Changes in Carbohydrate and Ribulose Bisphosphate Carboxylase-Oxygenase Contents in Peach Leaves after Applications of Different Amounts of Nitrogen Fertilizer

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Summary

The effects of applying different amounts of nitrogen on the accumulation of nonstructural carbohydrate and ribulose biphosphate carboxylase-oxygenase (RuBisCO) and on some characteristics of peach leaves were determined on young trees. The leaf nitrogen contents of trees supplied 0 and 1.5g N/pot (N-0 and N-1.5, respectively) were lower than were those administered 9.0g/pot (N-9). On a dry weight (DW) basis, leaves sampled from N-0 trees and N-9 trees were 1.8% and 4.0%, respectively. The chlorophyll content per unit leaf area increased with increased nitrogen applied. The starch content on a dry weight basis was inversely proportional to the amount of nitrogen administered, whereas total soluble sugar per unit dry weight was proportional. The sorbitol and sucrose contents per unit leaf fresh weight were the lowest in leaves from N-0 trees. The RuBisCO content detected by immunoblotting after SDS-polyacrylamide gel electrophoresis as well as by immunostaining of sections under the light microscope increased with the increase in the amounts of nitrogen applied.

Introduction

Nitrogen is a very effective fertilizer for tree growth in most soil. Generally, the volume and capacity of sink organs with vigorous vegetative growth increase with increased soil application of nitrogen to fruit trees. Leaf starch content decreases with the application of nitrogen (DeJong et al., 1984; Nii et al., 1993), because the rate of export of photoassimilates from fully expanded leaves may be stimulated by vigorous vegetative growth. By contrast, with decreases in sink capacity due to limited application of nitrogen, starch increases in the leaves of fruit trees, whereas chlorophyll and the rate of photosynthesis (Pn) decreases (DeJong, 1982, 1983; DeJong et al., 1984; Nii et al., 1993). Although some information is available about the changes in starch content of leaves upon the application of nitrogen, the changes in contents of other carbohydrates in fruit trees have not been reported in detail. The photo-

synthetic activity of leaves is significantly related to the concentration of sugars translocated in the phloem. In general, there is a negative correlation between sucrose content and Pn in many plant species (Sawada et al., 1986, 1987; Tschaplinski and Blake, 1989).

Peach trees are woody species of *Rosaceae* in which the main translocatable carbohydrate is sorbitol (Webb and Burley, 1962; Williams et al., 1967; Bielecki, 1969). In the *Prunus* spp, there have been a number of reports on the relationship between Pn and the sorbitol content of leaves. Layne and Flore (1993) reported that the increase in Pn in partially defoliated plants was associated with reduced starch content in leaves and elevated sucrose and sorbitol contents in sour cherry trees. In a more recent study, they concluded that the diurnal decline in Pn in defoliated plants is associated with an increase in the contents of sucrose and sorbitol in leaves (Layne and Flore, 1995). They also noted that they were unaware of any other current reports that linked the content of sorbitol in leaves to the inhibition of photosynthe-

sis in source leaves.

Several researchers have indicated that there is a good correlation between leaf nitrogen content and the Pn in woody plants (Mooney et al., 1978; Gulmon and Chu, 1981; DeJong, 1982, 1983; DeJong et al., 1984; DeJong and Doyle, 1985; Williams and Smith, 1985; Nii et al., 1993). It is important to understand how changes in the anatomy and biochemical composition of leaves are related to the soil application of nitrogen. Ribulose biphosphate carboxylase-oxygenase (RuBisCO) is one of the most important photosynthetic enzymes in leaves and accounts for more than 50% of the soluble in chloroplasts. The RuBisCO content which changes with the accumulation of starch in the chloroplasts (Nii et al., 1995) has been estimated by immunostaining sections of leaves embedded in Technovit resin (Kuroiwa, 1991). Together with the changes in content of the RuBisCO protein that can be detected by immunoblotting after electrophoresis on polyacrylamide gels, results of immunocytochemical analysis are very useful for the characterization of leaf composition.

In the present experiments we applied different amounts of nitrogen to young peach trees and followed the changes in leaf area, leaf dry weight per unit leaf area (SLW), and chlorophyll content. We also measured contents of nonstructural carbohydrate, including starch and sorbitol, and monitored changes in the content of RuBisCO protein.

Materials and Methods

Plant materials

Three-yr-old peach trees (*Rrunus persica* (L.) Batsch. cv. Ohkubo) grown in the open air in 30-cm diameter pots (volume, 121) filled with sandy soil were treated on March 15 and May 15, 1994, with NH_4NO_3 fertilizer as follows: zero nitrogen per pot (N-0); 1.5 g/pot (N-1.5); 3.0 g/pot (N-3); 6.0 g/pot (N-6), and 9.0 g/pot (N-9). Other minerals applied to the trees were $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ (1.0 g per pot), K_2SO_4 (1.0 g), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g). Five plants were used per treatment, with the single exception that 10 plants were subjected to N-0 treatment. The trees which grew continuously until the middle of May were not pruned.

Mature leaves on the fifth to seventh nodal positions on the current shoots in the first growth cycle were sampled from each tree at 9 a.m. on June

14 and the shoot length recorded.

Quantitation of chlorophyll, carbohydrate, and nitrogen

Chlorophyll content was measured by using five leaf discs (8 mm in diameter). Chlorophyll was extracted with 100% ethanol and quantified by the method of Wintermans and De Mots (1965). Chlorophyll content is expressed per unit leaf area.

The total soluble carbohydrate content was determined with the anthrone reagent after extraction with 80% ethanol. To determine the starch content of leaves, the ethanol-insoluble residue was boiled to hydrate the starch grains and after cooling, it was incubated with glucoamylase for 2 hr. The mixture was then filtrated and an aliquot of the filtrate was analyzed with the anthrone reagent.

The sorbitol and sucrose contents were determined by HPLC (Nii et al., 1994). A 1.0-g (fresh weight) leaf sample was macerated in 5 ml of distilled water at 4°C. Three drops of 2% sulfosalicylic acid were added with a Pasteur pipette to the homogenate, which was then filtered through a 0.45- μm filter. A 10- μl aliquot of the filtrate was injected into an HPLC system equipped with a Shodex-CH-801 column (Showa Denko K. K., Japan). The column was eluted with water at a flow rate of 1 ml min⁻¹.

The nitrogen in leaves was quantitated by the Kjeldahl method.

Electrophoresis and immunoblotting for detection of RuBisCO protein

A 1.0 g (FW) leaf sample was macerated in a mortar with a pestle that had been chilled with liquid nitrogen with 5 ml of 50 mM Tris-HCl buffer (pH 8.0). The homogenate was centrifuged at 12,000 $\times$ g for 5 min at 4°C and the supernatant was subjected to SDS-PAGE (12.5% acrylamide) as described by Laemmli (1970). The proteins were separated on the gel, and blotted electrophoretically onto a cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). Antibodies against RuBisCO from spinach were prepared as described previously (Nishimura and Akazawa, 1974). Immunologic reactions were detected with a second antibody conjugated with alkaline phosphatase, as described previously (Tsugeki et al., 1993).

Immuno-localization of RuBisCO protein

For anatomical observations, the central area of the lamina was excised and fixed overnight in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C. The fixed specimens were dehydrated through a graded ethanol series and embedded in Technovit resin (Kulzer, Germany). To observe the RuBisCO protein, thin sections (0.5 μm) were immunostained with monospecific antibodies against RuBisCO proteins from spinach and with a second antibody conjugated with rhodamine. Immunostained sections were examined by epifluorescence microscopy (Hayashi et al., 1995).

Results and Discussion

The soil applications of different amounts of nitrogen resulted in a linear relationship between amounts applied and leaf N. Thus, leaf N were: 1.8% in N-0 trees, 2.2% in N-1.5 trees, 3.1% in N-3 trees, 3.6% in N-6 trees, and 4.0% in N-9 trees.

With increased application of nitrogen, the length of current shoots, leaf area, and chlorophyll content of peach trees increased but SLW was not obviously affected (Fig. 1, Table 1). Mean leaf area was smallest in N-0 and N-1.5.

The total soluble sugar content in leaves increased proportionately to the nitrogen added (Table 2), but starch content was inversely proportionate. Figure 2 shows the relationship between total soluble sugar and starch content and the leaf-N. These results are similar to those obtained with kiwifruit and peach trees (DeJong et

al., 1984, Nii et al., 1993); the leaves of nitrogen-deprived plants had lower leaf-N and chlorophyll but a higher starch content than did leaves of plants with sufficient N.

The sorbitol and sucrose content in leaves grown with sufficient nitrogen was higher than of N deprived leaves (Table 2). Similar results were reported in apple trees (Nii et al., 1997), but the differences were small. The volume and capacity of sink organs increased with shoot elongation

Fig. 1. Effects of differential applications of nitrogen on shoot elongation of peach trees. □, Average shoot length; ▨, total shoot length per tree. See text for definitions of treatments.

Table 1. Effects of differential applications of nitrogen on leaf characteristics of peach trees.

Amount of nitrogen (g per pot)	Leaf area (cm ²)	Dry weight per unit leaf area (mg cm ⁻²)	Total chlorophyll ($\mu\text{g cm}^{-2}$)	chl a / chl b
0	34.3	6.7	23.6	2.63
1.5	31.8	7.8	39.6	2.70
3.0	41.3	7.0	53.5	2.76
6.0	44.6	6.6	57.6	2.80
9.0	41.2	6.8	62.1	2.61
L.S.D. 5 %	6.4	0.8	4.7	0.20
1 %	8.7	1.0	6.4	NS

Values are means of results from five trees in each case, with the exception of trees that did not receive any added nitrogen. In the latter case, ten trees were examined.

Table 2. Effects of differential applications of nitrogen on the carbohydrate content of peach leaves.

Amount of nitrogen (g per pot)	Total soluble sugar (% DW)	Starch (% DW)	Sorbitol (% FW)	Sucrose (% FW)
0	6.5	8.2	2.5	0.7
1.5	6.7	8.1	3.0	1.3
3.0	6.4	3.1	3.5	1.2
6.0	7.6	2.1	3.5	2.1
9.0	7.5	2.5	3.5	2.1
L.S.D. 5 %	0.7	1.1	0.33	0.37
1 %	1.0	1.5	0.46	0.52

Values are means of results from five trees in each case, with the exception of trees that did not receive any added nitrogen. In the latter case, ten trees were examined.

Fig. 2. Relationship between contents of total soluble sugar and starch and the nitrogen content in leaves of peach trees. ○, Total sugar; ●, starch.

during the second growth cycle during June in the present experiment. Sorbitol content in mature leaves may increase with utilization of other soluble carbohydrates. Conversely, the vegetative growth in N-0 trees was less during June than N-3, N-6, and N-9 trees. The sorbitol content might be a reflection of a decreased capacity for sugar export from mature leaves and attributed to the weak sink activity. The sorbitol content of the

Fig. 3. Gel plate of peach leaf RuBisCO protein, revealed by immunoblotting. An equal volume of sample was loaded in each lane (a, N-0 leaves; b, N-1.5 leaves; c, N-3 leaves; N-6 leaves). Numerals on the left indicate molecular weights (mw) of pre-stained protein standards run that were subjected to electrophoresis in parallel with samples. Arrows indicate the L (Large, large arrow) and S (Small, small arrow) subunits of RuBisCO.

leaves of fruiting peach trees during the fruit-maturation stage was higher than that of the leaves of defruited trees (Nii, 1997). These results suggest that sorbitol in leaves increases with the increased demands created by the developing fruit and/or shoots.

Figure 3 shows gels which resulted from immunoblotting peach RuBisCO protein after isola-

Fig. 4. Immunofluorescence from palisade and spongy tissue of peach leaves after staining with antibodies against RuBisCO from spinach and rhodamine-conjugated second antibody. Arrows point to RuBisCO protein in the chloroplast. A. N-0 leaf. B. N-6 leaf. Bar = 20 μ m.

tion by SDS-PAGE. The immunoblot of peach leaf extract probed with monospecific antibodies against RuBisCO from spinach reveal two immunoreactive bands, which corresponded to the L and S subunits of RuBisCO, respectively. This demonstrates that extracts of peach leaves contained proteins that cross-reacted with antibodies against the enzyme from spinach and that the antibodies are applicable for cytochemical analysis of RuBisCO in peach leaves. These proteins were assumed to be components of RuBisCO. Immunoblot analysis also revealed that the contents of the immunoreactive proteins increased markedly as the leaves were supplied with more nitrogen.

The results of immuno-detection after SDS-PAGE were supported by the results of immunocytochemical staining of sections of leaves (Fig. 4). Mesophyll tissue from peach leaves, immunostained with antibodies against RuBisCO from spinach, revealed the distribution of RuBisCO in the chloroplasts. The intensity of immunoreaction increased with the increase in the amounts of nitrogen applied so that stronger immunoreactivity was detected in the greener and less starchy chloroplasts.

The changes in RuBisCO protein content in response to the application of nitrogen contribute to our understanding the physiological functions of

leaves (Evans, 1983, 1989; Evans and Terashima, 1988; Terashima and Evans, 1988; Makino et al., 1992). Increases in the RuBisCO protein content with increased contents of nitrogen in the leaves depend on the plant species; contents of RuBisCO protein were increased by applications of nitrogen in rice, spinach, bean, and pea, whereas it was independent of applied nitrogen in wheat (Makino et al., 1992). In the present studies, N-0 and N-1.5 resulted in decreased RuBisCO protein and chlorophyll contents and a concurrent increase in starch.

Acknowledgements

We thank Mr. E. Yokotani and Miss. N. Shinkai for their invaluable assistance. This work was supported in part by a grant from a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (Japan) and a grant (94-102) from the National Institute for Basic Biology (Japan).

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窒素施肥量の相違がモモ葉の炭水化物含量と RuBisCO タンパク質量に及ぼす影響

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摘 要

モモの幼木（‘大久保’3年生未結実樹）を用いて、窒素施肥量の相違が葉の形質と炭水化物含量、RuBisCO タンパク質量に及ぼす影響を調査した。無窒素区と1.5g/ポット区の葉の窒素含量（乾物重%）は窒素施肥量が多い処理区より低く、無窒素区が1.8%であるのに対して9g区は4.0%であった。単位面積当たりのクロロフィル含量は窒素施肥量の増加とともに増大した。無窒素区と1.5g区の葉のデンプン含量（乾物重%）は窒素施肥量の多い処理区の葉より有意に高

かった。葉の全糖含量は葉の窒素含量の増加とともにわずかに高くなる傾向がみられたが、デンプン含量に対する影響に比べて処理区間の差は小さかった。葉のソルビトールとスクロース含量（新鮮重%）は無窒素区でもっとも低かった。RuBisCO タンパク質量は、SDS-PAGEによる電気泳動法とハウレンソウ葉のRuBisCOの特異抗体を用いたイムノブロット法と抗体染色法によって検討した。その結果、RuBisCO タンパク質量は窒素施肥量の増加とともに増大した。