Effect of Potassium, Magnesium, and Calcium Deficiencies on Carbohydrate Pools and Metabolism in *Citrus* Leaves

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Abstract. Carbohydrate content and related enzyme activities were determined in leaves of rough-lemon (*Citrus volkameriana* Ten. & Pasq) plants grown under K, Mg, and Ca deficiencies. Starch content was lower and soluble sugar significantly higher in K-deficient than in control leaves. Magnesium- and Ca-deficient leaves, on the other hand, accumulated large amounts of starch. Electron micrographs also showed a reduction in the number and size of starch grains in chloroplasts of K-deficient leaves, while those of Mg- and Ca-deficient leaves were filled with large starch grains. Total amylase activity increased 3- to 7-fold in K-deficient leaves, with maltose as the major product and small amounts of maltotriose. Electrophoretic separation of amylase isozymes on native gels containing starch or amylopectin showed higher band intensities in K-deficient leaves. Examination of the capacity of leaf extracts to use amylopectin vs. β -limit dextrin indicated that mainly 8-amylase was involved. Acid invertase activity increased 7-fold in K-deficient leaves, while alkaline invertase showed little change.

In most higher plants, the principal end-products of leaf photosynthesis are starch and sucrose. However, species partition the photosynthate differently between starch and sucrose, and various factors affect this partitioning pattern (Huber, 1989). Starch is the major storage carbohydrate in *Citrus* (Sanz and Guardiola, 1988) and may comprise as much as 12.3% of leaf dry matter (Goldschmidt and Golomb, 1982).

The links between mineral deficiencies and carbohydrate metabolism are complex and poorly understood. Potassium deficiency resulted in increased soluble sugar accumulation in leaves of tomato (Wall, 1939) and wheat (Ward, 1960). Huber (1984) found that K-deficient soybean leaves contained less starch and higher hexose and sucrose as well as increased acid invertase activity. Accumulation of starch grains has been observed in chloroplasts of Mg- and Ca-deficient tomato and spinach plants (Vesk et al., 1966). Starch and sucrose accumulation has recently been described also in Mg-deficient *Phaseolus vulgaris* leaves (Fischer and Bremer, 1993).

In the present study we investigated the effects of K, Mg, and Ca deficiencies in *Citrus* on leaf carbohydrate pools. The results prompted a subsequent examination of some closely related enzyme systems. While the present study centered on rough lemon (*Citrus volkameriana*), supplementary data were obtained from experiments with calamondin [*Citrus reticulata* (*austera* x *fortunella* spp.)] and 'Shamouti' orange (*Citrus sinensis* L. Osbeck).

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Material and Methods

Plant material and growth conditions. Rough-lemon cuttings were grown in aerated nutrient solutions. The solutions were modifications of those used in Long Ashton (Hewitt, 1966). Full nutrient solutions consisted of 1 mM KNO₃, 0.9 mM MgSO₄·7H₂O, 3.8 mM Ca(NO₃)₂·4H₂O, and 0.8 mM Na₂HPO₄·2H₂O. Iron was added as Fe-EDTA (5.6 μ g Fe³⁺/ml). In K-deficient solutions, KNO₃ was replaced by 0.5 mM Mg(NO₃)₂·6H₂O, 0.4 mM Na₂HPO₄·2H₂O, and 0.2 (NH₄)₂HPO₄. In Mg-deficient solutions, Mg(NO₃)₂ was replaced by 0.6 mM KNO₃, 0.3 mM K₂SO₄, and 1.3 mM NaNO₃. In Ca-deficient solutions, Ca(NO₃)₂ was replaced by 1.9 mM Mg(NO₃)₂·6H₂O. All nutrient solutions included a complete complement of micronutrients (Hewitt, 1966). Calamondin seedlings were grown in sand cultures and fertigated with the same nutrient solutions. Plants were grown for 4 to 6 weeks in full nutrient solutions before transfer to deficient conditions. Plants were kept under deficient conditions at least 3 months until deficiency symptoms developed. Mature, fully expanded (2 to 3 months old) leaves with visible deficiency symptoms that had developed after the transfer to deficient conditions were used for the various analyses.

'Shamouti' orange leaves were collected from fruiting terminals of 30-year-old trees in a 5-year fertigation experiment; that experiment involves N-P-K, N-P, and N treatments in which the amounts of nutrients supplied are 220 kg N, 52 kg P, and 249 kg K/hectare year.

Leaves were collected in the morning and rinsed in deionized water and the mid vein was removed.

Mineral analysis. Samples of leaf tissue were dried in a forced-air oven at 65°C. Samples of 50 mg ground leaf powder were extracted with 10 ml deionized water according to Bar-Akiva

(1974). After 30 min of shaking, K, Mg, and Ca were analyzed by atomic absorption spectroscopy.

Soluble sugar and starch determination. Six 7-mm-diameter discs from fresh leaves were extracted with 3 ml 80% ethanol for 15 min at 80°C; the extraction was repeated five times. Supernatants were collected and brought to a volume of 15 ml. Soluble sugars were measured with anthrone (Schaffer et al., 1985). The insoluble leaf residue was boiled for 30 min with 1.0 ml 0.2 M KOH. Samples were cooled and adjusted to pH 4.5 with 0.4 ml 1.0 M acetic acid. After amyloglucosidase treatment soluble sugar was measured with anthrone (Schaffer et al., 1985).

Amylase determination and starch hydrolysis product analysis. Leaf tissues were extracted with an Ultra-Turrax homogenizer in an extraction buffer containing 20 mM tris-maleate (pH 6.2), 5 mM CaCl₂, and 0.04% Tween-20, using a 1 plant tissue : 10 buffer ratio. The extract was filtered through Miracloth and centrifuged at 40,000× g for 10 min. Supernatants were dialysed overnight against the same buffer, from which Tween-20 was omitted, with two buffer changes and clarified by centrifugation at 40,000× g for 10 min. The dialysate was used for total amylolytic enzyme assay according to a slightly modified version of Hammond and Burton (1983). All operations were carried out at 4°C. Amylase activity was assayed in 1 ml reaction mixtures containing 0.4% soluble potato starch solution improved by Lintner (Sigma), 2 mM acetate buffer (pH 5.3) and 500 µl of dialysate. The mixture was incubated for 45 min at 37°C. The reducing sugars released were determined according to Somogy (1952). Products of enzyme reactions were tentatively identified by paper chromatography according to Doehlert et al. (1982).

Gel electrophoresis of amylase isozymes. Leaf tissue was extracted according to Beers and Duke (1988) with the addition of 20% glycerol. The plant tissue-to-extraction-buffer ratio was 1: 10. The extract was filtered through Miracloth and centrifuged at 1000× g for 10 min. All operations were carried out at 4°C. Native gel electrophoresis was performed as described by Doehlert et al. (1982), except that 5% to 15% of linear-gradient polyacrylamide gels containing 0.2% soluble starch improved by Lintner (Sigma), 0.2% amylopectin, or 1.8% β-limit dextrin was used as substrates. Gels were stained with 2.5 mM I₂ and 0.5 M KI.

Invertase determination. Invertases were assayed according to Schaffer et al. (1987) with some modifications. Leaf tissue (2.0 g fresh weight) was extracted in 10 ml buffer containing 50 mM HEPES-NaOH (pH 7.5), 5 mM MgCl₂, 1 mM Na₂EDTA, and 0.05% Triton X-100. The extract was passed through Miracloth and centrifuged at 17,300× g for 20 min at 4°C. The supernatant was concentrated by the addition of (NH₄)₂SO₄ to 80% saturation. After centrifugation for 15 min at 20,000× g the precipitate was resuspended in 2 ml of extraction buffer and dialyzed overnight against 5 mM HEPES-NaOH (pH 7.5), 5 mM MgCl₂, and 1 mM Na₂EDTA. The invertase assay mixture contained 200 µl dialyzed extract, 200 mM sucrose, and 100 mM citrate buffer (pH 5.0) for acid invertase and 100 mM HEPES-NaOH (pH 7.5) for alkaline invertase. The reaction mixtures were incubated for 45 min at 37°C and the glucose and fructose released were determined with dinitrosalicylic acid.

Electron microscopy. Pieces of leaf tissue (about 1 mm square) were taken from the same leaves used for carbohydrate analyses. The pieces were fixed in 2% glutaraldehyde in 20 mM phosphate buffer (pH 6.8) for 1 h. Postfixation was done with 2% osmium tetroxide in 20 mM phosphate buffer (pH 6.8), followed by dehydration in acetone and embedding in Agar-100 (Orion and Frank, 1990). Sections were cut on an ultramicrotome (Ultratome III, LK13), stained with 2% uranyl acetate and lead citrate, and examined in an electron microscope (Jeol Gem 100 CX).

Results

In full nutrient solutions the concentrations of K, Mg, and Ca in leaves were in the optimum-high range (Embleton et al., 1973; Golomb, 1984). When grown in mineral-deficient solutions, tissue concentrations of these ions were significantly reduced (Table 1). Similar results were obtained with calamondin leaves (data not shown). In leaves from field-grown 'Shamouti' orange, K concentration was significantly lower in the N-P and N treatment than in the control leaves which had an optimal K concentration (data not shown).

The starch content of K-deficient rough-lemon leaves was lower, and that of Mg- and Ca-deficient leaves considerably higher than that of control leaves (Table 2). The concentration of soluble sugar was significantly higher in K-deficient than in control leaves (Table 2). The starch : sugar ratio was 0.4 for K-deficient leaves compared to 1.4 for control and 2.2 for Mg- and Ca-deficient leaves.

Decreased starch content of K-deficient leaves was also evident in electron micrographs (Fig. 1). Chloroplasts of K-deficient leaves were smaller and there were smaller and fewer starch grains. Chloroplasts of Mg- and Ca-deficient leaves, on the other hand, were packed with oversized starch grains.

Total amylase activity was significantly higher in extracts from K-deficient leaves than in controls for rough lemon and calamondin (Table 3). In Mg- and Ca-deficient leaves, total amylase activity was not significantly higher than in controls. Results were essentially the same when expressed on a protein basis (data not shown).

Chromatographic analysis of the amylolytic reaction products revealed the presence of glucose and maltose. Rapid accumulation of maltose was evident in the K-deficient treatment (Fig. 2). An additional faint spot closer to the front was tentatively identified as maltotriose. Prolonged incubation (20 h) of control and K-deficient extracts with pullulan substrate yielded large amounts of maltotriose, indicating the presence of debranching enzyme activity (data not shown).

Electrophoretic separation of amylolytic extracts on polyacry-

Table 1. Concentration of K, Mg, and Ca in full-nutrient-control rough-lemon leaves and in leaves from plants deficient in each respective element.

Nutritional treatment	Elemental concn (% dry wt)		
	K	Mg	Ca
Control	1.77 a ^z	0.31 a	0.35 a
Deficient	0.24 b	0.03 b	0.11 b

^zMean separation within columns by Duncan's multiple range test at *P* = 0.05.

Table 2. Starch, soluble sugar, and starch : soluble sugar ratio in rough-lemon leaves deficient in K, Mg, and Ca.

Treatment	Foliar concn (mg·g ⁻¹ leaf fresh wt)		
	Starch	Soluble sugar	Starch : soluble sugar
Control	37.2 ab ^z	25.0 b	1.4 ab
K deficiency	16.0 b	38.3 a	0.4 b
Mg deficiency	64.4 a	30.6 ab	2.2 a
Ca deficiency	65.6 a	31.0 ab	2.2 a

^zMean separation within columns by Duncan's multiple range test at *P* = 0.05.

Fig. 1. Electron micrographs of chloroplasts from rough-lemon leaves from different nutritional treatments. (a) Control leaf, (b) K-deficient leaf, (c) Mg-deficient leaf, (d) Ca-deficient leaf; sg = starch gran.

Table 3. Total amylase activity in rough-lemon and calamondin leaves deficient in K, Mg, and Ca.

Treatment	Amylase activity (mg reducing sugar/g fresh wt per h)	
	Rough lemon	Calamondin
Control	2.1 b ²	1.9 b
K deficiency	12.0 a	5.3 a
Mg deficiency	3.7 b	3.0 b
Ca deficiency	4.2 b	2.2 b

²Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

lamide gels containing starch or amylopectin as a substrate showed two groups of amylase isozymes: a slow (A) and a fast (B) migrating group (Figs. 3 and 4). Group B activity, which was rather weak in crude extracts, appeared clearly in $(\text{NH}_4)_2\text{SO}_4$ concentrated extracts (data not shown). With amylopectin as a substrate, group B stained red, whereas group A yielded bright, colorless bands (Fig. 4).

Group B activity did not appear on gels when β -limit dextrin was used as a substrate in spite of the high amount of protein applied (Fig. 4). In both groups of isozymes, extracts from K-deficient leaves had stronger amylase activity than controls with starch (Fig. 3) and amylopectin (Fig. 4). Group A isozymes digested P-limit dextrin, but no difference between K-deficient leaves and controls could be seen (Fig. 4).

Fig. 2. Paper chromatogram of amylolytic reaction products by rough-lemon leaf extracts with 0.2% starch as a substrate. Extracts were incubated for 15 min. Maltose (Mal) and glucose (Glc) markers (50 μg each) were used.

Acid invertase activity increased markedly in K-deficient leaf extracts compared to the control. In Mg- and Ca-deficient leaf extracts acid invertase activity was low and undetectable, respectively (Table 4). Alkaline invertase activity was less affected by nutritional treatments (Table 4).

Discussion

This study reports effects of K, Mg, and Ca deficiencies on carbohydrate metabolism in *Citrus* leaves. Potassium deficiency produced the strongest effects, resulting in less starch and more soluble sugar. Magnesium and Ca deficiencies resulted in marked increase in starch content, demonstrated both chemically and microscopically. Potassium deficiency also resulted in higher amylase and acid invertase activities. A K-deficiency-related increase of acid invertase activity in soybean leaves has been described previously by Huber (1984); however, no effect on amylase has yet been reported.

Using different amylase substrates we attempted to characterize the changes in amylase isozyme activity. Whereas starch and amylopectin are digested by α - and β -amylase, β -limit dextrin is not digested by β -amylase. When amylopectin is used as a substrate, β -amylase activity produces red stain, while α -amylase produces white, colorless bands (Kakefuda and Duke, 1984; Kakefuda et al., 1986).

Group B isozyme activity was undetectable with β -limit dextrin and produced red bands with amylopectin (Fig. 4), indicating that this group contained only β -amylases. Group A, on the other hand, contained α -amylase as demonstrated by the colorless bands obtained with amylopectin and by the digestion of p-limit dextrin (Fig. 4). The much weaker digestion of β -limit dextrin by group A, compared with amylopectin and starch, suggests that this group also contained β -amylases.

Table 4. Acid invertase and alkaline invertase activities in rough-lemon leaves deficient in K, Mg, and Ca.

Treatment	Invertase activity (mg glucose equivalent/g fresh wt per h)	
	Acid invertase	Alkaline invertase
Control	1.2 b ^z	3.9 bc
K deficiency	8.5 a	5.5 a
Mg deficiency	0.2 b	5.3 ab
Ca deficiency	nd ^y	3.7 c

^zMean separation within columns by Duncan's multiple range test at $P = 0.05$.

^ynd = Nondetectable.

Fig. 3. Amylase activity in rough-lemon and calamondin leaf extracts from different nutritional treatments. Crude extracts were separated on non-denaturing PAGE, containing Lintner soluble starch and stained with KI-I₂. The amount of protein per lane was 2.5 µg.

Stronger activity of K-deficient extracts was evident in both isozyme groups (A and B) with starch (Fig. 3) and amylopectin (Fig. 4). These differences could not be observed with P-limit dextrin (Fig. 4). This indicates that the K deficiency induced mainly β -amylase activity. The liberation of maltose as the major product of amylolytic activity in K-deficient leaf extracts (Fig. 2) also suggests a role for β -amylase (Ziegler and Beck, 1986).

The results of the present study as a whole indicate that K deficiency interferes with carbohydrate status and metabolism of mature *Citrus* leaves. Apparently, under K deficiency, the starch-soluble-sugar balance changes in favor of the latter component. It is, however, impossible at this time to suggest a specific metabolic reaction and identify it as the source of the interference. Starch degradation is one likely area for further exploration, but this might

Fig. 4. Amylase activity in leaf extracts of 'Shamouti' orange from N-P-K (1) N-P (2) and N (3) fertilization treatments. Crude extracts were separated on non-denaturing PAGE containing either amylopectin or β -limit dextrin. The amounts of protein per lane were 60µg in the amylopectin and 90 µg in the β -limit dextrin gel.

be a secondary effect and not the primary cause. It should also be mentioned that the role of β -amylases, which were found in many cases to be extrachloroplastic (Ziegler and Beck, 1986; Lin et al., 1988), in leaf-starch metabolism, is far from clear (Ghien et al., 1993). Caspar et al. (1989) proposed that high β -amylase activity could be induced in response to accumulation of soluble sugars and this possibility must be considered in our case as well. A similar response of β -amylase accumulation to sucrose and soluble sugars was reported in leaf petiole cuttings of sweetpotato (Nakamura et al., 1991). Huber (1984) suggested that the accumulation of soluble sugar was involved in maintenance of leaf osmotic potential in K-deficient leaves. Potassium also plays a critical role in the phloem translocation mechanism (Bhandal and Malik, 1988). The impairment of phloem transport under K deficiency may lead to the accumulation of soluble sugar in leaves. This perturbation of carbohydrate metabolism may possibly account for the reduction of *Citrus* fruit size under conditions of K deficiency (Chapman, 1968).

From a different perspective, the elevated level of invertase might have a developmental significance. In starch-storing plants, including *Citrus* (Schaffer et al., 1987), acid invertase activity declines to very low levels during the course of leaf development (Huber, 1989). The relatively high level of acid invertase in K-deficient *Citrus* leaves may reflect a metabolically juvenile condition. In view of the presumed role of Ca in the delay of leaf

senescence (Poovaiah, 1988), it might be argued that Ca deficiency enhances leaf senescence, as reflected by the accumulation of starch and the disappearance of acid invertase activity. The association of Ca deficiency with leaf senescence fits very well with the loss of chlorophyll, total protein, and Rubisco protein, which have been found to be characteristic of Ca deficiency in *Citrus* leaves (Lavon, 1992).

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