Indian J. Plant Physiol., Vol. 14, No. 3, (N.S.) pp. 229-238 (July-Sept., 2009)

COMBINED APPLICATION OF SERINE AND KINETIN REGULATES LEAF DISC SENESCENCE IN *SPINACIA OLERACEA* L.

D. MUKHERJEE* AND S. JAKHAR

Department of Botany, Kurukshetra University, Kurukshetra-136 119, India

Received on 5 June, 2008, Revised on 20 Sept., 2009

SUMMARY

Investigation was carried out to know whether serine can delay senescence and if it has any additive effect when applied in combination with kinetin (Kn) to leaf discs of senescent spinach leaves. The selected concentrations of serine and kinetin were 5 mM and 0.38 mM respectively and experiment was conducted under either 8 mmol photon m^{-2} s^{-1} or in dark for 6 days. The amount of protein along with pigments declined with time in untreated leaf discs and serine alone and in combination with Kn inhibited the degradation by controlling protease activity in light and dark. Progress of leaf disc senescence was characterized by an increase in total free amino acids; the increment was much greater in dark than in light. Protein bound amino acid content was also declined in control. However, serine individually and in presence of Kn brought down the accumulation of free amino acids and helped to retain bound amino acids significantly. This study reveals additive effect of serine with kinetin in delaying leaf senescence.

Key words: **Free and protein bound amino acids, kinetin, leaf discs, senescence, serine**

INTRODUCTION

Senescence is an important developmental process in plants which is highly regulated and genetically controlled that leads to whole plant, organ, tissue or cell death (Quirino *et al*. 2000, Chandlee 2001, Wilhelmova *et al*. 2004). During leaf senescence the reserved food material built up by the plant during its growth phase is mobilized into younger tissue (eg. growing leaves, flowers, fruits, and developing seeds) to prepare for the next generation and / or to allow plant survival under adverse environmental condition (Gan and Amasino 1995, Buchanan *et al*. 2000). The process of senescence has been studied in detached leaves, leaf discs, and attached leaves (Spencer and Titus 1972). The breakdown of chloroplast pigments results in yellowing of leaves, which is the most obvious symptom of leaf senescence catalyzed by numerous enzymes (Hortensteiner 2004, Beale 2005). During leaf disc senescence, these rapid changes occur immediately resulting in an accumulation of amino acids and a great reduction in protein and RNA synthesis (Wollgiehn 1967, Beevers 1968). Old leaves are able to synthesize protein almost as well as young leaves (Simon 1967) and do not accumulate amino acids until quite late in the senescence process (Plaisted 1958). Numerous reports show increased activity of proteases and protein degradation (Mukherjee and Rao 1993, Senyuk *et al*. 1998, Ramakrishna and Ramakrishna Rao 2005). There are different factors which initiate and / or modulate senescence rate both in attached and detached plant systems. Amongst these factors, there is a strong evidence for light to play an important role (Biswal and Biswal 1984, Ono *et al*. 2001).

*Corresponding author, E-mail: dibumukherjee@gmail.com

Very little work has been done so far to record changes in free amino acids and protein bound amino acids during senescence and to know their role in the regulation of senescence. One of the steps in plant senescence seems to be increased proteolysis and amino acid interconversion (Hurst and Clark 1993). Derbali *et al*. (1998) reported about 2-fold increment in total free amino acid content when broccoli plantlets were stored 8 days in anaerobic or aerobic conditions at 20 ^{0}C. However, quantities of individual amino acids were not measured by them. Role of amino acids to control senescence was observed in pea seedlings and oat leaves (Shibaoka and Thimann 1970, Martin and Thimann 1972). These workers have reported antagonistic behaviour between kinetin (Kn) and L-serine in senescence regulation. Exogenous application of several cytokinins such as kinetin, zeatin and benzylaminopurine (BA) has delayed the onset of senescence in leaves of tobacco (Singh *et al*. 1992). Reports do exist where DL-serine exhibited synergistic effect when applied along with kinetin in the regulation of senescence (Mukherjee and Ponmeni 2000).

Attempts have been made, therefore, in the present study to see how DL-serine and kinetin individually and in combination affect the protein loss, protease activity and amino acid changes during senescence in both light and dark, the latter is widely used as inducer of senescence for excised leaves (Pastori and del Rio 1997, Weaver *et al*. 1998).

MATERIALS AND METHODS

Leaf discs were cut from the senescent leaves of *Spinacia oleracea* L.cv. S-23 and were floated on 5 ml each of double distilled water (DDW)/ buffer (pH.5.2) / DL-serine (5 mM / kinetin (Kn, 0.38 μM) and a combination of both DL-serine and Kn placed in Corning Petri dishes of 9 cm diameter. Each Petri dish was lined with Whatman No.1 filter paper having 40 leaf discs, each one having an area of 0.6 cm^2 size. The samples were collected at 0, 2, 4 and 6-day to estimate changes in chloroplast pigments, protein and protease activity whereas amino acids were measured at 0, 2, and 6-day. The entire experiment was conducted both in dark and light in a growth chamber provided with a fluorescent tube of 8 μmol photon m^{-2} s^{-1} at two different times.

Estimation of chlorophylls and carotenoids : Leaf sample (200 mg) was grinded in chilled 80% acetone (AR grade) with a pinch of $CaCO_3$ and centrifuged at 2000 rpm for 5 min. The absorbance was recorded at 645 and 663 nm in case of chlorophylls and at 480 and 510 nm for carotenoids using UV-Visible Specord-205 (Analytic-Jena, Germany) spectrophotometer. The amount of chlorophyll was estimated using the formula of Arnon (1949) while that of carotenoids was calculated by the method of Holden (1965).

Protein estimation and measurement of protease activity : The total soluble proteins were estimated by the method of Bradford (1976) using Coomassie Brilliant Blue G-250. For protein extraction, 50 mg of fresh leaf tissue (earlier stored in a freezer) was homogenized in 80% ethanol after boiling in the same ethanol for 15-min. This was centrifuged at 10,000 g for 5 min. The residue was re-extracted with 5% perchloric acid followed by centrifugation at 10,000 g for 5 min. The residue was then diluted in 1N NaOH (5 ml) and kept in warm water (40-50 ^{0}C) with regular shaking for 30 min. The clear supernatant was used for further analysis.

The procedure for protease extraction was a slight modification of that described by Yomo and Varner (1973). The samples weighing 200 mg each were homogenized in 100 mM phosphate buffer of pH 7.2 and the final volume was raised to 25 ml. 1% casein (Sigma-make, USA) was prepared by dissolving 1g casein in 2 ml of 0.1 N NaOH and then final volume was made to 100 ml with 100 mM phosphate buffer of pH 7.6. To 1ml of casein, 1ml of enzyme extract was added and incubated for 3 h at 37 ^{0}C. The pH of the reaction mixture was 7.5. After incubation, 1ml of 16 % TCA was added to all reaction sets and centrifuged them. The residue was discarded. Out of the resultant 3 ml filtrate, 0.5 ml was taken to estimate protease activity by ninhydrin method, originally described by Yemm and Cocking (1955) and modified by Reimerdes and Klostermeyer (1976). The activity was expressed in μM lysine equivalent mg^{-100} dry wt. h^{-1}.

Extraction and estimation of free and protein-bound amino acids: The method of Steward *et al*. (1954) was followed with a slight modification for the extraction of free and protein bound amino acids. For each sample, 2 g of leaves was homogenized using a mortar and pestle in 60 m1 of 80 % ethyl alcohol and was centrifuged at 10,000 g for 10 min. Supernatant was collected in evaporating dish. The procedure was repeated thrice with the residue to guarantee the maximum recovery of amino acids and amides. The solution was kept in an evaporating dish, evaporated to dryness (using a cold current of air from a table fan at room temperature) followed by extraction with 5 ml of 20% ethyl alcohol. It was centrifuged for 10 min at 10,000 g. The clear liquid was decanted and stored in a sterilized bottle and kept in the freezer till it was subjected to chromatographic separation. Residue left after extraction of free amino acids was hydrolyzed with 6N HCl under reflux conditions at 110 ^{0}C for 18 h (Pirie 1955). This was then neutralized with an alkali and was kept under a cold current of air to evaporate. After evaporation, it was subjected to the same procedure as used for the extraction of free amino acids.

Two-dimensional paper chromatography was used for the separation of amino acids as described by Pal and Laloraya (1967). The detail method of estimation has been dealt elsewhere (Mukherjee and Laloraya 1979). Synthetic amino acids were used to prepare trace chromatograms, which were used for identification of spots. Except amino acids all data are based upon three uniform replicates, each one having three aliquots during the estimation. For amino acid extraction, only one sample was taken at each stage. However, the experiment has been repeated to confirm the result.

RESULTS

As leaf discs progressed to senescence, per cent reduction in total chlorophyll (Total Chl) was 88 in light and 76 in dark. Similarly, 83 and 74 percent decline could be recorded in carotenoids during light and drak, respectively. DL-serine (5 mM) alone and also in combination with Kn (0.38 μM) were able to reduce this loss with respect to control (Table 1). The degradation of pigments (total Chl and carotenoids) in leaf discs was more in light than in dark. The ratio of Chl a to Chl b has decreased as leaf discs passed through advanced stage of senescence in control. Serine was able to minimize the loss of total Chl. by about 17 and 7 percent during light and dark respectively; which is slightly higher as compared to its effect in retaining carotenoids. In combination with Kn, serine was able to reduce the pigment losses in greater magnitude. Amount of carotenoids decreased significantly in the leaf discs during 2, 4 and 6-day stages in control as well as treated (kinetin and serine) discs. These treatments were also able to maintain quantities of carotenoids at 6-day stage with respect to initial and control leaf discs.

Incubated leaf discs with double distilled water, under complete darkness showed about 70 per cent loss of protein after 6-day whereas discs kept under low photon flux density (PFD) had exhibited about 70 percent loss (Table 2). However, serine alone and in combination with Kn brought down the degradation markedly with respect to control in both light and dark. The retention of protein was greater in light than in dark. The specific activity of protease increased up to 968 percent between 0 to 6-day in control leaf discs in light whereas an increment of 1424 per cent was recorded during dark. The application of serine was able to check protease increment by about 214 and 160 per cent in light and dark respectively. It was again more effective in combination with Kn than alone in bringing down protease increment (Table 2).

Changes in free amino acids as shown in Fig. 1-2, an accumulation of about 154 and 212 per cent has been noticed in light and dark respectively in control set over a period of 6-day. Maximum increment was registered in iso-leucine (Ile) followed by methionine (Met), valine (Val), lysine (Lys) and histidine (His) during light whereas in dark, threonine (Thr), His and cysteic acid (Cys) disappeared in control at 6-day and maximum increment was seen in Ile followed by gamma amino butyric acid (γ-ABA), Leu-Phe, Gly-Ser, Val and Met at 6-day in control. DL-serine reduced the amount of Thr, Lys and other amino acids during 6-day whereas in combination with Kn, was able to reduce the amount of maximum number of amino acids such as Leu-Phe, Val, Met, Ile and His. DL- serine was quite effective in reducing the

Table 1. Effect of exogenously supplied serine and kinetin (individually and in combination) on the changes in Chl a, Chl b, total Chl and carotenoids (mg g^{-1} dry wt.)±S.E. in senescent leaf discs of *Spinacia oleracea* L.cv. S-23 maintained in 8 µmol photon m^{-2} s^{-1} light and dark.

	LIGHT				DARK			
Days	**Chl. a**	**Chl. b**	**Total Chl.**	**Carotenoids**	**Chl. a**	**Chl. b**	**Total Chl.**	**Carotenoids**
				INITIAL				
0 †	0.932±0.08	0.281±0.01	1.213±0.03	0.380±0.02	1.251±0.07	0.382±0.06	1.633±0.09	0.477±0.06
				CONTROL (DDW)				
2	0.437±0.03	0.197±0.04	0.643±0.07	0.206±0.07	0.533±0.01	0.196±0.08	0.729±0.06	0.257±0.01
4	0.280±0.01	0.121±0.01	0.401±0.04	0.169±0.08	0.423±0.03	0.149±0.02	0.572±0.02	0.237±0.03
6	0.105±0.01	0.040±0.01	0.145±0.07	0.063±0.03	0.310±0.05	0.075±0.01	0.385±0.01	0.120±0.01
				PHOSPHATE BUFFER (pH 5.2)				
2	0.495±0.03	0.203±0.04	0.698±0.86	0.212±0.08	0.597±0.01	0.208±0.09	0.805±0.03	0.235±0.02
4	0.346±0.01	0.181±0.09	0.527±0.82	0.181±0.09	0.453±0.09	0.179±0.06	0.632±0.01	0.220±0.05
6	0.216^{**aa}±0.01	0.100^{**aa}±0.00	0.316^{**aa}±0.84	0.136±0.07	0.330^{*}±0.07	0.113±0.05	0.443^{**aa}±0.02	0.124^{*a}±0.05
				SERINE ($5x10^{-3}$ M)				
2	0.523±0.01	0.213±0.04	0.736±0.17	0.223±0.09	0.627±0.07	0.212±0.01	0.839±0.09	0.243±0.08
4	0.368^{*aa}±0.03	0.196^{**}±0.01	0.564^{**aa}±0.18	0.193±0.07	0.565±0.01	0.193±0.01	0.758±0.05	0.225±0.05
6	0.240^{**aa}±0.03	0.113^{**aa}±0.06	0.353^{**aa}±0.17	0.121±0.06	0.360^{*}±0.07	0.139±0.05	0.499^{**aa}±0.03	0.145^{*a}±0.03
				KINETIN ($0.375x10^{-6}$ M)				
2	0.466±0.05	0.196±0.09	0.662±0.05	0.214±0.06	0.629±0.01	0.221±0.06	0.850±0.07	0.249±0.07
4	0.375^{*aa}±0.03	0.200^{**}±0.03	0.575^{**aa}±0.09	0.199±0.04	0.579±0.03	0.199±0.01	0.778±0.05	0.236±0.08
6	0.243^{**aa}±0.05	0.119^{**aa}±0.04	0.362^{**aa}±0.04	0.139±0.03	0.379^{*}±0.03	0.148±0.01	0.527^{**aa}±0.05	0.151^{*a}±0.06
				SERINE ($5x10^{-3}$ M) + KINETIN ($0.375x10^{-6}$ M)				
2	0.535±0.02	0.217±0.04	0.752±0.14	0.229±0.09	0.631±0.08	0.231±0.01	0.862±0.03	0.252±0.01
4	0.383^{*aa}±0.03	0.210^{**}±0.03	0.593^{**aa}±0.09	0.210±0.01	0.590±0.09	0.201±0.06	0.791±0.05	0.216±0.02
6	0.251^{**aa}±0.04	0.130^{**aa}±0.02	0.381^{**aa}±0.08	0.143±0.01	0.393^{*}±0.02	0.160±0.01	0.553^{**aa}±0.01	0.171^{*a}±0.07

* and ** ; a and aa – values significant at 5 % and 1 % due to day interval and treatment respectively.
† - Initial values with regard to light and dark are different because experiments carried out on different days.

concentration of total free amino acids by about 82 and 77 percent in light and dark respectively in comparison to control. In combination with Kn, the reduction in total amount was about 139 and 134 percent as compared with control in light and dark respectively. However, α-Ala initially present in trace amount but later disappeared in control at 6-day stage in both light and dark. In light, asparagine (Asn) and cysteic acid (Cys) were present in trace amount in O-day but later increased in 6-day in control. γ-ABA registered maximum accumulation followed by glutamic acid (Glu), Cys, Asn, glycine-serine (Gly-Ser) at 6-day control leaf discs in light. Serine was able to reduce the amount of aspartic acid (Asp), Cys, Glu, Gly-Ser, Asn, glutamine (Gln) and in combination with Kn the effect is more pronounced in light. However, the trend in dark was slightly different where serine could not check the increment of Gly-Ser, and Gln, individually and also in combination with Kn. Cys though present in light at all the stages but in dark it was only found at 0 and 2-day in control whereas treated samples had only trace amounts.

The distribution and quantities of protein amino acids have been depicted in Fig. 3 and 4 for experiments conducted in light and dark respectively. Total amount of protein amino acids declined dramatically as leaf discs

Table 2. Protein content [mg/100mg dry.wt.] and protease activity [µM lysine equivalent mg^{-100} dw h^{-1}] in senescent leaf discs of *Spinacia oleracea* L.cv. S-23 after treatments with serine and kinetin (individually and in combination) in 8 µmol photon m^{-2} s^{-1} light and in dark. Means ± SE, n=3x3, * and ** -values significantly different from control at 5% and 1% level; a and aa– values significantly different due to treatment at 5% and 1% level.

Treatments	Protein		Protease Activity	
	Light	Dark	Light	Dark
Initial value †	19.43±1.72	21.93±1.30	4.34±0.06	3.16±0.23
Values after 2-day				
Control (DDW)	10.20±0.36	9.16±0.93	18.06±0.36	18.17±1.30
Buffer (pH 5.2)	10.88±0.34	9.73±0.53	16.36±0.70	17.40±0.96
Serine (5x 10^{-3} M)	11.96±0.72	10.36±0.64	15.09±0.36	16.10±0.87
Kinetin (0.375 x 10^{-6}M)	12.46±0.96*	11.28±0.72*	16.09±1.00*	15.70±0.88*a
Serine (5x10^{-3}M)+ Kinetin (0.375 x 10^{-6}M)	13.16±0.37*	12.16±0.96*	15.08±1.08*	16.00±0.70*a
Values after 4-day				
Control (DDW)	7.86±0.02	6.19±0.86	29.10±1.89	30.13±1.90
Buffer (pH 5.2)	8.96±0.10	6.99±0.30	27.30±0.95	28.36±1.39*
Serine (5x 10^{-3} M)	9.86±0.80	7.63±0.63*	25.86±0.32*	27.10±1.32*
Kinetin (0.375 x 10^{-6}M)	10.73±0.97*	7.42±0.25	23.17±0.42*	25.30±0.56*a
Serine(5x10^{-3} M)+ Kinetin (0.375 x 10^{-6}M)	11.30±0.86*a	8.73±0.32*a	21.10±0.36**a	22.10±0.36**a
Values after 6-day				
Control (DDW)	5.82±0.03	5.06±0.37	46.37±1.05	48.17±2.26
Buffer (pH 5.2)	6.76±0.05	5.93±0.08	43.17±1.10*	46.30±2.10*
Serine (5x 10^{-3} M)	8.10±0.07*	6.73±0.06*	37.07±1.78*a	43.10±1.72*a
Kinetin (0.375 x 10^{-6}M)	9.26±0.08*a	9.26±0.09*a	34.30±1.05*a	40.09±0.66**a
Serine (5x 10^{-3} M)+ Kinetin(0.375 x 10^{-6}M)	10.03±0.79**a	10.03±0.05**a	31.73±1.00**aa	39.36±0.76**aa

* and ** ; a and aa – values significant at 5 % and 1 % due to day interval and treatment respectively.
† - Initial values with regard to light and dark are different because experiments carried out on different days.

began to senesce in untreated control. In light, the percent decline in total amount was more than in dark, the reduction in the former amounted to 50 percent as compared with 40 percent in the latter. Overall, serine prevented reduction in total protein amino acids by about 21 and 16 percent in light and dark respectively. Significant and greater control was exerted by combined treatment of serine and Kn than serine or kinetin alone in raising the values of protein amino acids in both light and dark.

In light, maximum decrease could be seen in proline (Pro) followed by α-Ala, Leu-Phe, Val, His, Lys, tyrosine (Tyr), Ile, Glu and Thr in control. Plants assimilate inorganic nitrogen into Glu, Gln, Asp and Asn. These compounds are used to transfer nitrogen from source organs to sink. In the present study, the contents of these amino acids were mostly higher in leaf disc kept in control. Notable exceptions were Met, Gly-Ser and Cys which increased at 6-day (Fig. 3). In dark, Pro was present at 0- day, disappeared at 6-day in control whereas its presence was recorded in trace amount in treated ones at 6-day and the concentration of the rest of the amino acids during 6-day in control declined to a greater extent as compared to initial stage (Fig. 4). Maximum reduction was seen in α-Ala followed by His,

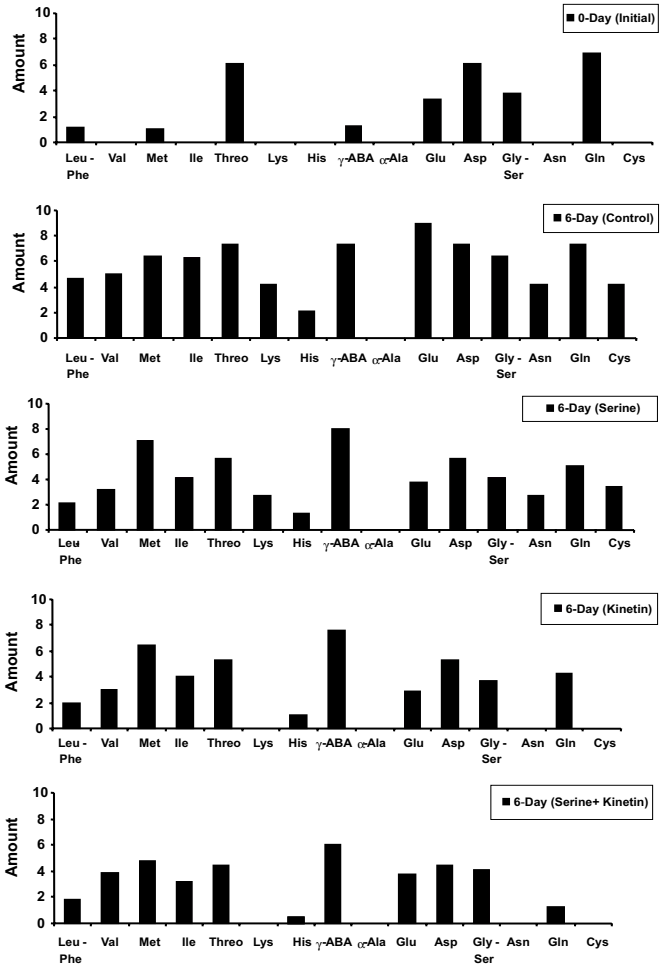

Fig. 1. Changes in free amino acids [mg g^{-100} (dw)] during progress of leaf disc senescence in *Spinacia oleracea* L. after treatments with serine (5 mM) and kinetin (0.38 µM) (individually and in combination) in 8 µmol photon m^{-2} s^{-1} PFD

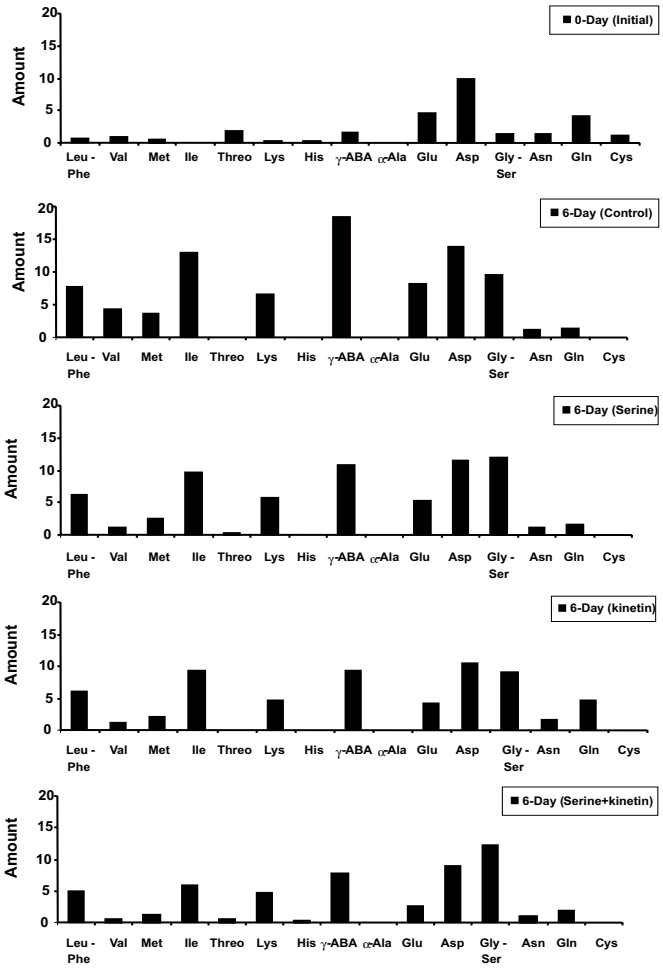

Fig. 2. Changes in free amino acids [mg g^{-100} (dw)] during progress of leaf disc senescence in *Spinacia oleracea* L. after treatments with serine (5 mM) and kinetin (0.38 µM) (individually and in combination) in dark

Cys, Gly- Ser, Leu- Phe, Thr, Asp, Glu, Val, Tyr, Ile and Met over a period of 6-day in control. Serine was able to increase the amount of most of the protein amino acids compared to control at 6-day whereas it was not able to do so for Met, Ile and Thr (Fig. 4). Again, in combination with kinetin the magnitude of retention was found to be higher. The total concentration of protein amino acids were higher than free amino acids at 0, 2 and 6-day by about 19, 7 and 4 times respectively in light whereas the values in dark were about 18, 6.5 and 3.5 times greater respectively in control leaf discs.

DISCUSSION

Many workers have demonstrated that the degradation of chlorophylls can be minimized by kinetin treatments (Paranjothy and Wareing 1971, Hukmani and Tripathi 1994). It has been also reported that higher PFD may cause photo-oxidative damage and induce leaf senescence (Prochazkova and Wilhelmova 2004). In the present investigation, Chl a lost preferentially to Chl b during light which could be attributed to its conversion to Chl b by chlorophyllide a oxygenase (CAO) activity

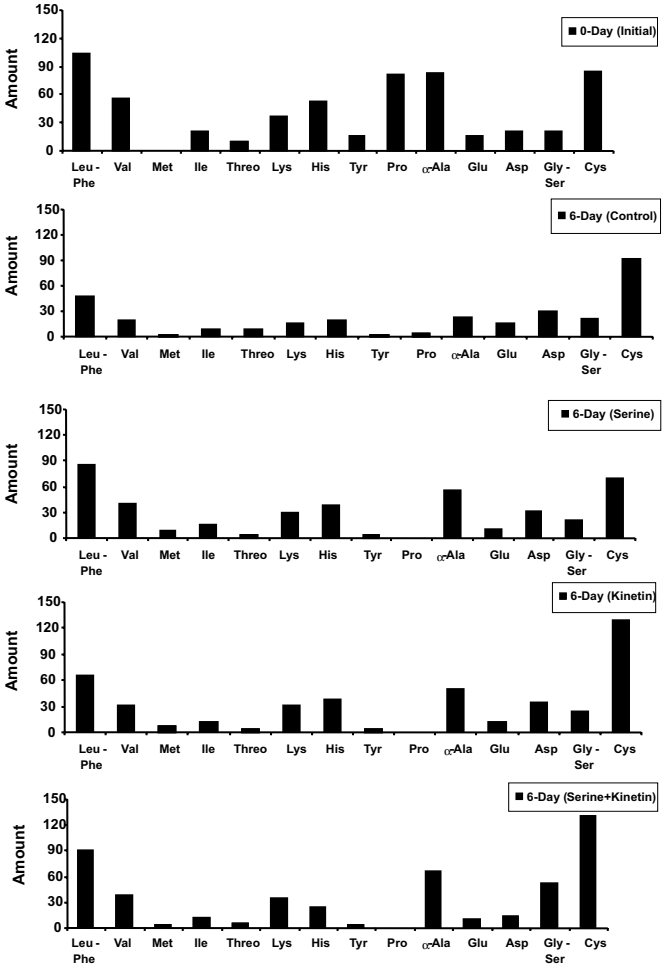

Fig. 3. Changes in protein amino acids [mg g^{-100} (dw)] during progress of leaf disc senescence in *Spinacia oleracea* L. after treatments with serine (5 mM) and kinetin (0.38 µM) (individually and in combination) in 8 µmol photon m^{-2} s^{-1} PFD

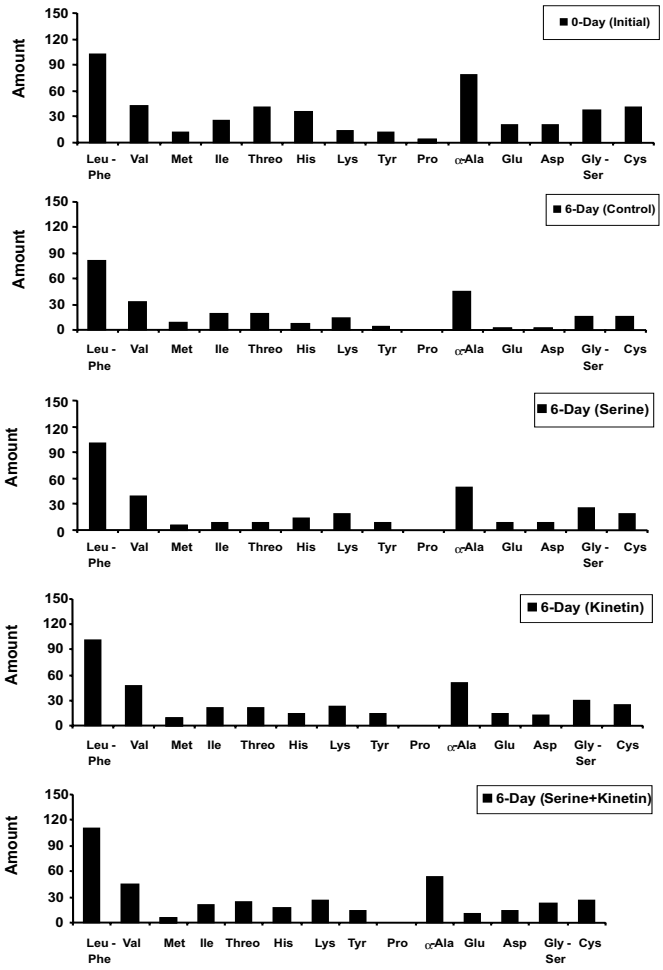

Fig. 4. Changes in protein amino acids [mg g^{-100} (dw)] during progress of leaf disc senescence in *Spinacia oleracea* L. after treatments with serine (5 mM) and kinetin (0.38 µM) (individually and in combination) in dark

(Tanaka *et al*. 1998). An investigation with *Arabidopsis* by Masuda *et al*. (2003) have revealed that CAO mRNA levels decrease when plants were transferred from low light to high light conditions thereby increasing the Chl a/b ratio. By contrast, over expression of the CAO gene decreased the Chl a/b ratio. Our study also exhibited that serine can reduce the breakdown of chloroplast pigments including carotenoids as was reported earlier in pigeonpea (Mukherjee and Ponmeni 2000). It has to be mentioned here that lipoxygenase is one of the important enzyme in the senescing leaves responsible for carotenoids' cleavage (Ben-Aziz *et al*. 1971) with reduced levels of zeaxanthin, antheraxanthin and violaxanthin. During foliar senescence, ABA can be derived from caroteniods as suggested by Afitlhile *et al*. (1993) while working on *Hordeum vulgare* leaves.

The fall in protein level in the leaf discs of *Spinacia oleracea* showed a similar pattern as observed during floral and leaf senescence of other plants (Smart 1994, Jones *et al*. 1995, Celikel and van Doorn 1995, Stephenson and Rubinstein 1998). Protease activity increased markedly in controls whereas the effectiveness of kinetin is very clear specially between 2-4 and 4-6 day stages. Kinetin was most effective to reduce protease activity followed by serine both in light and dark. However, the reduction was greater in light. Increased proteolysis and decline in the level of total RNA, protein

and lipids are the common biochemical changes witnessed during leaf senescence studies (Wittenback 1979, Hortensteiner and Feller 2002, Jakhar and Mukherjee 2006, Mukherjee and Kumar 2007). The combined application of kinetin and serine shows additive effect of this growth regulator when present alongwith this amino acid.

One of the steps in plant senescence seems to be increased proteolysis and amino acid interconversion (Hurst and Clark 1993). This study has revealed the increment in the free amino acid pool in control leaf discs of spinach both in light and dark and also showed the effectiveness of kinetin and serine in bringing down this level. Derballi *et al*. (1998) reported that total free amino acid content increased 2-fold when broccoli were stored for 8 days. Hansen *et al*. (2001) have also reported an increase of 23 per cent in the quantity of free amino acids while decrease of 12 per cent in protein amino acids. γ-ABA accumulation accounted for most of the increase in free amino acids in their study.

A common response of plants to environmental stress is an accumulation of amino acids. Plants appear to preferentially accumulate proline (Aspinall and Paleg 1981) in response to environmental stress, but also other amino acids especially those derived from aspartic acid, including Asn, Ile, Leu, Met and Val (Fukutoku and Yamada 1981, Handa *et al*. 1983). In our study, all free amino acids exhibited increment during senescence while treatments could bring down their levels appreciably. Similarly, as the degradation of protein was minimized by kinetin and serine; levels of most of the individual protein amino acids were higher in these cases in comparison to control leaf discs. α-Alanine could not be traced in the free pool of amino acids but it was present in considerable amount as protein amino acid. Kao (1980) had reported that exogenously applied Glu, Gln, Asp, Asn, Gly, Ser, His, Tyr, Phe, Try, Val, Leu, Ile, Lys and Thr promoted chlorophyll degradation. In the present study, exogenously applied serine instead of promoting chlorophyll and caroteniod degradation helped to minimize their breakdown. Further, the effect of kn in bringing down pigment destruction and protein degradation was further intensified by the additive effect of serine when both were together as reflected in the decreased protease activity and the amino acid pattern as discussed above.

ACKNOWLEDGEMENTS

Financial assistance in the form of a Research Project No. F.3-6/2004(SR) from UGC, New Delhi to DM and Project Fellow to SJ are gratefully acknowledged.

REFERENCES

Afitlhile, M.M., Dent, R.M. and Cowan, AK. (1993). Changes in carotenoid composition in senescing leaves of *Hordeum vulgare* L.CV. Dyan *J. Plant Physiol.* **142:** 43-49.

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts: Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol*. **24:** 1-15.

Aspinall, D. and Paleg, L.C. (1981). Proline accumulation-Physiological aspects. In: L.G. Paleg and D. Aspinall (eds.), The Physiology and Biochemistry of Drought Resistance in Plants, pp. 205-240. Academic Press, Australia.

Beale, S.I. (2005). Green genes gleaned. *Trends Plant Sci*. **10:** 309-312.

Beevers, L. (1968). Growth regulator control of senescence in leaf discs of nasturtium *(Tropaeolum majus)*. In: F. Wightman and G. Setterfield (eds.), Biochemistry and Physiology of Plant Growth Substances, pp. 1417-1435. The Runge Press Ltd, Ottawa.

Ben-Aziz, A, Grossman, S., Ascarelli, I. and Budowski, P. (1971). Carotene - bleaching activities of lipoxygenase and haeme proteins as studied by a direct spectrophotometry method. *Phytochemistry* **10:** 1445-1452.

Biswal, U.C. and Biswal, B. (1984). Photocontrol of leaf senescence. *Photochem Photobiol*. **39:** 875- 879.

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.

Buchanan - Wollaston, V., Morris, K.A, Mackerness, S., Page, T., John, C.F., Murphy, AM. and Carr, J. (2000). Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant* J. **23:** 677-685.

Celikel, F.G. and van Doorn, W.G. (1995). Solute leakage, lipid peroxidation and protein degradation during the senescence of *Iris tepals. Physiol Plant.* **94:** 514-521.

Chandlee, J.M. (2001). Current molecular understanding of the genetically programmed process of leaf senescence. *Physiol Plant.* **113:** 1-8.

Derbali, E., Makhlouf, J. and Vezina, L.P. (1998). Biosynthesis of sulphur volatile compounds in broccoli seedlings stored under anaerobic conditions. *Postharvest Biol.* Technol. **13:** 191-204.

Fukutoku, Y. and Yamada, Y. (1981). Diurnal changes in water potential and free amino acid contents of water - stressed and non-stressed soybean plants. *Soil Sci Plant Nutr.* **27:** 195-204.

Gan, S. and Amasino, R.M. (1995). Inhibition of leaf senescence by auto regulated production of cytokinin. *Science* **270:** 1986-1988.

Handa, S., Bressan, R.A., Handa, A.K., Carpita, N.C. and Hasegawa, P.N. (1983). Solutes contributing to osmotic adjustment in culture plant cells adapted to water stress. *Plant Physiol.* **73:** 834-843.

Hansen, E.M., Sorensen, H. and Cantwell, M. (2001). Changes in acetaldehyde, ethanol and amino acid concentrations in broccoli florets during air and controlled atmosphere storage. *Postharvest Biol. Technol.* **22:** 227-237.

Holden, M. (1965). Chemistry and biochemistry of plant pigments. In: T.W. Goodwin (ed.), pp. 462-468. Academic Press, New York.

Hortensteiner, S. (2004). The loss of green color during chlorophyll degradation - a prerequisite to prevent cell death? *Planta.* **219:** 191-194.

Hortensteiner, S. and Feller, U. (2002). Nitrogen metabolism and remobilization during senescence. *J. Exp. Bot.* **53:** 927-937.

Hukmani, P. and Tripathy, B.C. 1994 Chlorophyll biosynthesis reactions during senescence of excised barley (*Hordeum vulgare* L. cv. IB 65) leaves. *J. Plant Physiol.* **105 :** 1295- 1300.

Hurst, P.L. and Clark, C. J. (1993). Post harvest changes in ammonium, amino acids and enzymes of amino acid metabolism in asparagus spear tips. *J. Sci. Food Agric.* **63:** 465-471.

Jakhar, S. and Mukherjee, D. (2006). Chloroplast pigments, free and bound amino acids, activities of protease and peroxidase during development and senescence of attached nodal leaves of *Cajanus cajan* L. *J. Plant Biol.* **33:** 125-132.

Jones, M.L., Larson, P.B. and Woodson, W.R. (1995). Ethylene-regulated expression of a carnation cysteine proteinase during flower petal senescence. *Plant Mol. Biol.* **28:** 505-512.

Kao, C.H. (1980). Senescence of rice leaves. IV. Influence of benzyladenine on chlorophyll degradation. *Plant Cell Physiol.* **21:** 1255-1262.

Martin, C. and Thimann, K.V. (1972). The role of protein synthesis in the sensescence of leaves. II The influence of amino acids on senescence. *Plant Physiol.* **50:** 432-437.

Masuda, T., Tanaka, A. and Melis, A. (2003). Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll a oxygenease (CAO) and Lhcb gene expression. *Plant Mol. Biol.* **51:** 757-771.

Mukherjee, D. and Kumar, R. (2007). Kinetin regulates plant growth and senescence of leaves, flowers and pods of *Cajanus cajan* L. *Biol. Plant.* **51:** 80-85.

Mukherjee, D. and Laloraya, M.M. (1979). Nitrogen and free amino acids changes during seedling growth in *Bauhinia purpurea. J. Indian Bot. Soc.* **58:** 75-82.

Mukherjee, D. and Ponmeni, G. (2000). Senescence regulation by serine and its synergistic action with kinetin in pigeonpea leaf discs. In: P.S. Basu, M.A. Choudhuri, K. Gupta, A.K. Mukherjee (eds.), Recent Trends of Researches in Microbiology and Plant Physiology in India, pp. 101-106. University of Burdwan, Burdwan, India.

Mukherjee, D. and Rao, K.U.M. (1993). Protease activity in leaves, flowers and pods of *Cajanus cajan* during maturation and senescenc. *Plant. Physiol. Biochem.* **20:** 45-48.

Ono, K., Nishi, Y., Watanabe, A. and Terashima, I. (2001). Possible mechanisms of adaptive leaf senescence. *Plant Biol.* **3:** 234-243.

Pal, R.N. and Laloraya, M.M. (1967). Nitrogen metabolism of *Tamarindus indica.* Changes in γ-methylene glutamine and its corresponding acid γ-methylene glutamic acid during seedling growth. *Plant Physiol.* **20:** 789-801.

Paranjothy, K. and Wareing, P.F. (1971). The effect of abscisic acid, kinetin and 5-flurouracil on ribonucleic acid and protein synthesis in senescing radish leaf discs. *Planta.* **99:** 112-119.

Pastori, G.M. and del Rio, L.M. (1997). Natural senescence of Pea Leaves (An Activated oxygen-mediated function forPeroxisomes). *Plant Physiol.* **113:** 411-418.

Pirie, N.W. (1955). Proteins. In: K. Paech and M.V. Tracey (eds.), Modern Methods of Plant Analysis, 4. pp. 23-68 Springer-Verlag, Berlin.

Plaisted, P.H. (1958). Some biochemical changes during development and aging of *Acer platanoids* L. leaves. *Contrib. Boyce Thompson Inst.* **19:** 245-254.

Prochazkova, D. and Wilhelmova, N. (2004). Changes in antioxidative protection in bean cotyledons during natural and continuous irradiation-accelerated senescence. *Biol. Plant.* **48:** 33-39.

Quirino, B.F., Noh, Y.S. Himelblau, E. and Amasino, R.M. (2000). Molecular aspects of leaf senescence. *Trends Plant Sci.* **5:** 278-282.

Ramakrishna, V. and Ramakrishna Rao, P. (2005). Purification of acidic protease from the cotyledons of germinating Indian bean (*Dolichos lablab* L. var Lignosus) seeds. *Afr. J. Biotech.* **4:** 703-707.

Reimerdes, E.H, and Klostermeyer, H. (1976). Determination of proteolytic activities on casein substrates. In : S.P. Colowick, N.O. Kaplan (eds.), Methods in Enzymology, pp. 26-28. Academic Press, New York.

Senyuk, V., Rotari, V., Becker, C., Zakharov, A., Horstmann, C., Muntz, K. and Vaintraub, I.A. (1998). Does an asparaginyl-specific cystein endopeptidases trigger phaseolin degradation in cotyledons of kidney bean seedlings? *European. J. Biochem.* **258:** 546-548.

Shibaoka, H. and Thimann, K.V. (1970). Antagonism between kinetin and amino acids. Experiments on the mode of action of cytokinins. *Plant Physiol.* **46:** 212-220.

Simon, E.W. (1967). Types of leaf senescence. *Symp. Soc. Exp. Biol.* **21:** 215-230.

Singh, S., Letham, D.S. Zhang, X. and Palni, L.M.S. (1992). Cytokinin biochemistry in relation to leaf senescence. Effect of nitrogenous nutrients on cytokinins levels and senescence of tobacco leaves. *Physiol. Plant.* **84:** 262-268.

Smart, C.M. (1994). Gene expression during leaf senescence. *New Phytol.* **126:** 419-448.

Spencer, P.W. and Titus, John. S. (1972). Biochemical and enzymatic changes in apple leaf tissue during autumnal senescence. *Plant Physiol.* **49:** 746-750.

Stephenoson, P. and Rubinstein, B. (1998). Characterization of proteolytic activity during senescence in daylilies. *Physiol Plant.* **104:** 463-473.

Steward, F.C., Wetmore, R.H., Thompson, J.F. and Nitsch, J.P. (1954). A quantitative chromatographic study of nitrogenous components of shoot apices. *Amer. J. Bot.* **41:** 123-134.

Tanaka, A., Ito, H., Tanka, N.K. Yoshida, K. and Okada, K. (1998). Chlorophyll a oxygenase (CAO) is involved in chlorophyll b formation from chlorophyll a. *Proc. Natl. Acad. Sci.* USA **95:** 719-723.

Weaver, L.M. Gan, S., Quirino, B. and Amasino. R.M. (1998). A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatments. *Plant Mol. Biol.* **37:** 455-469.

Wilhelmova, N., Prochazkova, D., Machackova, I., Vagner, M., Srbova, M. and Wilhelm, J. (2004). The role of cytokinins and ethylene in bean cotyledon senescence. The effect of free radicals. *Biol. Plant.* **48:** 523-529.

Wittenbach, V.A. (1979). Ribulose bisphosphate carboxylase and proteolytic activity in wheat leaves from anthesis through senescence. *Plant Physiol.***64:** 884-887.

Wollgiehn, R. (1967). Nucleic acid and protein metabolism of excised leaves. *Symp. Soc. Exp. Biol.* **21:** 231-246.

Yemm, E.W. and Cocking, E.C. (1955). The determination of amino acids with ninhydrin. *Analyst.* **80:** 209-213.

Yomo, H. and Varner, J.E. (1973). Control of the formation of amylase and protease in the cotyledons of germinating peas. *Plant Physiol.* **51:** 708-713.