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SENESCENCE REGULATION IN LEAF DISCS OF *RAPHANUS SATIVUS* L. BY PLANT GROWTH REGULATORS IN DARK

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SUMMARY

From mature and fresh leaves of *Raphanus sativus* L. cv. Chetki long, discs were punched out and treated with two different concentrations of kinetin (KN, 0.375 and 3.75 µM) and a morphactin (MOR, CME 74050, 3.64 and 36.4 µM) in order to make a comparative assessment of plant growth regulators (PGR's) with regard to senescence regulation. A gradual breakdown of chlorophylls, carotenoids, proteins and an increment in protease and peroxidase activity were noticed. Total sugars also registered an increasing trend. Applications of both PGR's could delay senescence by minimizing degradation of chloroplast pigments and bringing down protease and peroxidase activity as well as sugar accumulation. Protein breakdown was reduced markedly by only KN.

Key words: **Enzymatic activity, kinetin, morphactin, *Raphanus sativus*, senescence**

INTRODUCTION

Leaf senescence is the last phase of the leaf development comprising several complex biochemical and physiological events that occur in an ordered sequence (Biswal and Biswal 1988, Smart 1994). Situations of abiotic and biotic stresses as well as plant hormones play an important role in promoting senescence and programmed cell death (Thimann 1980, Nooden 1988, Lim *et al.* 2003, Guo *et al.* 2004). Leaf senescence brings about degradation of photosynthetic pigments, proteins, lipids, nucleic acids and essential cellular metabolites as well as extensive disruption of internal structures and cellular organelles, which are highly controlled by genes (Buchanan-Wollaston 1997, Thomas and Howarth 2000, Eckhardt *et al.* 2004).

Light is one of the important factors of senescence. Alterations in the duration, intensity, quality and interaction of light with other environmental variables can bring about changes in senescence pattern. On the contrary, darkness is widely used as inducer of senescence for excised leaves (Park *et al.* 1998, Weaver *et al.* 1998, Hodges and Forney 2000) where as light delays senescence (Stoddart and Thomas 1982) by maintaining the rate of photosynthesis (Goldthwaite and Leatsch 1967, Thimann *et al.* 1977).

Plant growth regulators such as kinetin, morphactins and salicylic acid (SA) are well known in delaying senescence (Smart *et al.* 1991, Gan and Amasino 1995, Robson *et al.* 2004). Morphactins are auxin transport inhibitor and delay senescence in soybean and increase the number of pods (Nooden and Nooden 1985). Cytokinins regulate a number of growth and developmental processes in plants, such as stimulating cell division, maintaining plant vigour and delaying plant senescence (Gan and Amasino 1997) by their ability to promote the transport, accumulation and retention of metabolites in tissues and organs, besides protecting membranes against degradation (Leshem 1988). It was, therefore, thought to carry out a comparative study of

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the senescence regulation in leaf discs of *Raphanus sativus* by using different concentrations of kinetin and morphactin during dark as this kind of investigation has not been made earlier. Alterations in the amount of chlorophylls, carotenoids, proteins, total soluble sugars and specific activity of proteases and peroxidases were recorded which are amongst the reliable senescence markers.

MATERIALS AND METHODS

Seeds of *Raphanus sativus* were germinated and plants were grown in experimental cage having length, breadth and height of 12m x 12m x 2.5m respectively in university botanical garden, Kurukshetra. Inside cage, nine experimental plots were prepared. Prior to sowing, seeds were dipped in double distilled water (DDW) for about 24 hours during first week of December and May in two experimental plots, each possessing an area of 1x3 m^2. After about two months, leaves of radish were collected in the morning for sampling. Leaf discs were punched out from washed and dried mature leaves and floated on 6 ml of different concentrations of kinetin (0.375 and 3.75 µM) and morphactin (3.64 and 36.4 µM) in corning Petri dishes each lined with Whatman No. 1 filter paper having 55 leaf discs each with area of 0.6 cm^2. Samples were collected at 0, 2, 4 and 6 day under dark condition in a growth chamber.

***Chlorophyll and carotenoid estimation*:** The amount of samples used for an extraction ranged from 50-100 mg depending upon availability and requirements. Chilled 80 percent acetone (AR grade) and a pinch of $CaCO_3$ were used during extraction and the absorbance was recorded at 480, 510, 645, and 663 nm using an UV-vis spectrophotometer (Specord-205 Analytik Jena, Germany). The pigments were estimated by the formulae and method of Arnon (1949) and Holden (1965).

***Protein estimation and protease activity*:** Protein was estimated by the method of Bradford (1976) using coomassie brilliant blue G-250 dye. The ninhydrin method was followed for the estimation of protease activity originally described by Yemm and Cocking (1955) and modified by Reimerdes and Klostermeyer (1976). The protease activity was expressed in µM lysine equivalent per 100 mg weight of the sample per hour.

***Peroxidase activity*:** The total peroxidase activity was measured by the method of Maehly (1954) using guaiacol and H_2O_2. Specific activity of peroxidase was expressed as mg^{-1}protein min^{-10}.

***Total soluble sugars*:** The total soluble sugars were measured following the method of Hart and Fisher (1971). Amount of reducing and non reducing sugars were calculated against a standard curve of glucose. Three replicates were used for each biochemical analysis.

RESULTS AND DISCUSSION

Results of present study have been summarized in Fig. 1-4. During dark, chlorophyll and carotenoid content exhibited decline from the initial to 6-day stage, the amount of decline was much greater in the former than latter (Fig. 1). Generally it has been noticed that chl-a declines more rapidly than chl-b (Maunder *et al.* 1983, Moore 1986, Kurra-Hotta *et al.* 1987). However, the degradation of chl-b was much higher than chl-a in all control samples, whereas kinetin (KN) and morphactin (MOR) treated leaf discs reversed the trend up to 4 and 6-day respectively (Fig. 2). At 6^{th} day, KN (0.375µM) treated leaf discs exhibited highest degradation of chl b than chl a in comparison to MOR treated leaf discs. MOR (3.64 µM) concentration was found to be more effective in retaining chl b than chl a (Fig. 2). Both plant growth regulators reduced the degradation of total chlorophylls and carotenoids appreciably. Kinetin also showed its effectiveness by delaying the loss of chlorophyll and proteins in senescing leaf discs (Paranjothy and Wareing, 1971, Martin and Thimann 1972 a,b, Beevers 1976).

Chl-a, chl-b, total chlorophyll and carotenoid content exhibited a decline of 85.54, 95.72, 89.72 and 84.86 percent respectively from initial to 6-day stage in control whereas KN apperciably brought down the degradation up to 55.05, 72.79, 62.33 and 66.29 percent respectively of chl-a, chl-b, total chlorophyll and total carotenoid. Effective role of kinetin to control degradation of

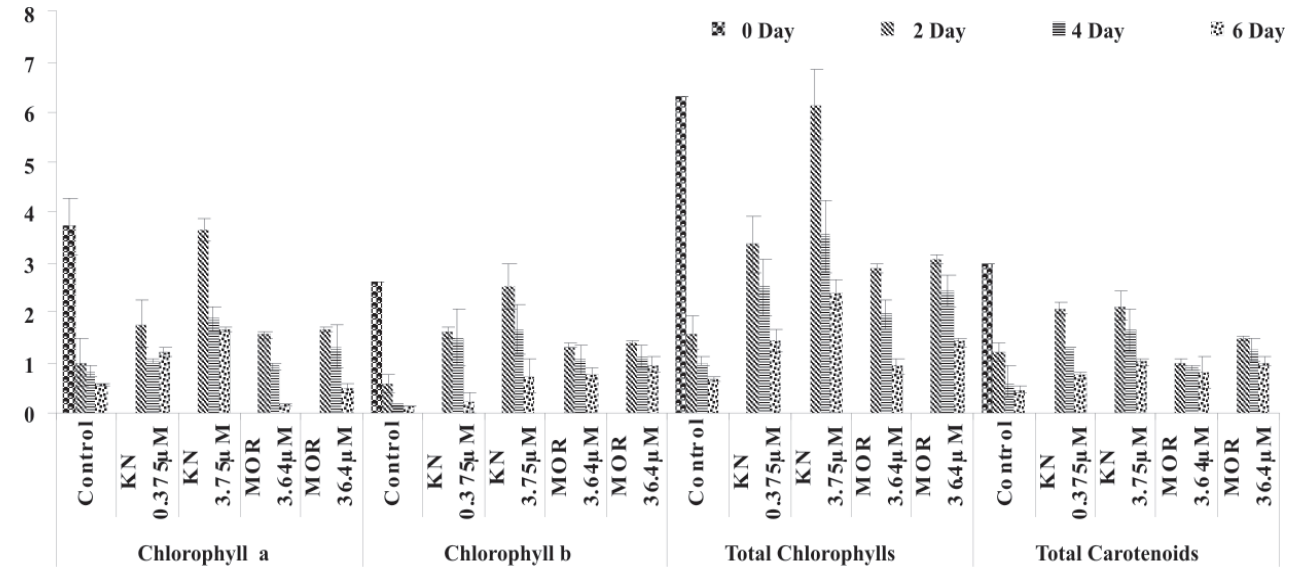

Fig. 1. Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids (mg/100 mg dry wt.) in *Raphanus sativus* during dark

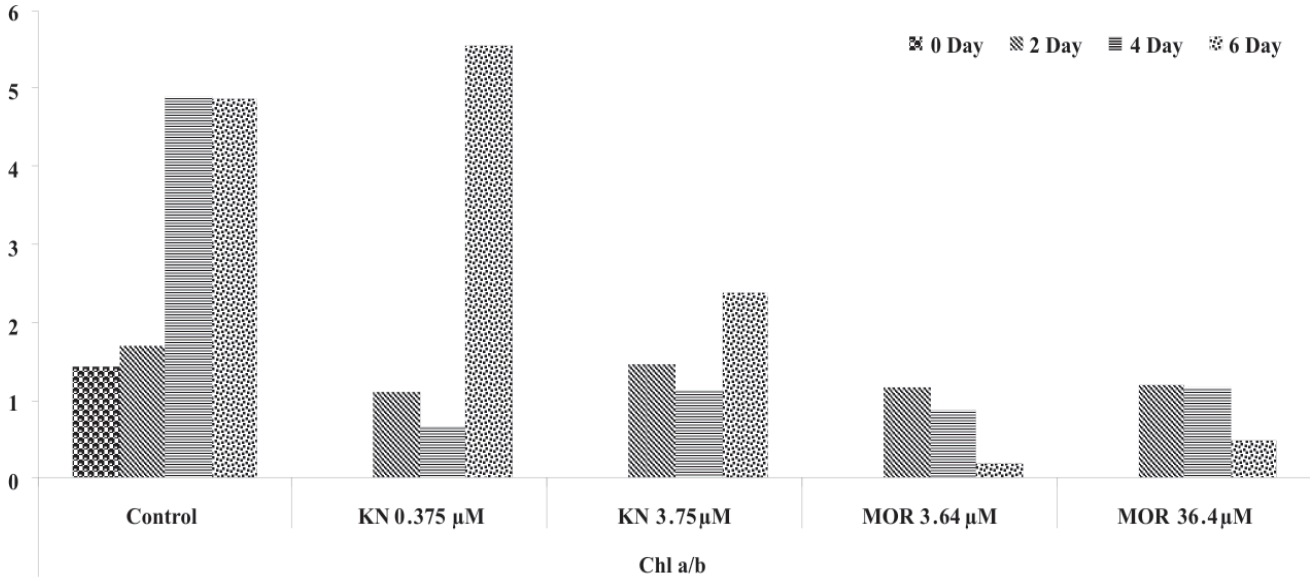

Fig. 2. Chlorophyll a/b ratio in *Raphanus sativus* during dark

chloroplast pigments was also noticed in pigeon pea (Rao and Mukherjee 1990, Ponmeni and Mukherjee 1997). Tetley and Thimann (1974) while working on oat leaf senescence and the regulation by cytokinins have also reported that in dark, rate of decline of chlorophyll was slightly higher than that of carotenoid.

The decrease in chlorophyll may be due to the increase in peroxidase activity as noticed in the present study where advancement of senescence was characterized by a steady increase in peroxidase activity (Fig. 3). Percent increment was reduced by KN (3.75 µM) from 1333.15 to 614.87. For reducing peroxidase

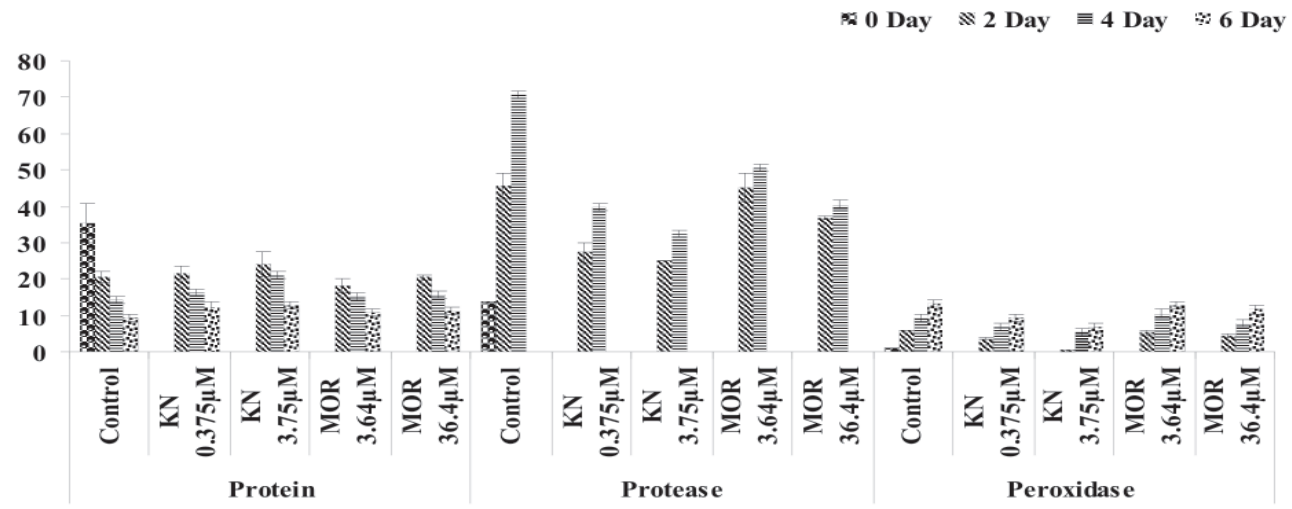

Fig. 3. Protein content (mg/100 mg dry wt.), protease activity (µM Lysine equivalent/100 mg dry wt hr^{-1}) and peroxidase activity (mg^{-1} protein 10 min^{-1}) in *Raphanus sativus* during dark

activity best result was noticed with KN, followed by MOR treatments and higher concentration of both plant growth regulators performed better (Fig. 3). Peroxidase is one of the important enzymes, predominantly found in plants, which bleaches chlorophyll in the presence of H_2O_2 and certain phenolics such as 2,4-dichlorophenol, p-coumaric acid, phenol, p-hydroxyphenyl acetic acid, resorcinol, etc. (Matile 1980, Huff 1981, Martinoia *et al.* 1982, Kato and Shimizu 1985). The enzyme is responsible for the lipid peroxidation of the membrane (Barber and Thompson 1980).The increment in specific peroxidase activity is well marked with the progress of senescence (Grover and Sinha 1985, Ponmeni and Mukherjee 1997). Wilhelmova (1998) has stated that increase in the peroxidase activity is associated with cell wall rigidification.

Leaf disc senescence of *R. sativus* exhibited regular degradation of protein content and increment in protease activity (Fig. 3). With the progress of senescence, percent degradation of protein content was 41.95, 59.61 and 73.69 at 2, 4 and 6-day respectively. Among both plant growth regulators, kinetin (KN) was found to be quite effective in retaining proteins in comparison to control. Application of KN appreciably brought down this degradation by 73.69 to 63.65 % (Fig. 3). However, marked change could not be seen after MOR treatments. Protein degradation has been considered to be one of the important events during leaf senescence (Osborne 1962, Fletcher 1969, Shibaoka and Thimann 1970, Martin and Thimann 1972 b, Tetley and Thimann 1974). Enzymes associated with the process are known as proteases and hydrolases (Richter 1978). Kinetin was effective in retarding protein degradation thereby confirming findings of earlier workers (Richmond and Lang 1957, Banerji and Laloraya 1963, Paranjothy and Wareing 1971, Martin and Thimann 1972a, b, Beevers 1976). Present findings also indicate that protein loss is associated with the increment in protease activity (Fig. 3) which was particularly higher in the advanced stages of senescence. Here not only KN but MOR could also reduce the enzymatic activity. KN reduces the enzymatic activity from 421.44 to 137.6%, whereas MOR showed its effectiveness by slowing down the activity from 421.44 to 198.87%. Numerous reports are available where increase in the activity of proteases has been correlated with the breakdown of proteins (Mukherjee and Rao 1993, Senyuk *et al.* 1996, Ramakrishna and Ramakrishna Rao 2005).

The distribution of sugars in radish leaf discs point out a gradual accumulation of both reducing and non reducing sugars (Fig. 4). During first 2 days, reducing sugar increment was higher than non reducing sugars.

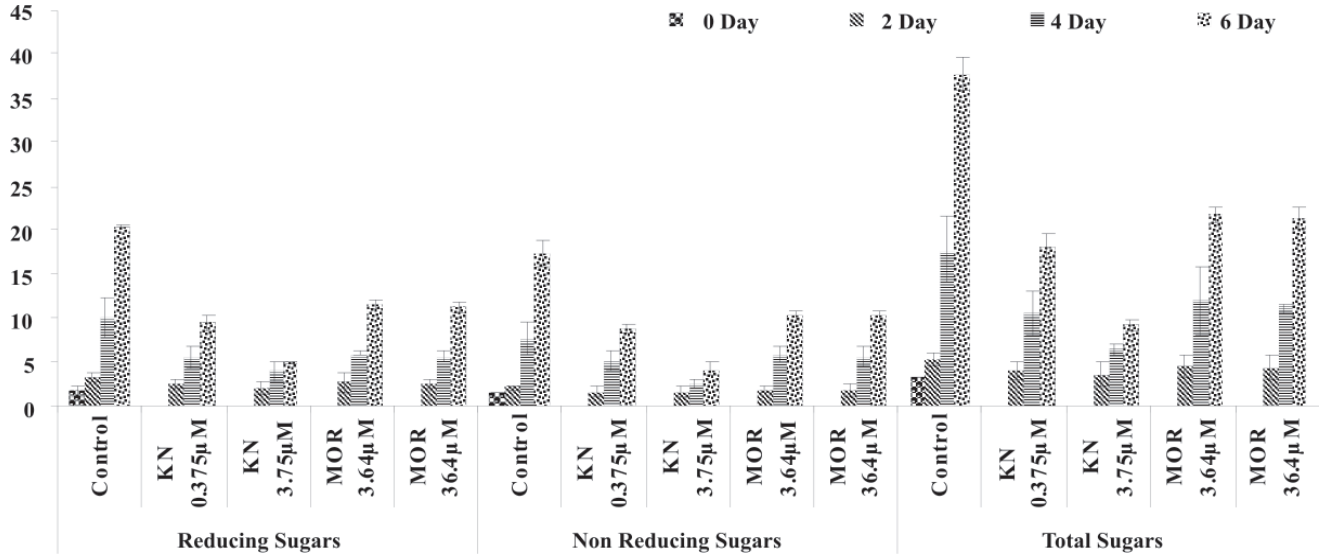

Fig. 4. Reducing sugars, non reducing sugars and total sugars (mg/100 mg dry wt.) in *Raphanus sativus* during dark

The percent increment gap narrowed down at later stage and amount of reducing sugars still maintained higher values. From initial values onwards percent increments in reducing sugars were 92.84, 496.21 and 1108.28, whereas in non reducing sugars, percent increments were 32.32, 385.58 and 996.52 respectively. A sharp 12-fold increment was noticed in the quantity of reducing sugars during 6-day in control. Both growth regulators were found to reduce the accumulation of reducing and non reducing sugars very efficiently. However, best results were obtained again with KN followed by MOR and in each case the higher concentration has better power in curtailing the accumulation of sugars (Fig. 4). Increment in reducing sugars was constantly higher than non reducing in leaf discs maintained in continuous dark. Total sugar increment was maximum at 6 day which was curtailed remarkably by KN and MOR. KN curtailed sugar accumulation from 1045.13 to 314.67 percent whereas in case of MOR from 1045.13 to 394.16% (Fig. 4).

It has been postulated that degradation of chloroplast pigments and loss in photosynthetic efficiency may lead to sugar starvation which may act as a signal for the induction of leaf senescence (Hensel *et al.* 1993). However, studies of Rolland *et al.* (2002) revealed that the accumulation of glucose and sucrose repress the transcription of the photosynthetic genes thereby indicating role of accumulating sugars in the regulation of senescence. Data on sugars in the present investigation clearly demonstrate the pattern of accumulation instead of their decline with the progress of leaf disc senescence. Some other studies with senescent leaves have been found to accumulate glucose and fructose without any sign of sugar starvation (Wingler *et al.* 1998, Quirino *et al.* 2001, Stessman *et al.* 2002).

From overall results and discussion it can be concluded that both selected plant growth regulators were effective in delaying leaf senescence in *Raphanus sativus* during dark condition. However, the most effective among the two was kinetin followed by morphactin.

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