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## Regulation of nitrate uptake in a whole plant perspective

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## Summary

When leafy vegetables, like spinach and lettuce, are grown in greenhouses during winter and early spring, they may accumulate a high amount of nitrate in the leaves. A high nitrate content in vegetables is undesirable because it may be harmful for the consumer. Nitrate itself is not toxic, but it is easily reduced to the toxic compound nitrite. At present, standards for maximum acceptable nitrate concentrations in vegetables are being set in the European Community. During winter, the nitrate concentration in lettuce and spinach in The Netherlands often exceeds the permitted level, which puts a high pressure on efforts to lower the nitrate content in these vegetables. There is a need for more knowledge of the physiological processes that lead to the accumulation of nitrate in the leaves, like the nitrate uptake by the roots, the assimilation of nitrate to amino acids and proteins and the relation of these processes with the C metabolism and plant growth.

During the winter period, a high nitrate concentration in the leaves results mainly from the poor light conditions. The combination of a low light intensity and a short daylength restricts the production of organic osmotic solutes, like soluble sugars and malate, and nitrate serves as an osmotic alternative. It accumulates in the vacuoles of the leaves during the night. Previous research revealed that the accumulation of nitrate in the leaves was accompanied by a stimulation of the net nitrate uptake rate by the roots. It was hypothesised that the need for nitrate for osmotic purposes in the shoot might regulate the net nitrate uptake rate by the roots via the rate of nitrate translocation from root to shoot. In this model, nitrate itself functions as the signal that informs the roots about the nitrate requirements of the shoot. The model was based on net nitrate uptake measurements. The net nitrate uptake rate is the result of two opposite nitrate fluxes, namely nitrate influx and efflux. Whether the observed rapid increase of net nitrate uptake rate was due to an increase of nitrate influx, a decrease of nitrate efflux or both could not be established at that time.

Nitrate influx and efflux are both rapid and of the same order of magnitude. Therefore, they can only be separated in short-term experiments (time-scale of minutes) and by use of proper tracers. The available isotopes are the stable  $^{15}\text{N}$

and the radioactive  $^{13}\text{N}$ . Until recently, technical limitations of detection restricted application of  $^{15}\text{N}$  to experiments with a time-scale of several hours, which is too long for studies on nitrate influx and efflux. The radioactive  $^{13}\text{N}$  offers the right sensitivity, but due to its short half-life (10 min) it can only be used near  $^{13}\text{N}$  production sites, which are limited in number. In the Positron Emission Tomography Center of the Groningen University Hospital, approximately 10 km from the laboratory where the present investigations were carried out,  $^{13}\text{N}$  can be produced in sufficient amounts. This enabled the a detailed analysis of nitrate influx and efflux in spinach.

The aim of the investigations was twofold. Firstly, to clarify the physiological role of nitrate influx and efflux in the regulation of net nitrate uptake rate in spinach, grown under winter light conditions. Secondly, to examine the role of nitrate itself, as well as of reduced N, in the control of nitrate influx and efflux. The ultimate goal was to further increase our knowledge of the physiological causes of nitrate accumulation in leaves in relation to nitrate uptake by the roots. The obtained fundamental knowledge may be useful for finding ways to lower the nitrate content in commercially grown vegetables.

All experiments are carried out with plants that were grown in hydroculture. Two spinach cultivars were used, Subito and Glares, which differ in the extent of nitrate accumulation in the leaves.

The nitrate accumulation in the leaves is the result of complex interactions between N and C metabolism. Therefore, the investigations were started with an analysis of N and C metabolism in relation to the net nitrate uptake rate in the two cultivars (Chapter 2). There were marked differences between Subito and Glares in N investment in organic and inorganic fractions. In Subito, the nitrate concentration was higher and the reduced N concentration was lower than in Glares. This was accompanied by a lower *in vivo* nitrate reductase activity in Subito. Furthermore, in Subito, less C was directed towards structural C compounds than in Glares. The differences between the cultivars were maximal in the morning. The differences in nitrate concentration were osmotically compensated for by differences in concentrations of soluble sugars and malate. In Subito, the osmotic function of nitrate was more important than in Glares. It was

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postulated that the higher demand for nitrate for osmotic purposes in Subito might have driven increased leaf expansion compared to Glares, resulting in a higher specific leaf area. Net nitrate uptake rate and relative growth rate (RGR) were very closely coupled and the cultivars did not differ in this respect.

With  $^{13}\text{N}$  and  $^{15}\text{N}$ , nitrate influx and efflux were studied in both cultivars in relation to the plant's N demand for growth, defined as RGR times plant N concentration. The main differences in net nitrate uptake rate, either RGR or cultivar dependent, occurred in the morning and were primarily caused by large variations in nitrate influx. But nitrate efflux also varied in a time- and growth-dependent way. The experimental net nitrate uptake data agreed very well with the N demand for growth, provided that physical manipulations of the plants in the last hours prior to the uptake measurements were avoided.

In Chapter 4 the effects of physical manipulation of the plants on the nitrate fluxes were studied. Nitrate influx and efflux were both subject to changes after physical handling. The impact of the handling and the time course of the recovery depended on the severity of the disturbance. Even after a gentle transfer of the plants from one beaker to another, a normal procedure in nutrient uptake studies, recovery was mostly not complete within two hours. A model with a key role for stretch-activated ion channels and for variation of the cytoplasmic  $\text{Ca}^{2+}$  concentration is presented to explain the observations.

The influence of the nitrate concentration in the nutrient solution on nitrate influx and efflux was studied in undisturbed plants, grown on different nitrate concentrations (Chapter 5). The nitrate concentration in the uptake solution was the same as in the growth solution. Under these conditions, nitrate influx, efflux and net nitrate uptake rate were independent of the external nitrate concentration. RGR-related signals predominantly determined the nitrate fluxes. With increasing RGR, the efflux:influx ratio substantially declined. Thus, the efficiency of the net nitrate uptake process increased with increasing RGR. There were indications that an increase of the flux of reduced N from shoot to root resulted in a decrease of nitrate influx.

A sudden increase of the nitrate concentration in nutrient solution led to an instantaneous increase of nitrate influx (Chapter 6). The stimulation of nitrate influx was linearly dependent on the final external nitrate concentration and lasted several hours. The enhancement of nitrate influx was accompanied by an

increase of the rate of nitrate translocation from root to shoot, which resulted in an increase of the leaf nitrate concentration. The largest part of the surplus of incoming nitrate, however, was removed from the roots by nitrate efflux. The results are in accordance with the hypothesis that nitrate efflux is regulated by a nitrate signal. After several hours, feedback responses resulted in a gradual decrease of nitrate influx. A model is presented in which i) the rate of nitrate influx per nitrate transporter depends on the external nitrate concentration, and ii) the needed number of transporters per unit of plant weight is adjusted to the N demand of the plant by a reduced N signal.

The results of the experiments described in Chapter 7 confirm that an increase of the flux of reduced N from shoot to root results in a decrease of nitrate influx.

The present study shows that nitrate influx and efflux together regulate net nitrate uptake rate in hydroponically grown spinach plants. Both nitrate fluxes vary in dependence of internal and external conditions, thereby providing a flexible and sensitive nitrate uptake system, which continually adjusts the net nitrate uptake rate to the requirements of the plant for N for growth. Reduced N predominantly regulates nitrate influx, probably via changes in number of nitrate transporters. These changes occur on a time-scale of hours. Nitrate efflux provides a mechanism for a rapid (time-scale of minutes) response to changing internal or external conditions. Nitrate itself is the most likely candidate for regulation of nitrate efflux.

It is shown that the plant's need for nitrate and its uptake are very strictly coupled. Therefore, a change in net nitrate uptake rate will be accompanied by a change in RGR. For commercial spinach production this implicates that it will be difficult to manipulate the nitrate concentration in the plants without a substantial decrease in yield. There are some ways to reduce the nitrate content of plants grown in winter (Chapter 8), of which additional illumination during the last day(s) prior to harvest probably will be most successful, but which will increase the price of the product. In view of the new European standards for maximum permitted nitrate concentrations in vegetables it may be worthwhile to reconsider the economic costs associated with the suggested measures.

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