

Nitrate Reductase Activity in the Course of Cucumber Leaf Ontogenesis

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Abstract. Nitrate reductase activity in the leaves of young plants of cucumber, *Cucumis sativus* L. cv. Bílská, as determined both *in vitro* and *in vivo* and expressed in terms of fresh weight, gradually changes in dependence on the ontogenetic development of the plants, reaching its maximum before full expansion of the leaves.

The enzyme nitrate reductase (E.C.1.6.6.1) occurs in green, as well as non-green parts of plants, both in roots and leaves. Its activity depends on the age of the plant and its organs (WALLACE 1975, PATE 1968). Within the above-ground plant parts it varies with leaf insertions. In radish or cucumber cotyledons maximum nitrate reductase activity was correlated with maximum weight of the organ. On the other hand, in wheat and foxtail leaves this maximum persisted for a longer time (HAGEMAN and HUCKLESBY 1971). These authors also refer to the fact that in certain species the extractibility of nitrate reductase decreases with the leaf age. This is in accordance with the findings of SCHRADER *et al.* (1974) and other authors who pointed out the lesser suitability of the *in vitro* method of nitrate reductase assay in such cases.

Our experiments aimed at investigating the changes in nitrate reductase activity in the course of ontogenesis of young cucumber leaves, using both *in vitro* and *in vivo* methods for its estimation. The results of these two procedures are compared in the Discussion.

MATERIAL AND METHODS

Pre-germinated seeds of cucumber (*Cucumis sativus* L., cv. Bílská) were planted into plastic boxes (200 × 100 × 70 mm), six seeds per box, containing purified large-grain quartz sand. Every fourth day the plantlets were watered with a nutrient solution prepared as recommended by the Vegetable Research Institute in Olomouc (DUFEK 1972), supplemented with magnesium sulfate (3.0 mM) and sodium molybdate (0.017 mM). Nitrogen was present in the

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form of KNO_3 (11 mM). In addition to this, the plants were watered, if necessary, with distilled water. Throughout the experiment they were kept under constant conditions at $20 \pm 1^\circ\text{C}$ with a 12-h light period (2800 lx).

Nitrate Reductase Assay *in vitro*

For the determination of nitrate reductase activity in a crude leaf homogenate a slightly modified method according to FILNER (1966) was employed. Freshly sampled leaves of an exact weight, rinsed in distilled water and wiped, were homogenized in a mortar under cooling with an extraction buffer containing phosphate buffer (0.1 M) pH 7.5 with 0.1 mM dithiothreitol at a ratio of 1 : 5 (g fresh weight : ml) (instead of Tris buffer and cysteine as given in the original Filner's procedure), and with a small amount of sea sand. The homogenization was performed as rapidly as possible and the homogenate obtained was filtered through a 4 fold gauze. The filtrate was immediately centrifuged in PVC tubes at $20\,000 \times g$ at $1-4^\circ\text{C}$ for 15 min in a K-70 Janetzki centrifuge. The supernatant was immediately used for nitrate reductase assay. The reaction mixture (FILNER 1966) consisted of 0.5 ml 0.1 M phosphate buffer pH 7.5, 1 ml 0.1 M KNO_3 , 0.1 ml 1.0 mM NADH, 0.2–0.3 ml crude enzyme preparation. In the blank NADH was replaced by distilled water. The incubation took place in the dark at 25°C and was stopped by the addition of 1.0 ml of 1% sulfanilamide in 1.5 N HCl and 1.0 ml of 0.02% N-1-naphtylethylenediamine dihydrochloride (NICHOLAS and NASON 1960). The enzyme activity ($\mu\text{moles of the NO}_2^- \text{ formed} \times 0.20 \text{ g fresh weight}^{-1} \times \text{time}^{-1}$) was measured at 540 nm using a SP 500 spectrophotometer. Each measurement was repeated 3–4 times.

Nitrate Reductase Assay *in vivo*

The procedure employed was a modified method of JAWORSKI (1971). From freshly sampled leaf blades, rinsed with distilled water and wiped with gauze, discs 7 mm in diameter were cut (outside the region of vascular bundles). The weighed sample (0.2–0.4 g) was immediately transferred into a 25 ml Erlenmayer flask with 5.0 ml of incubation medium and corked. The incubation solution (5 ml) consisted of 15 mM KNO_3 , 0.15% (v/v) n-1-propanol and phosphate buffer pH 7.5, instead of 20 mM KNO_3 and 5% n-1-propanol, as given in the original procedure by JAWORSKI (1971). The incubation took place in the dark at 25°C for the period required during which the flasks were shaken occasionally. After the termination of the incubation period, from each flask 1 ml of the solution was pipetted into a test tube on addition of 1 ml of 1% sulfanilamide in 1.5 N HCl and 1 ml 0.02% N-1-naphtylethylenediamine dihydrochloride (NICHOLAS and NASON 1960). For the following 20–30 min the stain was stabilized and its absorbance was measured at 540 nm similarly as given in the procedure *in vitro*. The enzyme activity was again expressed in $\mu\text{moles NO}_2^- \times 0.20 \text{ g fresh weight}^{-1} \times \text{time}^{-1}$.

To obtain an average sample for both procedures the leaves of 6–10 plants from the same planting and the same insertion levels were taken. In the samples thus obtained the nitrate reductase activity was measured with an error of less than $\pm 5.5\%$ (BLÁHOVÁ 1978). The leaf insertion levels were numbered in an acropetal direction (1st leaf insertion level = the oldest leaves).

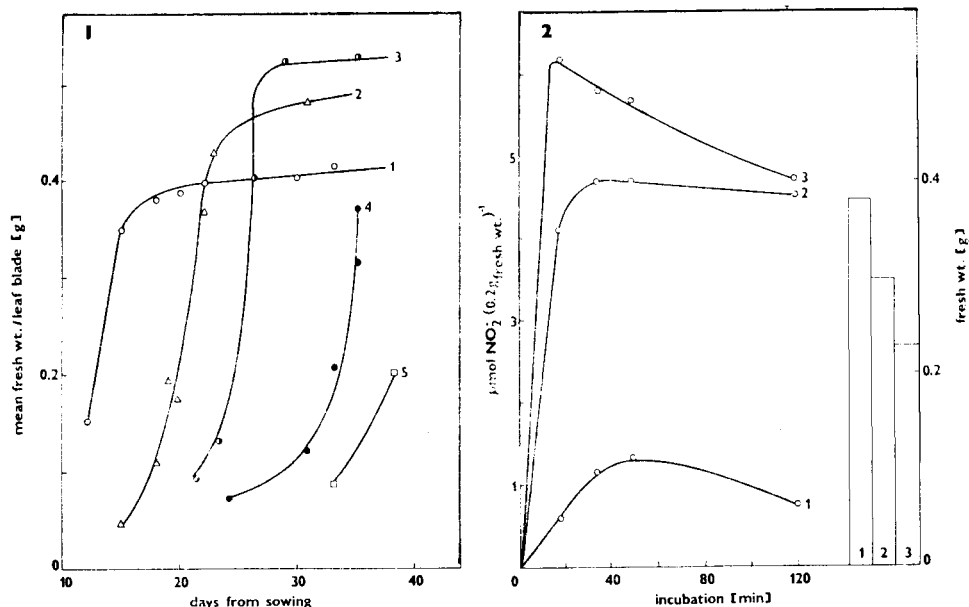


Fig. 1. Mean fresh weights of the leaf blades of the 1st–5th insertion level (1–5). The plants were cultivated as mentioned in Material and Methods.

Fig. 2. The incubation curves of nitrate reductase activity [$\mu\text{moles of NO}_2^-$ formed per 0.2 fresh weight] as measured *in vitro* in crude extracts from the 1st–3rd leaves (1–3) and mean fresh weights of their leaf blades [g]. The plants were cultivated for 27 days before the experiment.

RESULTS

In the course of plant cultivation the changes were examined in mean weights of the leaf blades sampled during ontogenesis of leaves of individual insertion levels (Fig. 1). The growth curves thus obtained gave information, in the period investigated, of the development of leaves from the 1st–4th insertion level; the 5th leaf began growing.

The enzymic nitrate reduction was found to vary considerably with the insertion levels of the leaves. Nitrate reductase activity as determined using the *in vitro* method (Fig. 2) was high in the youngest (3rd) leaf, whereas in the oldest (1st) one it was already very low. Similar measurements were performed by the *in vivo* method in leaf discs (Fig. 3) with identical results: the highest nitrate reductase activity was shown in two youngest leaves (3rd and 4th) that in the first leaf being low. It should be noted that the curves of nitrate reductase activity obtained by the two methods in the presence of the same substrate concentrations differed in their courses (Figs. 2, 3, respectively).

The relationship of weights of developing leaves and their nitrate reductase activity is shown in Fig. 4A,B. In the third leaves where expansion growth has already been completed as well as in the developing fourth leaves, the *in vivo* method revealed the highest nitrate reductase activity (Fig. 4A). A more detailed investigation of ontogenesis of the first leaf (Fig. 4B) showed the period of maximum nitrate reductase activity to occur about the 20th day after planting; later on this activity, as measured by the *in vitro* method,

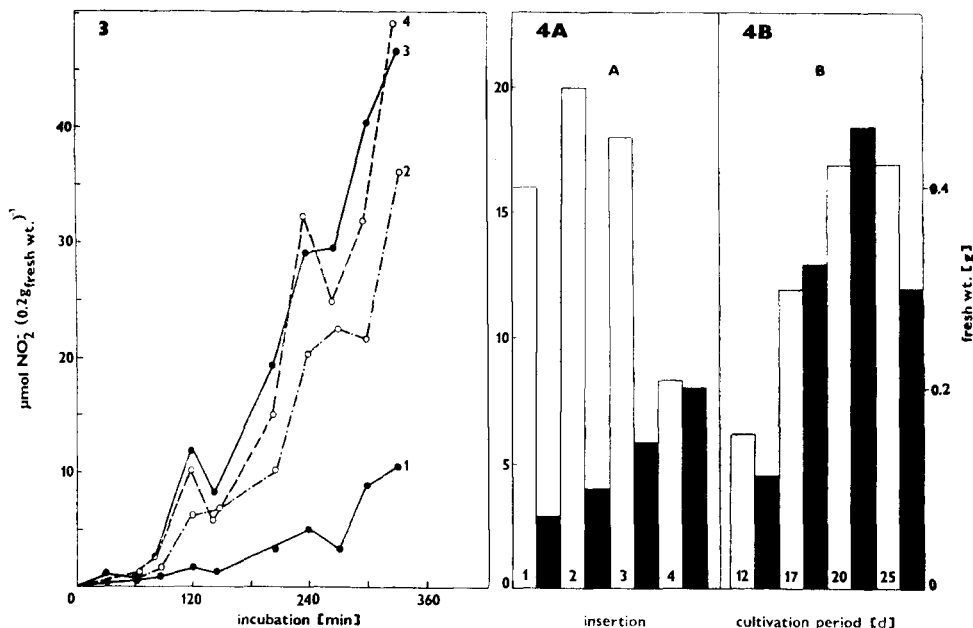
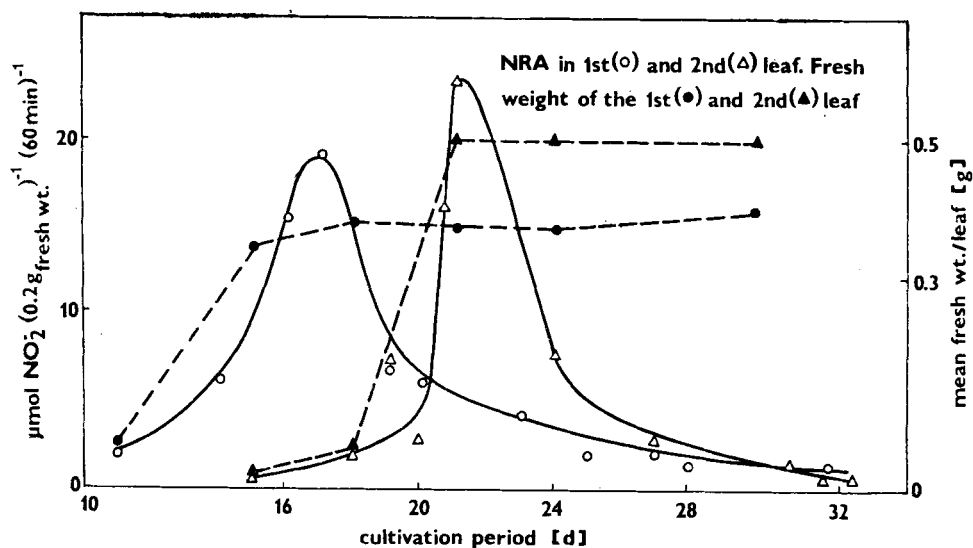


Fig. 3. The incubation curves of nitrate reductase activity [μ moles of NO_2^- formed per 0.2 g fresh weight] as measured in the leaf discs (*in vivo*) in the 1st–4th leaves (1–4). The plants were grown for 25 days before the experiment.

Fig. 4 A. *In vivo* nitrate reductase activity [μ moles of NO_2^- per 0.2 g fresh weight per 120 min] in the leaf discs from the 1st–4th leaves (full column) and mean fresh weights of their leaf blades (blank column). The plants were grown for 30 days before the experiment.

Fig. 4 B. *In vitro* nitrate reductase activity [μ moles of NO_2^- per 0.2 g fresh weight per 60 min] in crude extracts from the 1st leaves during their ontogenesis (full column), and mean fresh weights of the 1st leaves during their development (blank column). The plants were grown for 12–27 days.

decreased. In this connection the low nitrate reductase activity in the first leaf in 25-day plants (Fig. 3), in 27-day plants (Fig. 2), and in 30-day plants (Fig. 4A) may be compared. It is clear that the results obtained by the two methods are identical. A more detailed analysis of the course of nitrate reductase activity of the first leaf in dependence on its ontogenesis is shown in Fig. 5, which summarizes the measurements by the *in vivo* method. The region of maximum nitrate reductase activity was located between the 15th and 17th day after planting, or, more exactly, in the period just before the termination of expansion growth. Following the termination this activity decreased rapidly. The same figure shows a similar course in nitrate reductase activity during ontogenesis of the second leaf: the maximum was again found at the time of completion of leaf expansion, followed by a drop. Analogous curves resulting from the sums of *in vitro* measurements (Fig. 6), demonstrate the changes in nitrate reductase activity in the first and second leaf during their ontogenesis. In view of the data mentioned one may state that nitrate reduction by nitrate reductase is high in young leaves, decreasing rapidly after the completion of their expansion growth to occur intensively at the next insertion level (compare Figs. 1, 5, 6).



Figs. 5. The changes of nitrate reductase activity as determined by the *in vivo* method [NRA, $\mu\text{mol NO}_2^-$ per 0.2 g fresh wt. per 60 min] in the 1st and 2nd leaf during ontogenesis [mean fresh weights of the leaves in g].

DISCUSSION

Until recently, nitrate reductase activity has been assayed by the *in vitro* method comprising the use of extracts from severely damaged cells. Quite often the *in vivo* method could have given better results, if the leaf segments or discs were vacuum or chemically infiltrated (JAWORSKI 1971 *etc.*). In the

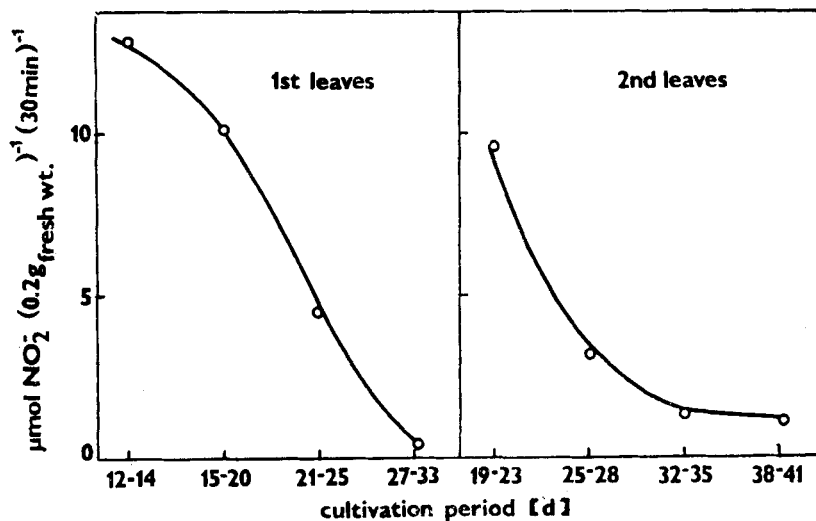


Fig. 6. The changes of nitrate reductase activity determined by the *in vitro* method [$\mu\text{mol NO}_2^-$ per 0.2 g fresh wt. per 30 min] in the 1st and 2nd leaf in the course of the cultivation period (12–41 days).

in vivo method changes in membrane permeability may occur, but those in the compartmentation of cell structures are not so heavy as is the case with the *in vitro* method. Moreover, there is no need to supplement endogenously NADH (into the incubation medium) as an electron donor for nitrate reduction, as in the *in vitro* method (BRUNETTI and HAGEMAN 1976, DUKE *et al.* 1978). The finding of HAGEMAN and HUCKLESBY (1971) and other authors, that on ageing of leaves the extractability of nitrate reductase is deteriorated, may be a further reason in favour of the application of the *in vivo* method.

It follows from the results of our experiments that for the determination of nitrate reductase activity in leaves of young cucumber plants, both methods, *in vitro* as well as *in vivo*, were applicable. However, the particularities of both procedures must be respected, *e.g.* especially the length of incubation of leaf discs must take at least 60 min, as suggested by the shape of our incubation curves at a substrate concentration of 15 mM (Fig. 3). A slower release of the reduction product (NO_2^-) into the incubation medium, or a lower rate of substrate uptake by the cells of leaf discs was also stated by other authors (*e.g.* STRETTER and BOSSLER 1972) and this is one of the drawbacks of the *in vivo* method. The slow rate of nitrite production during the initial 60 min of leaf disc incubation and the exponential shape were characteristic of our incubation curves (compare Figs. 2, 3).

In spite of the differences in the curves obtained by the *in vitro* and *in vivo* methods, identical conclusions for the ontogenetical studies mentioned could be drawn from our experiments.

Our experiments confirmed the positive correlations between the development of the leaf blade and nitrate reductase activity, and, on the other hand, a decrease in nitrate reductase activity during the process of leaf senescence. The maximum activity of this enzyme was shown to occur approximately at the time when the leaf ceased growing. These findings are in good agreement with those of DUDEL and KOHL (1974) who observed maximum nitrate reductase activity in the first leaf of barley closely before the termination of its elongation growth, this being followed by a drop in nitrate reductase activity and by nitrate accumulation. Similar results were also obtained by TEARE *et al.* (1974). Figs. 5 and 6 show an intense decrease in senescing cucumber leaves of the first and second insertion level. These results correspond to the finding of DI MARCO *et al.* (1976) in wheat.

In the course of ontogenesis of the entire plant correlations exist between the metabolism and development of leaf insertions. Our results show that the second fully developed leaf exhibited a higher nitrate reductase activity than the first leaf (Fig. 5).

From the point of view of nitrate reductase activity the correlations between photosynthetic processes, inorganic nitrogen assimilation and protein synthesis must be taken into account. Thus the curves representing chlorophyll accumulation during the development (ŠESTÁK 1977) have an almost identical shape with the curves for nitrate reductase activity in the present paper. The activities of photosystems I and II underwent considerable changes in the course of leaf ontogenesis as well (ŠESTÁK 1977).

The synthesis and metabolism of proteins in pea leaves was investigated by PATE (1968) in relationship to nitrate reductase activity. The protein production and conversion were high especially at the period of a high

nitrate reductase activity in young leaves. Similar results were obtained by ANDREEVA (1969) who dealt with amino acid and protein synthesis in dependence on the rate of photosynthetic CO₂ assimilation at various phases of leaf ontogenesis.

Generally one may state that in plant physiological and biochemical experiments the ontogenetic aspects of metabolic processes must be respected. The case of the enzyme nitrate reductase demonstrates a possible source of errors which may occur if an exact ontogenetic characterization of the plant or its organs is neglected.

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