences in scale). Each symbol represents the mean of five replicates. Equations from regression analyses are: *A* ($y = 0.36\,x^2 - 7.28\,x + 56.33$; $r^2 = 0.97^{**}$); *B* ($y = 7.69\,x + 54.48$; $r^2 = 0.91^{**}$); *C* ($y = 5.03\,x + 100.85$; $r^2 = 0.94^{**}$); *D* ($y = 1.60\,x + 61.45$; $r^2 = 0.97^{*}$); *E* ($y = 0.01\,x^2 - 0.27\,x + 1.48$; $r^2 = 0.98^{*}$); *F* ($y = 0.02\,x + 0.50$; $r^2 = 0.87^{*}$). $^{*}p < 0.05$, $^{**}p < 0.01$.

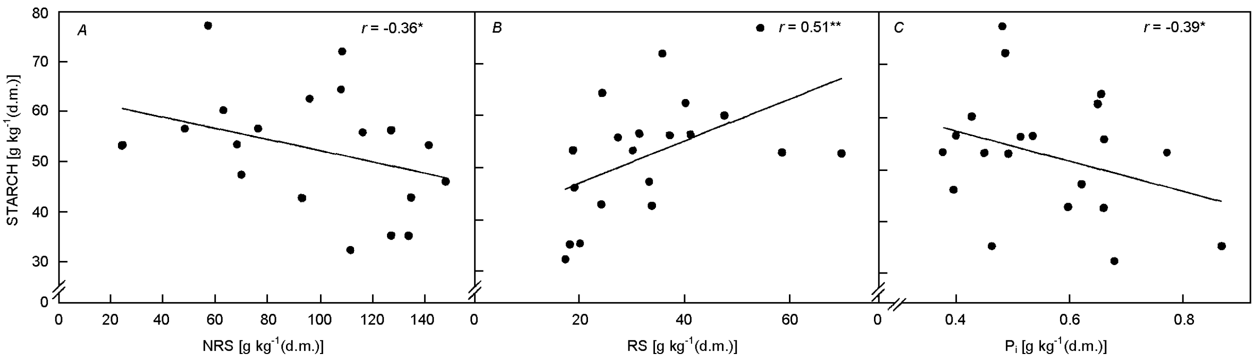

Fig. 3. Correlations between starch and non-reducing saccharides (NRS) (*A*), reducing saccharides (RS) (*B*), and inorganic phosphate (P_i) (*C*) in leaves of cassava plants. $^{*}p < 0.05$, $^{**}p < 0.01$.

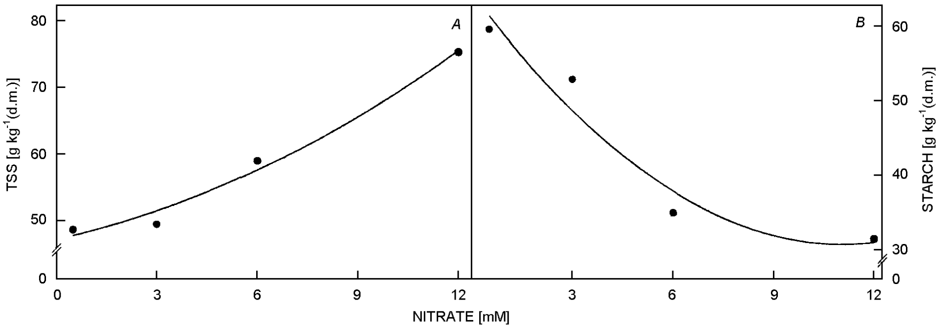

Fig. 4. Contents of total soluble saccharides (TSS) (*A*) and starch (*B*) as a function of NO_3^- applications in absorbing roots of cassava plants. Each symbol represents the mean of five replicates. Equations from regression analyses are: *A* ($y = 2.53\ x + 44.56$; $r^2 = 0.97^{**}$); *B* ($y = -2.51\ x + 58.60$; $r^2 = 0.83^{*}$). $^{*}p < 0.05$, $^{**}p < 0.01$.

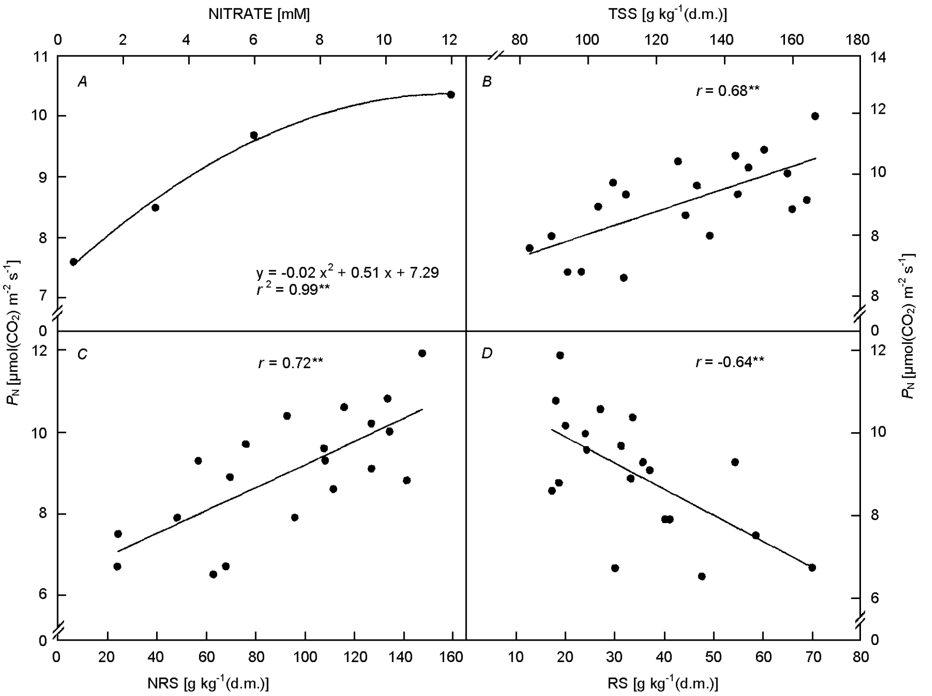

Fig. 5. Net carbon assimilation rate (P_N) as a function of NO_3^- applications (*A*), and correlations between P_N and total soluble saccharides (TSS) (*B*), non-reducing saccharides (NRS) (*C*), and reducing saccharides (RS) (*D*) in cassava plants. For (*A*), each symbol represents the mean of five replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

Absorbing roots accumulated less soluble saccharides and more starch as the N-supply decreased (Fig. 4*A,B*). A similar trend was observed for tuberous roots (Table 2). Conversely to what happened in leaves, N-deficiency did not cause an accumulation of reducing saccharides in tuberous roots (Table 2). The amplitude of changes in content of total non-structural saccharides (soluble saccharides plus starch), as a function of the N-treatments, was narrow; it was about 15 % for absorbing roots (data not shown) and 19 % for tuberous roots (Table 2). These findings imply that root growth was unlikely to be restricted by carbon availability. Nonetheless, since root growth declined with the severity of N-stress (Fig. 1), the absolute amounts of carbon partitioned to roots should have decreased accordingly. Altogether, these results suggest a limited capacity to use carbon skeletons for growth (this should have been to a great extent a consequence of restricted protein synthesis, which was depressed by up to 80 % in cassava plants grown at 0.5 mM NO_3^- relative to N-replete controls; Cruz 2001). That, in turn, would lead to a limited capacity for saccharide export from source leaves. This implies that the lower formation of sucrose in the source would be controlled by the decreased ability of sink tissues to use source-produced assimilates.

Carbon assimilation: P_N increased with increasing N-supply, tending to level off when NO_3^- application theoretically exceeded 9 mM (Fig. 5*A*). However, the amplitude of variation in P_N when comparing plants at the extreme N-supplies was relatively narrow (26.4 %). Yet, P_N on a whole-plant basis must have decreased considerably, taking into account that shoot growth declined remarkably with the severity of N-deficiency.

At biochemical level, decreases in P_N in cassava plants fed with inadequate N-supply are likely to be associated, at least in part, with decreased content of proteins, a large fraction of them probably linked to the biochemistry of photosynthesis, and declined carboxylation capacity of RuBPCO (Cruz 2001). In addition, changes in saccharide status as well as in P_i, as found in this work, should have also contributed to depressed P_N under N-limited conditions. Since the sinks could not use assimilates as fast as they were produced, the accumulation of saccharides in the source may cause directly or indirectly a feedback inhibition of photosynthesis-related gene expression and thereby regulate source strength (Stitt *et al.* 1995, Thomas *et al.* 1995). The positive correlations between P_N and soluble saccharides (Fig. 5*B*) or non-reducing saccharides (Fig. 5*C*), and the negative correlation between P_N and reducing saccharides (Fig. 5*D*) circumstantially suggest that hexoses might have in part negatively regulated P_N. In effect, Foyer (1988) showed that glucose, unlike sucrose, was effective in causing feedback inhibition to P_N. On the other hand, Sheen (1990) noted that glucose, fructose, and sucrose inhibited the expression of seven photosynthesis-related genes; however, sucrose probably affected gene expression indirectly through products from its cleavage (Jang and Sheen 1994). Finally, a direct effect of starch alone on P_N seemed unlikely because of the missing correlation between them ($r = -0.29, p > 0.10$; values not shown).

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