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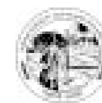

SHORT COMMUNICATION

RETARDATION OF SENESCENCE OF DETACHED LEAVES BY METHYLGLYOXAL

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In the present study the effect of methylglyoxal on detached leaf senescence of six plant species, viz. carrot, solanum, papaya, basil, ribbed gourd and rice was investigated. When the detached leaves were kept for a fixed time period, the methylglyoxal treated leaves could significantly arrest senescence in all the plant species tested, which was evident by the retention of structural integrity as well as of chlorophyll and protein contents. However, pyruvate had a very mild effect though it has structural similarity with methylglyoxal. Comparison of activity profiles of peroxidase, glyoxalase I and glucose-6-phosphate dehydrogenase in freshly excised, senesced and methylglyoxal treated leaves showed that the enzyme activities of methylglyoxal treated leaves closely resembled that of the freshly excised leaves.

Key words: **Kinetin, leaf senescence, methylglyoxal, pyruvate**

Senescence in plant is the final event in growth and development which is highly regulated, genetically programmed process dominated by catabolic pathways and ultimately leads to the death of a particular organ or whole plant. The phenomenon is associated with structural, biochemical and molecular changes that bear the hallmarks of programmed cell death (Nooden 1988). Situations of abiotic and biotic stresses as well as plant hormones play an important regulatory role in promoting senescence and programmed cell death (Thimann 1980, Nooden 1988, Guo and Gan 2005). From a researcher's viewpoint the study of senescence of leaves has a great advantage over study of the senescence of any other plant parts (Thimann 1980). Senescence in detached leaves can be rapidly induced in a number of ways and this induced senescence does not differ much from natural senescence. Leaf senescence brings about degradation of photosynthetic pigments, proteins, lipids, nucleic acids and other essential cellular metabolites as well as extensive disruption of internal structure and cellular organelles, which are highly controlled by genes (Buchanan-Wollaston 1997, Thomas and Howarth 2000, Eckhardt *et al.* 2004)

Cytokinins are involved in the differentiation of calluses for the regeneration of plantlets through organogenesis as well as in the retardation of leaf senescence (Skoog and Miller 1957, Skoog 1973, Kim *et al.* 2006). Recently, it has been observed that methylglyoxal (MG), a normal metabolite can completely replace kinetin, a cytokinin to initiate differentiation of plantlets from calluses of *Solanum nigrum* and *Daucus carota* (Roy *et al.* 2004). Methylglyoxal has a strong anticancer property (Szent-Györgyi 1979, Ray *et al.* 1991), similar to cytokinins (Honma and Ishii 2002). Interestingly, despite their structural dissimilarity, the effect of kinetin and MG are functionally similar. In the present study an attempt has been made to investigate the role of MG in arresting senescence in detached leaves.

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The experiment was carried out with leaves of six plant species, namely carrot (*Daucus carota* L.), solanum (*Solanum nigrum* L.), basil (*Ocimum sanctum* L.), papaya (*Carica papaya* L.), ribbed gourd (*Luffa acutangula* L.), and rice (*Oryza sativa* L.). Whole leaf laminas of carrot, solanum, and basil were taken. Whereas 2 cm x 2 cm leaf pieces cut from the leaf laminas of papaya and ribbed gourd were used. In case of rice, pieces of leaf blades (approximately 8 cm from tip) were taken.

Glass distilled water was taken in 100 ml conical flasks, each containing 30 ml of water and closed with cotton plugs and sterilized by autoclaving. Required aliquots of filter-sterilized MG or pyruvate from 100 mM stock solution were then added to each flask aseptically to obtain the desired concentration of the compounds. One set of 'untreated' flasks, i.e. without the addition of any test compound and one set of flask treated with 5 ppm kinetin were maintained in parallel for comparison with the treated samples of carrot and solanum.

Mature leaves of carrot, solanum and basil or leaf pieces of papaya, ribbed gourd and rice were surface sterilized by 0.1% $HgCl_2$ for 3-4 minutes and washed repeatedly with autoclaved distilled water. The leaf samples were transferred to each flask in the laminar hood under aseptic conditions. The flasks were then kept in dark at 24±2°C to induce senescence. Observations from the point of appearance of visible signs of senescence were noted. Freshly excised leaves were considered as control samples in all the experiments. Each experiment was repeated at least four times.

Using the method of Arnon (1949) chlorophyll was estimated in 80% acetone extracts by measuring the optical density at 652 nm. For protein estimation, protein precipitated with trichloroacetic acid (5%) was dissolved in 0.5 N NaOH and estimated as described by Layne (1957) with BSA as a standard.

The crude enzyme fractions were prepared from different plant leaves by crushing them in mortar and pestle in 0.2 M sodium phosphate buffer, pH 6.8, containing 1 mM EDTA. These were then homogenized in a glass-teflon Potter-Elvehjem homogenizer and centrifuged at 10,000 g for 15 minutes. The pellets were discarded and the supernatants were used as the crude enzyme preparations.

Peroxidase was assayed by measuring increase in absorbance at 460 nm due to the formation of oxidized o-dianisidine (Chen and Asada 1989). Glyoxalase I was assayed by measuring the change in absorbance at 240 nm due to formation of the thioester lactoylglutathione from GSH and MG (Mannervik *et al*. 1982). Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically by monitoring the rate of NADPH formation at 340 nm in the presence of glucose-6-phosphate and NADP (Steinbach *et al*. 1982). The specific activities for all the enzymes were expressed as unit per mg of protein. We measured chlorophyll and protein contents and assayed the enzyme activities at that time when the visible signs of senescence of the untreated leaves became prominent.

Detached leaves of all the plant species incubated in the dark in absence of MG or kinetin showed signs of senescence. The progress of senescence was accompanied by brown or dark brown patches in all the leaf samples of carrot, solanum, basil, papaya, ribbed gourd and rice. Visible signs of disintegration were also more or less common for all the leaf samples except for rice, which showed only yellowing of leaves. Leaf samples incubated in the dark in presence of MG however, retained their green appearance with little or negligible brown colour. These leaves also retained their structural integrity as compared to the senesced leaves.

It was observed that the decrease in chlorophyll contents was maximum in the untreated leaves as compared to the MG treated leaves, which showed very little chlorophyll loss. Moreover this loss was similar to that of kinetin treated leaves wherever tested (Table 1). A comparison of the amount of protein present in the freshly excised leaves (control), treated and untreated leaves indicated a similar pattern to that of chlorophyll retention (Table 1).

Visible signs of chlorophyll loss and browning appeared in the leaf pieces of ribbed gourd after 72 hours of incubation. Leaf pieces kept in 0.25 mM and 0.5 mM MG did not show any such symptoms of senescence. After 96 hours, the untreated leaf pieces showed much

Table 1. Effect of different treatments on chlorophyll and protein contents in different leaves

Plant species/ Treatment	Chlorophyll (μg/g fw)	Protein (mg/g fw)
Carrot (48 h)		
None (fresh leaves)	309 ± 2.5 (100)	5.37 ± 1.1 (100)
None (untreated)	84± 1.1 (27)	0.75 ± 0.04 (14)
MG (0.25 mM)	186 ± 2.3 (60)	1.42 ± 0.04 (26)
MG (0.5 mM)	272 ± 1.2 (88)	3.61 ± 0.03 (67)
MG (1.0 mM)	81 ± 1.1 (26)	0.62 ± 0.01 (11)
Kinetin (5 ppm)	267 ± 2.1 (86)	3.87 ± 0.2 (72)
Solanum (96 h)		
None (fresh leaves)	325 ± 4.2 (100)	6.39 ± 0.33 (100)
None (untreated)	92 ± 1.3 (28)	0.83 ± 0.02 (13)
MG (0.25 mM)	271 ± 2.3 (83)	3.82 ± 0.07 (60)
Kinetin (5 ppm)	280 ± 1.9 (86)	4.51 ± 0.04 (71)
Pyruvate (0.25 mM)	10 ± 1.1 (3)	1.1 ± 0.02 (20.5)
Basil (144 h)		
None (fresh leaves)	781 ± 17.10 (100)	26.19 ± 2.16 (100)
None (untreated)	353 ± 7.22 (45)	10.09 ± 0.05 (39)
MG (0.1 mM)	382 ± 8.37 (49)	12.27 ± 0.06 (46)
MG (0.25 mM)	697 ± 15.54 (89)	22.8 ± 0.05 (68)
MG (0.5 mM)	657 ± 16.32 (84)	20.55 ± 0.07 (78)
MG (1 mM)	279 ± 10.44 (36)	8.63 ± 0.06 (33)
Pyruvate (0.1 mM)	507 ± 11.45 (65)	13.76 ± 0.04 (53)
Pyruvate (0.25 mM)	373 ± 8.12 (48)	11.25 ± 0.05 (43)
Pyruvate (0.5 mM)	287 ± 7.23 (37)	8.78 ± 0.06 (34)
Papaya (144 h)		
None (fresh leaves)	2520± 97.12 (100)	18.15 ± .08 (100)
None (untreated)	1352 ± 72.29 (59)	9.12 ± 0.05 (50)
MG (0.5 mM)	2150 ± 78.46 (85)	13.65 ± 0.03 (75)
MG (1.0 mM)	2284 ± 91.06 (94)	16.11± 0.07 (89)
Pyruvate (1.0 mM)	1248 ± 55.24 (50)	6.81 ± 0.02 (38)
Ribbed gourd (120 h)		
None (fresh leaves)	1196 ± 85.09 (100)	29.49 ± 0.28 (100)
None (untreated)	527 ± 32.47 (44)	11.47 ± 0.13 (38)
MG (0.25 mM)	625 ± 17.84 (52)	18.28 ± 0.14 (62)
MG (0.5 mM)	945 ± 42.29 (78)	23.12 ± 0.26 (78)
Rice (120 h)		
None (fresh leaves)	856 ± 19.09 (100)	37.26 ± 2.10 (100)
None (untreated)	35 ± 2.12 (4)	3.71 ± 0.03 (10)
MG (5 mM)	282 ± 7.35 (33)	4.58 ± 0.02 (12)
MG (10 mM)	433 ± 12.09 (11)	4.87 ± 0.03 (13)

MG: methylglyoxal, Values in parenthesis indicate retention (%), Chlorophyll and protein contents were estimated at indicated time (h).

more browning and also signs of structural disintegration. The leaf samples maintained in presence of 0.25 mM MG had started undergoing chlorophyll loss with hardly any sign of tissue disintegration during this time period. Leaves kept in 0.5 mM MG remained intact and nicely green. Signs of senescence became more prominent after 120 hours with further loss of chlorophyll and structural disintegration in untreated leaf pieces of ribbed gourd. Brown colour indicating loss of chlorophyll was increased in leaves kept in 0.25 mM MG after 120 hours. The leaf samples were also structurally disintegrated to some extent during this period. The leaf samples kept in 0.5 mM MG looked much fresh and intact with limited loss of chlorophyll (Fig. 1). Maximum protective effect of MG vis-à-vis retardation of senescence was, thus, observed in case of 0.5 mM MG treatment.

Since pyruvate is also an important metabolite in the glycolytic pathway and is structurally similar to MG, so

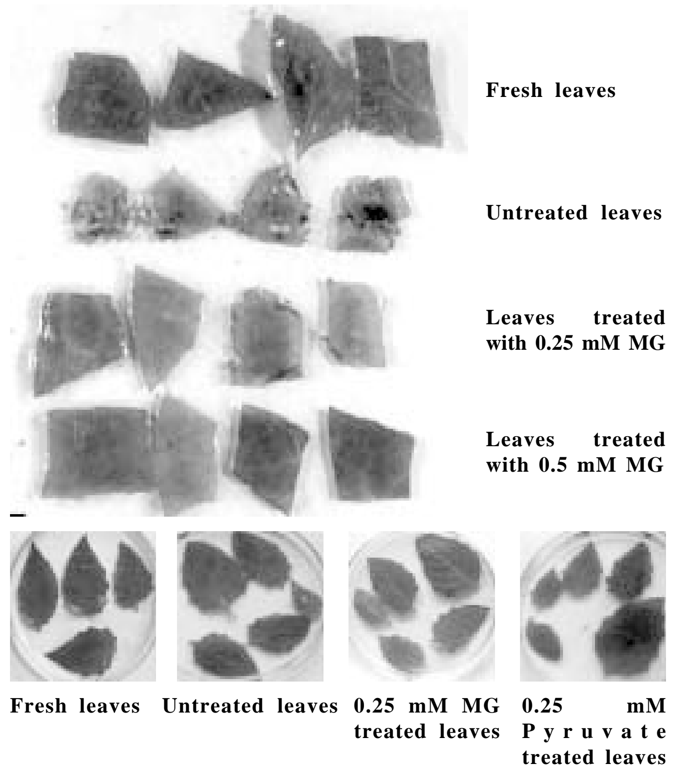

Fig. 1. Effect of different concentrations of methylglyoxal and pyruvate on senescence of detached leaves of ribbed gourd and solanum

we compared the effect of MG to that of pyruvate on senescence of solanum, basil and papaya. The optimum concentration for MG that was effective in retarding senescence of detached solanum leaves was 0.25 mM, while 0.25 mM pyruvate failed to prevent senescence of solanum leaves (Fig. 1). Similar results were obtained when detached leaves of basil and papaya were kept with graded concentrations of pyruvate (0.1-1 mM). It was observed that pyruvate at these concentrations could not retain the structural integrity and chlorophyll contents of these leaves to any significant extent (Table 1). However, pyruvate (0.1 mM) could marginally retain these features in detached basil leaves. These results indicate that pyruvate is devoid of any significant antisenescence effect. Therefore, the antisenescence effect of MG is presumed not to be merely by providing carbon and/or energy source.

The effect of various concentrations of MG on senescence of detached leaves of carrot, basil, papaya and rice was investigated in a manner similar to that of ribbed gourd and solanum as described above. In these cases also, MG could significantly protect the leaves from senescence. The optimum concentrations of MG for carrot, basil, papaya and rice were found to be 0.5, 0.25, 1.0 and 10 mM respectively. The chlorophyll as well as the structural integrity of MG-treated leaves of these plant species was retained up to 48, 144, 144 and 120 hours respectively. In contrast the untreated leaves suffered chlorophyll loss and were disintegrated much earlier.

Three enzymes, viz. peroxidase (Px), glyoxalase I and glucose-6-phosphate dehydrogenase were assayed in the leaves during senescence at the same time when chlorophyll and protein were measured. The results are depicted in Table 2. Large increases in the specific activity of Px were observed in the untreated senesced leaves than freshly excised leaves. The increase in the enzyme activity was however much less in detached leaves kept in the dark in presence of MG. Untreated, senesced leaves of ribbed gourd recorded 280% increase in the specific activity of Px compared to control. The increase was 200% and only 40% in presence of 0.25 mM and 0.5 mM MG respectively. This trend was persistent in all the plant species tested except solanum where MG failed to minimize the increased level of Px (360% as compared to 300% in senesced leaves).

Activities of glyoxalase I were assayed in the leaves of solanum, papaya, ribbed gourd and basil. Glyoxalase I activity was drastically reduced in all the senesced leaves as compared to that of the freshly excised leaves. The maximum reduction was for papaya (92%), followed by ribbed gourd (73%) and basil (60%). For solanum, the activity was not detectable. The optimum concentrations of MG that could retain chlorophyll and protein were also able to retain the enzyme activity of glyoxalase I. The retention was 82%, 84%, 66% and 71% for solanum (0.25 mM, MG), papaya (1 mM, MG), ribbed gourd (0.5 mM, MG) and basil (0.5 mM, MG) respectively.

Glucose-6-phosphate dehydrogenase was assayed in solanum, papaya and ribbed gourd and the activities of different leaves did not indicate any pattern in contrast to the other two enzymes tested. In case of solanum and ribbed gourd, the activity was drastically reduced in senesced leaves while, it was significantly retained in MG (0.25 mM and 0.5 mM) treated leaves (92% and 76% respectively). In case of papaya, both senesced and MG-treated (1 mM) leaves showed rise in the activity of the enzyme (220% and 240%, respectively).

The results obtained clearly indicate that MG could significantly arrest senescence in detached leaves of the six species tested. Similar to the effects of kinetin, chlorophyll breakdown and proteolysis were significantly prevented by MG, concomitant with the retention of cellular integrity of the detached leaves. However, in comparison to kinetin, higher concentration of MG was needed to observe the desired effects.

The activity profiles of the enzymes studied in this work are also to a great extent consistent with the antisenescence effect of methylglyoxal. It has been reported by several workers that Px is increased during senescence (Kar and Mishra 1976, Miidla *et al*. 1987, Krishnan *et al*. 1999, Veljovic-Jovanovic *et al*. 2006). Accumulation ROS during senescence (Leshem 1981, Mahalingam and Fedoroff 2003) could act as a trigger for Px activity (Polle 1997, Bohnert and Sheveleva 1998),

Table 2. Effect of different treatments on activity profile of peroxidase, glyoxalase I and glucose-6-phosphate dehydrogenase

Leaves / enzymes	Specific activities of the enzymes		
	Fresh leaves	Untreated leaves	MG (mM) treated leaves
Peroxidase			
Solanum	0.69 ± 0.02	2.76 ± 0.03	3.16±0.04 (0.25)
Papaya	2.5 ± 0.01	12.5 ± 0.4	8.4±0.02 (0.5)
			7.5 ± 0.03 (1.0)
Ribbed gourd	5.0 ± 1	19.0 ± 2	14.7±1 (0.2)
			7.0 ± 1 (0.5)
Basil	0.1 ± 0.01	0.15 ± 0.02	0.11± 0.01 (0.5)
Rice	4.2 ± 0.8	10.5 ± 0.9	3.7 ± 0.2 (10)
Glyoxalase I			
Solanum	0.085 ± 0.005	n.d.	0.07 ± 0.003 (0.25)
Papaya	0.38 ± 0.002	0.03 ± 0.001	0.26±0.001 (0.5)
			0.32 ± 0.002 (1.0)
Ribbed gourd	0.044 ± 0.007	0.012 ± 0.003	0.019±0.002 (0.25)
			0.029 ± 0.001 (0.5)
Basil	0.082 ± 0.006	0.033 ± 0.001	0.058 ± 0.003 (0.5)
Glucose-6-phosphate dehydrogenase			
Solanum	0.012 ± 0.001	n.d.	0.011 ± 0.001 (0.25)
Papaya	0.025 ± 0.002	0.08 ± 0.001	0.081±0.002 (0.5)
			0.085 ± 0.003 (1.0)
Ribbed gourd	0.021 ± 0.002	0.006 ± 0.001	0.0075±0.001 (0.25)
			0.016 ± 0.002 (0.5)

n.d. - not detectable, One unit of the activity of the enzyme is defined as the unit required to utilize/ produce 1µmol of substrate or product per minute. The specific activity of the enzyme is defined as the unit of activity per mg protein. The values in the parentheses indicate concentration of MG in mM.

which in turn could remove hydrogen peroxide. In the present work we have also observed remarkable increase in the activity of Px in the detached leaf samples incubated in water. The extent of increase was, however, much lower in leaves in presence of MG. This indicates that MG can delay the onset of oxidative burst by prolonging the life of leaves under excised condition and keeping Px close to control levels. In case of solanum, the level of Px was more or less similar in untreated and MG-treated leaves. The reason for this phenomenon is difficult to explain. The actual level of Px in MG-treated leaves was found to be well below the corresponding values for most of the plant species tested. Apart from the comparative study, the actual levels of enzyme activity also become important while discussing such wide varieties of plant species.

Glyoxalase I activity has been reported to decrease with age (Armeni *et al*. 1998, Kuhla *et al*. 2007). Recently, it has been found that glyoxalase system is involved in imparting resistance to crop plants under abiotic stress conditions such as salinity, drought, cold, osmotic stress etc (Espartero 1995, Singla-Pareek *et al*. 2003, Yadav *et al*. 2005). Although the physiological significance of the glyoxalase system has not been clearly defined in plants, but it is assumed as a “marker for cell growth and division” (Paulus *et al*. 1993). In the present study a drastic reduction in the activity of

glyoxalase I during detached leaf senescence was observed. This reduction in the activity might have resulted from the cellular disintegration of the senesced leaf samples tested. Application of MG to the detached leaves had not only delayed the senescence process but also prevented the loss of activity owing to the retention of cellular integrity by MG. In the present study, the concentrations of MG used for all plant species (except rice) were more or less in the same range as reported in different plant species (Yadav *et al.* 2005). The higher concentration of MG (10 mM) to arrest senescence in rice might be due to the fact that the texture of rice leaves is much tough and rigid, for which penetration of MG was not that easy as that of other plant species.

In plants, glucose-6-phosphate dehydrogenase isozymes are present in cytosol and plastids (Hauschild and Schaewen 2003). It had been reported that different stimuli differentially affect these two isozymes. The differential effect of MG on glucose-6-phosphate dehydrogenase of different plant species might be due to relative levels as well as sub-cellular localization of the enzyme in a particular plant species (Hauschild and Schaewen 2003, Palma *et al*. 2006). Several studies suggest that leaf senescence is regulated by the coordinated expression of specific genes, many of which are senescence associated (Buchanan-Wallaston 1997). So, it is quite possible that MG may have some effect in the expression of at least some of these genes. Senescence is associated with the increase in free radicals and subsequent lipid peroxidation. Methylglyoxal has the potential to affect active state of macromolecules (Szent-Györgyi 1979) and can also scavenge these senescence-enhancing events. In addition, methylglyoxal might also inhibit the chlorophyll catabolyzing enzymes and can increase chlorophyll retention in the leaves by facilitating the binding of chlorophyll with chlorophyll-binding proteins.

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