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SENESCENCE-DEPENDENT CHANGES IN SOME METABOLIC PROCESSES AFFECTED BY 1,1′-POLYMETHYLENEBIS(3-ARYLSUBSTITUTED)UREAS IN BARLEY (*HORDEUM VULGARE* L.) LEAF SEGMENTS

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Summary. Dark-senescence-induced metabolic changes in barley leaf segments (*Hordeum vulgare* L., cv. Alfa) influenced by 15 synthetic bis-urea derivatives of aliphatic diamines were investigated. Leaf segments were incubated in test solutions for 24, 48, 72 h and chlorophyll, protein, free amino acids contents, catalase, guaiacol-peroxidase and ribonuclease activities were measured. Results indicated that bis-(phenyl- or halogenophenyl)ureas′ derivatives of aliphatic diamines protected the leaf segments from the senescence symptoms. Furthermore, it appeared that among bis-(phenyl)-ureas′ derivatives, more active were these with a longer aliphatic chain while among bis-(4-halogenophenyl)ureas – those with a shorter polymethylene chain. The nature of halogen atom determined the mode of action of the test compounds – the bis-(4-fluorophenyl)ureas-induced protection was mediated in general by increased antioxidative enzyme activities and that of the bis-(4-chlorophenyl)ureas was mediated probably by a declined concentration of AOS, thus decreasing substrate-enzyme activation.The protective effect of the test compounds on the two basic processes of senescence was realized by means of different mechanisms: the prevention of Chl degradation correlated with greater antioxidative enzyme activities, whereas the prevention of protein degradation – with lower H_2O_2-detoxifying enzyme activities.

Key words: bis-ureas, dark-induced leaf senescence, *Hordeum vulgare*

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Abbreviations: AAC – amino acid content, AOS – active oxygen species, CAT – catalase, Chl – chlorophyll, DPU – 1,3-diphenylurea, GPX – guaiacol peroxidase, IA – initial activity, IC – initial content, Put – putrescine

Introduction

Senescence in plants is characterized by the breakdown of cell wall components and membrane, leading to the loss of tissue structure and cellular compartmentation and ultimately, homeostasis (Thimann, 1980; Thompson et al., 1987a,b; Nooden, 1988). Plant senescence is known to consist of genetically driven degradative processes which lead to the death of cells, organs, and organisms. The degradative processes are accompanied by the decay of chlorophyll and the onset of proteolysis, which are also more rapid in darkness (Martin and Thimann, 1972; Trippi and DeLuca D'Oro, 1985; Kanazawa et al., 2000). Altered membrane fluidity and permeability, mainly caused by free radical-induced lipid peroxidation, is another inherent feature of the deteriorative changes associated with senescence in plants, both in the light and in darkness. Radical species derived from molecular oxygen as well as singlet oxygen are responsible for the oxidative processes which can damage proteins, chlorophyll, lipids, polysaccharides, and DNA with numerous consequences (Borraccino et al., 1994).

Plant cells possess a variety of defense strategies against oxidative injury. Such strategies involve specific detoxifying enzymes, such as superoxide dismutase and catalase, which decompose superoxide radicals and hydrogen peroxide, respectively, as well as various antioxidants and quenchers, including vitamin E (a-tocopherol), vitamin C (ascorbic acid), glutathione, etc. (Leung et al., 1981). The ability of a plant to detoxify the active oxygen species is believed to be a tolerance mechanism (Lee et al., 1984; Castillo and Greppin, 1988). Reinforcement of the defense mechanisms in plants against oxidative damages in cases when plants are subjected to abiotic stress may be successfully reached by the application of plant growth regulators. Thus, senescence-asssociated parameters can effectively be retarded by the cytokinin group of plant growth regulators whose reported mode of action includes interaction with tRNA metabolism, effects on membrane permeability to mono- and di-valent ions, and localized induction of metabolic sinks (Letham, 1978; Kao, 1980). Di- and polyamines, as well as their synthetic analogues, are involved in the control of several stress-related phenomena, such as senescence, wounding, heat and salinity, both in plant organs and in isolated tissues and cells (Alexieva, 1994). Polyamines may stabilize both nucleic acids and membrane functions during senescence (Naik and Srivastava, 1978; Popovic et al., 1979; Cheng et al., 1984).

For the last 3 years in our laboratory have been synthesized certain 1,1′-polymethylenebis(3-substituted)ureas which are derivatives of both active structures – diphenylurea (cytokinin functional moiety) and aliphatic diamines (Yonova and Ionov, 1999).

This study aims to characterize the possible senescence-retarding effect of these compounds like the parent active structures. Therefore, we examined the time-dependent changes in the levels of chlorophyll, protein and free amino acid contents, and in the activities of catalase, guaiacol peroxidase and ribonuclease in dark-senescing barley leaf segments as affected by the synthetic bis-urea's compounds. Some important chemical structure – senescence-retarding effect relationships are discussed.

Materials and Methods

Plant material and aging treatments

Barley seeds (*Hordeum vulgare* L., cv. Alfa), soaked 2 h in water, were sown on the vermiculite with tap water and grown under controlled conditions at a day/night temperature of 24±1 °C with a 16 h photoperiod and photon flux density of 120 µmol.m^{-2}.s^{-1} for 7 days. Segments of the first leaf, cut 3 cm long from 5 mm below the leaf tip, and then repeatedly washed with distilled water. Ten segments were floated with abaxial side downward on 5 ml of test solution in a Petri dish which were kept covered at 24±1 °C for 24, 48 and 72 h in darkness. The test solutions used were either 1 mM KH_2PO_4–Na_2HPO_4 buffer, pH 5.8 (control) or 1 mM solution of the investigated compounds, prepared in the same buffer and 0.1% (v/v) Tween 20. Three replicates for each treatment were performed, and all experiments were repeated at least twice.

Chemicals

15 Bis-arylcarbamoyl-substituted biogenic and non-biogenic diamines were tested as senescence-retarding agents. The compounds synthesized and characterized earlier in our laboratory (Yonova and Ionov, 1999) have the following common formula

$$ArNHCO\text{-}NH(CH_2)_nNH\text{-}CONHAr,$$

where Ar = C_6H_5- , 4-FC_6H_4- , and 4-ClC_6H_4- ; n = 2,3,4,5,6.

(1) Ar = C_6H_5-, n=2	(6) Ar = 4-FC_6H_4-, n=2	(11) Ar = 4-ClC_6H_4-, n=2
(2) Ar = C_6H_5-, n=3	(7) Ar = 4-FC_6H_4-, n=3	(12) Ar = 4-ClC_6H_4-, n=3
(3) Ar = C_6H_5-, n=4	(8) Ar = 4-FC_6H_4-, n=4	(13) Ar = 4-ClC_6H_4-, n=4
(4) Ar = C_6H_5-, n=5	(9) Ar = 4-FC_6H_4-, n=5	(14) Ar = 4-ClC_6H_4-, n=5
(5) Ar = C_6H_5-, n=6	(10) Ar = 4-FC_6H_4-, n=6	(15) Ar = 4-ClC_6H_4-, n=6

Determination of chlorophyll and free amino acid contents

At the time of measurement 10 segments (~0.250 g) were blotted and extracted by boiling in 80% (v/v) ethanol for 10 min, repeatedly (Gepstein and Thimann, 1981).

The ethanolic extracts were combined and made to 10 ml. Chlorophylls *a* and *b* were quantified and expressed as mg Chl.segment^{-1} by transferring 600 ml aliquots of the ethanolic extract into 4.2 ml of 80% (v/v) acetone and determining the absorbance at 663 and 645 nm according to Mackinley (1941). Chlorophyll *a*/*b* ratio was then calculated.

All subsequent handling of the extracts for the determination of free amino acid content was performed as described previously (Palmer, 1985). The remaining ethanolic extracts were evaporated to dryness in vacuo at 40 °C and the residue taken up in 0.1 N HCl. The solution was adjusted to pH 6.0 with NaOH and diluted to ca 25 mg (fresh weight).ml^{-1}. AAC in 100 ml aliquots of the aqueous solution was determined by the ninhydrin method (Spies, 1957).

Determination of enzyme activities

For extraction of enzyme protein 10 segments were homogenized at 0–4 °C in 5 ml of 0.1 M phosphate buffer, containing 0.1 mM EDTA at pH 6.8. The homogenate was centrifuged at 12 000 × g for 20 min and the supernatant obtained was used as enzyme extract. Catalase [EC 1.11.1.6] activity was determined by following the consumption of hydrogen peroxide (extinction coefficient 40.0 mM^{-1}.cm^{-1} – Kato and Shimizu, 1987) at 240 nm for 2 min. The 3 ml reaction mixture contained 50 mM phosphate buffer, pH 6.8, 75 µl of enzyme extract, and the reaction was initiated by adding 22.5 µl of 30% (w/v) hydrogen peroxide. The decrease in hydrogen peroxide was followed as a decline in A_{240} using a UV-VIS spectrophotometer model Shimadzu connected to a recorder. Guaiacol peroxidase [EC 1.11.1.7] activity was based on the determination of guaiacol oxidation (extinction coefficient 26.6 mM^{-1}cm^{-1}) at 470 nm by hydrogen peroxide (Decker, 1977). The reaction mixture contained 1.2 ml of 100 mM phosphate buffer, pH 6.8, 50 µl of enzyme extract, 600 µl of 1% (w/v) guaiacol (Fluka Chemika AGOH-9470 Buchs), and 150 µl of 100 mM hydrogen peroxide. The increase in A_{470} was followed for 2 min. The specific activities of catalase and guaiacol peroxidase were expressed in micromoles of decomposed H_2O_2 [µmol.mg^{-1} protein.min^{-1}] and micromoles of oxidized quaiacol [µmol GDHP.mg^{-1} protein.min^{-1}], respectively under the assay conditions.

Ribonuclease [EC 3.1.27.5] activity was assayed with purified baker's yeast RNA (Fluka, Biochemika) according to Altman et al. (1977). The reaction was carried out for 60 min at 37 °C, and terminated by adding cetyl-trimethylammonium bromide at a final concentration of 0.5% (w/v). After centrifugation, the supernatant was appropriately diluted and the absorbance was measured at 260 nm. Activity was expressed in units of [ΔA_{260}.segment^{-1}.h^{-1}]. All estimations were repeated three times. The *in vitro* activity of the enzymes was not influenced by any of the compounds investigated.

Total soluble protein content in the enzyme extracts of barley leaf segments was determined according to Bradford (1976) using bovine serum albumin as a standard.

At the start of all experiments, leaf senescence parameters were determined and the experimental parameters were expressed as % of the initial values.

Statistical analysis

The data presented in this paper are expressed as means ±SD. Statistical tests were carried out by using the Fisher's Least Significant Difference – test. Differences between means were considered significant at $P \leq 0.05$ and $P \leq 0.01$.

Results

Biochemical parametres of the leaf senescence

The rate of leaf senescence in non-treated (control) and treated barley leaf segments was estimated through determining the quantitative changes of chlorophyll and free amino acids which are the two main biochemical indicators of the aging process.

Our results showed noticeable differences in the capability of the compounds used to delay the Chl loss during fast aging of leaf segments in the dark. These differences depended on the available substituent in the benzene ring and the length of the polymethylene chain. 4-Fluoro- and chloro-substituted bis-phenylureas' derivatives retarded Chl degradation during the aging period more strongly than the unsubstituted bis-phenylureas and the control (about 15% more Chl than the control after 72 h) (Table 1). The lowest rate of Chl loss was observed in leaf segments treated with bis-(4-fluorophenyl)ureas' derivatives of 1,4-diaminobutane and 1,6-diaminohexane especially during the first 24 h (only 3–7% Chl loss compared to 34% of the control). The applied compounds influenced differently both Chl *a* and *b* losses. Chl degradation in the senescing leaf segments treated with bis-phenylureas¢ derivatives predominantly started with Chl b degradation (an increased Chl *a*/*b* ratio) similarly to the control, whereas the treated ones with bis-(4-halogenophenyl)ureas' derivatives showed initially Chl *a* decrease similarly to the standards (DPU and Put).

We determined the effect of test compounds on the quantitative changes of total soluble proteins in the senescing leaf segments (Table 2). Generally, the rate of protein loss was in accordance with the rate of amino acids accumulation. Bis-(phenyl)ureas' derivatives which prevented proteolysis in the senescing tissues, retarded the protein loss – 98–114% protein content, whereas the control segments had 79% referred to the initial protein content, at the end of day 3.

Our results showed also an increased content of total soluble protein in the bis-(4-fluorophenyl)ureas-treated leaf segments (152–185% referred to IC protein) during the first incubation day that quickly decreased afterwards. The high rate of protein loss was in correlation with the high rate of proteolysis in these aging leaf segments.

Table 1. Effect of some 1,1′-polymethylenebis(3-arylsubstituted)ureas on the time-dependent changes in Chl (*a*+*b*)content and Chl *a*/*b* radio in barley leaf segments induced to senesce in darkness. Segments were floated on phosphate buffer (control) or on test solutions for 24, 48 and 72 h. Data are expressed as µg.segment^{-1} and as % of the initial values: Chl (*a*+*b*) = 127±2.10 µg.segment^{-1} ; Chl *a*/*b* = 1.94.

Treatments		Chlorophyll (*a*+*b*)/segment						Chlorophyll *a*/*b*		
Test compound	Conc.	24 h		48 h		72 h		24 h	48 h	72 h
		µg ± sd	%	µg±sd	%	µg±sd	%			
Control		84±0.89	66	70±0.91	55	53±0.85	42	1.99	1.91	1.90
1	1 mM	104±1.70	82	66±0.79	52	48±0.87	38	2.07	1.92	1.90
2	1 mM	92±1.60	72	67±1.09	53	45±0.25	35	1.99	1.88	1.84
3	1 mM	85±2.40	67	64±1.33	51	52±0.84	41	1.99	1.95	1.99
4	1 mM	89±2.30	70	67±1.10	53	53±0.91	42	2.03	1.94	1.83
5	1 mM	95±2.00	75	69±1.17	54	56±1.60	44	2.06	2.00	1.85
6	1 mM	105±1.66	83	100±0.63	79	72±0.57	57	1.84	1.75	1.99
7	1 mM	110±0.69	87	79±0.72	63	72±1.03	57	1.75	1.79	1.89
8	1 mM	117±3.71	93	87±1.22	69	72±0.56	57	1.73	1.73	1.88
9	1mM	111±1.16	88	87±1.07	69	67±0.67	53	1.74	1.66	1.73
10	1 mM	123±1.52	97	93±3.09	73	72±1.04	57	1.70	1.77	1.85
11	1mM	107±1.13	85	79±0.49	62	63±0.76	50	1.85	1.84	1.85
11	0.1mM	116±1.13	92	86±0.46	68	64±0.79	50	1.87	1.86	1.82
12	1 mM	85±0.73	67	79±0.82	63	53±0.45	42	1.79	1.88	1.89
13	1 mM	84±0.52	66	81±1.02	64	60±1.18	48	1.83	1.86	1.88
14	1 mM	81±1.51	64	74±2.01	58	68±0.63	53	1.80	1.82	1.85
15	1 mM	91±1.03	72	82±0.89	65	57±0.77	45	1.78	1.81	1.88
Standards										
DPU-	1 mM	95±0.56	75	78±0.47	62	56±0.79	44	1.78	1.83	1.93
Put-	5 mM	111±1.43	88	87±3.42	69	61±3.02	48	1.83	1.79	1.92
		LSD5% = 3.936		LSD1% = 5.284						

Table 2. Effect of some 1,1´-polymethylenebis(3-arylsubstituted)ureas on the time-dependent changes in soluble protein content in barley leaf segments induced to senesce in darkness. Segments were floated on phosphate buffer (control) or on test solutions for 24, 48 and 72 h. Data are expressed as μg.segment^{-1} and as % of the initial value: protein 318±2 μg.segment^{-1}.

Test compound	Conc.	24 h		48 h		72 h	
		μg ± sd	%	μg±sd	%	μg±sd	%
Control		271±15	85	254±7.2	80	250±12	79
1	1 mM	237±15	75	255±3.3	80	316±25	99
2	1 mM	310±15	98	286±5.5	90	310±19	98
3	1 mM	306±8.0	96	268±16	84	288±7.5	90
4	1 mM	220±5.0	69	264±19	83	333±4.0	105
5	1 mM	314±19	99	318±4.0	100	363±13	114
6	1 mM	587±6.0	185	340±2.0	107	206±19	65
7	1 mM	520±14	164	278±20	88	125±20	39
8	1 mM	512±11	161	324±30	102	301±25	95
9	1 mM	492±48	155	381±39	120	182±18	57
10	1 mM	483±39	152	416±5.0	131	310±23	98
11	1 mM	382±20	120	467±13	147	445±25	140
11	0.1 mM	323±2.0	102	435±29	137	480±5.0	151
12	1 mM	334±12	105	239±15	75	206±4.0	65
13	1 mM	321±4.7	101	197±4.0	62	225±12	71
14	1 mM	229±9.0	72	161±1.2	51	179±14	56
15	1 mM	172±10	54	134±12	42	135±1.2	43
Standards							
DPU	1 mM	215±7.0	68	200±25	63	225±12	71
Put	5 mM	413±2.3	130	435±14	137	326±24	103
		LSD 5% = 66.3391		LSD 1% = 93.1146			

Although bis-(4-chlorophenyl)ureas-treated leaf segments showed lower AACs compared with bis-(phenyl)- and bis-(fluorophenyl)ureas-treated ones, net protein in these tissues was lower than that in the latter treated tissues. The compound 11 was the only exception. At the end of day 3, leaf segments treated with this compound had 140% protein and 123% AAC compared to the control – 79% protein and 316% AAC, and the corresponding analogues: compd. 1 – 99% protein and 248% AAC, and compd. 6 – 65% protein and 472% AAC referred to the initial values.

Chl degradation and protein hydrolysis in the aging leaf segments did not pass to the same rate. Generally, bis-(4-fluoro- and chlorophenyl)ureas protected Chl degradation more strongly than proteolysis at the end of day 3 similarly to DPU and even 1,5-fold more strongly. The chlorophyll/protein ratio was the highest in the leaf segments treated with compounds 7 (1.46), 9 (0.93), 14 (0.95) and 15 (1.05). Bis-phenyl-

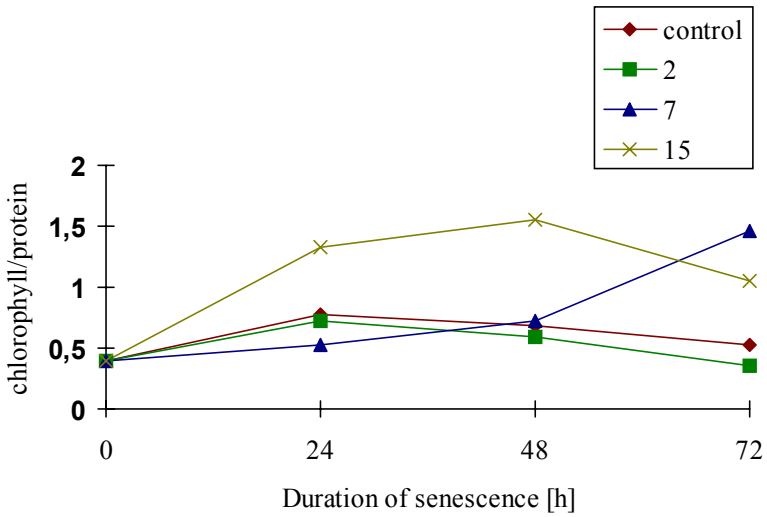

Fig. 1. Time-dependent changes in the chlorophyll/protein ratio during dark senescence of barley leaf segments: effects of compounds **2** (1 mM), **7** (1 mM) and **15** (1 mM).

ureas' derivatives of diaminoalkanes protected proteolysis more strongly than Chl degradation at day 3 like Put and even 1,3-fold more (Chl/protein = 0.36–0.46) (Fig. 1).

The quantity of free amino acids in the treated leaf segments increased with advancing of senescence like the control and the standards (Table 3). We observed differences in the proteolysis rate and in the absolute content of free amino acids depending on the compounds type. Bis-(4-fluorophenyl)ureas' derivatives which showed the lowest rate of Chl degradation exhibited the highest rate of proteolysis during the first 24 h and the AAC was higher than that of the respective bis-(phenyl)ureas (1.3–1.9-fold) and the control (1.1–1.5-fold) at the end of day 3. Bis-(4-chlorophenyl)ureas' derivatives demonstated the lowest rate of proteolysis during the first 24 h which later increased, but AAC was lower than that of the respective bis-(phenyl)ureas (1.1–2.0-fold) and the control (1.3–2.5-fold) at the end of day 3.

Putrescine induced higher rate of proteolysis (about twise) compared to DPU. The quantity of free amino acids in the test compounds-treated leaf segments (with the exception of fluoro-substituted derivatives) was lower than in Put-treated segments and higher than in DPU-treated segments at the end of the third aging day.

Antioxidant protective enzymes

We investigated the effect of the test compounds on the time-dependent changes in the specific activities of H_2O_2-scavenging enzymes – catalase and guaiacol peroxidase – in the aging leaf segments. In the control leaf segments, the GPX activity increased and the CAT activity declined with advancing of senescence.

Table 3. Effect of some 1,1´-polymethylenebis(3-arylsubstituted)ureas on the time-dependent changes in the levels of free amino acid contents in barley leaf segments induced to senesce in darkness. Segments were floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72 h. Data are expressed as µmol leucine eqv.segment^{-1} and as % of the initial values: AAC 2.237±0.26 µmol leucine eqv.segment^{-1}.

Test compound	Conc.	24 h		48 h		72 h	
		µg±sd	%	µg±sd	%	µg±sd	%
Control		3.49±0.10	156	5.75±0.18	257	7.08±0.36	316
1	1 mM	6.25±0.34	280	4.84±0.04	216	5.54±0.52	248
2	1 mM	4.76±0.42	213	5.69±0.05	255	7.83±0.30	350
3	1 mM	4.75±0.40	212	4.55±0.22	203	6.94±0.16	310
4	1 mM	3.72±0.21	166	5.12±0.06	229	5.51±0.24	247
5	1 mM	4.90±0.25	219	5.07±0.24	227	5.82±0.14	260
6	1 mM	6.04±0.31	270	8.84±0.40	395	10.56±0.39	472
7	1 mM	4.84±0.18	216	5.10±0.06	228	8.59±0.18	384
8	1 mM	6.20±0.12	277	7.32±0.49	327	8.48±0.28	379
9	1 mM	5.73±0.12	256	5.67±0.37	254	7.28±0.38	325
10	1 mM	7.65±0.19	342	7.40±0.31	331	7.65±0.35	342
11	1 mM	3.26±0.07	146	4.48±0.04	200	2.76±0.17	123
11	0.1 mM	2.84±0.04	127	3.53±0.14	158	5.32±0.11	238
12	1 mM	2.09±0.15	93	4.88±0.07	218	4.63±0.11	207
13	1 mM	2.96±0.05	132	4.78±0.19	214	4.83±0.19	216
14	1 mM	2.09±0.07	93	3.08±0.12	138	5.05±0.35	226
15	1 mM	2.96±0.13	132	4.10±0.34	184	5.37±0.11	240
Standards							
DPU	1 mM	2.44±0.09	109	2.98±0.20	133	4.33±0.28	194
Put	5 mM	3.60±0.08	161	6.17±0.03	276	7.48±0.57	334
		LSD 5% = 0.9390;		LSD 1% = 1.2590			

Bis-(phenyl)ureas' derivatives progressively increased the GPX activity similar to the control. The CAT activity was stimulated only during the first 24 h, afterwards it decreased and at the end of day 2 it was lower than in the control segments (133%). Only compound 1 maintained CAT activity (184%) higher than the control at day 2 (Figs. 2A and 3A).

Bis-(4-fluoro- and chlorophenyl)ureas' derivatives increased the GPX activity in the leaf segments over the whole studied period and especially after the second day. The activity reached its maximum at day 3 of senescence (2–3-fold more than the control) (Fig. 2B and C). The CAT activity was significantly inhibited during the first 24 h (40–80% compared with the IA) and increased during the second day especially in chloro-derivatives-treated segments (3–8-fold compared to the first day)

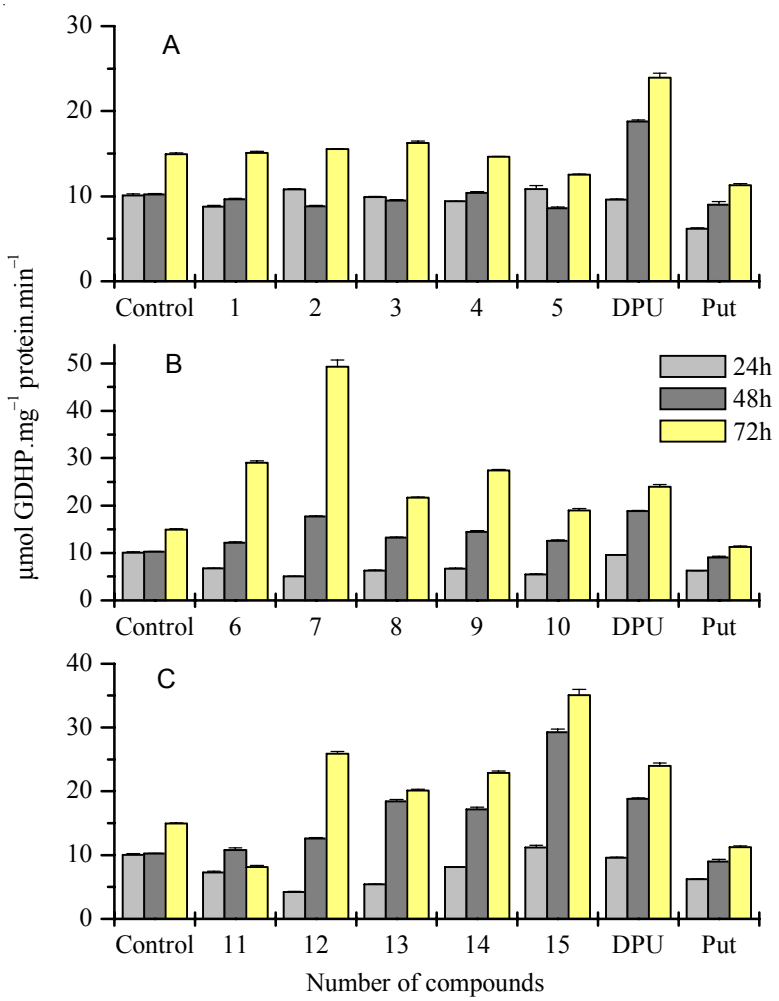

Fig. 2. Comparative time-dependent changes in the levels of guaiacol peroxidase activity during dark senescence of barley leaf segments for 24, 48 and 72 h: the effect of compounds N 1-15 at 1.0 mM. The initial GPX value was 2.503+0.35. Each value is a mean of 3 replicates. LSD 5% = 1.1047; LSD 1% = 1.4828

like DPU. The CAT activity in fluoro-derivatives-treated segments was generally lower than in chloro-derivatives-treated segments but it reached its relative maximum during the third day (3–6-fold compared to the first day) (Fig. 3B and C). The compd. 7 – treated leaf segments demonstrated the highest CAT activity at day 3 of senescence (198%) which was higher than the control (123% compared with the IA) and the standards. The compd. 11 – treated leaf segments showed the lowest CAT activity during the whole aging period and the lowest GPX activity at the end of day 3.

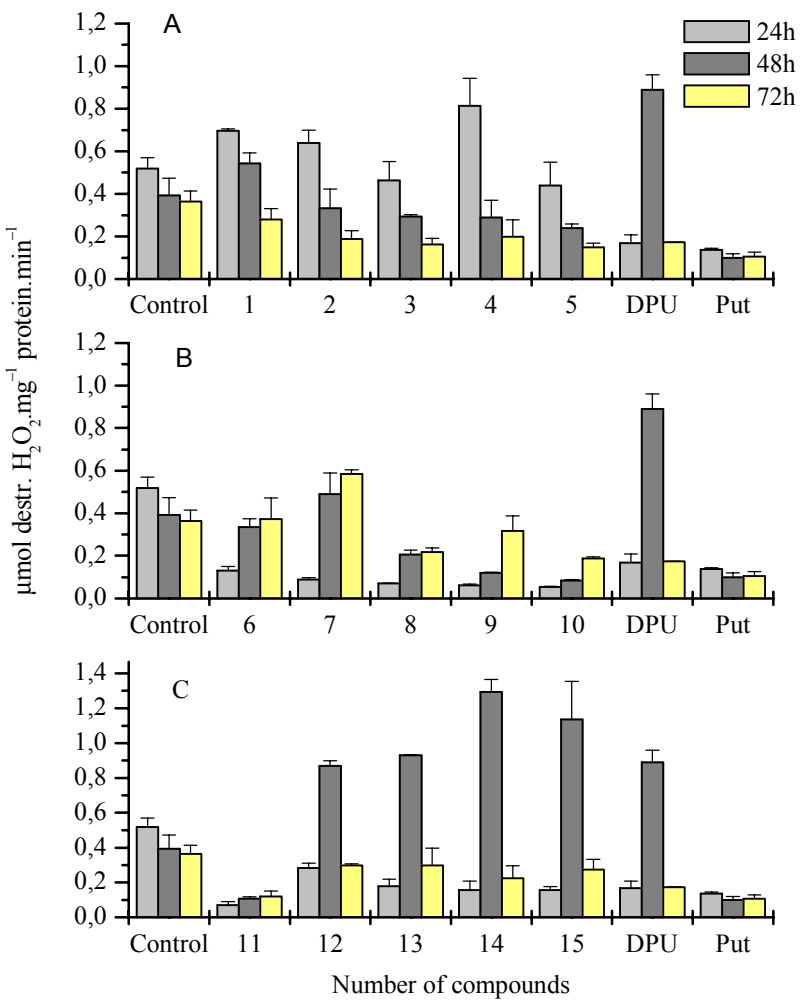

Fig. 3. Comparative time-dependent changes in the levels of catalase activity during dark senescence of barley leaf segments for 24, 48 and 72 h: the effect of compounds N 1–15 at 1.0 mM. The initial CAT value was 0.295+0.0072. Each value is a mean of 3 replicates. LSD 5% = 0.2126; LSD 1% = 0.2917

Total ribonuclease activity

Leaf senescence involves a sequence of degradative events which results in the breakdown of the main macromolecules as proteins and nucleic acids. Ribonuclease increases in activity rapidly in plant tissues in response to cutting, water stress, mechanical injury, and also during senescence of attached or detached leaves (Wyen et al., 1971) or cotyledons (Altman, 1982).

Bis-(phenyl)ureas' derivatives of diaminoalkanes especially those with an even number of methylene groups (n=2,4,6) inhibited the RNase activity (10–31% and 22–43% compared with the IA and the control, respectively) in senescing leaf segments during the first 24 h. At the end of day 2, the activity slightly increased compared with the control (the exceptions were compds. 1 and 5 with 50% and 30%, respectively over the control values) and significantly declined at the end of day 3 (twice lower than the control) (Table 4).

Bis-(4-fluorophenyl)ureas' derivatives increased the RNase activity (20–40% compared to the control) in the treated segments during the first 24 h. Then the activity decreased below the control and at the end of day 3 it increased again reaching the control values. However, the RNase activity in the leaf segments treated with compds. 6 and 10 continued to decrease also during the third aging day (51 and 38%, respectively below the control values) (Table 4).

Table 4. Effect of some 1,1′-polymethylenebis(3-arylsubstituted)ureas on the time-dependent changes in total ribonuclease activity in barley leaf segments induced to senesce in darkness. Segments were floated on phosphate buffer (control) or on test solutions for 24, 48 and 72 h. Data are expressed as average values (ΔA_{260}.segment^{-1}.h^{-1}) and as % of the initial value: RNase activity = 18.610.

Test compound	Conc.	24 h		48 h		72 h	
			%		%		%
Control		20.771	112	22.875	123	24.818	133
1	1 mM	14.238	77	32.289	174	11.718	63
2	1 mM	20.370	110	23.639	127	11.450	62
3	1 mM	16.825	90	23.810	128	14.704	79
4	1 mM	18.715	101	22.522	121	14.231	76
5	1 mM	12.740	69	29.194	157	13.928	75
6	1 mM	28.427	153	21.520	116	15.340	82
7	1 mM	21.090	113	21.826	117	24.920	134
8	1 mM	24.983	134	21.777	117	24.745	133
9	1 mM	26.750	144	16.680	90	26.600	143
10	1 mM	27.013	145	15.495	83	17.710	95
Standard							
Put	5 mM	16.079	86	25.953	139	19.961	107
		LSD 5% = 5.4360;		LSD 1% = 7.4662			

Discussion

Senescence is the result of complex changes in basic plant metabolism. In higher plants, various degradative phenomena associated with free radicals (FRs) have been

implicated in the senescence process (Dhindsa at al., 1981; Leshem et al., 1986; Thompson et al., 1987a). Free radical-mediated reactions have direct or indirect effects on breakdown of membranes, nucleic acids, polysaccharides and proteins (Halliwell and Gutteridge, 1986; Thompson et al., 1987a). It is proposed that the loss in membrane integrity that occurs with progressive aging (Knowles and Knowles, 1989) is most likely due to a shift in the equilibrium between FRs production and consumption toward production.

In plants, senescence-associated parameters can effectively be retarded by cytokinins (CK) and polyamines. Leaf senescence is accompanied by loss of enzymatic scavenging capacity expressed as decreased levels of superoxide dismutase (SOD) and catalase. Concerning the effect of cytokinin on these enzymes, conflicting evidence exists. Kinetin inhibits senescence at least in part by maintaining high levels of SOD and catalase (Dhindsa et al., 1982). However, Leshem et al. (1982) found that in pea foliage, cytokinin decreases SOD in senescing or stress environments. Investigations relating to harmful effects on plants of ozone have also indicated that the cytokinins benzyladenine and zeatin or antioxidants such as sodium benzoate or propyl gallate have an attenuating action (Pauls and Thompson, 1982). These results are interpreted in the light of a radical scavenger role to protect plants against ozone. Lee and Chen (1982) have shown that a cytokinin-like substituted phenylurea compound has a senescence-retarding effect and an antiozonating action and their interpretation is that FR metabolism is involved.

To clarify the changes brought about by the different treatments, leaf segments senescing in the dark for 24, 48, 72 h periods were assayed for Chl, free amino acids, soluble protein, as well as cytosolic catalase and guaiacol peroxidase, and ribonuclease activities. Analysis of these senescence parameters in barley leaf segments incubated in our compound solutions demonstrated, for what we believe to be the first time, that the senescence-dependent changes were differentially affected by various bis-ureas, perhaps due to their different composition and structure. They have both activity-determining structural components – phenylurea and aliphatic diamines which have a senescence-retarding effect (4-XPh)Ph-NHCO-NH-(CH_2)n-NH-CONH-Ph(4-XPh).

The treated with bis-phenylureas of diaminoalkanes leaf segments showed greater and faster loss of chlorophyll than of protein during the early stages of senescence. Chl loss increased continuously with advancing of senescence while the protein loss was stopped. During the first 24 h the compounds increased amino acid accumulation which did not reflect in the protein content; a direct correlation existed only for the bis-phenylurea' derivative of 1,2-diaminoethane. The protein loss was 21% in control segments while in treated segments – 2–10%, at day 3 of senescence. Treatment with bis-phenylureas' of 1,5-diaminopentane and 1,6-diaminohexane (comps. 4 and 5) resulted even in a slight increase (5 to 14%) over the initial protein content. Probably these compounds were able to keep the protein synthetic capacity in senescing leaf segments after 3 days of senescence (Lamattina et al., 1985). The time-dependent

changes in GPX activity in the treated segments were not significant as compared to the control and these in comps. 4- and 5-treated segments were lower than the control at day 3 of senescence.The specific activity of catalase increased much more in the treated segments than in controls during the first 24 h, then quickly declined proportionally to the increase in the number of methylene groups in the hydrophobic chain of the compounds.

Therefore, the basic property of bis-(phenyl)ureas' derivatives is the prevention of protein degradation. The protein content in all treated tissues was greater than in the control at the end of the third aging day (by 11–35%). The results regarding the increase in the activity of H_2O_2-scavenging enzymes during the early stages of senescence suggest that a complex and specific enzymatic antioxidant defense system is operative and could represent an induced protective reaction to an increased rate of generation of reactive species (Bartoli et al., 1995). The total RNase activity in the treated segments was inhibited during the first and especially the third aging day. The comp. 2-treated segments demonstrated the lowest activity.

The rate of Chl degradation in the leaf segments treated with bis-(4-fluorophenyl)-ureas' derivatives (comps. 6–10) was almost equal in all variants. The Chl quantity diminished with advancing of senescence but remained 15–30% higher than that of the control during the whole examination period. The elevated protein content in the treated tissues during the first aging day is probably a result of the synthesis of certain non-enzymatic short-living proteins which are perhaps associated with the supply of energy and membrane transport during the early stages of tissue senescence (Makovetzki and Goldschmidt, 1976). The rate of proteolysis in all treated tissues was higher than in the control tissues and decreased with increasing the number of methylene groups in the aliphatic chain. The greatest accumulation of free amino acids was found in tissues treated with the compds. 6 and 7, which correlated with the greatest induced protein content in the same tissues during day 1. The specific activities of the defense antioxidative enzymes (GPX and CAT) in the treated tissues were significantly low at day 1. GPX activity considerably increased during the next days and at the end of day 3 it was up to three times higher than the control in all treated tissues. The segments treated with compds 6 and 7 had the highest GPX and CAT activities. Total RNase activity in the treated tissues was inhibited at day 2 and by the end of the experiment compd. 6-treated tissues had the lowest activity.

The results obtained suggest the existence of a lag-phase for bis-(4-fluorophenyl)-ureas' action, i.e. their protective action is probably connected with mechanisms that lead either to activation or de novo synthesis of defense systems. Increased enzyme activities in the treated tissues are not limiting factors for the process of proteolysis but the protection of Chl degradation is mediated by increased antioxidative enzyme activities similar to kinetin action (Dhindsa et al., 1982; Kuroda et al., 1990).

The proteolysis rate in the tissues treated with bis-(4-chlorophenyl)ureas' derivatives of diamines was significantly lower than in the control during the whole aging

period as compared to the fluoro-derivatives. Among all chloro-derivatives, compd. 11 demonstrated the greatest protective effect in regard to protein hydrolysis. The tissues treated with this compound retained the protein content or even increased it over the initial value which directly correlated with the lowest free amino acid content. Unlike compd. 6-treated tissues (fluoro-derivative with two methylene groups), the compd. 11-treated tissues (chloro-derivative with two methylene groups) had the lowest GPX and CAT activities. The former showed increasing protection of the Chl loss with advancing of senescence while the latter – decreasing protection but it strongly prevented the protein hydrolysis. Compound 15 (chloro-derivative with six methylene groups) prevented the Chl degradation which was attributed to the high levels of GPX and CAT activities while the respective fluoro-derivative (compd. 10) showed approximately the same protection in both processes by low levels of the enzyme activities. The above mentioned two couples of compounds whose only difference is the halogen nature demonstrated differential protection of the basic biochemical processes.

On the basis of the results obtained in this work, it seems clear that the investigated compounds possess physiological effects similar to the diamines rather than the active cytokinins in this model system. Therefore, the mode of their action is suchlike, i.e. they could act as direct free radical scavengers (amine → amide) (Leshem, 1984). However, the test compounds manifested different anti-senescence effects with regard to tendency and magnitude. Therefore, this suggests that senescence-retarding effects of different compounds could be achieved through several adaptive mechanisms and the modulation of antioxidative enzyme levels could only be part of the whole mechanism.

Conclusions

This study demonstrates that bis-(phenyl/halogenophenyl)urea' derivatives of aliphatic diamines protect barley leaf segments from the dark-senescence induced metabolic changes. Furthermore, it appears that among bis-(phenyl)ureas¢ derivatives, more active are these with a longer polymethylene chain while among bis-(4-halogenophenyl)ureas' – those with a shorter polymethylene chain. The nature of the halogen atom determines the mode of action – bis-(4-fluorophenyl)ureas-induced protection is mediated in general by increased antioxidative enzyme activities while bis-(4-chlorophenyl)ureas-induced protection is mediated probably by a declined concentration of AOS, thus decreasing substrate-enzyme activation. The protective effect of the test compounds on the two basic processes of senescence is realized by means of different mechanisms: the prevention of Chl degradation correlats with greater antioxidative enzyme activities, whereas protein hydrolysis prevention is connected with low activities of H_2O_2-detoxifying enzymes.

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References

Alexieva, V., 1994. Effect of exogenous putrescine and its synthetic structural analogues on leaf senescence. Compt. rend. Acad. bulg. Sci., 47(9), 57–60.

Altman, A., R. Kaur-Sawhney, A W. Galston, 1977. Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. Plant Physiol., 60, 570–574.

Altman, A., 1982. Retardation of radish leaf senescence by polyamines. Physiol.Plant., 54, 189–193.

Bartoli, C. G., M. Simontacchi, J. J. Guiamet, E. Montaldi, S. Puntarulo, 1995. Antioxidant enzymes and lipid peroxidation during ageing of *Chrysanthemum morifolium* RAM petals. Plant Science, 104, 161–168.

Borraccino, G. L., L. Mastropasqua, S. De Leonardis, S. Dipierro, 1994. The role of the ascorbic acid system in delaying the senescence of oat (*Avena sativa* L.) leaf segments. J. Plant Physiol., 144, 161–166.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–254.

Castillo, F. J., H. Greppin, 1988. Extracellular ascorbic acid and enzyme activity related to ascorbic acid metabolism in *Sedum album* L. leaves after ozone exposure. Environ. Exp. Bot., 28, 231–238.

Cheng, S. H., Y. Y. Shyr, C. H. Kao, 1984. Senescence of rice leaves. VII. Effects of 1,3-diaminopropane, spermidine and spermine. Bot. Bull. Acad. Sin., 25, 191–196.

Decker, L. A., 1977. Peroxidase. In: Worthington Enzyme Manual. Worthington biochemical corp., Freehold New Jersey, 66–70.

Dhindsa, R. S., P. L. Plumb-Dhindsa, D. M. Reid, 1982. Leaf senescence and lipid peroxidation: Effects of some phytohormones, and scavengers of free radicals and singlet oxygen. Physiol. Plant., 56, 453–457.

Dhindsa, R. S., P. L. Plumb-Dhindsa, T. A. Thorpe, 1981. Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot., 32, 93–101.

Gepstein, S., K. V. Thimann, 1981. The role of ethylene in the senescence of oat leaves. Plant Physiol., 68, 349–354.

Halliwell, B., J. M. C. Gutteridge, 1986. Iron and free radical reactions: two aspects of antioxidant protection. Trends Biochem. Sci., 11, 372–375.

Kanazawa, S., S. Sano, T. Koshiba, T. Ushhimaru, 2000. Changes in antioxidative enzymes in cucumber cotyledons during natural senescence: comparison with those during dark-induced senescence. Physiol. Plant., 109, 211–216.

Kao, C. H., 1980. Senescence of rice leaves. IV. Influence of benzyladenine on chlorophyll degradation. Plant Cell Physiol., 21, 1255–1262.

Kato, M., S. Shimizu, 1987. Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. Can. J. Bot., 65, 729–735.

Knowles, N. R, L. O. Knowles, 1989. Correlations between electrolyte leakage and degree of saturation of poplar lipids from aged potato (*Solanum tuberosum* L.) tuber tissue. Ann. Bot., 63, 331–338.

Kuroda, M., T. Ozawa, H. Imagawa, 1990. Changes in chloroplast peroxidase activities in relation to chlorophyll loss in barley leaf segments. Physiol. Plant., 80, 555–560.

Lee, E. H., J. A. Jersey, C. Gifford, J. Bennett, 1984. Differential ozone tolerance in soybean and snapbean: Analysis of ascorbic acid in O_3 susceptible and O_3 resistant cultivars by HPLC. Environ. Exp. Bot., 24, 331–334.

Lamattina, L., R. P. Lezica, R. D. Conde, 1985. Protein metabolism in senescing wheat leaves. Determination of synthesis and degradation rates and their effects on protein loss. Plant Physiol., 77, 587–590.

Lee, E. H., C. M. Chen, 1982. Studies on the metabolisms of ozone tolerance: Cytokinin-like activity of N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N´-phenylurea, a compound protecting against ozone injury. Physiol. Plant., 56, 486–491.

Leshem, Y. Y., J. Wurzberger, A. A. Frimer, G. Barness, I. B. Ferguson, 1982. Calcium and calmodulin metabolism in senescence: interaction of lypoxygenase and superoxide dismutase with ethylene and cytokinin. In: Plant Growth Substances (Ed. P. F. Wareing), Acad. Press, New York, 569–578.

Leshem, Y. Y., 1984. Interaction of cytokinins with lipid-associated oxy free radicals during senescence: a prospective mode of cytokinin action. Can. J. Bot., 62, 2943–2949.

Leshem, Y. Y., A. H. Halevy, C. Frenkel, 1986. Free radicals and senescence. In: Processes and control of plant senescence (Eds. Y. Y. Leshem, A. H. Halevy, C. Frenkel), Elsevier, New York, 100–166.

Letham, D. S., 1978. The Cytokinins. In: Phytohormones and related compounds. A comprehensive treatise. Eds. D. S. Letham, P. B. Goodwin, T. J. V. Higgins, Elsevier/North-Holland Biomedical Press, Amsterdam, Vol. I, 205–263.

Leung, H. W., M. J. Vang, R. D. Masvis, 1981. The co-operative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. Biochim. Biophys. Acta, 664, 266–272.

Mackinley, G., 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem., 140, 315–322.

Makovetzki, S., El. E. Goldschmidt, 1976. A requirement for cytoplasmic protein synthesis during chloroplast senescence in the aquatic plant. Plant Cell Physiol., 17, 859–862.

Martin, C., K. V. Thimann, 1972. The role of protein synthesis in the senescence of leaves. I. The formation of protease. Plant Physiol., 49, 64–71.

Naik, B. I., S. K. Srivastava, 1978. Effect of polyamines on tissue permeability. Phytochemistry, 17, 1885–1887.

Nooden, L. D., 1988. The phenomenon of senescence and aging. In: Senescence and Aging in Plants. Eds. L. D. Nooden and A. C. Leopold, Acad. Press, San Diego, 2–50.

Palmer, C. E., 1985. The effect of ABA on aminonitrogen and protein content of potato plants in relation to the inhibition of nitrate reductase activity. Plant Cell Physiol., 26, 1083–1091.

Pauls, K. P., J. E. Thompson, 1982. Effects of cytokinins and antioxidants on the susceptibility of membranes to ozone damage. Plant Cell Physiol., 23, 821–832.

Popovic, R. B., D. J. Kyle, A. S. Cohen, S. Zalik, 1979. Stabilization of thylakoid membranes by spermine during stress-induced senescence of barley leaf discs. Plant Physiol., 64, 721–726.

Spies, J. R., 1957. Colorimetric procedures for amino acids. Methods Enzymol., 3, 468–472.

Thimann, K. V., 1980. The senescence of leaves. In: Senescence of Plants. Ed. K. V. Thimann, CRC Press Inc., Boca Raton, Florida, 85–115.

Thompson, J. E., R. L. Legge, R. F. Barber, 1987a. The role of free radicals in senescence and wounding. New Phytol., 105, 317–344.

Thompson, J. E., G. Paliyath, J. H. Brown, C. L. Duxbury, 1987b. The involvement of activated oxygen in membrane deterioration during senescence. In: Plant Senescence: Its Biochemistry and Physiology. Eds. W. W. Thomson, E. Nothnagel, R. Huffaker, 146–155.

Trippi, V. S., M. De Luca d'Oro, 1985. The senescence process in oat leaves and its regulation by oxygen concentration and light irradiance. Plant Cell Physiol., 26, 1303–1311.

Wyen, N. V., S. Erdei, G. L. Farkas, 1971. Isolation from *Avena* leaf tissue of a nuclease with the same type of specificity towards RNA and DNA. Accumulation of the enzyme during leaf senescence. Biochim. Biophys. Acta 232, 472–483.

Yonova, P., I. Ionov, 1999. Synthesis of 1,1'-polymethylenebis(3-substituted)ureas and related compounds of potential biological interest. Compt. rend. Acad. bulg. Sci., 52(3-4), 53–56.