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Research Article

The Low Above-zero Temperature Effect in the Zone of Roots on Nitrate Reductase Activity in Pea Organs in the Process of Vegetating

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Abstract: Experiments with pea (*Pisum sativum* L.) cv. *Marat*) in a phytotron chamber showed that the process of nitrate assimilation in pea organs depends on temperature in the zone of the roots and on the phase of development of the plants. The potential of tissues of different organs for nitrate reduction was compared to the amount of nitrates which they accumalated. A decrease in nitrate reductase activity during certain phases of development is assumed to be related to the process of formation and functioning of nodules.

Key Words: nitrate reductase activity, nitrate, nitrogen metabolism, root nodule.

Vejetatif Gelişim Sırasında Kök Bölgesindeki Düşük Sıcaklığın Bezelye (*Pisum sativum* L. cv. *Marat*) nin Farklı Organlarının Nitrat Peduktaz Aktivitesi Üzerindeki Etkisi

Özet: Bitki büyüme kabininde bezelye (*Pisum sativum* L. cv. *Marat*) üzerinde yapılan denemeler bezelyenin organlarında nitrat özümlenmesi işleminin kök bölgesindeki sıcaklığa ve bitkilerin gelişim evresine bağlı olduğunu göstermiştir. Farklı organların dokularındaki nitrat indirgeme potansiyelleri biriktirdikleri nitratların miktarıyla karşılaştırılmıştır. Belli gelişme evrelerinde nitrat reduktaz aktivitesindeki azalmanın nödüllerin oluşum işlemi ve işlevi ile ilişkili olduğu düşünülmektedir.

Anahtar Sözcükler: nitrat redüktaz aktivitesi, nitrat, azot metabolizması, Kök nodülü.

Introduction

The availability of nitrates for plants is determined by the level of nitrate reductase (NR) activity. Variations in activity of this enzyme are used to deduce the effect of physiological factors on nitrate reduction, on the intensity of oxidized nitrogen assimilation in plants, and on the productivity and thermoresistance of plants (Alekhina & Klyuikova, 1988; Izmailov, 1986). NR is a genetically inherited enzyme (Eilrich, 1973; Tokarev, 1976). On the basis of the NR activity in roots and leaves, Pate (1980) classified the types of plants in three groups: those reducing NO_3^- primarily in roots, primarily (or only) in leaves, and in both organs. In the last case, according to Klyuikova and Alekhina (1983), the involvement of roots and shoots in NO_3^- reduction depends on physiological conditions.

Environmental factors determine in many respects the realization of the inherited abilities of plants to assimilate nitrogen. The temperature factor fluctuates greatly during the period of growth. The effect of this factor on nitrogen assimilation has been studied by many researchers; however, evidence for the effect of low temperatures on the NR activity in plants is ambiguous. Some researchers reported inhibition of nitrate reduction in roots at low temperatures (Kondratiev, Vasyukov & Aladina, 1983; Makhnovskaya & Babenko, 1980); on the other hand, a decrease in nitrate transport in the above-ground organs, and an increase in nitrate reduction and in the NR activity in roots with decreasing temperature were observed (Alekhina & Klyuikova, 1986;Macduff & Trim, 1986). There are also data suggesting no changes in NR activity in roots with decreasing temperature

(Klyuikova & Alekhina, 1991). Data on the reduced temperature effect on NR activity in the above-ground organs are also ambiguous (Klyuikova & Alekhina, 1983; Kondratiev, Vasyukov & Aladina, 1983; Makhnovskaya & Babenko, 1980; Kalinina, Alekhina & Primak, 1982). Kalinina, Alekhina and Primak (1982) reported that in the presence of a low-temperature stress, the involvement of roots and shoot in nitrogen assimilation was redistributed.

In bean plants, methods of utilizing inorganic nitrogen are complicated because of the ability of plants to fix atmospheric nitrogen, and of the interrelationship between two symbiotic partners. Although a large number of papers have addressed the issue of the assimilation of nitrate nitrogen in bean plants, its role in nitrogen nutrition, particularly at low above-zero temperatures, still remains unclear.

The objective of this work was to show how the NR activity and the nitrate content changed in pea organs under the influence of external (reduced temperature) and internal (ontogenesis stages) factors.

Materials and Methods

Plant material and growth conditions. The experiments were carried out in a phytotron chamber. Pea plants (*Pisum sativum* L.) cv. *Marat* were grown in vessels of volume 5 kg of dry sand. Inoculation was performed with *Rhizobium leguminnosarum biovar. viceae,* strain 250a, when sowing seeds. Experimental and control plants were grown, respectively, at low (8±1°C) and optimum (22±1°C) temperatures of the substrate (river sand), and at the same air temperature (23±1°C/15±1°C, day/night) for both control and experimental plants. The temperature in the zone of roots was maintained at a constant level by immersing the vessels into thermostatically controlled baths. The Gelrigel medium (Grodzinsky & Grodzinsky, 1973) with a complete set of microelements was used as the nutritient solution. Nitrogen was applied to the medium at the rate of 1/5 of its full volume in the medium. Watering was performed with tap water to create 60% moisture of the total moisture capacity of sand. The illumination (xenon lamps, 300 W/m^2 irradiation, 16/8 h photoperiod) and air humidity (60%) were controlled. The NR activity was determined in vitro (Voronova, Reimers & Khavkin, 1976). Samples used to analyze the NR activity were taken every 4 h after the onset of light innduction when a rapid increase in activity of the enzyme ceased.

Enzyme extraction. The enzyme extract was prepared by homogenizing plant tissue in four volumes of 0.25 M potassium phosphate buffer (ph 8.8), consisting of 0.01 M of 2-mercaptoethanol. One percent of casein was added to the medium to be homogenized for the stabilization of NR. The homogenate was centrifuged in a refrigerated centrifuge at 22 000 x g for 25 min, and the supernatanat obtained in this manner was used to determine the activity of NR. All steps for the preparation of the enzyme extract were carried out at 4°C.

Enzyme assays. The reactive mixture consisted of 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.6), 0.1 ml of 0.1 M potassium nitrate, and 0.4 ml of enzyme extract in the final volume of 1 ml. The reaction started when 0.02 ml of NADH (10mg/ml) was added to the reactive mixture. The reaction proceeded at 27°C for 15 min; after that the enzyme activity was stopped on addition of 0.02 ml of oxaloacetic acid (13mg/ml). Seven minutes later, a staining technique was used by adding 1 ml of sulfanilamide (1% in hydrochloric acid), and 1 ml of 0.02% N-(1-Naphthyl) ethylenediamine dihydrochloride ($C_{12}H_{14}N_2$. 2HCl). Staining was carried out for 30 min. The samples were then centrifuged at 2 000 x g for 15 min. Changes in optical density of supernatant at 540 nm were measured. The control mixture was without NADH. The NR activity was calculated in nmol of nitrite produced in an hour on 1g fresh tissue, on the root, and on all metabolically active leaves of a single plant.

The nitrate content in plants was determined colorimetrically with salicylic acid (Cataldo et al., 1975).

The dry and fresh weights of plant organs were measured simultaneously in both temperature treatments.

Results are represented as the arithmetical mean over three biological replications of the same-aged plants taken from three vessels with a density of 5 plants per vessel. Triple analytical replication was used. The standard deviation did not exceed 10%.

Results and Discussion

A different nitrate-reducing ability of pea roots and leaves was revealed in the expriments during pea growth

Table 1. The activity of nitrate reductase during pea growth under optimum and low temperatures in the zone of roots

Phase of development	Nitrate reductase activity					
	8°C			22°C		
	roots	leaves	nodules	roots	leaves	nodules
2 leaves	32.4 6.0	2.9 0.6	-	82.8 40.2	57.0 18.6	-
3 leaves	198.0 90.6	9.6 3.6	-	114.6 126.6	34.2 18.6	-
4 leaves	126.0 67.2	4.2 1.8	-	117.6 256.2	48.6 68.4	16.8
5 leaves	63.0 84.0	0	-	0	0	43.8
6 leaves	47.4 54.0	0	162.0	not determined		
7 leaves	202.8 571.2	0	119.4	24.0 71.4	0	48.0
8 leaves	not determined			45.6 143.4	0	55.2
Flowering	59.4 154.2	0	132.6	30.6 64.8	0	21.6
Fruit formation	20.4 59.4	0	136.2	31.8 93.6	0	121.2

Note: Above the line and for nodules, nmol NO_2^-/hour on 1g fresh tissue; below the line, nmol NO_2^-/hour on plant

at optimum and low above-zero temperatures. A calculation of the NR activity per 1 g of fresh weight and for plant organs (Table 1) showed that NR activity in the roots varied during the growing period at both optimum and reduced temperatures. Thus, in control plants, the NR activity in the roots increased and reached its maximum in the phase of four leaves, disappeared in the phase of five leaves, and recovered in the following stages without reaching its maximum level, however. At the low temperature, maximum enzyme activity was observed in the phase of seven leaves and was almost twice as high as that at optimum temperature. A decrease in NR activity at the low temperature is characteristic for the 6th leaf phase.

Both methods of calculation showed that the enzyme activity in leaves was lower than that in roots, especially at the low temperature, and, starting with the 5th leaf phase, the activity completely disappeared and did not reappear until the end of growth in experimental and control plants.

Nodules were formed in the phase of four leaves at 22°C and in the phase of six leaves at 8°C. The NR activity in nodules of both treatments was recorded until the end of growth. Furthermore, at the low temperature it was high in all phases of development.

Calculations of the nitrate content per 1 g of fresh weight (Table 2) showed that at the optimum temperature, the nitrate content was higher in the root tissue than that in the leaf tissue during early stages of development (up to the 4th leaf), while in all subsequent stages the nitrate content in the leaf tissue increased and was more than twice as high as that in the root tissue. During fruiting, the nitrate content in the leaf tissue was an order of magnitude higher than that in early stages of plant development. The nitrate content in the root tissue

Table 2. The content of nitrate in pea plants during growth under optimum and low temperatures in the zone of roots

Phase of development	Nitrate content			
	8°C		22°C	
	roots	leaves	roots	leaves
2 leaves	3.16 3.82	5.73 4.11	2.89 2.55	0.82 0.37
3 leaves	2.16 3.71	4.73 4.89	2.53 3.05	0.87 0.60
4 leaves	2.71 5.53	5.47 8.25	not determined	
6 leaves	2.98 5.08	4.71 4.26	2.32 5.45	2.58 4.19
8 leaves	2.48 6.61	4.03 4.84	not determined	
Onset of flowering	2.26 7.29	3.66 10.81	2.47 18.23	7.16 29.92
Flowering	1.69 6.03	9.53 17.11	2.98 17.16	8.06 26.52
Fruit formation	4.24 20.48	11.68 18.85	3.61 16.48	8.69 38.95

Note: Above the line, nmol NO_3^- on 1g freh tissue, below the line, nmol NO_3^- on plant.

also increased by the end of growth in comparison to the other stages, although this increase was not as high as that in the leaf tissue.

At the low temperature, the nitrate content in the leaf tissue was higher than that in the root tissue during all phases of development. During fruiting, the nitrate content increased by a factor of 1.5 to 3 in tissues of both organs as compared to the other plant phases. A decrease in temperature did not markedly affect the nitrate content in the root tissue as compared to control plants; however, it resulted in an increase in nitrate content in the leaf tissue, especially in early stages of development.

Calculations of the nitrate content per root and for all leaves of a single plant also showed that at the optimum temperature the nitrate content in the roots was much higher during early stages of development (up to the 6th leaf) than that in the leaves. In subsequent phases of development, the nitrate content in the leaves increased and exceeded that in the roots more than two-fold. By the end of growth, the nitrate content increased 3-6-fold in the roots and 1-2 orders of magnitude in the leaves as compared to early stages of development.

At the low temperature, the roots and leaves did not change greatly in nitrate content, except for the flowering stage with a higher nitrate content in the leaves. During fruiting, the nitrate content increased both in roots and in leaves as compared to the other stages.

A decrease in temperature in the zone of roots resulted in a reduced nitrate content in the roots during the flowering stage as compared to control plants; during fruting, however, the nitrate content in the root exceeded that in the control plants. At the low temperature the nitrate content in the leave was higher during early stages of development than that in control plants, the difference smoothed out in the phase of six leaves, and the content was lower in all subsequent phases.

The root fresh weight during plant growth (Table 3) increased gradually at the optimum temperature and

Table 3. Fresh and dry weight of pea organs during growth under optimum and low temperatures in the zone of roots

Phase of development	Fresh and dry weight, g/plant			
	8°C		22°C	
	roots	leaves	roots	leaves
2 leaves	1.21 0.064	0.72 0.130	0.88 0.051	0.44 0.065
3 leaves	1.72 0.100	1.03 0.173	1.20 0.061	0.69 0.093
4 leaves	2.05 1.109	1.52 0.257	2.57 0.170	1.43 0.185
6 leaves	1.70 0.105	0.91 0.151	2.34 0.130	1.63 0.217
8 leaves	2.67 0.149	1.19 0.240	4.62 0.219	2.47 0.314
Onset of flowering	3.22 0.232	1.40 0.319	7.41 0.471	4.18 0.773
Flowering	4.65 0.312	1.57 0.408	5.76 0.495	3.26 0.685
Fruit formation	5.40 0.423	1.64 0.425	4.58 0.524	4.50 0.878

Note: Above the line, fresh weight; under the line, dry weight.

reached its maximum by the start of flowering, and it was observed to decrease in all subsequent phases. At the low temperature, the fresh weight of the root increased throughout the growing season without reaching maximum values of control plants, however. The fresh weight of the leaves increased in both control and experimental plants until the fruiting stage. At the low temperature, however, the fresh weight increased slowly and was lower by a factor of nearly 3 than at optimum temperature. Therefore, the nitrate content, calculated for all leaves of a single plant in the second period of growth at the low temperature, decreased because of a decrease in leaf fresh weight, while the nitrate content, calculated per unit fresh weight (1 g) of the leaf tissue, increased and exceeded the control value.

The dry weight of the roots and leaves increased gradually during growth in both control and experimental plants. At the low temperature, the accumulation of dry weight of the roots and leaves began to lag behind the control plants, starting with the fourth to sixth leaf stage, and was more pronounced in the leaves. The dry weight of the leaves was twice as low at the low temperature as at the optimum temperature by the period of fruiting.

Results obtained in this study suggest that out of the two parameters of the nitrogen metabolism, the ability to reduce and the ability to accumulate nitrates, the former is more dynamic in the root tissue at the background of ontogenetic and temperature changes.

While the leaf tissue has an extremely low ability to reduce nitrates, it accumulates a significant amount of nitrates, especially at the low temperature and with the age of plants in both temperature regimes.

By analyzing changes in the NR activity and in the nitrate content in root tissue during growth of plants at the optimum and low temperaters, one can find a direct interrelationship between them. According to Klyuikova and Alekhina (1992), nitrate accumulation and reduction processes appear to have a complex and relatively independent regulation.

It should be noted that the process of assimilation of mineral nitrogen in bean plants is influenced by the nitrogen fixation system of plants. For instance, Li and Jresshoff (1991) investigated the effect of nodules on the nitrate metabolism in soybean leaves and found that the product of metabolic nitrogen fixation could directly affect the constitutive NR activity of leaves by inhibiting it. They consider ureide-allantoic acid to be such a nodule metabolite which competitively totally inhibited the constitutive NR in in vitro experiments. Some researchers reported the effect of nodulation on the expression of the NR activity in soybeans (Conejero, Tirado & Robin, 1986) and pea (Ligero et al., 1987). However, its physiological mechanism remains unclear. It is interesting that in our experiments the disappearance of the NR activity in the leaf tissue and its decrease in the root is time-coincident with nodule formation, which also suggests a relationship between the two systems of assimilation of inorganic nitrogen in bean plants; this problem needs further elucidation, however.

Conclusion

1. Pea root and leaf tissues differ in their ability to reduce and accumulate nitrates during the growing period at optimum and low temperatures. Nitrates were mainly reduced in the roots where the NR activity was observed until the end of growth, while the enzyme activity was negligibly low in the leaves, especially at the low temperature, and was detected only during early phases of development. The leaf tissue has an enhanced property of accumulating nitrates. The nitrate content here is higher than that in the root tissue, and it increases by the end of growth, while in the root tissue it does not change significantly.

2. The ability of the root to reduce nitrates during growth is variable: in some instances it increases and reaches maximum values, and sometimes it decreases and even completely disappears. It was found that a decrease or the disappearance of the NR activity was time-coincident with the phase of nodule formation.

3. The low temperature effect causes an increase in maximum NR activity in the roots, a still greater decrease in the already low NR activity of the leaves during early phases of development, and an increase in enzyme activity in nodule tissue. The nitrate content in the roots does not change greatly at a low temperature, whereas in the leaves it increases drastically.

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