Pak. J. Bot., 40(5): 1993-2004, 2008.

INVESTIGATION ON THE RELATIONSHIP BETWEEN SENESCENCE AND NITRIC OXIDE IN SUNFLOWER (*HELİANTHUS ANNUUS* L.) SEEDLINGS

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Abstract

In this study, the effects of NO (nitric oxide) on senescence occurring in the cotyledons of *Helianthus annuus* L. (sunflower) seedlings, were examined. Following germination of the sunflower the seedlings were grown in perlit for 10 days and then transferred into hydroponic culture containing ¼ Hoagland solution. At day 23 approximately when senescence started occurring, 0.1, 1, 10, 100 and 400 μM concentrations of Sodium nitroprusside prepared in ¼ Hoagland solution used as NO donor were applied to the seedlings. The cotyledons of both control and experimental groups were harvested when the avarage green area in the control group reached 50%. Effects of NO in senescence process were determinated by measuring parameters such as Peroxidase activity, amount of chlorophyll and total soluble protein.

When compared with the control cotyledons, senescence was observed to be delayed at lower SNP concentrations, especially at 0.1 μM. As SNP concentrations were elevated chlorophyll amount gradually decreased, being under the control value at 400 μM SNP, while total soluble protein reached a maximum at 10 μM SNP and Peroxidase activity showed an increase parallel to increment concentrations. It would suggest that exogenously applied SNP may play a role in regulating senescence process in *Helianthus annuus* seedlings, depending on concentration.

Introduction

Nitric oxide (NO) is an atom small colorless gaseous, molecule that is formed by the union of a nitrogen and an oxygen atom and may be dissolved both in water and in oils. The fact that it has an unshared electron in its outer orbit both gives it the radical property and allows it to diffuse easily through membranes (Lancaster, 1997; Stöhr & Ullrich, 2002). It has been detected that NO is produced in cytosol, nucleus, peroxisome matrix and chloroplast. There are two main metabolic pathways involving in NO synthesis: As enzymatic and non–enzymatic. It is known that, in enzymatic way some enzymes such as nitric oxide synthesis (NOS), nitrate reductase (NR), xanthine oxidoreductase (XOR), Peroxidase and cytocrom P450 play a role (Delledonne *et al*., 1998; Durner *et al.*, 1998; Yamasaki & Sakihama, 2000; Beligni & Lamattina, 2001; Corpas *et al.*, 2002; Rockel *et al.*, 2002; Lamattina *et al.*, 2003; Del Rio *et al.*, 2004). However, in non enzymatic way, NO is synthesized by the reduction of nitrite by carotenoids at low pH or in the presence of light (Cooney *et al.*, 1994; Henry *et al.*, 1997; Horemans *et al.*, 2000; Beligni *et al*.., 2002; Stöhr & Ullrich, 2002). Two of the most important non-enzymatic NO production processes are nitrification and denitrification (Bollmann *et al.*, 1999). Also, NO is non enzymaticly produced when NO donors like Sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) are exogenously applied (Leshem, 1996). Exogenous NO affects several aspects of plant growth and development (Ribeiro *et al.*, 1999), and can affect the responses of plants to pathogens (Durner *et al.*, 1998), light (Beligni &

Lamattina, 2000), gravity (Pedroso & Durzan, 2000) and oxidative stress (Beligni & Lamattina, 1999). Also possible roles of NO in inhibition of catalase, ascorbate Peroxidase and aconitase enzymes in plants (Clark *et al.*, 2000), lignification of cell wall (Ferrer & Ros Barcelo, 1999), arrangement of ion channels in guard cells (Garcia Mata *et al.*, 2003), functions of chloroplasts and mitochondria (Yamasaki *et al.*, 2001), cell death (Pedroso *et al.*, 2000a), senescence (Leshem, 1996; Leshem & Haramaty, 1996; Hung & Kao, 2003) and wound signal were revealed.

The senescence that takes place in cell, tissue, organ and the whole plant during the normal growth process of the plant includes very complicated catabolic reactions at molecular level and is dependent on energy input (Taiz & Zeiger, 2002). The complex interaction of both internal and environmental factors is seen in senescence process in the plants (Nam, 1997; Noodén *et al.*, 1997). While internal factors include plant growth regulators (phytohormones) and age, stress factors such as excess temperature changes, darkness, lack of minerals, high light, aridity and pathogen infections are environmental factors (Hensel *et al.*, 1993). The genetic factors are also one of the most important internal factors. A lot of special genes (SAGs: senescence-associated genes) that play a role in the arrangement of senescence have been defined recently (Buchanan-Wollaston, 1997; Weaver *et al.*, 1997; Quirino *et al.*, 2000). Senescence, as we all know, is a death state in which so many well organized facts play a role. During this process changes at structural, biochemical and molecular level occur in all organs of the plant including cotyledons. Butler (1967) who explored the structural changes during senescence has stated that chloroplasts get damaged at first, then the number of free ribosomes is reduced, the tonoplast becomes permeable, plasma and nucleus membranes are damaged and endoplasmic reticulum loses its functions.

The first sign of senescence is the increased level of photosynthesis and the decreased respiration level. Although photosynthesis gradually slows down and finally stops, mitochondria and nucleus remain functional and respiration continues until the end of the senescence (Wareing & Phillips, 1973; Mondall *et al.*, 1978; Srivastava, 2002), which is not in fact a totally demolishing process. An important amount of protein is synthesized along with secondary products during senescence (Srivastava, 2002).

NO is an endogen factor that regulates maturation and senescence process in higher plants (Leshem *et al.*, 1998). It has been observed that NO delays senescence in pea leaves (Beligni & Lamattina, 2000; Leshem & Haramaty, 1996). It has been detected that NO concentration is lower in ripe fruits than green fruits and in senescencing flowers than younger ones. NO was reported to extend life span after the harvest and delay senescence when applied exogenously to flowers, fruits and vegetables (Leshem *et al.*, 1998). The occurrence or delay of senescence in various plants is probably related with the production of free ethylene or the negative correlation between ethylene and NO (Leshem *et al.*, 1998; Magalhães *et al.*, 2000). NO is produced more than ethylene in the developmental process of plant organs from anthesis to senescence (Magalhães *et al.*, 2000). The younger plants and tissues produce more NO than ethylene not only under stress conditions, but also under normal conditions (Leshem *et al.*, 1998). Under the light of this information it is stated that NO is a senescence delaying agent in plants. However, in spite of all these data, the role of NO in senescence process has not been completely understood yet. Further research is needed to determine how and at what stages NO affects senescence.

The aim of the present study is to clarify the effects of NO molecule, on which scientists have focussed in recent years, on sunflower plant (*Helianthus annuus* L.) which is of economical importance in Turkish agriculture.

Materials and Methods

Plant material: Sunflower (*Helianthus annuus* L.) seedlings which belong to Compositae (Asteraceae) family were purchased from MAY seed company (AS 6310) and used as experimental material. The reasons for choosing this plant are that it shows monocarpic senescence, it becomes experimental material in short terms, it exhibits epigeic germination, it may be grown in water culture (hydroponic culture) and it has previously been used in studies about both NO and senescence.

Plant growth conditions: The seeds that have been left under running tap water for 24 hours were germinated in 9 cm diameter Petri dishes containing 5 ml distilled water (for 48 hours at 25˚C). After germination, they were planted in perlite and watered with ½ Hoagland (nutrient solvent) every other day for 10 days. Then they were transferred into ¼ Hoagland water culture containers. The germinated seeds were grown in a growth chamber (12 hours of light period, 6000 lux illumination and 25±2°C room temperature).

NO treatments: Sodyum nitropurissid (SNP) which is a NO donor was used to apply to the plants (in the experiments). The *H. annuus* seeds that were grown in perlite were transferred to water culture containers that contain ¼ Hoagland solutions when they were 10 days old. After the beginning of senescence in the cotyledons (app. 23 days) the control group plants were placed in the containers containing ¼ Hoagland, while the experimental group plants were transferred into the containers including different SNP concentrations prepared with ¼ Hoagland (0.1, 1, 10, 100 and 400 μM SNP). When 50% senescence was observed in the control group, the cotyledons were harvested and the experiments were performed.

Detection of senescence degree: The degree of senescence was determined by "Plastocron Index" method which was developed by Lindoo & Noodén (1976) for soybean. With this method which was commonly used by several researchers (Sağlam, 1989; Lohman *et al.*, 1994) senescence was visually observed without interfering the plant or the cotyledons. In the "Plastocron Index" method the senescence degree of the cotyledons of a plant was determined by taking the ratio of the green area of cotyledons into consideration. The equation given below was used for calculating the senescence degree of the cotyledons of the plants that make up the experiment and control groups in terms of average green area (Sağlam, 1989):

$$\text{AGA } \% = \frac{(n_1 \text{ x } 1.00) + (n_2 \text{ x } 0.75) + (n_3 \text{ x } 0.50) + (n_4 \text{ x } 0.25) + (n_5 \text{ x } 0.00)}{\Sigma \text{ n}}$$

where
AGA = average of green area of the cotyledons,
n1 = the number of cotyledons the whole of which is green,
n2 = the number of cotyledons of which 75 % is green (25 % yellow),
n3 = the number of cotyledons of which 50 % is green (50 % yellow),
n4 = the number of cotyledons of which 25 % is green (75 % yellow),
n5 = the number of cotyledons of which 0 % is green (dried in appearance),
Σn = the total number of cotyledons ($n_1 + n_2 + n_3 + n_4 + n_5$).

Determination of chlorophyll amount: Total chlorophyll was determined according to Arnon (1949). After the fresh weights of the cotyledons that were harvested at the day that 50% senescence was observed in the control group were recorded the cotyledons were extracted in a mortar containing 3 ml 80 % acetone and $CaCO_3$. After centrifugation at 3000 g, +4˚C for 10 minutes the supernatant was measured for the volume. Then supernatants were spectrophotometrically measured at 645 and 663 nm absorption values were put in their places in the Arnon formula and total chlorophyll amount in 1 milliliter was calculated as mg.

Arnon Formula

$$\text{Total chlorophyll (C)} = 20{,}2 \times D_{645} + 8{,}02 \times D_{663}$$

Determination of total soluble protein amount: For the quantitative determination of total soluble protein amount Bradford' s (1976) Dye-binding method was employed. The cotyledons fresh weights were recorded and extracted in 3 ml 0.1 M Sodium phosphate buffer (pH 7.0) +4˚C in a cold mortar. Homogenate was centrifuged at 13.000 rpm, +4˚C for 30 minutes. Bio-Rad previously prepared with Comassie Brillant Blue G-250 was diluted at ¼ ratio and 0.1 supernatant was added in to 5 ml of it. Distilled water 0.1 ml was added to 5 ml buffer to use as blank. The samples and blank were kept in dark at room temperature for 20 minutes. Then they were measured at 595 nm with a spectrophotometer. The obtained absorption values were calculated according to bovine serum albumin (BSA) protein standard which has been previously prepared and the amount of total protein was estimated as µg/ml.

Spectrophotometric determination of peroxidase activity: The method developed by Birecka *et al.* (1973) was used in the experiments to determine Peroxidase activity. The cotyledons fresh weights were recorded were extracted in 3 ml 0.1 M Sodium phosphate buffer (pH 7.0) +4 ˚C in a cold mortar. Homogenate was centrifuged at 13.000 rpm, +4 ˚C for 30 minutes. Fresh buffer was prepared by adding 5 mM H_2O_2 and 15 mM guaiacol to 0.1 M Sodium phosphate buffer. 60 µl supernatant was put into 1 ml of this buffer. Measurements were made at 470 nm wavelength at 15 sec intervals for 2 minutes (with the spectrophotometer**)**. The Peroxidase enzyme activity was stated as ΔA / g fresh weight / minute.

Statistical calculations: Standard errors indicated by bars in the graphics and standard deviation were calculated with the following formulas.

$$\text{Standard error} = \sigma / \sqrt{n} \qquad \sigma = \sqrt{\sum (x_i - \bar{x})^2 / n}$$

σ: Standard deviation.
xi: Values of the variants from the first to n
$\bar{x}$: Mean of the variants from the first to n
$\sum(xi-\bar{x})2$: Total of the squares of the difference of the values from the first to n from the mean.
n: Number of the samples.

As the number of the samples was more than 30, total of the squares of the differences of the variants from the mean was divided by the number of samples (n) for the calculation of standard deviation.

Results

Cotyledon senescence was delayed in seedlings that were grown at 0.1, 1 and 10 μM SNP concentrations compared to the control group showing 50% cotyledon senescence (Fig. 1). 100 μM SNP concentration exhibited more or less the same senescence level with the control group, whereas 400 μM SNP concentration senescence was observed to be faster.

While 30% increase was observed in the chlorophyll amount of 0.1 μM SNP group, 3% increase was seen in the chlorophyll amount of 1 μM SNP applied cotyledons when compared to the chlorophyll amount of the control group. Contrary to the 0.1 ve 1 μM SNP groups, 8% decrease was observed in the chlorophyll amount of 10 μM SNP group, while 17% decrease in 100 μM SNP group and 22% decrease in 400 μM SNP group when compared to the chlorophyll amount of in the control group.

As seen in the accompanying Fig. 3 when the total soluble protein amounts of the experimental and control group cotyledons were compared increases of 2, 9 and 25% were observed at 0.1, 1 and 10 μM SNP concentrations, respectively, whereas 5% decrease at 100 μM SNP and 7% at 400 μM SNP were found.

When the experimental series were compared with the control group for Peroxidase enzyme activity a decrease of 12% was found in 0.1 μM SNP group while 1% increase at 10 μM SNP, 4% at 100 μM SNP and 10% at 400 μM SNP concentrations were observed.

Variations in the chlorophyll and total soluble protein amount and Peroxidase enzyme activity of the cotyledons were time to time compatible with plastocron index while they were incompatible at other times.

Discussion and Conclusions

The cotyledons of sunflower seeds undergo senescence gradually, turn yellow and finally die. During this process changes at biochemical level take place in cotyledons as well as morphological and anatomical changes. In this study, the effects of different SNP concentrations that were applied exogenously to sunflower seeds, tosenescence process were evaluated on the basis of various parameters such as chlorophyll content, total soluble protein amount and Peroxidase enzyme activity.

It has been determined from the results of Plastocron index that senescence begins on day 23. Some researchers have reported that senescence begin on the 19^{th}, 20^{th} and 22^{nd} day in sunflower seeds (Sağlam, 1989; Cevahir, 1992). This fact reveals that the degree of senescence is related to environmental factors including conditions of growth chamber as well as internal factors. No information is available in the relation between cotyledon senescence in sunflower seeds and NO, many data about the relation between senescence process and some plant growth regulators exist (Taiz & Zeiger, 2002; Srivastava, 2002; Noodén, 1988; Grbic & Bleecker, 1995; Arteca, 1996; Creelman & Mullet, 1997; Kappers *et al.*, 1998; Zavalet-Mancera, 1999). This study provided evidence that NO, which we think it may be a growth regulator, delays the cotyledon senescence in sunflower at lower concentrations (0.1, 1 ve 10 μM), and induces it at higher concentrations (100 ve 400 μM).

Determination of the senescence degree:

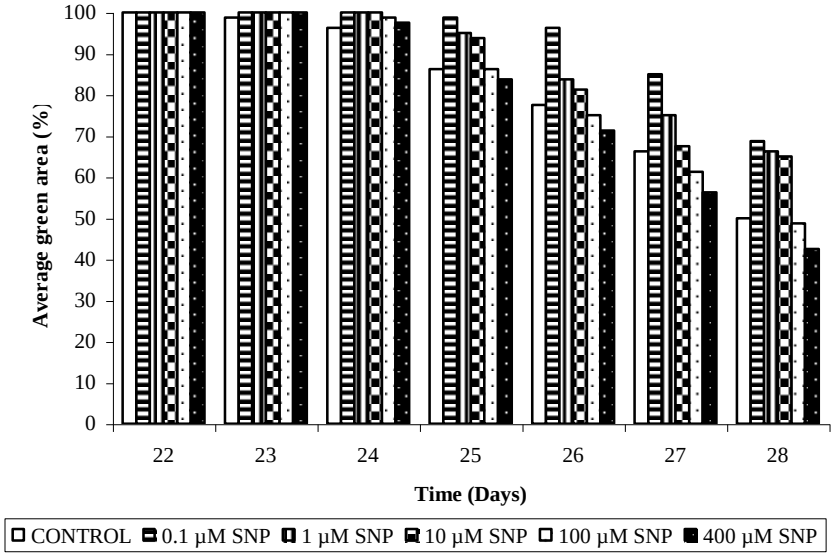

Fig. 1. The comparison of the percentages of the average green areas of the cotyledons of the control and experimental groups (0.1, 1, 10, 100 and 400 µM SNP).

Chlorophyll amount:

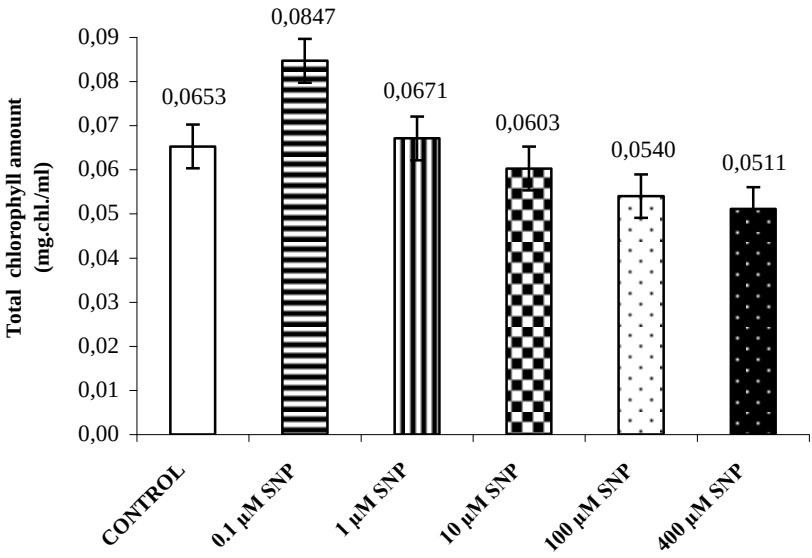

Fig. 2. The comparison of the chlorophyll amount of the cotyledons of the control and experimental groups (0.1, 1, 10, 100 and 400 µM SNP) plants that were harvested at the day of 50% senescence ($p<0,05$).

Total soluble protein content:

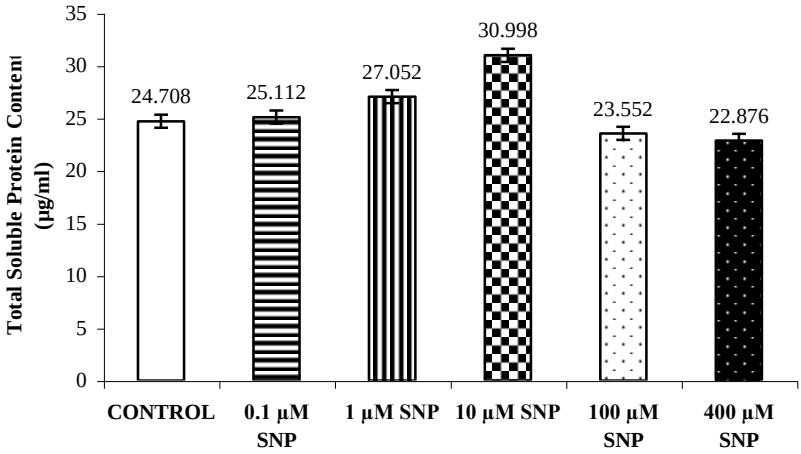

Fig. 3. The comparison of the total soluble protein amount of the cotyledons of the control and experimental groups (0.1, 1, 10, 100 and 400 µM SNP) plants that were harvested at the day of 50% senescence ($p<0,05$).

Peroxidase activity:

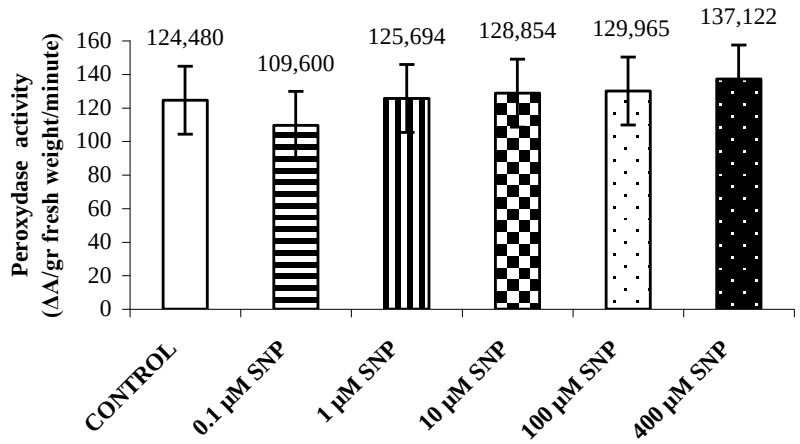

Fig. 4. The comparison of Peroxidase activity of the cotyledons of the control and experimental groups (0.1, 1, 10, 100 and 400 µM SNP) plants that were harvested at the day of 50% senescence ($p<0,05$).

Plastocron index:

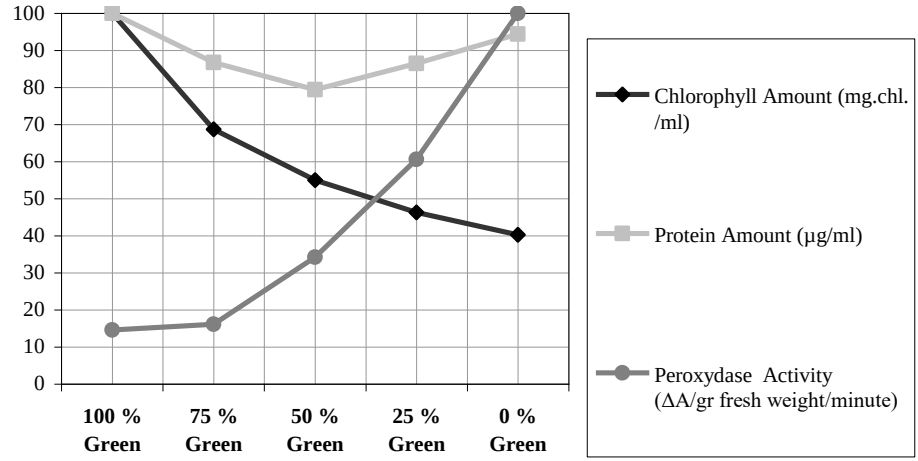

Fig. 5. The comparison of plastocron index showing (various phases of senescence of the control of the cotyledons and experimental groups (0.1, 1, 10, 100 and 400 µM SNP) plants that were harvested at the day of 50% senescence, amount of chlorophyll and total soluble protein and Peroxidase activity.

From the observations of chlorophyll amounts of the cotyledons of the sunflower seeds subjected to SNP, application of 0.1 µM SNP is the most effective concentration giving rise to increased chlorophyll content by 30%. Although a very slight increase similar to the control has been observed with 1 µM SNP concentration, the chlorophyll amount is less than the control (at other concentrations). Because senescence is induced by 100 and 400 µM SNP applications, decrease in the chlorophyll amount at these concentrations may be explained with chlorophyll destruction, according to Plastocron index results, senescence is delayed at 10 µM SNP concentration. This is confirmed by chlorophyll amount which is decreased by 8% although it is not as much as with 100 and 400 µM.

However, this fact is not parallel to the information that chlorophyll destruction begins in the senescence at first, making us think that 10 µM SNP concentration affects pigment content of chloroplasts by a different mechanism. Laxalt *et al*., (1997) suggested that NO that serves as a protective molecule in potato leaves infected with *Phyptophthora infestans* prevents loss of chlorophyll by either protecting chloroplast membranes against toxicity of reactive oxygen species or affecting any step in chlorophyll synthesis and destruction process. While chlorophyll amount at lower (0.1 and 1 µM SNP) and higher (100 and 400 µM SNP) concentrations are coherent with the findings of the above mentioned studies, decreased chlorophyll content at 10 µM SNP supports our hypothesis that this concentration affects different processes.

When the total soluble protein amounts of the cotyledons are taken into account, protein amount seems to increase in parallel with increasing of concentrations, by reaching a peak at 10 µM SNP, and to suddenly decline below the control at 100 µM SNP, by gradually decreasing at 400 µM SNP. While some proteins are degraded during

senescence new protein are synthesized, as stated by Lohman *et al*., (1994). Increased protein amount at 0.1, 1 and 10 μM SNP concentrations that delay senescence according to Plastocron index is probably due to synthesis of new proteins and slowed or arrested protein degradation. Affected regulation of senescence by changes in total soluble protein amount is supported by current literature. In the light of these data, we may say that NO is effective in protein amount during senescence process either alone or with other factors. Decreased protein amount (at 100 and 400 μM SNP concentrations) may be result of a decrease in the present protein amount and slower synthesis of new proteins as senescence develops. Or, it may be because of NO which elevates its concentration by combining with some substances such as proteins including Fe when it occurs at high concentrations in the cell. Sankhla *et al*., (2003) reported that NO decreases ethylene production which delays senescence, by interacting with proteins, which include metal and thiol, such as signal proteins, receptors, enzymes and transcription factors since it is a highly active molecule.

As to Peroxidase activity, a 12% decrease was observed at only 0.1 μM SNP concentration while a gradual increase was seen at other concentrations. Changes in Peroxidase enzyme activity are accepted as a parameter of senescence (Parish, 1968; Grover & Sinha, 1985; Mukherjee & Rao, 1993). However Patra & Mishra (1979) and Yanova *et al*., (2001) oppose that they cannot be senescence indicator. The facts that senescence is delayed at 0.1 μM SNP concentration with lower POD activity, and POD activity is increased at higher concentrations, depending on senescence may render this anti oxidant enzyme a senescence indicator.

Effects of NO on senescence process were studied by a limited number of researchers. The common point resulted from the current studies is that exogenous SNP application may delay senescence. Results of the present study are in accordance with the studies by Leshem & Haramaty (1996), Beligni & Lamattina (2000), Leshem *et al*., (1998) stating that senescence may be delayed by exogenous NO applications. However, delay of senescence is directly related to concentration of SNP which is exogenously applied. The present study reveals that duration of delay increases as SNP concentrations are lowered.

As a result, it may be stated that exogenously applied NO plays a role in cotyledon senescence that takes place during normal development period of sunflower, and lower concentrations (0.1, 1 ve 10 μM SNP) delay senescence while higher concentrations (100 ve 400 μM SNP) induce it. In the light of the result of the present study, one may say that NO regulates senescence process by interacting with other plant growth substances on the basis of NO-ethylene relationship rather than affecting senescence signal receptor system.

Acknowledgements

This work was supported by Research Fund of the Istanbul University. Project number T-328/03112003.

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(Received for pulication 5 January 2008)