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Full Length Research Paper

Investigating the effect of growth regulator, furolan on the nucleic acid content of winter wheat grains

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The germinating seeds of three winter wheat varieties namely Batko, Diya and Krasnodar 99 were investigated for the effect of growth regulator, Furolan on their nucleic acid contents which were determined by the following protocol procedures including; affinity chromatography on poly (U) sepharose, thermal chromatography and electrophoresis methods after isolation of the total RNA. Results showed that the degree of polyadenylation of mRNA in the experimental variant actually exceeded the control in Batko, Krasnodar and the 99 varieties. In both Diya and Batko varieties, the content of 25S rRNA fraction in ripened wheat grain was increased by Furolan. In Batko variety, Furolan actually reduced the content of long-living rRNA (18S) fraction resulting in a lower growth intensity of wheat sprouts in experimental variant in comparison with the control. In Diya's variety the increase in the content of the fraction of long-living rRNA in experimental variant was in conformity with the activation of growth shoot systems of their sprouts. The ratio of 25S/18S rRNA fractions is suggested to be a useful cultivar trait.

Key words: Furolan, winter wheat, nucleic acids, total RNA, 25S/18S rRNA.

INTRODUCTION

It is known that accumulation of proteins in cells during growth is an indication of the active processes of formation of cellular structures, thus forming basically complex proteins, nucleoproteins, lipoproteins, structural proteins of ribosomes mitochondria and plastid. Further increment of proteins in the cell is mainly due to simple proteins, the quantity of which is increased during cell growth and at the end accumulates reaching the sum more than half of the general content of protein in a cell. Thus, during the process cell of growth, activity of proteolytic enzymes gradually increases and reaches a maximum in the cells which have completed growth (Konarev, 1980). Synthesis and accumulation of proteins in cells during their growth are closely connected with synthesis of nucleic acids. During germination, there is an increase in the contents of the axial parts of the sprouts. Growth points of sprout contain increased quantity of nucleic acids and these are connected more intensively with the growth processes (Bulko, 1968). Thus, during germination of seeds of wheat and peas, the quantity of nucleic acids (DNA and RNA) (Ovcharov, 1974) increased considerably in their sprouts. There is an assumption that swelling of seeds is the starting moment for activation of reserve mRNA in dry germ cells and this induces synthesis of enzymes and other proteins which ensure germination at different stages (Ovcharov, 1974). Physiologically active substances seem to influence the contents of nucleic acids in zones of growth. It has been suggested, that rootlets of germ cells, sprouting within 48 hrs, is the point of preliminary synthesis RNA (Vecher, 1978). The synthesis of specific RNA in reserve tissues attracts special interest during germination. The synthesis of RNA begins early enough in reserve tissues as in the growing cells of axial organs, however, the massive synthesis of proteins, providing mobilization of reserves of the seeds (hydrolytic enzymes, amino-transferase, etc.) is connected with RNA, and is formed 2 − 4 days later after the beginning of germination. Most of the details of the process of synthesis of nucleic acids and proteins in axial organs of sprouts were investigated in cereals, especially wheat (Obrucheva, 1965, Bulko, 1968 and Osborne, 1977) and beans, particularly, in peas (Gumulevskaya, 1975; Sutcliffe, 1977). In dry seeds there

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are also other components of the system of protein synthesis and RNA: RNA-polymerases I−III, tRNA, including specific met−tRNA, enzymes of activation of amino acids and aminoacyl – tRNA, initiation and elongation factors. The most discussed topic in the last one and a half decades is about the presence of mRNA in mature dry seeds. In the opinion of some researchers, the presence of mRNA in dry seeds has been proven but others believe, that synthesis of protein during germination demands synthesis of a new mRNA. In all these disputes, it may be concluded that mRNA is present in seeds at resting stage, but other mRNA's are synthesized at the initial stages of germination of seeds (Dure, 1977; Dure, 1979). In the opinion of some Belgian researchers studying formation of mRNA− protein complexes in embryogenesis and the fate of this mRNA during germination; formation of large quantity of reserved mRNA and its storage of information have, first of all, adaptive value which increased the chances of plants to normally develop under adverse conditions of germination. The aim of this research in line with the trend of studies outlined above is to further investigate the way and manner growth regulator, Furolan influences the contents of nucleic acids of wheat during germination process of the crop.

MATERIAL AND METHODS

Methods of investigation of nucleic acids, Isolation and determination of total RNA

Isolation of the total RNA was carried out by phenol detergent method (Bray, 1979).Wheat grain was ground in liquid nitrogen up to powder state and filled with 5 volumes of extractive buffers (200 mm Tris -HCl; pH 8.5; 50 mm $MgCl_2$; 60 mM KCl). After the removal of starch in the buffer, sodium dodecyl sulphate (SDS) was added up to final concentration of 1 %. The homogenate was twice extracted at a room temperature with equal volume of a mixture of phenol-chloroform (1:1) for denaturation and removal of the greater part of proteins. The water phase was separated by centrifugation at (3000g, 20 minutes). Nucleic acids were precipitated with 2.5 volumes of ethanol with addition of sodium acetate up to the concentration of 0.3 M (2 hours, -20°C). The precipitate was separated by centrifiguration at (5000g, 30 mins) and re-dissolved in twice-distilled water (1 ml on 1g vegetative tissue), mixed with the same quantity de-proteinizing solution (8M lithium chloride, 8M urea, 4mM Na_2-EDTA) for sedimentation and cleaning of RNA. The mixture was left to stand for a night at +4°C and centrifuged at (3000g, 30 mins). The deposit was washed out with 70 % ethanol, dissolved in minimal volume of sterile twice-distilled water and centrifuged at (3000g, 30 mins) for separation of polysaccharides. Concentration and purity of RNA was determined by spectrophotometry according to the standard method from the calculation of 1 OE_{260} =40 mc.g/ml (Brooker et al., 1977). The high-polymer yield of RNA usually comprised about 1mg/1g fresh weight of the tissue at the ratio A_{260} / A_{280} > 1.8 and A_{260} / A_{230} > 1.9.RNA thus isolated could actively be translated in non-cellular system of protein synthesis. RNA may be stored in liquid nitrogen for not more than three months.

Isolation of poly (A) content of mRNA by the method of affinity chromatography on poly (U) sepharose

The water solution of total RNA was calcinated (180°C, 4) celite in the ratio 1 g celite: 4 mg RNA. Portion of the experimental samples was warmed at 65°C for 5 mins and cooled at room temperature. All the samples were mixed with celite for 5 mins at room temperature. Solution of RNA was decanted with celite. Lastly the sample was rinsed with buffer solution (20 mM Tris-HCl, pH 7.5; 0.5 M LiC1; 1mM EDTA-Na_2; 0.1 % SDS) until zero absorption at 260 nm. The combined solution of RNA was subjected to two-cycle affinity chromatography on poly (U) sepharose (Brooker et al., 1978; Osborne, 1977). Isolation of poly (A) content of mRNA was carried out as follows: to a solution RNA (1 mg/ml) was added equal volume of the buffer A (40 mM Tris -HC1; pH 7.5; 1 M LiCl; 2 mM EDTA-Na_2; 0.2 % SDS). Solution was applied twice to poly (U) sepharose at a room temperature at a speed of 30 ml/hr. The column was rinsed with buffer B (20 mM Tris-HC1; pH 7.5; 0.5 M LiCl; 1 mM EDTA-Na_2; 0.1) until the zero absorption at 260 nm. For removal nonspecific retained RNA on poly (U) sepharose the column was rinsed with the same buffer diluted five times with double-distilled water. Poly $(A)^+$ mRNA was eluted from column of the buffer C (10 mM of zither-phosphate solution,
pH 7.5; 1 mM Na_2-EDTA, 0.05 % SDS). The quantity of RNA was determined by spectrophotometry. In case of two-cycle chromatography; to poly $(A)^+$ mRNA was added equal volume of the buffer A and the cycle of chromatography was carried out.

Determination of the degree of polyadenylation of mRNA by thermal chromatography method

Correlation of mRNA molecules with long and short poly (A) sequences on the 3' end was determined by thermal stepped elution poly $(A)^+$ mRNA from the column of poly (U)-sepharose at temperatures 35°C eluting buffer containing 10 mM Tris-HCl, pH 7.5; 1 mM EDTA-Na_2; 0.05 % SDS) and 65°C (10 mM zither-phosphate buffer, pH 7.5, containing 1 mM EDTA-Na_2; 0.05 % SDS) (Gumulevskaya, 1975). The quantity of RNA in each fraction was determined by spectrophotometry as described above. Correlation of the quantity of RNA of

Table 1. Stability of mRNA of winter wheat grains.

Variety	mRNA, % of Total RNA			Degree of polyadenylination of mRNA, $(A)_n$65°C/$(A)_n$35°C		
	Control	Furolan	± Control	Control	Furolan	± Control
Batko	2.5 ± 0.30	1.2 ± 0.20	-1.3	1.59 ± 0.09	4.00 ± 0.10	2.41
Diya	1.7 ± 0.40	1.3 ± 0.20	-0.4	1.32 ± 0.07	1.42 ± 0.30	0.10
Krasnodar 99	1.2 ± 0.40	1.1 ± 0.30	-0.1	0.93 ± 0.20	1.20 ± 0.30	0.37
$LSD_{0.95}$		0.25			0.26	

±* indicates standard deviation (SD).

Table 2. Contents of the fraction of rRNA in winter wheat grain, in relative unit.

Variety	Control			Furolan			± Control		
	25S	18S	25S / 18S	25S	18S	25S / 18S	25S	18S	25S / 18S
Batko	24.22 ± * 0.30	27.64 ± 2.00	0.88 ± 0.17	6.77 ± 0.60	18.67 ± 1.00	0.36 ± 0.12	-17.45	-8.97	-0.52
Diya	12.93± 0.20	22.86 ± 2.00	0.57 ± 0.05	10.03 ± 0.30	26.83 ± 2.00	0.37 ± 0.02	-2.9	3.97	-0.20
$LSD_{0.95}$							8.95	4.86	0.33

±* indicates standard deviation (SD).

high- and low-temperature fractions – $(A)_n$65° / $(A)_n$35°C, was used as the index of the degree of polyadenylation mRNA. Index of stability of mRNA is defined as the ratio of poly $(A)^{++}$ mRNA (double purification) to poly $(A)^{+}$ mRNA (single purification). The percentage of poly $(A)^{++}$ mRNA to poly $(A)^{+}$ mRNA is calculated , assuming the quantity of the latter as 100 %.The average arithmetic result of two parallel determinations was accepted as the final result of determination.

Separation of RNA by agarose gel

For separation of RNA in agarose gel; 2 % agarose gel was prepared by the following technique and used: 400 mg of agarose powder was weighed into a 100 ml glass; in the same glass are added 18 ml H_2O and 2 ml 10xTris-Borate-EDTA (TBE); the suspension was heated up in a bath with boiling water or in a microwave until the agarose was melted; the solution was cooled up to 50°C to which was added etidium bromide up to final concentration of 0.5 mc.g/ml (in 20 ml − 4 µl); on an equal surface a cover from immunological tablet was fixed; then a comb was placed by means of clips so that between the bottom hollows and the main gel remained a layer of agarose of thickness 0.5 – 1.0 mm; warm solution of agarose in good form was poured out; after that as the gel completely hardened (between 30 - 45 mins at room temperature) the comb was carefully removed. Gel was placed in a chamber for electrophoresis. Sufficient quantity of electrophoresis buffer (1x TBE) was added so that the gel was closed by a layer of buffer of thickness, 1 mm.

Samples for application of gel was prepared by the following technique: in test tubes with products of amplification was added 40 µl chloroform, well shaken, centrifuged for 5secs. In a micro centrifuge. The manipulation for this was made, so that the oil covering a water phase was lowered downwards into the test tubes, and from the surface it was easy for the water phase to be taken up. In the hollow of the immunological tablet was poured in 2 µl (6x TBE) buffers for application in the

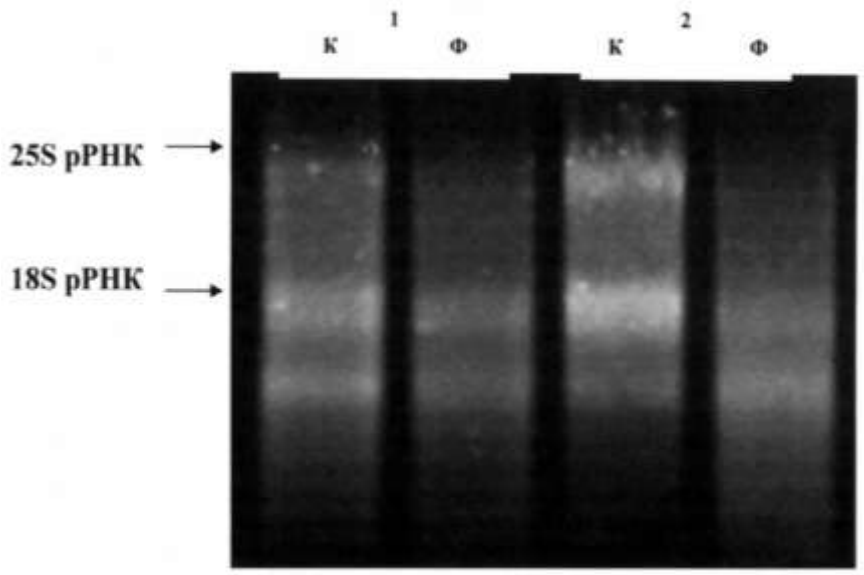

Figure 1. Elecrophoregram of rRNA (25S and 18S) of ripened winter wheat grain in agarose gel; where : 1 – Diya; 2 – Batko; К – Control; Ф –Furolan; 25SpPHK– 25S rRNA;18pPHK− 18S rRNA.

hollows. 10 µl of water phase was taken from the test tubes with products of amplification, mixed in the immunological tablet with the buffer for application. Samples were introduced in the hollows of gel under electrophoresis buffer. Electrophoresis was carried out for duration of 15 mins at a pressure of an electric field 3-5 V/cm. The gel was analyzed in UV-light in trans-illuminator and photographed (Maniatis et al., 1984).

STATISTICAL ANALYSIS

Data were statistically analyzed by using computer software Microsoft Excel and SPSS ver.10.00

RESULTS AND DISCUSSION

Influence of Furolan on the contents of nucleic acids in winter wheat grain

It had been earlier established that treatment of winter wheat plants with Furolan caused an increase in the stability of mRNA in the ripened grain (Surkova, 2005). Results of determination of mRNA stability in wheat grain during the wax ripeness phase are presented in Table 1. Varieties of winter wheat studied differ among themselves in mRNA content in the grain and its degree of polyadenylation characterized this index as a cultivar trait. Furolan, as earlier established, accelerates the ripening of grain which, due to the lower content of RNA especially in early ripening variety of Batko. This explains the decrease in content of mRNA in winter wheat grain especially in Batko variety. The degree of polyadenylation of mRNA in the experimental variant actually exceeded the control in the Batko and Krasnodar 99 varieties. Increase in the number of molecules having relatively longer poly (A) -sequence at the 3 '-end of the mRNA, affirms to the high translation activity and longer life-time of the mRNA that enables the full realization of the protein synthesizing systems. Furolan, as earlier ascertained, activates the synthesis of the protein thereby increasing its content in winter wheat grain (Nenco, 2006). Determination of stability of long-living rRNA of winter wheat grains was also investigated in this study. Results of the electrophoresis of rRNA are presented in Table 2 and in Figure 1. Diya's variety was almost twice lower in content of 25S rRNA, in comparison to Batko, but content of 18S rRNA showed tendency to increase. Hence, rRNA of Diya's variety had relatively more concentration of rRNA fraction as reflected in more intensive sprout growth in Diya variety than Batko variety. In both varieties, Furolan increased the content of 25S rRNA fraction in ripened winter wheat grain. In Batko variety Furolan actually reduced the content of rRNA (18S) fraction; however the value of the ratio of 25S/18S fractions increased. This was in conformity with the observation of more intense sprout growth in the experimental variant of Batko variety than in the control. In Diya's variety it was observed that increase in the content of fraction of long-living rRNA of its experimental variant conformed to the activation of growth shoot systems of the sprouts. From this study, the ratio of 25S/18S rRNA fractions was reaffirmed as cultivar trait. The change in ratio of 25S/18S rRNA fractions apart from leading to an increase in the long-living fraction (18S) of

the experimental variants also brought about the activation of germination of seeds. Furthermore, increase in the contents of 18S fractions resulted in more intensive growth of the shoot systems of sprouts.

CONCLUSION

Treatment of winter wheat plants with Furolan in this study resulted in an increase in the mRNA stability in the ripened grain. Increase in the number of molecules having relatively longer poly (A) -sequence at the 3 '-end of the mRNA affirmed the high translation activity and longer life-time of the mRNA that ensured the full realization of the protein synthesizing systems. The ratio of 25S/18S rRNA fractions in this study was reaffirmed as a cultivar trait.

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