ANTISENESCENCE EFFECT OF 2-PYRIDYLUREAS WITH UN- AND CYCLIC- UREIDO GROUP

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Dedicated to the memory of Acad. E. Karanov

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Summary. The potential antisenescence effect of eight synthetic urea compounds was investigated in excised barley (*Hordeum vulgare* L.) leaves which were induced to senesce by incubation in complete darkness. The compounds having uncyclic ureido group showed higher chlorophyll retention activity than those with cyclic ureido group. Compounds *1*, *6* and 4PU had long-term protecting effects on chlorophyll degradation whereas the effects of compounds *2* and *5* were short-term. These effects were mediated by increased H_2O_2-scavenging enzyme activities. The chlorophyll retention activity of the more active compounds (*1, 2, 5, 6, 8*) was lower compared to the standard 4PU at the end of the third aging day. Treatment of leaf segments with compounds *1* and *8* resulted in an increased carotenoids content after 48 h. In addition, it was higher compared to the 4PU-treated leaf tissues. Our results provide information about the relationship between the antisenescence effect of synthetic urea compounds and the activity of some antioxidative enzymes. We suggest that the activities of the antioxidative enzymes as well as the balance between H_2O_2-generating and H_2O_2-scavenging enzymes are important parameters that could provide evidence about the senescence-retarding effect of the tested compounds. The structure - activity relationships for the screened compounds was also studied. The presence of unsubstituted 2-pyridyl ring and of 5-chloro- or (3,5-dichloro)-2-pyridyl ring contributed to the higher activity of the tested compounds.

Key words: antioxidative enzymes, barley leaf antisenescence bioassay, dark-induced senescence, stress-markers, ureas

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Abbreviations: AOS-active oxygen species, AsPO-ascorbate peroxidase, CAT-catalase, Chl-chlorophyll, FW-fresh weight, GPO-guaiacol peroxidase, SOD-superoxide dismutase, 4PU-N-phenyl-N'-(4-pyridyl)urea, THF-tetrahydrofuran

INTRODUCTION

Foliar senescence is a pre-programmed stage in the development of the plant and it is subjected to direct physiological and genetic control (Thomas and Stoddart, 1980; Leshem et al. 1986). Cytokinins constitute a major class of plant growth regulators which are known to retard the process of senescence including protein, nucleic acids, and chlorophyll degradation in excised leaf tissues (Sabater, 1985; Davies, 1987). Cytokinin activity possessed by at least four different classes of compounds: purines, modified purines, heterocyclic ureas (and amides) and aminopyrimidines has been reported (Matsubara, 1980). Among urea derivatives tested, N-phenyl-N'-(4-pyridyl)urea (4PU) exhibits strikingly high cytokinin activity comparable to 6-benzylaminopurine (BAP). Moreover, an electronegative chlorine atom introduced at the 2nd position of the pyridyl ring increases strongly the activity (4PU-30) (Isogai, 1981). It is well known that the 2-chloroethyl group, included as a substituent to biologically active substrates (carriers) attributes different physiological action (Белоусова и др. 1977). Thus, the growth regulating chemical EDU, N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea showed much higher effectiveness in arresting senescence and plant protection against ozone injury than kinetin (Lee and Chen, 1982; Miller et al. 1994), but it was not a protectant against UV-B damage in cucumber leaves (Krizek et al. 2001).

Compounds having other ring substituents, such as triazoles and imidazoles combined with ureido- or carbamoyl-groups, have been synthesized and evaluated as cytokinins and plant antisenescence agents (Fawzi and Quebedeaux, 1976; Cavender et al. 1988).

Senescence is characterized by a cessation of photosynthesis, disintegration of organelle structures, intensive loss of chlorophyll and proteins, and dramatic increase in lipid peroxidation and membrane leakage (Buchanan-Wollaston, 1997). The changes in the photosynthetic parameters are found mainly for differentiated leaves and for longer periods of senescence. However, it has been shown (Ananieva et al., 2005) for intact zucchini cotyledons that the short term induced senescence (24 h dark treatment) does not result in any appreciable changes in the functional activity of PSII and the net photosynthetic rate. It is firmly established that lipid peroxidation is due mainly to the strong enhancement of active oxygen species (AOS) generation which takes place in plant tissues during the senescence process (Leshem, 1988; Nooden et al., 1997). However, plants possess enzymatic and nonen-

zymatic antioxidative defence systems providing adequate cellular protection against AOS.

Recently, volatile compounds, particularly heterocyclic flavor chemicals, have been found to protect lipids from oxidation (Macku and Shibamoto, 1991; Eiserich and Shibamoto, 1994). The antioxidative activity of these compounds (imidazoles, thiazoles, oxazoles, furanones and thiophenes) determined toward the oxidation of hexanal to hexanoic acid was not as strong as that of the known antioxidant α-tocopherol (Shaker et al., 1995). Krivenko et al. (2000) tested seven arylthioureas with alkyl, aryl and heterocyclic subtituents as antioxidants. The results showed that the sterically hindered arylthioureas have the highest antioxidative activity. Darlington et al. (1996) found that the synthetic antioxidant Ambiol [2-methyl-4-(dimethylaminomethyl)-5-hydroxybenzimidazole dihydrochloride] applied by seed treatment of two dicotyledons increased their growth under drought conditions.

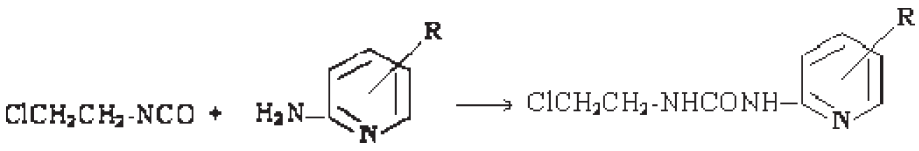
It could be expected that exogenously applied heterocyclic ureas/thioureas might be able to retain the senescence process probably due either to an inhibition of the oxidative degradation process or to stimulation of the antioxidative defence system.

Here, we studied the ability of eight 2-pyridylureas to delay senescence in excised barley leaves and the relationship between their antisenescence and oxidant protection effects. In this respect, we determined the time-dependent changes in the contents of chlorophyll, carotenoids, hydrogen peroxide, malondialdehyde and in the activities of catalase, ascorbate- and guaiacol-peroxidases, and superoxide dismutase during senescence.

MATERIALS AND METHODS

Synthesis of chemicals

The method used to prepare N-(2-chloroethyl)-N'-(2-pyridyl)ureas is outlined in the following scheme:



1 - R = H	4 - R = 4,6-Me_2
2 - R = 4-Me	5 - R = 5-Cl
3 - R = 5-Me	6 - R = 3,5-Cl_2

The compounds *1* - *3* were reported earlier (Vassilev et al., 1984) while compounds *4* - *6* are newly synthesized.

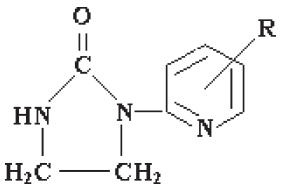
General procedure. The reaction was carried out in a medium of dichloromethane (20 ml for 0.01 mol 2-aminopyridines) with the addition of 5% mol excess of 2-chloroethylisocyanate by stirring in an ice-water bath for 1h. The reaction products crystallized quickly and were removed by filtration, washed with 3-5 ml of cooled ethanol and dried in a vacuum pump at room temperature. The crude products were purified by recrystallization from ethanol.

N-(2-chloroethyl)-N'-(4,6-dimethyl-2-pyridyl)urea (*4*): yield 98%, mp.112°C. Anal. Calcd for $C_{10}H_{14}N_3OCl$: C, 52.75; H, 6.15; N, 18.46. Found: C, 52.80; H, 6.13; N, 18.39.

N-(2-chloroethyl)-N'-(5-chloro-2-pyridyl)urea (*5*): yield 99%, mp.172°C. Anal. Calcd for $C_8H_9N_3OCl_2$: C, 41.03; H, 3.85; N, 17.95. Found: C, 41.20; H, 4.04; N, 17.81.

N-(2-chloroethyl)-N'-(3,5-dichloro-2-pyridyl)urea (*6*): yield 81%, mp.137°C. Anal. Calcd for $C_8H_8N_3OCl_3$: C, 35.75; H, 2.98; N, 15.64. Found: C, 35.62; H, 3.14; N, 15.68.

The second set of compounds, 1-(2'-pyridyl and 4'-methyl-2'-pyridyl)-2-oxo-imidazolidines, was obtained by intramolecular N-alkylation of compounds *1* and *2* using a phase transfer catalyst - pulverized KOH THF as a solvent and triethylbenzy-lammonium chloride as a catalyst.



7 - R = H
8 - R = 4-Me

The preparation of compounds *7* and *8* has been previously described (Yonova and Stoilkova, 2005).

Barley leaf antisenescence bioassay

Barley (*Hordeum vulgare* L. cv. Alfa) seeds were planted in wet vermiculite, placed in a growth chamber (24°C, 16-h days, 19°C night, relative humidity 50% and light intensity of 120 μmol.$m^{-2}s^{-1}$ PFD), and watered three times a day with tap water. Ten 3-cm long leaf segments, cut from 7-day-old primary barley leaves starting 0.5 cm below the tip were placed in a Petri dish (10-cm i.d.) containing 5 ml test solution. The test solutions used were as follows: phosphate buffer containing 0.02% Tween-80 (1 mM KH_2PO_4 - Na_2HPO_4, pH 5.8, control), 1.0 and 0.1 mM solutions of the tested compounds (*1* - *8*) and 4PU used as a standard (*9*), prepared in the same buffer

with 0.02% Tween-80. The Petri dishes were kept in the dark at 24±1°C. The leaf segments were taken at regular intervals (24, 48 and 72 h) for determination of chlorophyll, carotenoids, hydrogen peroxide and malondialdehyde levels as well as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (AsPO) and guaiacol peroxidase (GPO) activities.

Biochemical analyses

Fresh plant material (10 segments of 0.250g FW) was immediately extracted and assayed according to the appropriate methods listed below. The content of chlorophylls a, b and carotenoids was determined spectrophotometrically after extraction of 0.1 ml aliquots of buffer homogenate in 1.9 ml 80% (v/v) acetone at 4°C in the dark for 1 h and subsequent centrifugation at 4000g for 10 min. The optical density (OD) of the supernatants was read at 663, 645 and 460 nm, respectively (Mackinney, 1941). Hydrogen peroxide content was measured spectrophotometrically after reaction with 1 M KJ for 1 h in darkness and its amount was calculated using a standard curve (Alexieva et al., 2001). Lipid peroxidation was estimated based on determination of malondialdehyde content (MDA - a product of lipid peroxidation) using the thiobarbituric acid reaction (Dhindsa at al., 1981).

Enzyme analyses

Leaf segments (about 0.250g FW) were homogenized in 0.1 M K-phosphate buffer, pH 7.0, containing 1.0 mM Na_2-EDTA and 1% (w/v) polyvinylpirrolidone. The extracts were centrifuged at 14 000g for 30 min and the supernatant was used as a crude enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0-4°C. Enzyme activities were determined spectrophotometrically at 25°C according to the following protocols: SOD (Beuchamp and Fridovich, 1971), CAT (Beers and Sizer, 1952), AsPO (Nakano and Asada, 1987) and GPO (Dias and Costa, 1983). Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The data are presented as average values of at least 9 replicates, obtained from three independent experiments. The significance of differences was determined by the Student's t-test, and $P \leq 0.05$ and 0.01 were considered significant. The differences of variances were checked by Fisher's F - test.

RESULTS AND DISCUSSION

Pigment content and level of oxidative parameters

The effect of compounds *1* - *8* on chlorophyll and carotenoid contents is illustrated in Fig. 1 and 2. Among the compounds with uncyclic ureido group, compound *1* possessing an unsubstituted 2-pyridyl ring showed the highest chlorophyll retention activity since the chlorophyll content in the senescing leaf segments was higher compared to the control segments (36% at 0.1 mM and 49% at 1 mM after 48 and 72 h incubation, respectively). Compounds *2*, *3* and *4* containing one or two CH_3 groups on the 2-pyridyl ring delayed the chlorophyll loss only after 24 h incubation (13 - 26% Chl over the control level). The 5-CH_3 isomer (*3*) reported by Vassilev et al. (1984) to possess high cytokinin-like activity in the Amaranthus bioassay at an optimum concentration of 0.1 mM showed slight activity in the barley leaf antisenescence bioassay. Compound *5* with one chlorine atom on the 2-pyridyl ring retarded the chlorophyll loss in the aging leaf segments only during the first 24 h (20% and 37% at 0.1 and 1 mM, respectively) while the retarding effect of compound *6* with two chlorine atoms was higher compared with the control during the later aging periods (26% Chl at 0.1 mM and 39% Chl at 1 mM after 48 h incubation; 18% Chl at 0.1 mM and 23% Chl at 1 mM after 72 h incubation over the control).

Cyclization of the ureido group in the imidazolidinone ring (compounds *7* and *8*)

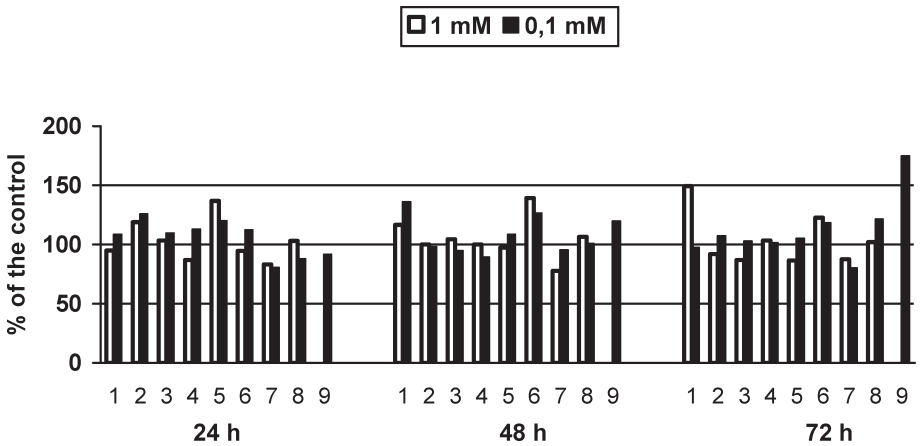


Fig. 1. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in **total chlorophyll** content during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (the average value was 69.73 ± 1.19, 47.0 ± 1.80 and 36.0 ± 1.23 μg Chl.segment^{-1} at 24, 48 and 72h, respectively (n=8)). Chlorophyll content on day zero was 86.964 ± 1.29 μg Chl.segment^{-1}.
Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

resulted in decreased chlorophyll retention activity. This effect was more pronounced after treatment with compound *7* when compared with compound 1. Compound *8* at 0.1 mM showed significant activity only after 72 h incubation (21% Chl over the control).

The chlorophyll retention activity of the more active compounds (*1*, *2*, $4^{0.1}$, *5*, *6*) was higher till the end of the second day and lower at the end of the third aging day in comparison with the standard 4PU (9) (0.1 mM). The activity of 4PU increased with senescence progression (75% Chl over the control at day 3).

In general, the time-course changes in carotenoids content in the treated leaf segments (Fig. 2) was similar to the chloropyll content changes. Carotenoid content was significantly lower compared to the chloropyll content during the respective senescing period in the case of compounds *1*, *5*, *6* applied at a concentration of 1 mM. The content of carotenoids in the compound 6-treated leaf segments increased even during the first 24 h and reached its maximum by the end of the third aging day (1 mM, 29% over the control at 72h) while the retarding effect of this compound on Chl degradation was optimal (39%) after 48h incubation. The compound *8*-treated leaf segments had the highest carotenoids content (~50%) at day 2 of senescence. Treatment of leaf segments with compounds *1* and *8* resulted in an increased carotenoids content after 48 h which was higher compared with the 4PU-treated leaf tissues (28%).

Under dark senescence, the H_2O_2 level in the treated leaf segments was either

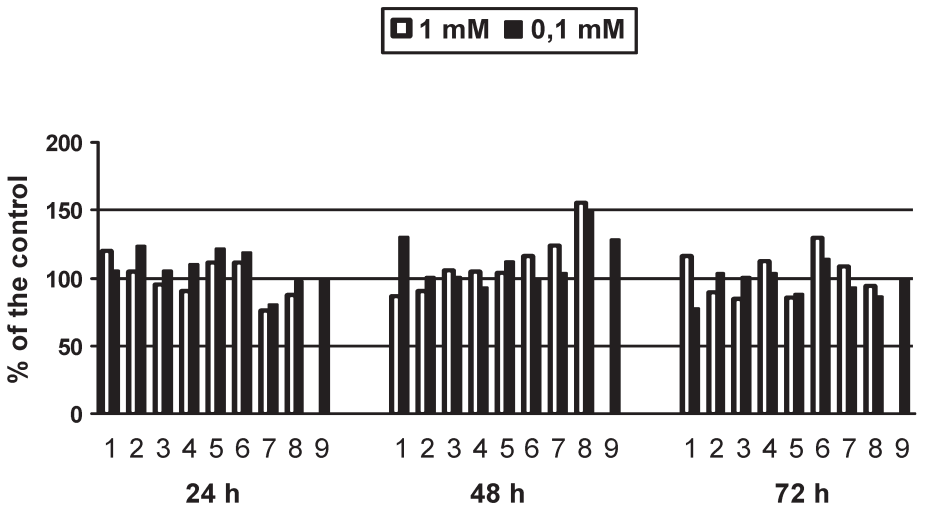


Fig. 2. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in **total carotenoids** content during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (the average value was 4.44±0.37, 5.86 ± 0.43 and 4.86 ± 0.32 μg Carot.segment^{-1} at 24, 48 and 72h, respectively (n=8)). Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

similar or lower compared to the control. A relatively small increase in H_2O_2 content (10-27%) was observed in the *1*, *2*, *5*, *6*-treated leaf segments during different senescing periods (Fig. 3).

The test compounds *2* (0.1 mM), *3* (1 mM), *5* (1 mM), *6* (0.1 and 1 mM) and *8* (0.1 and 1 mM) caused an increase in MDA content of senescing leaf segments during the first 24 h (28-88%), followed by a decrease reaching the control levels. No decline was observed only in the compound *8* (1 mM)-treated segments during the whole senescing period (Fig. 4).

The levels of H_2O_2 and MDA in the 4PU-treated leaf segments were always below the control values during the whole period of senescence.

Antioxidant enzyme activities

The tested compounds *1* - *8* promoted strongly the specific catalase activity in the aging leaf segments (2- to 5-fold higher compared to the control). In all cases, CAT activity of the leaf segments treated with 1 mM of compounds *1* - *8* was higher than that of the 0.1 mM-treated tissues. CAT activity increased even during the first 24 h, reaching to the maximum value at 48 h (at 72 h for *4*- and *7*-treated tissues) and decreased during day 3 of senescence but still the values remained 1.5-2.8-fold higher than the controls (Fig. 6).

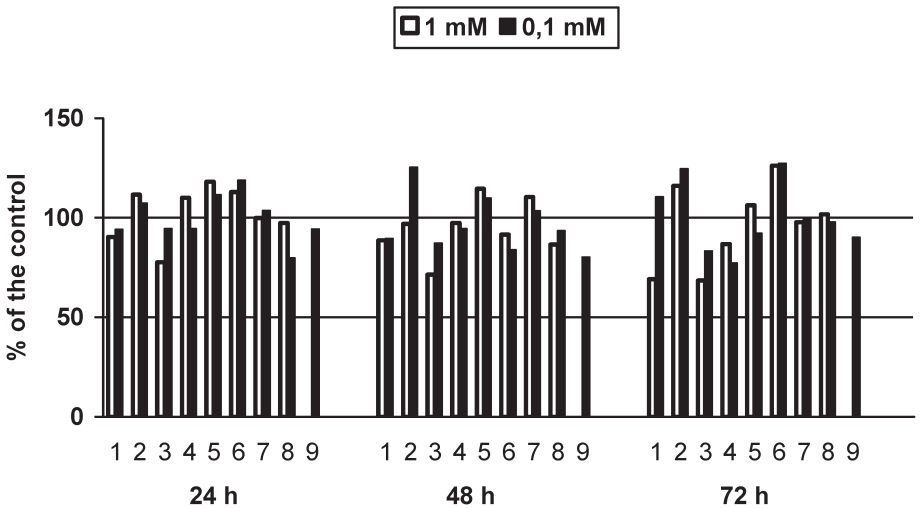


Fig. 3. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in $\mathbf{H_2O_2}$ levels during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (the average value was 23.72±4.45, 24.15 ± 4.79 and 17.87 ± 3.11 nmol H_2O_2.segment^{-1} at 24, 48 and 72h, respectively (n=8)). Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

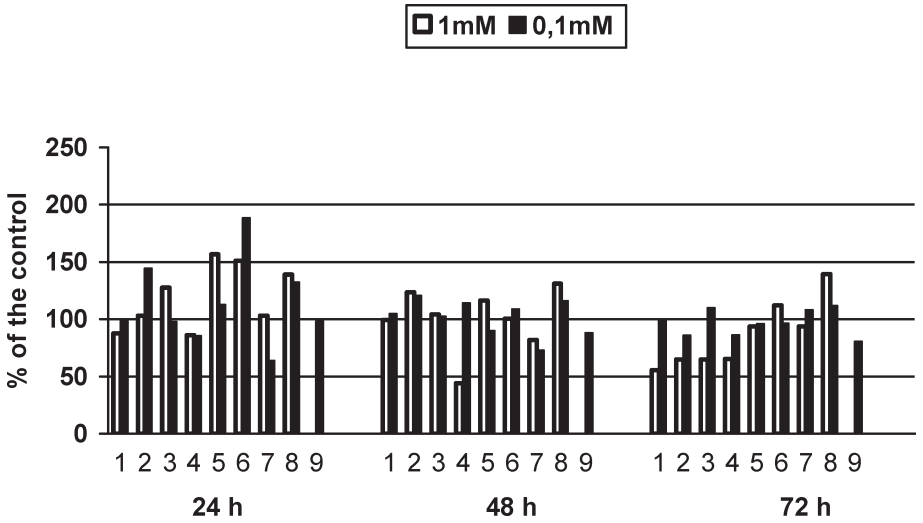


Fig. 4. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in **MDA** levels during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (the average value was 1.41±0.103, 1.28 ± 0.098 and 1.14 ± 0.083 nmol MDA.segment^{-1} at 24, 48 and 72h, respectively (n=8)). Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

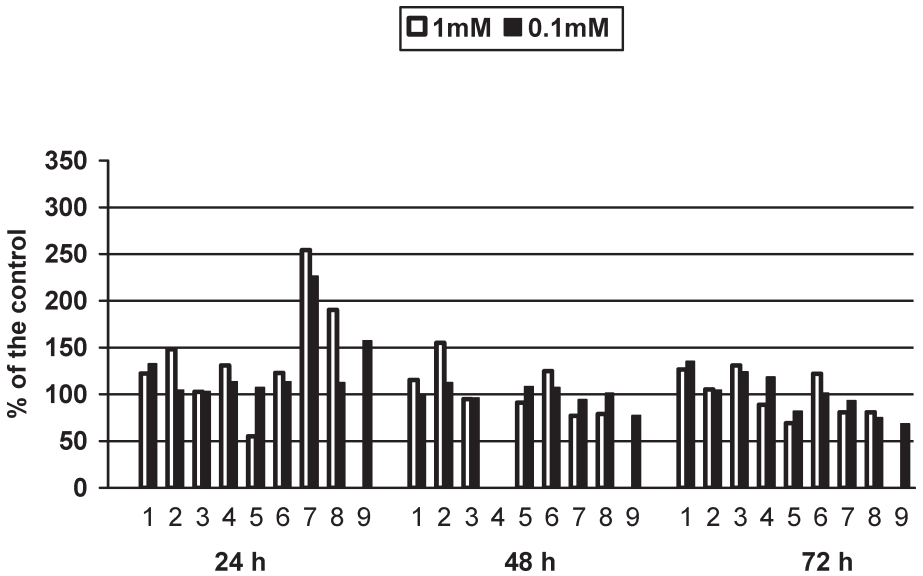


Fig. 5. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in **SOD** activity during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (specific SOD activity was 13.2 ± 0.98, 21.8 ± 2.72 and 33.0 ± 3.70 units.mg $^{-1}$protein at 24, 48 and 72h, respectively (n=4)). Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

Compounds *1*, *2*, *8* and 4PU caused a short-term increase in GPO activity (about 20%) of aging leaf segments. Compound *6* (1 mM) increased significantly the GPO activity at day 2 (52% over the control). Longer exposure to compound *7* (1 mM) led to a moderate increase in the enzyme activity in the aging tissues (30% at days 2 and 3) (Fig. 7).

A high stimulation of AsPO activity (up to 2-fold) was observed in the aging leaf segments treated with unmodified- and Me-substituted-pyridyl derivatives (*1*, *2*, *3*, *4*, *7* and *8*) during the first 24 h. It decreased during the second 24 h and by the end of day 3 it was below the control value (with the exception of the compound *7*-treated tissues). The aging leaf segments treated with Cl-substituted-pyridyl derivatives (*5* and *6*) showed a moderate increase (15-46%) in the enzyme activity during the whole period of senescence. The *5*-treated tissues demonstrated higher AsPO activity at day 2 (31% and 46% at 0.1 and 1.0 mM, respectively), (Fig. 8).

The total SOD activity of the treated aging leaf segments increased during the first 24 h by about 30% after treatment with compounds *1*, *4*, *6* (1 mM) and over 50% wiht *2*, 4PU, *7*, *8* at 1 mM. Further, SOD activity decreased significantly whereas only the *2*-treated segments preserved high activity at 48 h (55%). SOD activity was about 30% over the control in the *1*, *3*, *6* (1 mM)-treated leaf segments during the third day of the aging period. (Fig. 5)

The 4PU-treated leaf segments showed only a short-term increase in the activity of all antioxidative enzymes (SOD, AsPO, CAT, GPO).

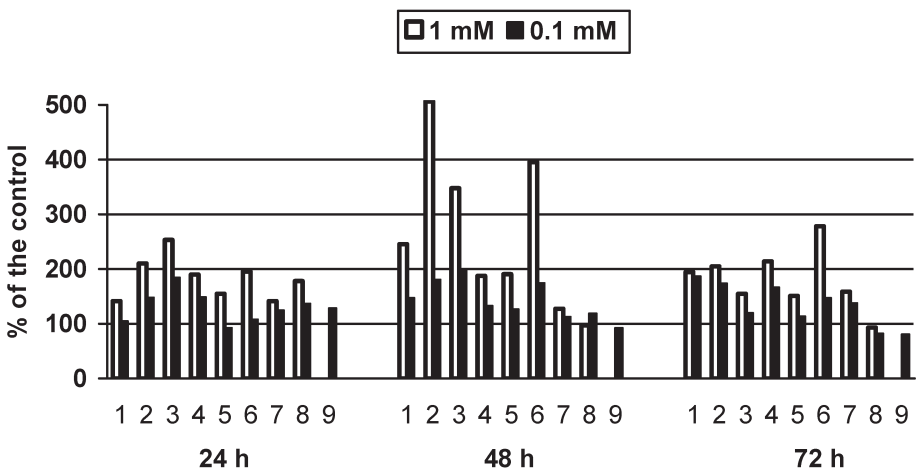


Fig. 6. Effect of compounds *1* – *8* and 4PU (*9*) on the time-dependent changes in **CAT** activity during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (specific CAT activity was 118.0 ± 13.31, 153.0 ± 13.92 and 233.7 ± 17.24 μmol^{1}(H_2O_2).min $^{-1}$.mg $^{-1}$protein at 24, 48 and 72h, respectively (n=6)).
Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

Imbalance between SOD activity and H_2O_2-scavenging activity during dark senescence

Kanazawa et al. (2000) showed that the changes in the activity of the antioxidative enzymes during dark-induced senescence in cucumber cotyledons were generally different from those found during natural senescence. These authors found that the SOD/CAT ratio increased in the late stages of both natural and artificial senescence while SOD/AsPO and SOD/GPO ratios increased during artificial senescence but decreased during natural senescence. These findings suggest that the increase in the SOD/CAT ratio, but not the SOD/peroxidases ratios, could be used as a general index in senescent cells.

Thus, we further examined the changes in the activity ratio of SOD to H_2O_2-scavenging enzymes (catalase and peroxidases) (Table 1).

Our results showed that the activity ratios depended on the type and concentration of the compound tested as well as the stage of senescence. In most cases, the treated leaf segments showed much higher peroxisomal catalase activity and the SOD/CAT ratio was usually less than 1.00. GPO was activated to a lower extent than AsPO and the SOD/GPO ratio was higher than 1.00 during the whole senescence period. An increase in the SOD/AsPO ratio was observed only for the segments treated with *1* (1.48) and *3* (1.64) at day 3, and with *5*-0.1 mM (1.55) at day 1.

Compounds *1* and 4PU showed a similar behaviour during the investigated period, but the antisenescence effect of 4PU was higher than that of compound *1* at the

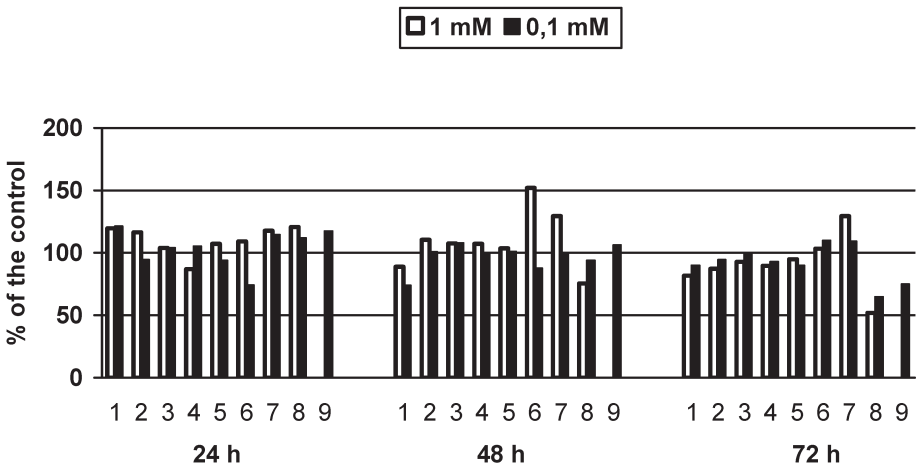


Fig. 7. Effect of compounds *1 – 8* and 4PU (*9*) on the time-dependent changes in **GPO** activity during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (specific GPO activity of the control was 10.38 ± 1.37, 18.39 ± 3.42 and 25.0 ± 3.51 μmol.min $^{-1}$.mg $^{-1}$protein at 24, 48 and 72h, respectively (n=8)).
Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

Table 1. Ratios of the activity of SOD relative to that of H_2O_2-scavenging enzyme during dark-induced senescence of barley leaf segments, 1 day, 2 days and 3 days after.

Variant	SOD / CAT			SOD / AsPO			SOD / GPO		
/Conc.[a)]	1 day	2 day	3 day	1 day	2 day	3 day	1 day	2 day	3 day
1 - 0.1	1.26	0.67	0.72	1.09	0.7	1.35	1.08	1.33	1.50
1.0	0.90	0.47	0.65	0.71	0.74	1.48	1.02	1.30	1.55
2 - 0.1	0.70	0.62	0.60	0.49	0.89	1.03	1.10	1.11	1.13
1.0	0.71	0.31	0.51	0.96	1.28	1.08	1.27	1.40	1.20
3 - 0.1	0.56	0.48	1.03	0.89	0.73	1.16	0.99	0.89	1.25
1.0	0.41	0.27	0.85	0.75	0.75	1.64	0.99	0.88	1.41
4 - 0.1	0.76	-	0.71	0.83	-	1.34	1.07	-	1.27
1.0	0.69	-	0.42	0.92	-	0.83	1.51	-	1.00
5 - 0.1	1.16	0.85	0.72	1.55	0.83	0.91	1.14	1.07	0.91
1.0	0.36	0.48	0.46	0.59	0.62	0.56	0.51	0.88	0.73
6 - 0.1	1.04	0.61	0.69	1.28	1.21	1.17	1.52	1.22	0.92
1.0	0.63	0.32	0.44	1.06	1.09	1.29	1.13	0.82	1.18
7 - 0.1	1.82	0.83	0.68	1.27	0.75	0.69	1.97	0.95	0.85
1.0	1.80	0.60	0.51	1.23	0.49	0.45	2.16	0.59	0.63
8 - 0.1	0.82	0.85	0.91	0.68	0.76	0.84	1.00	1.08	1.16
1.0	1.06	0.81	0.87	0.90	0.58	0.89	1.57	1.05	1.56
4PU[b)]	1.22	0.83	0.85	1.07	0.85	0.87	1.34	0.73	0.92

a) concentration of test compounds in mM; b) the concentration of 4PU is 0.1 mM.

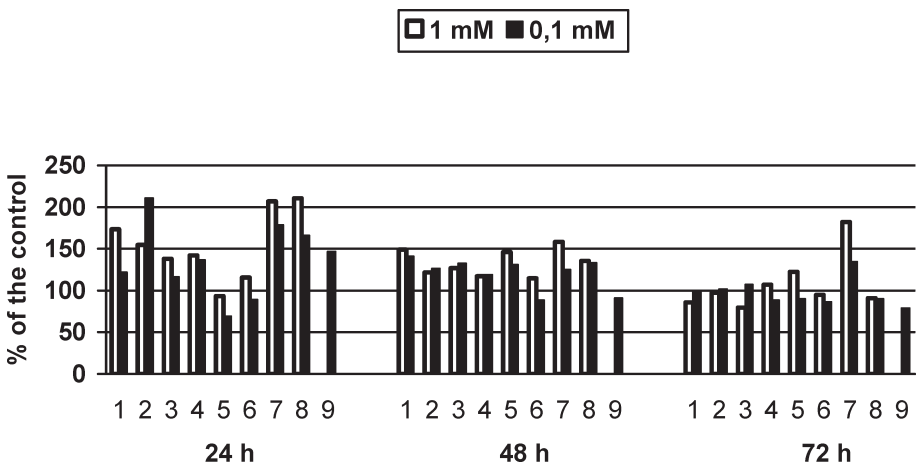


Fig. 8. Effect of compounds *1 – 8* and 4PU (*9*) on time-dependent changes in AsPO activity during dark senescence of barley leaf segments floated on phosphate buffer (control) or on test compound solutions for 24, 48 and 72h. Data are expressed as percent of the control (specific AsPO activity of the control was 1.37 ± 0.18, 1.81 ± 0.25 and 2.76 ± 0.22 μmol.min $^{-1}$.mg $^{-1}$protein at 24, 48 and 72h, respectively (n=8)).
Variant means were significantly different ($p \leq 0.05$) from the control by the Fisher's exact test.

end of day 3. They had equal SOD/CAT and SOD/AsPO activity ratios which decreased with progression of senescence. However, the third SOD/GPO activity ratio declined in the case of 4PU and increased for compound *1* during senescence. Probably, this may explain the different degree of the antisenescence effect of the two compounds.

The values of the three activity ratios for the 6-treated segments decreased up to day 2 and increased weakly during day 3 compared to day 2. Compound *6* manifested chlorophyll retention activity during 2 and 3 days with maximum activity at the end of day 2.

The three activity ratios for the compound *7*-treated segments had significantly high values at day 1 - 1.82, 2.16 and 1.27 for SOD/CAT, SOD/GPO and SOD/AsPO ratios, respectively. It is possible that this imbalance during the onset of senescence may contribute to the lack of chlorophyll retention activity of compound *7*.

In general, it could be suggested that chlorophyll retention activity correlates either with low values of the three activity ratios (< 1) or with the ability of the compounds to maintain the tissues in a reduced state. Therefore, not only SOD/CAT ratio could account for the chlorophyll retention activity of the different compounds. Other activity ratios (SOD/AsPO and SOD/GPO) could also contribute to this effect.

The lack of chlorophyll retention activity for compound *5* (1.0 mM) at days 2 and 3 of senescence was surprising since the values of the three activity ratios remained as low as at day 1. However, SOD activity was significantly inhibited during day 1 and thereby enhanced superoxide radicals can lead to high levels of lipid peroxidation. That is quite possible because the MDA content in compound *5*-treated segments was high (57%). The activities of the three H_2O_2-scavenging enzymes were strongly stimulated (over 2-fold higher than SOD activity) and as a result the values of SOD/CAT, SOD/GPO and SOD/AsPO ratios were around 0.5. Obviously, the balance between the antioxidative enzymes was disturbed in this case.

Therefore, we found that the activities of the antioxidative enzymes and the balance between them are important factors for demonstration of the senescence-retarding effect of the tested urea compounds. The most active compound 4PU, a well known cytokinin and used as a standard, was responsible for the balance between H_2O_2-generating enzyme and H_2O_2-scavenging enzymes in the senescing segments, since the values of the three activity ratios were approximately 0.85 during the second and the third aging days.

Structure – antisenescence activity relationship of compounds *1–8*

Dark-induced senescence of detached leaves or leaf segments is well suited to examine the effect of synthetic compounds on senescence as well as to study various physiological and biochemical changes associated with senescence. Although these induction methods may cause effects similar to those occurring during natural senes-

cence, significant differences have been found with respect to gene expression (Weaver et al., 1998) or to a number of antioxidative enzymes (Kanazawa et al., 2000). In addition, the levels of the physiologically active cytokinin bases, ribosides and nucleotides decreased during both senescence processes. In contrast with natural senescence, the storage cytokinin O-glucosides decreased under dark conditions (Ananieva et al., 2004).

Increased carotenoid content is an important response of cells to senescence or stress. Carotenoids are natural antioxidants that act as defence agents to protect chlorophyll against the AOS damages.

The barley leaf antisenescence bioassay is sensitive to structural variation of tested compounds. In fact, the same bioassay with wheat leaves has been used to develop quantitative structure-activity relationship for the antisenescence activity of a number of N-(2-substituted-4-pyridinyl N-oxide)-N'-arylureas (Henrie et al., 1988) and 4,5-disubstituted imidazoles (Cavender et al., 1988).

We found that among the compounds displaying chlorophyll retention activity during different stages of senescence (*1*, *2*, *5*, *6*, *8*), four of them contain an uncyclic ureido group similar to the 4PU standard and one-cyclic ureido group.

The leaf segments treated with compounds *7* and *8* containing cyclic ureido group showed less catalase and higher peroxidases activities at days 2 and 3 of senescence than those treated with compounds *1- 6* containing uncyclic ureido group. In comparison with compounds *1- 6*, treatment with compound *7* led to an increase in carotenoid content (by 24%) while compound *8* showed a significant increase (to 50%). Compound *1* increased carotenoid content by 31% only on the 2nd day. Compound *1* having an unsubstituted pyridine ring as well as compound *7* showed the highest antisenescence effect whereas compound *7* had no activity. Therefore, the cyclization of the ureido group in an imidazolidinone ring modified the mode of the antisenescence action compared to that of the compounds with uncyclic ureido group.

Among compounds *2*, *3* and *4*, possessing one or two CH_3 groups (an electron-donating group), the most active member of this series was the 4-methyl isomer (*2*). The latter had a short-term protecting effect on chlorophyll degradation in the senescent segments. This compound caused a moderate increase in carotenoids content (23%) and a higher increase in CAT and especially AsPO activities on day 1 of senescence.

Both compounds *5* and *6*, containing one or two electron-withdrawing Cl atoms on the pyridine ring were the most active compounds among all tested compounds. Compound *6* with two Cl atoms had a long-term protecting effect on chlorophyll degradation while compound *5* with one Cl atom showed a short-term effect. Carotenoids content in senescing segments exceeded the control by 29% at day 3 upon compound *6*-treatment, and by 21% at day 1 in the case of compound *5*-treatment. The onset of senescence in the compound *5*- and compound *6*-treated segments was accompanied by a slight increase in H_2O_2 content and a significant increase in MDA

levels. Catalase activity was 3-fold higher and peroxidases activities were 2-fold higher than the SOD activity in the compound *5*-treated segments on the first day. Higher H_2O_2-scavenging activity was also observed in the compound *6*-treated segments - catalase activity increased 3-fold compared to SOD activity while the peroxidase activity balanced the SOD activity after day 1. Evidently, the highly activated state of the H_2O_2-scavenging enzymes contributed to the elimination of the consequences of the high extent of lipid peroxidation (51-57% over the control) occurring in the tissues during day 1 of senescence.

Our results indicated that the presence of CH_3 group(s) (compounds *2*, *3*, *4*), the steric size of which is considered to be similar to that of a Cl atom (compounds *5*, *6*) did not provide the same level of antisenescence activity. A similar trend was found for the herbicidal activity of 4-substituted 4-oxazolin-2-one compounds (Kudo et al., 1998).

The high antisenescence effect of 4PU could be accounted for by its cytokinin activity. The exogenous cytokinin can probably compensate the declined levels of the endogenous physiologically active cytokinins in senescing tissues. The cytokinin-like activity demonstrated by compound *1* in this and other cytokinin bioassays (Vassilev et al., 1984) suggests that compounds *1*- *8* represent a new class of cytokinin mimics.

CONCLUSIONS

The results presented here, indicated that the response of senescing leaf tissues was differently affected by the tested compounds. Compounds *1*, *6* and 4PU had a long-term protecting effect on chlorophyll degradation whereas compounds *2* and *5* demonstrated a short-term effect. In addition, compound *8* showed activity only on day 3. This effect was mediated by strongly increased H_2O_2-scavenging enzymes activities, the peroxisomal catalase activity being mainly affected. The cyclization of ureido group in an imidazolidinone ring modified the level and mode of antisenescence action compared to compounds with an uncyclic ureido group. It is difficult for us to explain the mechanism of the antisenescence action of the tested compounds since the cellular changes associated with senescence are a more complex situation that is not completely understood.

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