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Comparative study of nitrogen and oxygen metabolism enzymes in Yugoslav cultivars of alfalfa (*Medicago sativa* L.)

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Abstract

Four Yugoslav cultivars of alfalfa were investigated in order to determine nitrogen fixing (nitrogenase), nitrogen assimilation (nitrate reductase, glutamine synthetase, glutamate dehydrogenase) and antioxidant (superoxide dismutase, catalase, peroxidase) enzymes activities. The level of lipid peroxidation and protein content were also investigated. On the basis of the results obtained a resistant cultivar with high nitrogen fixing and a cultivar with high nitrogen assimilation abilities were chosen. The cultivar with high nitrogen fixing ability had high activities of nitrogenase, superoxide dismutase, peroxidase and catalase, and also a low level of lipid peroxidation. The cultivar with high assimilation ability had high activities of nitrate reductase, glutamine synthetase and glutamate dehydrogenase and high soluble protein content.

Introduction

Leguminous plants are supplied with nitrogen necessary for protein biosynthesis in two ways: (*a*) by reduction of nitrate from the soil catalysed by nitrate reductase (NR, EC 1.6.6.1) and its further incorporation in ketoacids catalysed by glutamine synthetase (GS, EC 6.3.1.2) and glutamate dehydrogenase (GDH, EC 1.4.1.2); (*b*) from the atmosphere by reduction of the molecular nitrogen catalysed by nitrogenase (NG, EC 1.18.2.1) (Henson *et al.* 1982).

In nodules, the main product of N_2 fixation is NH_3. The obtained NH_3 reaches the cell cytosol of the host plant by active transport and serves as substrate in biosynthesis of amino acids and proteins (Bergensen 1965, Kennedy 1966, Matsumato *et al.* 1977, Trung-Chanh *et al.* 1986).

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Abbreviations : NR - nitrate reductase; GS - glutamine synthetase; GDH - glutamate dehydrogenase; NG - nitrogenase, AA - amino acids; SOD - superoxide dismutase; P - peroxidase; C - catalase; LP - lipid peroxidation, MDA - malonyldialdehyde, TBA - thiobarbituric acid, LSD - least significant difference

The joint action of the mentioned enzymes improves nitrogen uptake from the soil and atmosphere, which according to the metabolic pathway ($N_2 \rightarrow NO_3^- \rightarrow NH_4 \rightarrow$ AA $\rightarrow$ proteins) could be very important in alfalfa breeding for high protein content.

The oxygen consumption process by cells is coupled with danger, because oxygen can be partially reduced and can generate several toxic intermediates. The first one is the superoxide anion (O_2^-) which is the precursor of more potent oxidants, such as OH^- and H_2O_2 (Melhorn and Wellburn 1988, Hippeli and Elstner 1989) and activated forms of oxygen (Halliwell and Guteridge 1986, Ishii 1987). Toxic intermediates of oxygen inhibit chloroplast development, decrease seed viability and root growth, stimulate leaf absicission, and damage membranes of leaves and roots by their peroxidation (Cheeseman *et al.* 1988).

A primary defence is provided by superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (P, EC 1.11.1.8) and catalase (C, EC 1.11.1.6) with remarkable efficiency (Kenematsu and Asada 1989).

On the other hand, one of the most intriguing characteristics of nitrogen-fixing organisms is rapid and irreversible inactivation by oxygen of the key enzyme of the nitrogen fixing process, nitrogenase (Puppo and Riguard 1986).

In this paper nitrogen-fixing and nitrogen-assimilation enzymes and enzymes included in oxygen defence system of alfalfa are compared in order to evaluate a possible correlation. The aim of this study was also to evaluate genotypes which are able to synthetize a great quantity of proteins and at the same time have a good resistance to toxic action of oxygen radicals.

Material and methods

The Yugoslav cultivars of alafalfa (Medijana, Vršac, Banat and Bačka) were chosen for experiment. Activities of NR, GS, GDH, SOD, P and C, quantity of soluble proteins and lipid peroxidation (LP) level were determined in 90 d old leaves of alfalfa grown in field conditions. Nitrogenase activity was determined in roots of the same plants.

Nitrogenase activity in the rhizosphere was determined by the acethylene reduction assay on a gas chromatograph (Seetin and Barnes 1977). The roots of plants were taken from the pot and shaken slightly to remove most of the sand from the rhizosphere. The sand adhering to the root surface was preserved. The roots with the adhering sand were incubated with 10 % acetylene at 28 °C for 24 h. Nitrogenase activity was calculated from the concentration of the produced ethylene and was expressed in nmol C_2H_4 ml^{-1}.

Nitrogen assimilation enzymes: The activity of the *in vitro* NR transferase reaction of GS and NADH-dependent GDH was determined in a common extract from leaves according to the methods described by Coombs *et al.* (1982). The extract was prepared by the homogenization of 1.0 g fresh leaves in 10 ml extraction buffer (containing 50 µmol l^{-1} imidazole, 5 nmol l^{-1} 2-mercaptoethanol and 0.5 nmol l^{-1} EDTA, pH 7.2), followed by centrifugation at 10 000 *g* for 15 min.

Oxygen metabolism enzymes: The leaves were prepared for SOD measurement, by thorough grinding with a cold mortar and pestle until no fibrous residue could be seen; the grinding medium was 0.1 M K_2HPO_4 at the leaves: medium ratio of 1:5, centrifuged for 10 min at 15 000 *g*. A mixture of chloroform : ethanol (3:5) was added to the supernatant (20 µl) before measurement of the enzyme activity. The SOD activity was determined by the method based on the inhibition of the transformation of adrenaline to adrenochrome at pH 10.2 (Mirsa and Fridovisc 1972, Matkovics *et al.* 1977).

For the other enzymes, the leaves were used in the same way but the medium was 0.1 M phosphate buffer (pH 7) at the leaves : medium ratio of 1:5, centrifuged for 10 min at 15 000 *g*. The supernatant aliquots were used to measure enzyme activities. P and C activities were determined by the method of Simon *et al.* (1974).

Lipid peroxidation was measured at 37 °C as malonyldialdehyde (MDA) production at 533 nm with thiobarbituric acid (TBA). The total amount of TBA-positive substance is given as nmol MDA mg^{-1} (protein), as described by Placer *et al.* (1966) and Gidrol *et al.* (1989).

Soluble proteins were estimated by the method of Lowry *et al.* (1951). The statistical estimations were made by the LSD test.

Results and discussion

The range of nitrogenase activity (NG) was 18.75 - 58.50 nmol C_2H_4 ml^{-1}, of nitrate reductase (NR) 1.70 - 4.00 µmol NO_2 g^{-1} h^{-1}, of glutamine synthetase (GS) 700 - 1125 µmol γ-glutamylhydroxamate g^{-1} h^{-1} and of glutamate dehydrogenase (GDH) 43.00 - 82.50 nmol NAD^+ g^{-1} h^{-1}. Significant differences in mean values of NG, NR, GS and GDH activities were found among the cultivars used (Fig. 1).

Among the cultivars of alfalfa examined the cv. Medijana possessed the highest and the cultivar Bačka the lowest NG activity. The enzymes of nitrogen assimilation (NR, GS, GDH) reacted quite differently than NG, which is in connection with the Medijana's dominant nitrogen fixation mechanism in contrast to Bačka which had dominant mechanism of nitrogen assimilation. In the other two cultivars (Vršac, Banat) both mechanisms were responsible for nitrogen supply. Similar results were obtained earlier by examination of enzymes of nitrogen fixation and assimilation of *Medicago sativa*, *Glycine max* and *Lupinus albus* (Atkins *et al.* 1979, Van Berkum and Sloger 1983).

Comparing our results obtained by enzyme activities determination (NG, NR, GS and GDH) with the quantity of soluble proteins it is possible to estimate that the cultivar with the highest nitrogen assimilation enzyme activities had the highest proteins content and that the cultivar with the lowest enzyme activities had the lowest protein content (Fig. 1 and 3). This is in agreement with an earlier established hypothesis about the role of nitrogen assimilation enzymes in protein biosynthesis.

No correlation was observed between nitrogenase activity and protein content which can be ascribed to the substrate specificity of nitrogenase. Nitrogenase

catalyses symbiotic fixation of atmospheric nitrogen which is reduced in plant nodules to NH_4 and than transported to cell cytoplasm and mitochondria, where it enters the "active nitrogen pathway".

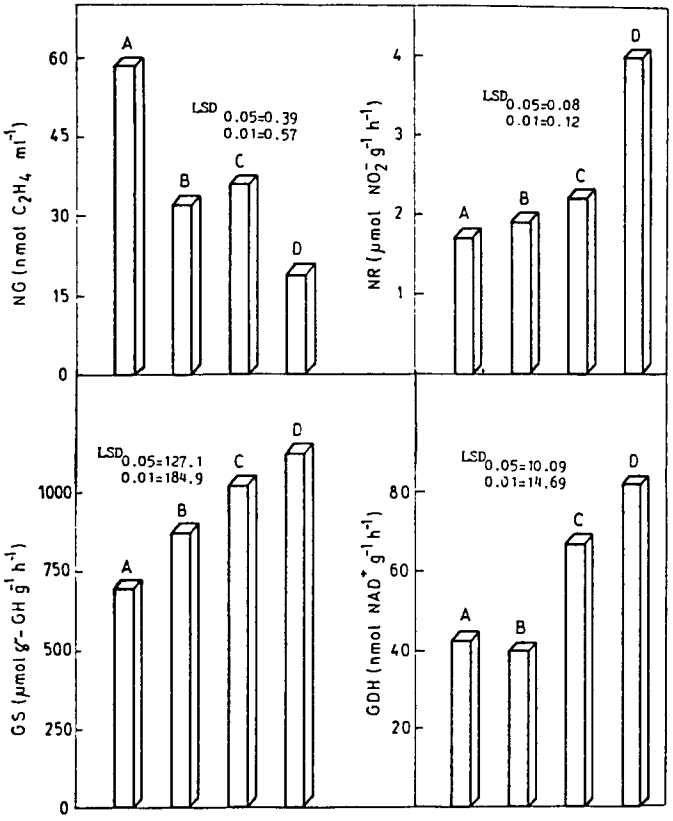

Fig. 1. Nitrogen fixation - nitrogenase (NG), and assimilation - nitrate reductase (NR), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) activities in examined cultivars of alfalfa (A - Medijana, B - Vršac, C - Banat, D - Bačka).

The superoxide dismutase (SOD) activity in the examined alfalfa cultivars ranged from 126.54 to 299.71 U g^{-1}, peroxidase (P) activity from 1.33 to 2.16 U g^{-1}, catalase (C) activity from 38.22 to 57.33 U g^{-1}, lipid peroxidation (LP) ranged between 27.23 and 50.76 nmol MDA g^{-1} (Fig. 2 and 3).

Significant differences in mean values of SOD, P and C activities, LP and protein content were found among the used cultivars.

Our results showed that the examined alfalfa cultivars had different susceptibility to the action of toxic oxygen species. The NS-Bačka exhibited a high P and C activities, though SOD activity and LP were low. Low SOD activity is in connection with a low quantity of generated O_2^- in 30 d old plants (Matkovics *et al.* 1989) and high quantities of different peroxides accumulated in young plants which had to be removed by the action of enzymes in order to avoid membrane peroxidation. On the

contrary, the cultivar Medijana had high SOD and C activies, low P activity and low LP level. High SOD and P activities prevented membrane damage due to O_2^- and peroxide elimination and degradation, which indicated the high resistance to the action of toxic oxygen species (Barta 1986). However, the low P activity could be compensated by the "peroxidase-like" action of catalase (Jones 1982).

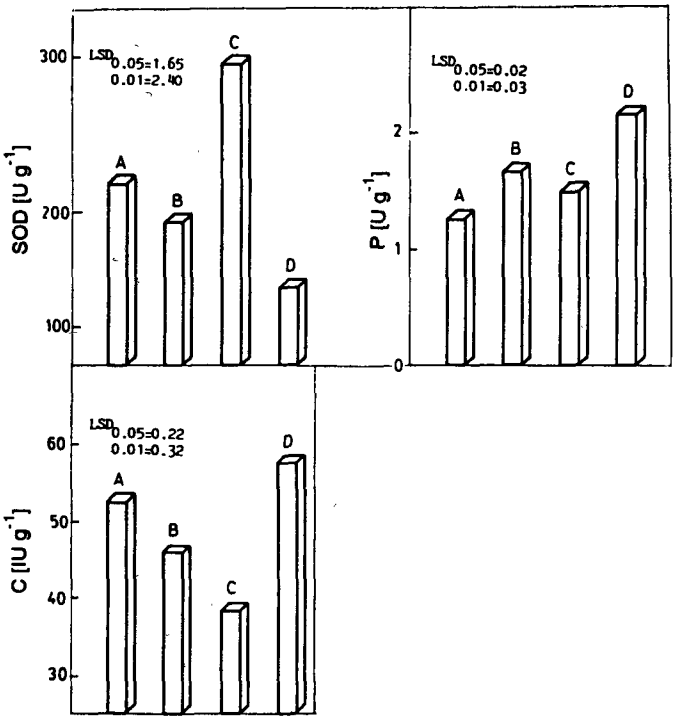

Fig. 2. Oxygen metabolism - superoxide dismutase (SOD), peroxidase (P) and catalase (C), enzyme activities in examined cultivars of alfalfa (A - Medijana, B - Vršac, C - Banat, D - Bačka).

The cv. Medijana had also high nitrogenase activity which is in agreement with the results of Buchanan (1977) and Puppo and Riguard (1986) who proved that NG could be inactivated in the presence of high O_2^- quantity. Concerning the high SOD and C activities in Medijana, it could be concluded that SOD and C scavenged toxic oxygen species. Their action enabled nitrogen fixation process due to the protection of nitrogenase activity.

The activities of investigated enzymes of oxygen metabolism and nitrogen assimilation were not correlated, which could be a possible consequence of the action of the reduction agent required for their substrate generation and possible competition for it in photosystem 2 (Halliwell and Gutteridge 1986).

Our results indicated that the cv. Medijana could be used in breeding oxygen resistant alfalfa hybrids with high nitrogen-fixing capacity and the cv. Bačka for the breeding hybrids with high protein content.

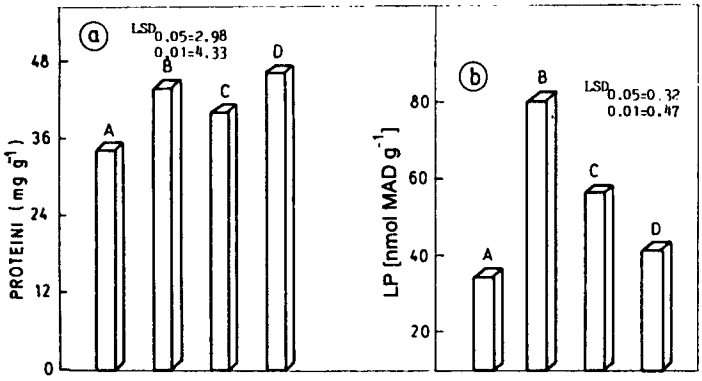

Fig. 3. Soluble proteins content (a), and lipid peroxidation level (b), in examined cultivars of alfalfa (A - Medijana, B - Vršac, C - Banat, D - Bačka).

References

Atkins, C.A., Pate, J.S., Layzell, B.D.: Assimilation and transport of nitrogen in nonnodulated (NO_3-grown) (*Lupinus albus* L.). - Plant Physiol. **64**: 1078-1082, 1979.

Barta, L.A.: Metabolic response of *Medicago sativa* L. and *Lotus corniculatus* L. roots to anoxia. - Plant Cell Environ. **9**: 127-131, 1986.

Bergensen, F.J.: Ammonia - an early stable product of nitrogen fixation by soybean root nodules. - Aust. J. biol. Sci. **18**: 1-9, 1965.

Buchanan, A.: The response of *Azotobacter chroococcum* to oxygen: Superoxide-mediated effects. - Can. J. Microbiol. **23**: 1548-1553, 1977.

Cheeseman, K.H., Beavis, A., Esterbaurer, H.: Hydroxyl radical-induced iron catalysed degradation of 2-deoxyribose. - Biochem. J. **252**: 649-653, 1988.

Coombs, J., Hall, O.D.: Techniques in Bioproductivity and Photosynthesis. - Pergamon Press, Oxford 1982.

Deroche, M.E., Babalar, M.: *In vivo* nitrate reductase activities in different organs of lucerne (*Medicago sativa* L.) plants: Effects of combined nitrogen during first vegetative growth and after shoot harvest. - Physiol. Plant. **70**: 90-98, 1987.

Gidrol, X., Sergihini, H., Nobhani, B., Mocquot, B., Mazliak, P.: Biochemical changes induced by accelerated aging in sunflower seeds. Lipid peroxidation and membrane damage. - Physiol. Plant. **76**: 591-697, 1987.

Groat, R.G., Vance, C.P.: Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). - Plant Physiol. **67**: 1198-1203, 1981.

Groat, R.G., Vance, C.P.: Root and nodule enzymes of ammonia assimilation in two 'plant-conditioned symbiotically ineffective genotypes of alfalfa (*Medicago sativa* L.). - Plant Physiol. **69**: 614-618, 1982.

Halliwell, B., Gutteridge, J.M.C.: Free Radicals in Biology and Medicine. - Claredon Press, Oxford 1986.

Henson, C.A., Collins, M., Duke, H.S.: Subcellular localization of enzymes of carbon and nitrogen metabolism in nodules of *Medicago sativa*. - Plant Cell Physiol. **23**: 227-235, 1982.

Hippeli, S.C., Elstner, E.F.: Disel-soot-catalyzed production of reactive oxygen species: Cooperative effects with bisulfite. - Z. Naturforsch. **44**: 514-523, 1989.

Ishii, S.: Generation of active oxygen species during enzymic isolation of protoplasts from oat leaves. - *In vitro* Cell. Develop. Biol. **23**: 635-658, 1987.

Jones, P.: Catalases and iron-porphyrin model systems: Roles of the coordination environment of iron in catalytic mechanisms. - In: Dunford H.B. (ed.): The Bilogical Chemistry of Iron. Pp. 427-438. 1982.

Kanematsu, S., Asada, K.: Cu-Zn-Superoxide Dismutases in rice: Occurrence of on active monomeric enzyme and two types of isozyme in leaf and non-photosynthetic tissues. - Plant Cell Physiol. **30**: 381-391, 1989.

Kennedy, I.R.: Primary products of symbiotic nitrogen fixation. II. Pulse labeling of *Serradella* nodules with $^{15}N_2$.- Biochim. biophys. Acta **130**: 295-303, 1966.

Lowry, O.H., Rosenbrough, N.J., Farr, L.A., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Matkovics, B., Gasić, O., Varga, S.I., Štajner, D., Kraljević-Balalić, M.: The antioxidant enzyme activities in wheat seeds and their F_1 hybrids. - Cereal Res. Commun. **17**: 113-119, 1989.

Matkovics, B., Novak, R., Duc Hanh, H., Szabo, L., Varga, S.I., Zelesna, G.: A comparative study of some more important experimental animal peroxide metabolisms enzymes. - Comp. Biochem. Physiol. **56B**: 31-34, 1977.

Matsumoto, T., Yatazawa, M., Yamamoto, Y.: Incorporation of ^{15}N into allantion in nodulated soybean plants supplied with $^{15}N_2$. - Plant Cell Physiol. **18**: 459-462, 1977.

Melhorn, H., Wellburn, R.A.: Ozone toxicity mechanisms in plants and animals. - In: Free Radicals: Chemistry, Pathology and Medicine. Pp. 253-270. Richelieu Press, London 1988.

Mirsa, H.D., Fridovics, T.: The role of superoxide anion in the autoxidation of epinephrine and a simple measurement for superoxide dismutase. - J. biol. Chem. **247**: 3170-3175, 1972.

Placer, Z.A., Cusman, L.L., Johnson, B.C.: Estimation of product of lipid peroxidation malonyldialdehyde in biochemical systems. - Anal. Biochem. **16**: 359-364, 1966.

Puppo, A., Riguard, J.: Superoxide dismutase: an essential role in the protection of the nitrogen fixation process. - FEBS Lett. **201**: 187-189, 1986.

Seetin, M.W., Barnes, D.K.: Variation among alfalfa genotypes for rate of acetylene reduction. - Crop Sci. **17**: 783-787, 1977.

Simon, L.M., Fatrai, Z., Jonas, D.E., Matkovics, B.: Study of metabolism enzymes during the development of *Phaseolus vulgaris*. - Biochem. Physiol. Pflanzen **166**: 389-393, 1974.

Trung-Chanh, T.A., Mohamed, A.F., Fercus, D.H.: Pathways of nitrogen metabolism in nodules of alfalfa (*Medicago sativa* L.). - Plant Physiol. **80**: 1002-1005, 1986.

Van Berkum, P., Sloger, C.: Interaction of combined nitrogen with the expression of root-associated nitrogenase activity in grasses and with the development of N_2 fixation in soybean (*Glycine max* L. Merr.). - Plant Physiol. **72**: 741-745, 1983.