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# COMPARATIVE ANALYSIS OF SOME BIOCHEMICAL AND ENZYMATIC CHANGES IN SENESCING BARLEY LEAVES BY USING 1,1´-POLYMETHYLENEBIS(3-ARYL-SUBSTITUTED)THIOUREAS

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**Summary**. The effects of 14 1,1´-polymethylenebis(3-arylsubstituted)thioureas on the kinetic changes in chlorophyll, protein and free amino acid contents, and in the activities of catalase and guaiacol peroxidase during senescence of barley leaf segments in the dark were investigated. The anti-senescence properties of bis-thioureas found expression predominantly in a significant inhibition of protein degradation. The degradation of chlorophyll-protein complex was, however, slightly protected. The length of the polymethylene chain was a determining factor for the effectiveness of bis-(phenyl)thioureas' derivatives of diaminoalkanes for the protein hydrolysis. Their protective effect on the protein loss increased with increasing the number of methylene groups in the aliphatic chain. The activity of catalase in treated tissues was very low while GPX activity increased during senescence period but remained lower than that of the control, and declined with increasing the length of polymethylene chain. The protein-protecting effect of bis-(4-halogenophenyl)thioureas' derivatives of diamines was higher than that of bis-(phenyl)thioureas, and was a function of the nature of halogen atom. Bis-(4-bromophenyl)thioureas manifested the highest effect. The protein content in the aging leaves increased in the following order F < Cl < Br and respectively decreased the accumulation of free amino acids. The GPX activity increased in the order Br < F < Cl, whereas CAT activity (relatively low activity, significantly lower than the control) increased in the order F < Cl < Br. The mode of action of the bis-(4-halogenophenyl)thioureas is probably associated with a decreasing peroxidative damage caused by reduced AOS accumulation

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which is proportional to the increase of electron-donor property of the halogen atoms.

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

***Abbreviations***: AAC – amino acid content, AOS – active oxygen species, APX – ascorbate peroxidase, CAT – catalase, Chl – chlorophyll, GPX – guaiacol peroxidase, DPU – 1,3-diphenylurea, Put – putrescine

## Introduction

Natural and artificial senescence has been studied in many laboratories by following the loss of Chl, proteins, and nucleic acids and the corresponding accumulation of free amino acids. Senescence is a multifactorial syndrome and free radicals and in particular active oxygen species seem to play an important role in aging processes (Fridovich, 1976). The connection between oxidative cellular damage and senescence, on one hand, and antioxidant defense mechanisms on the other, has been postulated in both animals and plants. A widely discussed theory of aging, first proposed by Szilard (1959) and extended by Orgel (1963), considers the aging process in terms of the accumulation of molecular damage to informational molecules. This damage may well be produced by an oxygen free radical, such as hydroxyl radical [$^{\bullet}$OH], which randomly alters DNA molecules or other components of the protein-synthesizing system (Harman, 1969; Emanuel, 1976; Chance et al.,1979). Reactions involving free radicals are implicated in wound healing (Salin and Bridges, 1981), senescence of seeds (Steward and Bewley, 1980; Puntarulo and Boveris, 1990), fruits (Brennan and Frenkel, 1977; Baker et al., 1978), carnation petals (Paulin et al., 1986; Droillard et al., 1987), salinity (Sreenivasulu et al., 2000), chemical stress (Levinsh et al., 1995), etc.

Senescence-associated symptoms can effectively be retarded by using some exogenous phytohormones – cytokinins (Vassilev et al., 1979; Dhindsa et al., 1982; Lee and Chen, 1982), gibberellic acid ($GA_3$) (Whyte and Luckwill, 1966) and other groups of plant growth regulators and suitable substances - polyamines (Altman, 1982; Alexieva, 1994a; Alexieva, 1994b), indole and phenolic derivatives (Karanov, 1972), piperidinoacetanilides (Karanov et al., 1980), antioxidants (Pauls and Thompson, 1982), etc. However, the mechanism of such inhibitory action of the plant growth regulators is not clearly understood (Rodoni et al., 1998).

In the recent 3 years have been synthesized in our laboratory certain 1,1′-polymethylenebis(3-substituted)ureas and thioureas which possess both phenylurea and aliphatic diamine active moieties. Phenylureas and aliphatic diamines are known to have senescence-delaying effects on plant tissues.

In our earlier work we reported on anti-senescence properties of some 1,1′-polymethylenebis(3-substituted)ureas in barley leaf segments by dark-induced senescence (Yonova et al., 2000).

The present study assessed the biochemical and enzymatic responses of senescing barley leaf segments to exogenously applied 1,1′-polymethylenebis(3-substituted) thioureas. We analyzed the time-dependent changes in chlorophyll, soluble protein and free amino acid levels, and in the activities of catalase and guaiacol peroxidase during the process of senescence in darkness. Some important chemical structure - senescence-retarding effect relationships were established.

## Materials and Methods

### Plant material and induction of senescence

Barley seeds (*Hordeum vulgare* L., cv. Alfa), soaked 2 h in water, were sown onto 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 primary leaves, 3 cm in length, starting 5 mm below the leaf tip, were harvested and then repeatedly washed with distilled water. Ten segments were floated with abaxial side downward on 5 ml of test solution in Petri dishes 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

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

$$ArNHCS\text{-}NH(CH_2)_nNH\text{-}CSNHAr,$$

where Ar=$C_6H_5$- , n=2,3,4,6,7; Ar = 4-F$C_6H_4$- , 4-Cl$C_6H_4$- and 4-Br$C_6H_4$-; n=2,4,6.

(1) Ar=$C_6H_5$-, n=2
(2) Ar=$C_6H_5$-, n=3
(3) Ar=$C_6H_5$-, n=4
(4) Ar=$C_6H_5$-, n=6
(5) Ar=$C_6H_5$-, n=7
(6) Ar=4-F$C_6H_4$-, n=2
(7) Ar=4-F$C_6H_4$-, n=4
(8) Ar=4-F$C_6H_4$-, n=6
(9) Ar=4-Cl$C_6H_4$-, n=2
(10) Ar=4-Cl$C_6H_4$-, n=4
(11) Ar=4-Cl$C_6H_4$-, n=6
(12) Ar=4-Br$C_6H_4$-, n=2
(13) Ar=4-Br$C_6H_4$-, n=4,
(14) Ar=4-Br$C_6H_4$-, n=6

### Determination of chlorophyll content and amino acid accumulation

After appropriate intervals of time, 10 segments (~ 0.250 g) were blotted and extracted by boiling in 80% (v/v) ethanol for 10 min repeatedly. The ethanolic extracts were

combined and made to 10 ml. Chlorophylls a and b were guantified and expressed as µg Chl.segment$^{-1}$ by transferring 600 µl 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 Arnon's method (1949). 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 content in 100 µl aliquots of the aqueous solution was determined by the ninhydrin method (Spies, 1957).

### Determination of enzyme activities

An enzyme extract was prepared by homogenizing 10 leaf segments in 5 ml of 0.1 M phosphate buffer, pH 6.8, containing 0.1 mM EDTA at 0–4 °C and centrifugation at 12 000 × g for 20 min. The assays of catalase [EC 1.11.1.6] activity (according to Kato and Shimizu, 1987) and guaiacol peroxidase [EC 1.11.1.7] activity (according to Decker, 1977) have been described in detail previously (Yonova et al., 2000). The specific activity of catalase and peroxidase were expressed in micromoles of decomposed $H_2O_2$ [µmol.mg$^{-1}$ protein.min$^{-1}$] and micromoles of oxidized quaiacol [µmol.mg$^{-1}$ protein.min$^{-1}$], respectively under the assay conditions. 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

Data presented in this report were 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

### Defining of the biochemical parameters regarding the leaf aging process

The obtained results showed that the bis-phenylthiourea derivatives of diaminoalkanes retarded the chlorophyll loss in the aging leaf segments during the first 24 h more compared to the control (13%). However, during the following aging periods their retarding effect decreased and was approximately equal to that of the control. The length of

**Table 1.** Effect of some 1,1´-polymethylenebis(3-arylsubstituted)thioureas on time-dependent changes of 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 compound 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 | 100±1.91 | 79 | 94±2.57 | 74 | 55±0.78 | 44 | 2.02 | 1.91 | 1.96 |
| 2 | 1 mM | 97±2.00 | 77 | 73±1.21 | 58 | 58±1.80 | 46 | 2.04 | 1.92 | 1.97 |
| 3 | 1 mM | 90±1.90 | 71 | 66±1.80 | 52 | 54±0.88 | 43 | 2.05 | 1.93 | 1.95 |
| 4 | 1 mM | 89±1.60 | 70 | 70±1.34 | 56 | 49±0.88 | 39 | 2.03 | 1.87 | 2.03 |
| 5 | 1 mM | 95±0.95 | 75 | 67±0.73 | 53 | 48±0.32 | 38 | 2.02 | 1.92 | 1.95 |
| 6 | 1 mM | 82±1.72 | 65 | 65±1.13 | 51 | 39±0.61 | 31 | 2.05 | 2.09 | 1.94 |
| 6 | 0.1 mM | 63±1.00 | 50 | 66±1.57 | 52 | 47±0.73 | 37 | 2.05 | 2.04 | 2.01 |
| 7 | 1 mM | 63±1.17 | 50 | 58±1.45 | 46 | 35±0.37 | 28 | 2.05 | 2.06 | 1.89 |
| 7 | 0.1mM | 88±1.53 | 70 | 59±0.74 | 47 | 41±0.77 | 32 | 1.98 | 1.98 | 1.88 |
| 8 | 1 mM | 87±0.81 | 68 | 81±0.92 | 64 | 52±0.42 | 41 | 1.98 | 1.91 | 2.09 |
| 8 | 0.1 mM | 82±0.63 | 65 | 72±0.44 | 57 | 45±0.33 | 35 | 1.95 | 1.95 | 1.99 |
| 9 | 1mM | 101±2.39 | 80 | 68±0.39 | 54 | 54±0.44 | 43 | 1.88 | 1.81 | 1.84 |
| 10 | 1mM | 94±1.57 | 74 | 67±1.22 | 53 | 65±2.02 | 51 | 1.87 | 1.83 | 1.78 |
| 11 | 1mM | 90±0.95 | 71 | 46±0.93 | 36 | 41±0.86 | 32 | 2.04 | 2.12 | 1.97 |
| 12 | 1mM | 93±0.64 | 73 | 74±0.87 | 58 | 56±0.82 | 45 | 1.97 | 1.96 | 2.03 |
| 13 | 1mM | 83±0.86 | 66 | 75±0.63 | 59 | 51±0.42 | 40 | 1.96 | 1.92 | 2.01 |
| 14 | 1mM | 91±0.64 | 72 | 81±0.36 | 64 | 59±1.52 | 46 | 1.95 | 1.94 | 1.82 |
| *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 |
| | | LSD 5% =3.0233; | | LSD 1% = 4.0588 | | | | | | |

the polymethylene chain was not a determining factor for the effectiveness of the compounds – all five derivatives reacted in almost one and the same way towards the Chl loss during the whole aging period. The only exception was the derivative of 1,2-diaminoethane. The Chl loss in the leaf segments processed with this derivate was 26% compared to 45% in the control segments at the end of the second day (Table 1).

Fluoro-, chloro- and bromo-substituted bis-phenylthiourea derivatives of diaminoalkanes demonstrated different behaviour in regard to the rate of Chl loss in the senescing leaf segments which was mainly determined by the nature of halogen atom and not by the length of polymethylene chain (Table 1).

Fluoro-derivatives caused faster Chl degradation during the first 24 h (until 50% compared to the initial Chl quantity and until 16% more than the control) whereas chloro- and bromo-derivatives - during the second and third 24h, respectively. By the end of the 3rd day, Chl quantity in the senescing segments processed with chloro- and bromo-derivatives was almost equal or to 9% more than that of the control. Chl quantity in the leaf segments processed with fluoro-derivatives was lower compared to the control (to 14%).

It was found that Chl degradation in the aging leaf segments treated with bis-thioureas' derivatives of diaminoalkanes (except for two compounds) started mainly with the Chl *b* degradation (increased Chl *a*/*b* ratio), similarly to the control. There were differences in the degradation rate of Chl *b* which depended on the type of the applied compounds. Bis-phenyl- and bis-(fluoro)phenylthioureas destroyed Chl *b* at a higher rate than bis-(chloro)phenyl- and bis-(bromo)phenylthioureas.

The content of total soluble protein in the senescing leaf segments treated with bis-thioureas' derivatives of diaminoalkanes declined with advancing senescence but remained higher than that of the control at the different aging stages (Table 2). Protein loss was not observed. There was even increased protein content in the leaf segments treated with bis-phenylthioureas' derivatives during the first 24 h which later slowly decreased. Protein content depended on the length of polymethylene chain in the tested compounds and was in the reverse relation to the accumulation of free amino acids. The compd. 5-treated leaf segments which had the lowest rate of protein hydrolysis contained the highest protein quantity (173, 152, 136% compared to 85, 80, 79% in the control at 24, 48 and 72 h of senescence, respectively).

Fluoro-, chloro- and bromo-substituted bis-phenylthioureas' derivatives of diaminoalkanes retarded protein degradation in the treated tissues much more strongly compared to the control but more weakly than the unsubstituted bis-phenylthioureas. Fluoro-derivatives showed the slightest protection compared to the other chloro- and bromo-derivatives. The content of total soluble protein in chloro- and bromo-derivatives – treated leaf segments increased during the first 24 h, then it decreased (by 26–40%) at day 2 and increased again at the end of day 3 (by 13–40%). This time-course dependency of the protein content changes correlated well with the kinetic alterations of amino acid accumulation in the treated senescing segments.

**Table 2.** Effect of some 1,1´-polymethylenebis(3-arylsubstituted)thioureas 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 compound solutions for 24, 48 and 72h. Data are expressed as µg.segment$^{-1}$, and as % of the initial values: protein 318±21 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 | 398±23 | 125 | 324±10 | 102 | 285±9.0 | 90 |
| 2 | 1 mM | 423±21 | 133 | 313±41 | 99 | 269±11 | 85 |
| 3 | 1 mM | 454±15 | 143 | 404±16 | 127 | 358±17 | 113 |
| 4 | 1 mM | 510±41 | 160 | 409±12 | 129 | 346±29 | 109 |
| 5 | 1 mM | 552±29 | 173 | 482±13 | 152 | 432±12 | 136 |
| 6 | 1 mM | 368±10 | 116 | 350±16 | 110 | 329±4.0 | 104 |
| 6 | 0.1 mM | 377±11 | 119 | 277±18 | 87 | 289±6.0 | 91 |
| 7 | 1 mM | 260±5.0 | 82 | 243±12 | 77 | 242±17 | 76 |
| 7 | 0.1mM | 253±2.0 | 80 | 280±22 | 88 | 267±9.0 | 84 |
| 8 | 1 mM | 380±4.0 | 119 | 365±11 | 115 | 356±19 | 112 |
| 8 | 0.1 mM | 337±4.0 | 106 | 437±17 | 138 | 335±40 | 105 |
| 9 | 1 mM | 256±21 | 80 | 324±18 | 102 | 387±33 | 122 |
| 10 | 1 mM | 452±17 | 142 | 233±18 | 73 | 389±55 | 122 |
| 11 | 1 mM | 395±16 | 124 | 367±9.0 | 115 | 324±30 | 102 |
| 12 | 1 mM | 400±25 | 126 | 318±16 | 100 | 380±26 | 120 |
| 13 | 1 mM | 437±17 | 138 | 260±2.0 | 82 | 398±23 | 125 |
| 14 | 1 mM | 395±16 | 124 | 302±19 | 95 | 344±10 | 108 |
| *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%=62.3205 | | LSD 1%=85.3680 | | | |

The experimental results in Table 3 showed that the accumulation of free amino acids in the treated leaf segments increased with advancing of senescence similarly to the control and the standards. Bis-phenylthioureas¢ derivatives of diaminoalkanes increased the proteolysis at the end of 2nd day and during the 3rd day its rate was either decreased or slightly increased. The absolute quantities of amino acids at the end of day 3 were lower (1.1–1.5 fold) than those of the control and Put. On the other hand, the quantities decreased with the extension of polymethylene chain. The lowest content of amino acids was observed after treatment with bis-phenylthiourea of 1,7-diaminoheptan (217% compared to 316% in the control and 334% of the Put).

Fluoro- and chloro-substituted bis-phenylthioureas' derivatives of diaminoalkanes increased the accumulation of amino acids in the aging leaf segments but at the

**Table 3.** Effect of some 1,1′-polymethylenebis(3-arylsubstituted)thioureas on the time-dependent changes in the levels of amino acid accumulation 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: AAs 2.237±0.26 µmol leucine eqv.segment$^{-1}$.

| Test compound | Conc. | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|---|
| | | µmol ± sd | % | µmol±sd | % | µmol±sd | % |
| Control | | 3.487±0.10 | 156 | 5.745±0.18 | 257 | 7.077±0.36 | 316 |
| 1 | 1 mM | 5.082±0.13 | 227 | 5.959±0.12 | 266 | 6.162±0.01 | 276 |
| 2 | 1 mM | 3.990±0.11 | 178 | 5.895±0.19 | 264 | 5.311±0.21 | 237 |
| 3 | 1 mM | 3.672±0.11 | 164 | 5.756±0.14 | 257 | 6.137±0.11 | 274 |
| 4 | 1 mM | 4.726±0.12 | 211 | 4.803±0.16 | 215 | 5.183±0.08 | 232 |
| 5 | 1 mM | 3.024±0.16 | 135 | 4.955±0.12 | 222 | 4.853±0.14 | 217 |
| 6 | 1 mM | 4.751±0.15 | 212 | 4.427±0.14 | 198 | 5.918±0.26 | 265 |
| 6 | 0.1 mM | 2.960±0.11 | 132 | 4.253±0.07 | 190 | 6.665±0.19 | 298 |
| 7 | 1 mM | 3.283±0.11 | 147 | 3.780±0.20 | 169 | 6.266±0.09 | 280 |
| 7 | 0.1mM | 4.403±0.19 | 197 | 2.512±0.09 | 112 | 6.914±0.21 | 309 |
| 8 | 1 mM | 1.543±0.05 | 69 | 2.910±0.04 | 130 | 3.631±0.02 | 162 |
| 8 | 0.1 mM | 1.790±0.19 | 80 | 4.451±0.35 | 199 | 4.975±0.17 | 222 |
| 9 | 1mM | 4.334±0.03 | 194 | 6.632±0.31 | 296 | 7.318±0.20 | 327 |
| 10 | 1mM | 4.004±0.49 | 179 | 8.792±0.48 | 393 | 5.615±0.24 | 251 |
| 11 | 1mM | 4.502±0.24 | 201 | 3.706±0.11 | 166 | 4.875±0.09 | 218 |
| 12 | 1mM | 1.990±0.05 | 89 | 2.885±0.18 | 129 | 3.532±0.20 | 158 |
| 13 | 1mM | 2.164±0.04 | 97 | 3.557±0.07 | 159 | 3.333±0.15 | 149 |
| 14 | 1mM | 1.890±0.05 | 84 | 3.632±0.05 | 162 | 3.581±0.08 | 160 |
| *Standards*: | | | | | | | |
| DPU | 1 mM | 2.437±0.09 | 109 | 2.984±0.20 | 133 | 4.328±0.28 | 194 |
| Put | 5 mM | 3.603±0.08 | 161 | 6.171±0.03 | 276 | 7.476±0.57 | 334 |
| | | LSD 5% = 0.7169; | | LSD 1% = 0.9556 | | | |

end of day 3 their quantities were lower than those of the control and Put. Generally, the rate of protein hydrolisis declined with the extension of polymethylene chain. The lowest content of amino acids was observed after treatment with the above derivatives of 1,6-diaminohexane.

Bromo-substituted bis-phenylthioureas' derivatives exhibited more different reaction in regard to this index. The rate of protein hydrolisis in the leaf segments treated with such compounds was much lower during the whole period of senescence. Actually proteolysis was present only during day 2 and the quantity of free amino acids was 1.6–2-fold lower than that of the control. This quantity remained the same at the end of day 3 and was lower compared to the control and the DPU (2 and 1.3-fold, respectively).

**Changes in activities of antioxidative enzymes during dark-induced senescence**

The examined bis-thioureas' derivatives of aliphatic diamines moderately promoted the specific peroxidase activity and strongly inhibited the specific catalase activity in the aging leaf segments.

Bis-phenylthioureas' derivatives induced a double increase (relative to the initial activity) in the GPX activity in the aging leaf segments during day 1 and another double increase during day 3 but the activity values remained lower than those of the control. The GPX activity decreased with increasing the length of polymethylene chain. The CAT activity was strongly inhibited (40–60% relative to the initial value at day 1) during the whole period of senescence and it was 3–6 times lower than that of the control at the end of day 3. The only exception was bis-phenylthiourea derivative of 1,2-diaminoethan (comp. 1) which increased the CAT activity up to day 2 (37% over the control) and then drastically decreased. (Figs. 1A and 2A)

Hence, the strong protective effect of compd. 5 in regard to the process of proteolysis is not a consequence of increased antioxidative enzyme activities. So, probably it either inhibits the proteases or reduces the quantity of free radicals which are substrates of the antioxidant defense enzymes.

Fluoro-, chloro- and bromo-substituted bis-phenylthioureas¢ derivatives of diaminoalkanes stimulated GPX activity (especially fluoro-derivatives on the first day) more strongly than the unsubstituted bis-phenylthioureas and at the end of day 3 the activity in the aging leaf segments was equal or lower compared to the control (bromo-derivatives had relatively the lowest activity) (Figs.1B and 2B). Generally, the CAT activity was strongly inhibited during the first 24 h (40–75% relative to the initial value) and remained at this level or continued to fall until the end of day 3. Bromo-derivatives showed a tendency to increase the CAT activity (by 32–50%) during day 3 but the values remained 1.6–2 times lower than those of the control. Two compounds demonstrated differences in their behaviour: i) chloro-derivative of 1,2-diaminoethane (compd. 9) significantly stimulated CAT activity during the first 24 h (288% relative to the initial value and 112% over the control), then declined trice and kept this activity up to day 3 of senescence; ii) fluoro-derivative of 1,6-diaminohexane (compd. 8) showed the greatest catalase activity at the end of day 3–116% compared to 123% in the control, relative to the initial activity.

## Discussion

In this study, we demonstrated, for what we believe to be the first time, the anti-senescent properties of 14 bis-thiourea derivatives, consisting of a polymethylene hydrophobic chain between the both thiourea bridges. Moreover, we examined the nature of *in vitro* senescence-dependent changes in some parameters affected by the various

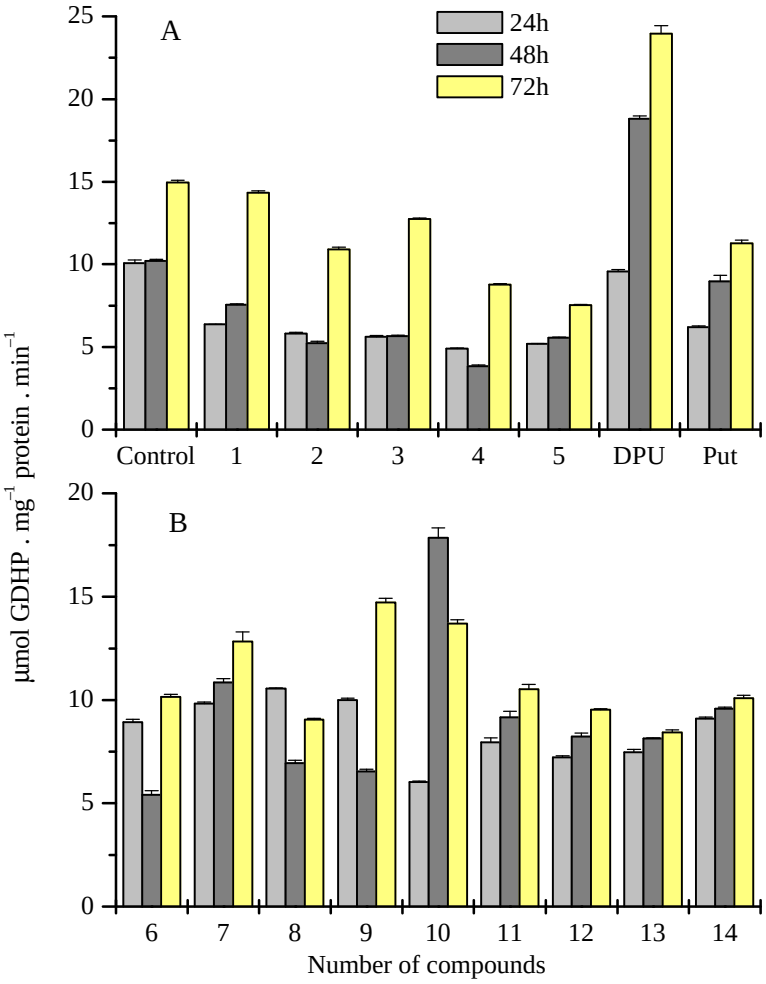


**Fig. 1**. Comparative time-dependent changes in the levels of guaiacol peroxidase activity during dark senescence of barley leaf segments for 24, 48 and 72 h: the effect of compounds No 1–14 at 1.0 mM. The initial GPX value was 2.503+0.35. Each value is a mean of 3 replicates. LSD 5 % = 0.6149; LSD 1 % = 0.8255.

compounds. The latters have two activity-determining structural components – phenylurea and aliphatic diamines. The former is known to have a senescence-retarding effect as cytokinins and the latter retards also senescence of detached plant leaves as polyamines.

Senescence is associated with a progressive decrease in protein and chlorophyll contents and a marked increase in protein degradation (Thimann 1980; Dhindsa et al., 1981; Miller and Huffaker,1985). A decline in catalase activity and a rise in peroxidase activity are characteristic responses of aged tobacco leaves (Kato and

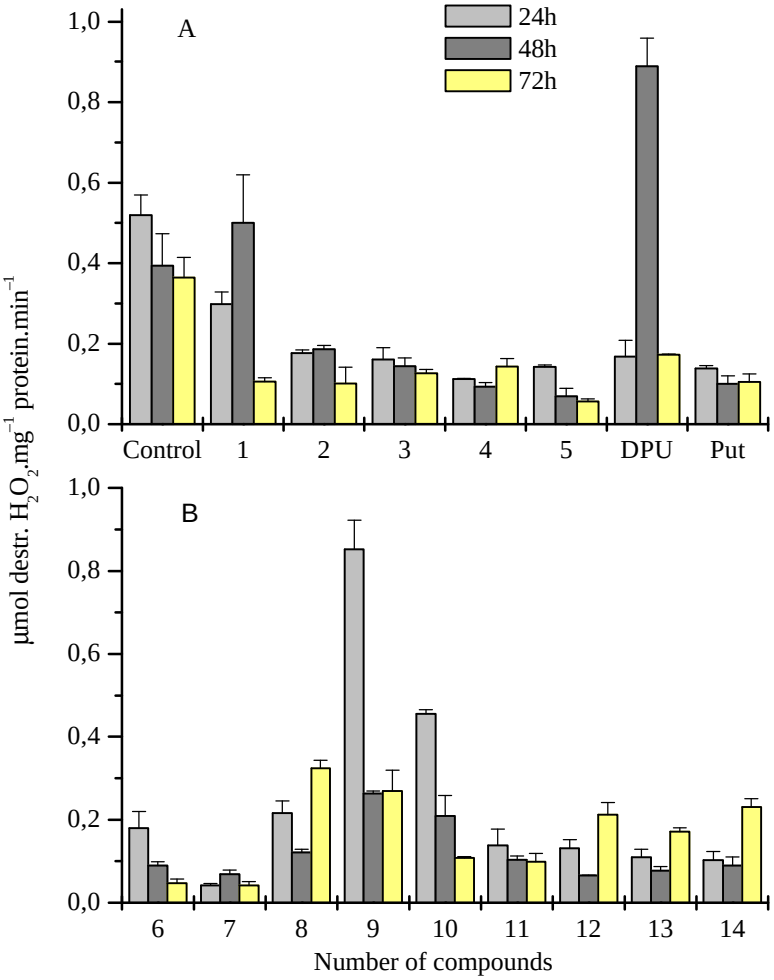


**Fig. 2**. Comparative time-dependent changes in the levels of catalase activity during dark senescence of barley leaf segments for 24, 48 and 72 h: the effect of compounds No 1–14 at 1.0 mM. The initial CAT value was 0.295+0.0072. Each value is a mean of 3 replicates. LSD 5 % = 0.1249; LSD 1 % = 0.1715

Shimizu, 1987). Both enzymes play a role in detoxifying $H_2O_2$, but their behaviour suggests quite different roles in senescence. The observed changes in the activities of catalase and guiaicol peroxidase showed a similar trend in our experiments.

Leaf senescence appears to require continued protein synthesis since the latter has been strongly inhibited by cycloheximide (Martin and Thimann, 1972; Thimann, 1980, Dhindsa et al., 1981, Lamattina et al., 1985, Lalonde and Dhindsa, 1990). It is not clear, however, whether this requirement is for the continued synthesis of the pre-existing proteins or for the synthesis of new ones. At present little is known about the identity of the proteins that are synthesized during leaf senescence.

Research evidence showed that anti-senescent properties of bis-thioureas found expression predominantly in a significant inhibition of the protein degradation. The treated tissues preserved protein content and kept low levels of amino acid accumulation. They even demonstrated a protein synthetic capacity during the whole senescence period. However, the degradation of chlorophyll was slightly protected. The Chl changes were less significant between treated and non-treated tissues over the investigated period.

In general, the high protein content in treated tissues correlated with a lower rate of proteolysis. Chloro-derivatives were the only exception. The higher protein content in treated tissues could possibly be attributed to a test-compounds-induced decrease in the free radical content, and particularly in hydrogen peroxide accumulation. We make this suggestion on the basis of the decrease in the activities of $H_2O_2$-scavenging enzymes.

The length of polymethylene chain was not a determining factor for the effectiveness of bis-phenylthioureas' derivatives of diaminoalkanes concerning Chl loss in the senescing leaf segments. It was a determining factor, however, for the protein hydrolysis. The protective effect of the compounds on the protein loss increased with the extension of polymethylene chain and similarly the capability for protein synthesis in the senescing leaves also increased. It was found that leaf senescence is associated with the synthesis of new proteins *in vivo* (Lalonde and Dhindsa, 1990) and in vitro (Malik, 1987). The rate of protein hydrolysis was in reverse relation to the length of polymethylene chain. The activity of catalase in treated tissues was very low while GPX activity increased during the senescence period, but remained lower than that in the control. The activity of GPX declined with increasing the number of methylene groups in the aliphatic chain.

The protein-protecting effect of bis-(4-halogenophenyl)thioureas' derivatives of diamines was higher than that of bis-(phenyl)thioureas. Bis-(4-bromophenyl)thioureas manifested the highest protein-protecting effect. The results showed that this effect was a function of the nature of halogen atom. The protein content in the aging leaves increased in the following order F < Cl < Br and respectively decreased the accumulation of free amino acids. The GPX activity increased in the order Br < F < Cl, whereas CAT activity (relatively low activity, significantly lower than the control) increased in the order F < Cl < Br.

The activities of AOS-scavenging enzymes (SOD, GPX, APX and CAT) usually decrease during dark-induced senescence (Dhindsa et al., 1981; Pastori and del Rio, 1994). Our results are thus consistent in demonstrating a significant decrease in catalase activity while that of GPX increased in the treated tissues with increasing duration of senescence but it remained less than in control. The decrease in the activities of $H_2O_2$-scavenging enzymes in the treated tissues suggests a less need to scavenge AOS. Therefore, the content of AOS (substrates of antioxidant defense enzymes) is less,

i.e. oxidative damage to macromolecules could be less. However, the test-compounds protected predominantly protein degradation but not chlorophyll degradation.

The greater protecting effect of bis-(4-bromophenyl)thioureas may be explained through the differences in the electronic properties of halogen atoms. In the order F < Cl < Br the electron-donor properties of the atoms increase because the energy needed for the separation of one electron from a negative ion decreases. This probably determines the greater reaction capability of bromo-derivatives compared to the other halogen derivatives.

## Conclusions

Results from the present study demonstrate that all bis-thioureas¢ derivatives of diaminoalkanes can protect proteolysis more strongly than Chl degradation and the protective effect increases with the extension of aliphatic chain. The protein-protecting effect of bis-(4-halogenophenyl)thioureas¢ derivatives of diamines is higher than that of bis-(phenyl)thioureas and it is a function of the nature of halogen atom. Bis-(4-bromophenyl)thioureas manifest the highest protein-protecting effect. The mode of action of the bis-(4-halogenophenyl)thioureas is probably associated with decreasing peroxidative damage caused by reduced $H_2O_2$ accumulation which is proportional to the increase of electron-donor properties of the halogen atoms.

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