International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 02-05-2021, Accepted: 15-05-2021, Published: 31-05-2021
www.botanyjournals.com
Volume 6, Issue 3, 2021, Page No. 485-488

Comparative study of some pyruvate and oxalacetate metabolism enzymes in common bean leaves under combined effect of radiation and salt

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Abstract

The dynamics of changes in activities of the enzymes pyruvate kinase (PK-ase, EC 2.7.1.40), NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37), and oxaloacetate decarboxylase (OAD-ase, EC 4.1.1.3) have been studied in the initial development phases of the common bean (*Phaseolus vulgaris* L.) plant leaves exposed to the combined effect of various radiation doses (5, 10, 50 Gy) and salt concentrations (1, 5, 10, 50 mM NaCl). It has been found that the combined effect of radiation (50 Gy) and salt (10-50 mM NaCl) causes a more active stimulating effect on common bean plants. At the intersection of energy exchange pathways, this effect forms inductive mechanisms that connect these pathways with substrates and intermediate metabolites and coordinate their functions mainly at the level of intermediate metabolites, proteins, pigments, activities of PK-ase, NAD-MDH, OAD-ase, and the H^+-pump. Inductive mechanisms arising under stress conditions cause temporary or permanent adaptive traits in the common bean plant.

Keywords: radiation, salt, stress, *Phaseolus vulgaris* L., PK-ase, NAD-MDH, OAD-ase, adaptation

Introduction

Plant metabolism is known to be a complex system of physiological and biochemical processes that depend on each other and abiotic factors of the external environment. Due to this system, the speed and direction of metabolic reactions are precisely coordinated, the stability and mobility inherent in metabolism are ensured. To understand the adaptive mechanisms of living organisms against the effects of extreme environmental factors, the responses of plants must be studied at different hierarchical levels, starting with the population and ending with the molecules [2]. Determination if stimulatory effects of radiation and salt stress are present or absent, detailed analysis of the mechanisms of the formation of radiobiological effects, finding causes of variability of the modifying factors and stimulating effect, their identification, assessment, and justification are some of the priority topics facing science [10]. Ivanishev claimed that an unknown way in the chloroplasts of C_3-plants implementing the conversion of C_4-dicarboxylic acids into pyruvate and CO_2 existed, and this process had a great physiological significance for plants [5]. He showed that the formed pyruvate could affect many processes related to the formation of lipids, some amino acids, as well as CO_2 in chloroplasts [8]. It is known that the participation of C_4-organic dicarboxylic acids in cell metabolism is important for the development of stress adaptive traits. The activity of enzyme systems that ensure the conversion of organic dicarboxylic acids plays an important role in the adaptation of the organism to the effects of stress. Considering their active role in C_3-plant metabolism, we have studied the dynamics of changes in the activity of the enzymes PK-ase, OAD-ase, and NAD-MDH involved in the metabolism of C_4- dicarboxylic acids in the common bean plant.

Being the main enzyme of glycolysis and carbon metabolism, PK-ase catalyzes the synthesis of pyruvate and ATP by transferring the phosphate group of phosphoenolpyruvate (PEP) to ADP in the presence of Mg^{2+} and K^+ ions [1]. NAD-MDH [14], widespread among animals, plants, and microorganisms and using NAD^+ as a cofactor, catalyzes the oxidation of L-malate to oxaloacetate (OA). OAD-ase catalyzes the reaction of the formation of pyruvate from OA, which is a C_4- dicarboxylic acid, thereby increasing its accumulation [6]. Thus, pyruvate is synthesized as a central substrate involved in energy metabolism as a result of several anabolic reactions in plant metabolism [7]. Thus, this problem still contains many unresolved questions. The information on the synthesis of C4-dicarboxylic acids in the cell cytosol, their transport to chloroplasts, and how they correlate with plant productivity and tolerance under the combined effects of radiation and salt is not sufficient yet.

Materials and Methods

The object of the research was common bean species (*Phaseolus vulgaris* L.) of the *Phaseolus* L. genus belonging to the legumes family (*Fabaceae* L.). All bean species are salt-sensitive and grow well at a temperature of 18-20 ºC.

The experiments were performed at the Experimental Science Department “Radiation sources of isotope origin” of the Institute of Radiation Problems of ANAS. Bean seeds were subjected to radiation of different doses by RUXUND 20.000-radiation device (Co^{60}-radiation source), were disinfected for 15 minutes in 3% H_2O_2 solution, washed with distilled water, dried, and placed in a dark chamber in Petri dishes to germinate. After 3-4 days, the obtained seedlings were transferred to 100 ml volumetric flasks containing 100 ml of NaCl solutions with concentrations of 0, 1, 5, 10, 50, 100, 150, 200, and 300 mM. Then, the

development of seedlings continued in an artificial climate chamber with a temperature of 20-30 ºC, the humidity of 60-70%, the light intensity of 40-50 klux, and the photoperiod of 14h/10 h (light/dark). The amount of pigments was determined by the spectrophotometric method in the 80% acetone extract (acetone: Tris-HCl buffer, 1 M, pH 7.8; v/v= 80:20) [13]. To determine the activity of the enzymes, the leaves were washed with distilled water and dried on filter paper, chopped, and homogenized using a mortar and a pestle in a homogenization medium specific for each enzyme. Homogenization medium for NAD-MDH: 20 mM $MgCl_2$·6H2O, 1mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 20% glycerin, 0.5% polyvinylpyrrolidone (PVP), 100 mM Tris-HCl, pH 8.0. Homogenization medium for OAD-ase: 10 mM NaCl, 1 mM EDTA-Na, 1 mM Na-ascorbate, 0.4 M sucrose and 0.1% polyethylene glycol (PEG), 50 mM Na-acetate acetic acid buffer, pH 6.8-7.0. Homogenization medium for PK-ase: 1 mM EDTA, 20 mM $MgCl_2$ · 6H2O, 0.5% PVP, 0.5% Triton X-100, 100 mM Na_2HPO_4 - NaH_2PO_4 buffer containing 5 mM DTT, pH 7.4. OAD-ase activity was determined in the medium containing 10 mM $MnCl_2$, 1 mM OA, 1 mM Na-ascorbate, 0.4 M sucrose, 0.1% PEQ and 5-10 µl enzyme extract under standard conditions at 30 ºC. The spectrophotometric measurements based on the decrease in absorption at 280 nm (Ultrospec 3300 pro, Amersham, USA) were made using 1 ml tubes. Divalent Mn^{2+} ions were added to the reaction medium as an activator. The activity unit of the enzyme was the amount of enzyme that catalyzes the decarboxylation reaction of 1 µM OA per minute at 30 ºC. PK-ase catalyzes the irreversible conversion of FEP and ADP to pyruvate and ATP. The enzyme activity was determined in a reaction medium containing 0.1 M Tris-HCl buffer (pH 7.6), 50 mM $MgSO_4$, or 0.2 M KCl, 10 mM PEP, 94 mM ADP, 12 mM NADH, 450 mg lactate dehydrogenase (LDH) (50 µl is taken from 35 mg /ml). The reaction was started by adding 60-80 µl of enzyme extract to the reaction mixture. The activity of the enzyme was calculated based on the change in optical density at a temperature of 25 ºC and a wavelength of 340 nm for 3 minutes. The extinction coefficient for NAD·H is 6.22 $mM \cdot cm^{-1}$ [9]. NAD-MDH activity was determined spectrophotometrically at 340 nm (Ultrospec 3300 pro, Amersham, USA). The reaction medium consisted of Tris-HCl buffer (pH 8.0) containing 15 mM OA, 10 mM $MgCl_2$·6H2O, 12 mM NAD·H, and 100 µM 5-10 µl of the enzyme preparation. The reaction started by adding a substrate (15 mM OA) to the reaction medium. Enzyme activity is measured by the change in optical density due to a decrease in the amount of NAD · H at 340 nm [11].

The total protein was determined on a spectrophotometer at a wavelength of 610 nm in the presence of 0.12% Coomassie Brilliant Blue G-250 solution [12]. Statistical processing of the results was performed according to Dospekhov [4].

Results and Discussion

The dynamics of changes in the activity of enzymes involved in the synthesis of pyruvate directly (PK-ase and ODA-ase) or indirectly (NAD-MDH) during the first 15 days of the initial phase of ontogenesis were determined in leaves of common bean seedlings germinated from seeds exposed to γ-radiation and cultivated at various NaCl concentrations (under conditions of dual stress). For this purpose, 3 optimal radiation doses (5, 10, 50 Gy) were selected, and seeds irradiated at each dose were planted separately at 1, 5, 10, and 50 mM NaCl concentrations, and some physiological and biochemical parameters were studied in the early stages of vegetation of the obtained seedlings. It was found that the amount of MDA, total proteins, and proline in the control samples first decreased slightly and then increased over time. Contrary to the control variant, total protein increased in each of the experimental variants as the dose and concentration of stress factors increased. Under these conditions, the change in the amount of proline occurred following the change in total protein (Table 1).

Table 1: Dynamics of changes in the amount of proteins, MDA, and proline in common bean leaves under the combined effect of radiation and salt

Variants	5-day-old			10-day-old			15-day-old		
	$C_{protein}$, mg/ml	**MDA**	**Proline**	**$C_{protein}$, mg/ml**	**MDA**	**Proline**	**$C_{protein}$, mg/ml**	**MDA**	**Proline**
Control	14.78	0.298	19.6	14.56	0.254	14.2	16.9	0.271	14.9
5 Gy + 1 mM	13.03	0.095	21.3	14.58	0.131	20.2	15.2	0.168	21.3
5 Gy + 5 mM	13.58	0.143	21.6	13.72	0.139	23.7	15.8	0.157	24.8
5 Gy +10 mM	13.12	0.166	22.0	13.99	0.172	26.9	14.9	0.151	28.1
5 Gy +50 mM	14.51	0.169	22.0	14.92	0.172	26.8	15.1	0.138	28.8
Control	14.78	0.298	19.6	14.56	0.254	14.2	16.9	0.271	14.9
10 Gy +1 mM	14.21	0.127	17.0	15.41	0.156	16.8	16.22	0.196	18.2
10 Gy +5 mM	14.21	0.162	16.5	15.62	0.169	17.1	15.49	0.145	18.1
10 Gy +10mM	14.44	0.177	16.0	14.93	0.196	17.6	15.11	0.133	18.8
10 Gy +50mM	14.51	0.181	16.6	14.90	0.175	17.6	14.86	0.131	19.4
Control	14.78	0.298	19.6	14.56	0.254	14.2	16.9	0.271	14.9
50 Gy +1 mM	17.22	0.223	19.1	16.11	0.285	18.6	14.54	0.227	19.7
50 Gy +5 mM	17.1	0.247	19.1	15.53	0.241	18.7	14.11	0.186	19.9
50 Gy +10mM	16.83	0.279	19.6	15.04	0.228	18.8	13.96	0.173	21.7
50 Gy+50 mM	16.47	0.296	19.9	16.72	0.243	19.2	16.21	0.132	22.1

Note: C_{H^+} - µequiv /hour, H_2O_2-µM/ml; C – Control, MDA- malondialdehyde - mM/ml;.Accuracy Index is less than 3%

As seen in the table, the amount of MDA in the variants [(5 Gr) +1 mM, +5 mM, +10 mM, +50 mM NaCl] initially increased slightly for 5, 10 and 15 days, but at higher doses and concentrations - [(10 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl]; [(50 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl] decreased by the 15th day of vegetation. The greatest decrease in MDA occurred on the 15th day of plant

development in experimental variants [(50 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl].

The obtained results allow estimating the stress level of the plant. As seen in Table 1, the amount of proteins and proline increased depending on time and experimental variants. The greater increase of these parameters on the 15th day of vegetation in the variants [(50 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl] can be attributed to the synthesis of stress proteins. The increase in the amount of MDA in the early stages of ontogenesis and the decrease on the 15th day can be explained by the activity of the antioxidant defense system. As can be seen, the parameters studied in the combination of 50 Gy + 50 mM NaCl increased more and showed stability. In this combination, over time, the H^+ -pump became more active (data not shown), the amount of MDA decreased, and the total amount of synthesized protein and proline increased. These traits suggest that the activation of the H^+ -pump accelerates mineral nutrition - ion exchange in the roots, increases total protein due to the synthesis of stress proteins. A decrease in the amount of MDA, a product of LPO, and an increase in the amount of proline lead to the optimization of other metabolic reactions in the plant under conditions of dual stress.

As seen in Table 2, plants grown in the 50 Gy + 5 mM NaCl combined medium gained more tolerance. This conclusion is based on the fact that the amount of carotenoids in plants increases over time.

Table 2: Dynamics of changes in the pigment amount in common bean leaves exposed to the combined effect of radiation and salt

Variants	5-day-old			10-day-old			15-day-old		
	a/b	(a+b)/C	C	a/b	(a+b)/C	C	a/b	(a+b)/C	C
Control	3.0	2.6	0.003	3.0	2.5	0.003	2.5	3.5	0.002
5 Gy+1 mM	1.5	3.3	0.003	1.8	4.6	0.003	1.5	5.0	0.002
5 Gy+5 mM	1.6	2.6	0.005	2.0	2.4	0.005	3.0	2.0	0.004
5 Gy+10 mM	1.8	3.5	0.004	2.6	2.7	0.004	2.0	4.0	0.003
5 Gy+50 mM	2.6	3.5	0.002	2.3	3.3	0.003	2.3	5.0	0.002
Control	2.0	3.0	0.003	3.0	2.6	0.003	2.0	3.0	0.002
10 Gy+1mM	1.2	2.2	0.005	1.8	0.7	0.015	2.0	3.0	0.003
10 Gy+5 mM	1.8	2.2	0.005	3.5	3.0	0.003	3.0	2.0	0.004
10 Gy+10 mM	1.8	5.5	0.002	8.0	8.0	0.001	3.0	4.0	0.002
10 Gy+50 mM	3.0	4.0	0.002	3.5	3.5	0.002	5.0	3.0	0.002
Control	1.5	2.08	0.004	2.7	3.33	0.008	2.3	1.7	0.005
50 Gy+1 mM	1.2	2.88	0.002	1.2	1.89	0.009	2.0	2.0	0.003
50 Gy+5 mM	0.9	3.37	0.002	1.2	1.92	0.003	0.6	0.3	0.010
50 Gy+10 mM	1.0	2.17	0.005	1.2	0.99	0.014	0.7	1.0	0.005
50 Gy+50 mM	1.1	2.10	0.006	0.5	1.30	0.005	0.4	1.1	0.004

Note: Gy-gray, C-carotenoids, unit, pigments-mM/ml, Accuracy Index is less than 3%

In these variants, the chl(a + b)/car ratio was highest (3.37) on the first 5 days of plant development and lowest (0.3) on the 15th day. This was due to a gradual decrease in chl(a + b) and an increase in the amount of carotenoids. A five-fold increase in the amount of carotenoids occurred on the 15th day compared to the 5th day in common bean leaves. All this suggests that a radiation dose of 50 Gy and a salt concentration of 5-50 mM play an optimizing role in plant adaptation. After determining the stress state of plants at the level of proteins, intermediate metabolites and pigments, dynamics in the activity changes of energy metabolism enzymes such as PC-ase, NAD-MDH and OAD-ase were determined under conditions of dual stress. Constant doses of radiation (5, 10, 50 Gy) were applied along with various NaCl concentrations (5, 10 and 50 mM NaCl) as follows: [(5 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl]; [(10 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl]; [(50 Gy) +1 mM, +5 mM, +10 mM, +50 mM NaCl] (Table 3).

According to Bound *et al*. plastid PK-ase plays a significant role in the stage of seed development while mPK-ase is activated after seed germination and plays an important role in the development of seedlings [3].

Table 3: Dynamics of changes in the activity of the enzymes PK-ase, OAD-ase, and NAD-MDH in common bean leaves exposed to the combined effect of radiation and salt

Variants	5-day-old			10-day-old			15-day-old		
	PK-ase	OAD-ase	NAD-MDH	PK-ase	OAD-ase	NAD-MDH	PK-ase	OAD-ase	NAD-MDH
Control	2.69	5.63	1.01	3.02	4.16	0.63	2.54	2.89	1.16
5 Gy+1 mM	3.53	8.65	2.32	3.26	5.83	1.74	3.08	4.71	1.65
5 Gy+5 mM	3.66	8.90	2.28	3.52	7.07	2.60	3.33	5.16	1.66
5 Gy+10 mM	3.96	9.50	2.59	3.84	8.42	2.92	3.64	7.41	1.94
5 Gy+50 mM	6.07	14.8	4.26	5.0	11.63	3.58	3.87	7.49	2.08
Control	2.69	5.63	1.01	3.02	4.16	0.63	2.54	2.89	1.16
10 Gy+1 mM	3.76	8.98	1.52	3.67	3.76	1.56	3.31	2.97	1.56
10 Gy+5 mM	4.12	9.23	1.67	3.58	3.73	1.66	3.54	3.30	1.83
10 Gy+10 mM	4.19	9.23	1.78	3.91	7.49	1.48	3.87	3.50	2.06
10 Gy+50 mM	4.29	9.23	1.90	3.78	7.60	2.15	3.78	3.56	2.55
Control	2.69	5.63	1.01	3.02	4.16	0.63	2.54	2.89	1.16
50 Gy+1 mM	2.71	7.18	1.95	3.40	4.83	2.48	3.60	4.59	3.18
50 Gy+5 mM	2.91	8.44	2.12	3.68	5.0	2.76	3.78	4.79	3.61
50 Gy+10 mM	3.15	8.62	2.35	3.91	6.19	3.13	4.18	6.06	4.22

50 Gy+50 mM	3.58	8.93	2.58	4.07	6.33	3.58	3.75	5.44	4.44

Note: CAT - μmol H_2O_2·mg^{-1}protein·min^{-1}; NAD-MDH - μmol OA·mg^{-}protein·min^{-1};PK-ase - μmol pyruvate· mg^{-1}protein·min^{-1}; OAD-ase - μmol OA mg^{-1}protein·min^{-1}; Accuracy Index is less than 3%

Under these conditions and in combined variants, PK-ase activity increased with increasing NaCl concentration and decreased over time. The highest PK-ase activity was observed at the 50 Gy + 50 mM NaCl exposure.

OAD-ase and NAD-MDH activities were also studied under the same conditions. As seen in Table 3, the activity of OAD-ase at the onset of stress was higher compared to the activities of the other enzymes. Besides, the activity of OAD-ase increased with increasing salt concentrations at each radiation dose and gradually decreased depending on the stage of plant development. The OAD-ase activity in the leaves of 15-day-old plants was 2-3 times lower than that in the leaves of 5- and 10-day-old plants.

The NAD-MDH activity increased depending on the NaCl concentrations and the duration of the initial stages of plant development. The enzyme PK-ase converts PEP, formed as an intermediate product in glycolysis, to pyruvate. Pyruvate is also formed from OA produced in the Krebs cycle as a result of the OAD-ase reaction. In the Krebs cycle, OA is synthesized from malate in the reaction catalyzed by the enzyme NAD-NDH. Pyruvate is the final product of several enzymatic reactions. This organic acid is also involved in some metabolic transformations as a common substrate, such as the first general pathway of catabolism, gluconeogenesis, amino acid metabolism, glyoxylate circulation, etc. Thus, 1) PK-ase activity increased over time in 5-, 10-, and 15-day-old plants under the combined effects of both stressors. This increase was mostly observed at the low dose radiation-5 Gy + 10 mM NaCl or the high dose radiation-50 Gy + low salt concentration-10 mM NaCl. 2) NAD-MDH activity increased with increasing salt concentration at each dose, and the highest activity was found at 50 mM NaCl 3) Although OAD-ase activity was highest on the 5th day of plant development, it decreased over time (days 10 and 15). The activity of the enzyme increased on the 5th, 10th and 15th days of plant development as the concentration of NaCl increased at all radiation doses.

Conclusion

We suggest that the combined effect of radiation and salt (50 Gy + 10-50 mM NaCl), which causes a stimulating effect on the bean plant, forms inductive mechanisms at the level of intermediate metabolites, proteins, pigments, activities of PK-ase, NAD-MDH, OAD-ase, and the H^+-pump. Inductive mechanisms formed in the bean plants under stress conditions, at the intersection of energy exchange pathways, manifest temporary or permanent adaptive traits by connecting these pathways with substrates and intermediate metabolites and coordinating their functions.

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