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BRIEF COMMUNICATION

Relationships between Nitrate Level, Nitrate Reductase Activity and Anaerobic Nitrite Production in *Pisum sativum* Leaf Tissue

V. ŠKRDLETA, ALENA GAUDINOVÁ and MARIE NĚMCOVÁ

Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha*

Abstract. Anaerobic nitrite production (the *in vivo* NO_3^--R activity) in an incubation medium lacking exogenous nitrate but containing 0.5% *n*-propanol and 0.1% Triton X-100 showed higher correlation ($y = ax^b$) with the level of endogenous nitrate in *Pisum sativum* L. leaves than the *in vitro* nitrate reductase activity. The *in vivo* NO_3^--R activity correlated well with the *in vitro* activity up to the 50 ppm NO_3^--N level of endogenous nitrate. The ratio *in vivo* : *in vitro* activity slightly decreased with increasing level of endogenous nitrate in leaf tissue.

The efforts to use the determination of nitrate reductase activity as a possible predictive test of crop yields (JOHNSON *et al.* 1976) is complicated by the action of exogenous factors, by stage of maturity and type of plant tissue, and by experimental conditions of the determination itself (JONES and SHEARD 1977a, b).

When studying the effect of short-time exposure of pea plants to nitrate on biological dinitrogen fixation (N_2), we also followed the changes in nitrate level in pea leaves, corresponding *in vitro* nitrate reductase (EC 1.6.6.1) activity and anaerobic nitrite production which is conventionally called *in vivo* nitrate reductase activity (*e.g.* JONES and SHEARD 1977a).

Four-week-old nodulated pea plants (*Pisum sativum* L., cv. Raman), precultivated in a minus-nitrogen nutrient medium in perlit saturated to its full water retention capacity (86—87% of humidity), were transferred to perlit saturated with a nutrient solution containing 280 ppm NO_3^--N (20 mM NO_3^-). *In vivo* and *in vitro* nitrate reductase activity (NO_3^--R) and the level of endogenous nitrate in leaf tissue were determined after 8 h and 1, 2, 3, 4 and 7 days of cultivation. Experimental plants were grown at constant temperature and light regimes — 23/17 °C, 16 h light ($E = 60$ J s^{-1} m^2). All leaves of each plant were used for the determination of both NO_3^--R activities and of endogenous nitrate in such a way that one leaflet of each leaf (leaflets from one plant were combined to one sample) was used for the determination of the *in vivo* NO_3^--R activity, the other leaflet was cut longitudinally into two halves, one half being used for the *in vitro* NO_3^--R

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* *Address:* Ke dvoru 16/15, 166 30 Praha 6-Vokovice, Czechoslovakia.

determination and the other for nitrate determination — always one combined sample from 10 plants. *In vitro* NO_3^--R activity was determined after homogenisation and extraction with Tris-HCl buffer pH 8.0 containing 3% bovine serum albumine (SCHRADER *et al.* 1974). Anaerobic nitrite production was determined in 0.05 M phosphate buffer pH 7.5 containing 0.5% *n*-propanol and 0.1% Triton X-100 (v/v). Whole intact leaflets (10 replicates), vacuum-infiltrated (5 × 1 min — vacuum × N_2 flushing cycle) were incubated

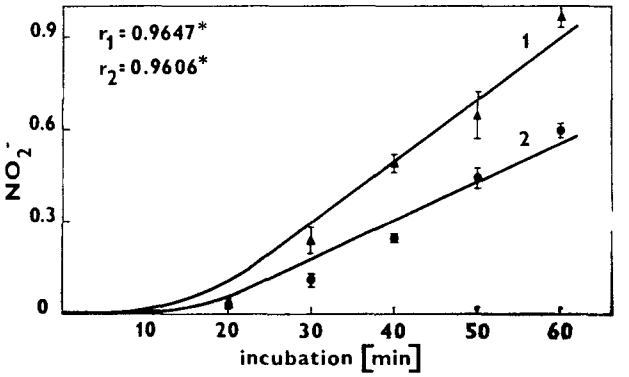

Fig. 1. Time course of anaerobic nitrite production (μmol NO_2^- g f. wt.$^{-1}$ min^{-1}) for younger (1) and elder (2) leaves of different *in vivo* NO_3^--R activity. Bars represent $\pm s_{\bar{x}}$; $n = 4$.

under N_2 atmosphere for 1 h in darkness at 25 °C. The nitrite formed was determined colorimetrically by adding 1% sulphanilamide in 1 N HCl and 0.02% N-(1-naphthyl)-ethylenediamine diHCl. Leaf tissue nitrate was determined with a Corning NO_3^- ion selective electrode (Orion Analyzer Application Bull. No. 7, Determination of nitrate in plant tissue). The results are expressed in ppm NO_3^--N per fresh weight unit. The results presented in this paper were obtained in three to four independent experiments.

The time course of anaerobic nitrite production in the above described medium by two leaflet samples differing in both maturity and enzyme activity is presented in Fig. 1. NO_2^- production is linear after an initial approx. 15 min lag phase. A similar lag phase was observed *e.g.* by STREETER and BOSLER (1972).

Fig. 2A, B, C demonstrates that the mathematical relation

$$y = ax^b \qquad (1)$$

complied best for the expression of the general relation between the *in vitro* and the *in vivo* NO_3^--R activity (Fig. 2A) and the relation between the level of endogenous nitrate and both nitrate reductase activities in pea leaf tissue (Fig. 2B, C), where y stands for nmol NO_2^- g f.wt.$^{-1}$ min^{-1}; x stands for NO_3^--N in ppm per fr. wt. basis (Fig. 2B, C) or nmol NO_2^- g^{-1} fr. wt. min^{-1} (Fig. 2A); a and b stand for regression coefficients and r^2 for the coefficient of determination.

However this relation approximated a linear relation in the case of the influence of endogenous nitrate on the *in vivo* NO_3^--R activity (Fig. 2B) and was correlated with the level of endogenous nitrate (in the range from 15.3

to 98.45 ppm NO_3^--N) much more closely than the *in vitro* NO_3^--R activity (Fig. 2C). Because anaerobic nitrite production was determined in a medium lacking exogenous nitrate we could consider the activity measured under such conditions as an "actual" activity in the sense that it better corresponds to the actual state of the tissue, above all to its *in situ* NO_3^- level (JONES and SHEARD 1977a). However it is necessary to point out that due to the application of *n*-propanol and Triton X-100 in the incubation medium, the concentration of nitrate available as substrate to the enzyme increased owing

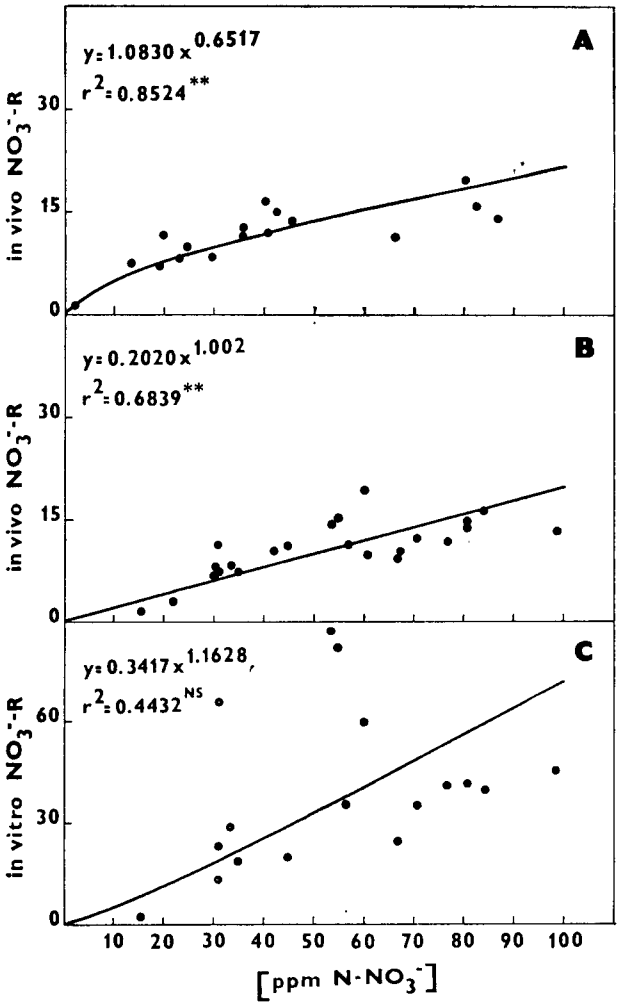

Fig. 2A. The relationship between *in vitro* NO_3^--R (x) and *in vivo* NO_3^--R activity (y) — (nmol NO_2^- g f. wt.$^{-1}$ min^{-1}) as tested by the power curve fit. Each point represents a mean from ten replications.
Fig. 2B. The relationship between NO_3^- level in leaflet tissue and *in vivo* NO_3^--R activity.
Fig. 2C. The relationship between NO_3^- level in leaflet tissue and *in vitro* NO_3^--R level.

to mixing of the storage and metabolic nitrate pools (*e.g.* FERRARI *et al.* 1973). This fact was obviously also reflected in a closer relationship between the endogenous NO_3^- level and the anaerobic nitrite production. In the experiments of JONES and SHEARD (1977a), the *in vivo* NO_3^--R activity in a medium lacking nitrate reached in the leaves of pea plants cultivated with 20 mM nitrate (the same concentration as in our experiments) in some cases 94% of the maximum nitrite production observed in an incubation medium containing 50 mM nitrate.

The mutual relationship between the *in vitro* and *in vivo* activity (Fig. 2A) was under our experimental conditions in extraordinarily good agreement especially at lower levels of endogenous nitrate in leaf tissue. The average ratio of *in vivo* : *in vitro* NO_3^--R activities was 0.35 ± 0.03 ($s_{\bar{x}}$) in the entire range of endogenous nitrate level and slightly decreased with increasing nitrate level or with increasing *in vitro* activity. This finding is in agreement with the results of JONES and SHEARD (1977b) obtained with both *Pisum arvense* L. and *Zea mays* L., as well as with the other four plant species investigated.

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