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Retardation of senescence by *meta*-topolin in wheat leaves

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Abstract

A new family of endogenous aromatic cytokinin have been discovered by Strnad et al. (1997) and named as *meta*-topolin (*m*T). The aim of this research is to reveal the relation of *m*T with senescence in excised wheat leaf segments. The effect of *m*T on protease activity, chlorophyll and soluble protein contents as senescence parameters have been investigated. Exogenous application of *m*T was effective in increasing chlorophyll content. Incubation of leaf segments in 0.25, 0.5 and 1.0 mM *m*T increased the chlorophyll a content by 14, 23, 41 % respectively compared to the control leaves on 10th day of incubation. Chlorophyll b also exhibited the results in the same manner. Various concentrations of *m*T had inhibitory effect on acid and neutral protease activities, specially neutral protease activity decreased gradually with the increasing concentration of *m*T. Fresh weight and soluble protein content exhibited linear stimulation by the application of increasing *m*T concentration. *m*T treatments prevented the DNA degradation during the senecence but these determinations did not depend on variation in concentration. A natural aromatic cytokinin *m*T is very active in the retardation of senescence. *m*T responded to senescence parameters very significantly. The results of this research is exhibited *m*T as a promising plant growth regulator in physiological studies.

Key words: Chlorophyll, *meta*-topolin, nucleic acid, protease, senescence

Meta-topolinin buğday yapraklarında senesensi geciktirmesi

Özet

Yeni bir aromatik sitokinin ailesi Strnad ve ark. (1997) tarafından keşfedilmiş ve *meta*-topolin (*m*T) olarak isimlendirilmiştir. Bu araştırmanın amacı buğday yapraklarından alınan segmentlerde *m*T in senesens ile ilişkisini ortaya koyabilmektir. Bu amaçla *m*T in senesens parametreleri olan klorofil yıkımına, proteaz enziminin aktivitesine ve protein içeriğine olan etkileri araştırıldı. Haricen uygulanan *m*T klorofil yıkımını önlemede etkin bulundu. Yaprak segmentlerinin 0.25, 0.5 ve 1.0 mM *m*T içinde inkübe edilmesi klorofil a içeriğini 10. günde kontrol yapraklarına oranla sırası ile % 14, 23 ve 41 oranında arttırdı. Klorofil b içeriğinde de aynı doğrultuda sonuçlar elde edildi. *m*T in farklı konsantrasyonlarının asit ve nötr proteaz aktivitesini inhibe ettiği, özellikle nötr proteaz aktivitesinin artan *m*T konsantrasyonu ile dereceli bir şekilde azaldığı belirlendi. Taze ağırlık ve protein içeriği de artan *m*T konsantrasyonu ile birlikte lineer bir yükseliş sergiledi. *m*T uygulamalarının senesens sırasında meydana gelen DNA yıkımını da önlediği belirlendi, fakat bu saptamalar konsantrasyon değişimine bağımlılık göstermedi. Doğal aromatik sitokinin *m*T senesensi geciktirmede çok aktif bulundu. *m*T senesens parametrelerine kayda değer bir şekilde cevap verdi. Bu araştırmanın sonuçları *m*T in fizyolojik araştırmalar için çok ümit verici bir bitki büyüme düzenleyicisi olduğunu ortaya koydu.

Anahtar sözcükler: Klorofil, *meta*-topolin, nukleik asit, proteaz, senesens

Introduction

A regulatory role of cytokinins in senescence comes from the senescence-delaying effects of cytokinin treatments. Because of the potential and realized benefits from delaying senescence of various tissues with cytokinin treatments numerous studies have concentrated around this subject (Naito et al., 1978; Gilbert et al., 1980; Nooden and Leopold, 1988; Kraus et al., 1993).

Cytokinin researches had focused for a long time on members of the isoprenoid class represented by zeatin, isopentenyladenin and related compounds. For this reason the aromatic 6-benzylaminopurine (BA) and its derivatives were thought to be purely synthetic cytokinins. But other cytokinins with an aromatic side chain have also been determined and identified in different plant tissues. Recently Strnad et al. (1997) have discovered a new family of endogenous aromatic cytokinin and named as *meta*-topolin (6-[3-hydroxylbenzyl-amino]purine) (*m*T). This compound was first detected in poplar leaves and Strnad et al. (1997) adopted the name topolin derived from "topol" the Czech word for poplar.

Plant senescence is initiated and accompanied by a series of degradative events. In leaves senescence is correlated with sharp increases in RNase and protease activities which may lead to nucleic acid and protein breakdown, followed by disintegration of chloroplast structure and ultimately chlorophyll loss (Thimann, 1980; Stoddart and Thomas, 1982).

The senescence of leaves involves changes in their photosynthetic apparatus. Because yellowing is so conspicious, chlorophyll breakdown has served as the major parameter for the measurement of leaf senescence (Gut et al., 1987; Jenkins et al., 1981; Young et al., 1991). Cytokinins are very effective in delaying this breakdown indicating that these hormones are somehow involved in maintaining the photosynthetic apparatus of plant organs. However it is now known that there are some exceptions that show poor correlation or no correlation at all between chlorophyll breakdown and the other characteristic symptoms of senescence (Thomas and Stoddart, 1980).

One of the early events in leaf senescence is the well documented rise in protease activity (Thimann, 1980). However little is known about the regulation of proteolysis itself during leaf senescence. The early observations (Richmond and Lang, 1957; Wollgiehn, 1967) that cytokinins exert parallel effects in maintaining protein and nucleic acid levels while inhibiting senescence has led to the generalization that cytokinins delay senescence by maintaining or promoting protein and nucleic acid synthesis.

The aim of this research is to reveal the relation of *m*T a new aromatic cytokinin, with senescence in excised wheat leaf segments. For this purpose we have investigated the effect of *m*T on chlorophyll breakdown, protease activity, and soluble protein content as main senescence parameters.

Material and methods

Plant material

Six first leaf segments (3 cm each) from 10 days old wheat (*Triticum aestivum*) seedlings were floated in various concentrations (0.25, 0.5 and 1.0 mM) of *m*T for 10 days in plant growth chamber (8000 lux light intensity, 12 h light, 12 h dark photoperiod and 25±2°C). Distilled water was used for control treatments.

Measurement of chlorophyll content

For chlorophyll determination leaf segments were homogenized in 80 % acetone. The samples were centrifuged at 4000 g for 5 min and the optical density of the supernatant was read at 663 and 645 nm using a spectrophotometer according to the Arnon (1949).

Initial values of each analysis were measured in leaf segments at the start of each experiment. We have expressed and discussed all results according to the final controls which mean incubated in distilled water for 10 days.

Measurement of soluble protein content

Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Determination of protease activity

Six leaf segments (3 cm each) were homogenized in a

prechilled mortar with 2 ml of cold 50 mM phosphate citrate buffer (pH 6.0). The homogenates were kept in the cold (4°C) for 0.5 h and then centrifuged at 12000 g for 15 min at 4°C. The clear supernatant fraction was assayed for protease activity using Azocoll (Calbiochem) as the substrate (Kaur-Sawhney et al., 1982). The final 1 ml reaction mixture contained 5 mg Azocoll, 0.8 ml of 50 mM phosphate-citrate buffer (pH 4.2 for acid protease and pH 6.6. for neutral protease) and 0.2 ml of the crude enzyme. The tubes stoppered, vortexed and floated in a water bath equipped with a shaker and maintained at 43°C for 3 h. Controls were similarly prepared, without enzyme. The reaction was terminated by immersing the tubes in an ice bath for 1 h, and the tubes were centrifuged to remove the undigested Azocoll. All data are expressed as A (520 nm) per g fresh weight. The absorbance of the supernatant fractions was measured at 520 nm. Each assay was replicated five times.

DNA extraction and electrophoresis

Approximately 100 mg frozen leaf segments were homogenized in liquid nitrogen by the addition of extraction buffer (15 % sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 250 mM NaCl). Initial control samples were not incubated in distilled water, they were extracted directly after excision of leaf segments (Walbot, 1988). These extracts were centrifuged at 6000 g for 5 min at 4°C and pellet was suspended in suspension buffer (20 mM Tris HCl, pH 8.0, 10 mM EDTA) after that 100 ml sodium lauryl sulfate was added and incubated at 70°C for 15 min, then 7.5 M ammonium acetate was added to the tubes and left in ice for 30 min. After this procedure extracts were centrifuged at 18 000 g for 5 min and 3 ml isopropanol was added to the supernatants and again were left in ice and DNA was precipitated by the centrifugation at 17 000 g for 5 min. After this procedure pellets were dissolved in Tris-EDTA buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA) and RNase (5 mg/ml) was added to the samples and left at 37°C for 5 min. Phenol-chloroform mixture was added to each tube and centrifuged again at 17 000 g for 10 min. Upper phases were transferred to the another tube and same procedure was repeated. Again upper phase was transferred to the new tube and sodium acetate (pH 5.2) was added to adjust the final concentration to 0.3 M and two volume cold ethanol was added to the extract and frozen in liquid nitrogen and left to -70°C for 30 min. After this process extracts were centrifuged at 18 000 g for 10 min and pellets were washed with 70 % cold ethyl alcohol, and evaporated after that dissolved in Tris-HCl buffer. Isolated DNA was determined spectrophotometrically at 260 nm.

Isolated DNA was analyzed by electrophoresis. 1.2 % agarose gels were prepared in tris acetate EDTA buffer (40 mM tris acetate, pH 8.2, 1 mM EDTA) Ethidium bromide (10 mg/ml) was added to the gels to be able to observe DNA under UV light. Loading buffer contained bromophenol blue (25 %) and sucrose (40 %). Electrophoresis was carried out with a current of 140 V and 70 mA for two hours.

Results and Discussion

In the present study it was established that the fresh weight of wheat leaf segments increased gradually with the increasing concentration of *m*T comparing with the final control leaves (Figure 1). Incubation of leaf segments in 0.25, 0.5 and 1.0 mM *m*T incresed the fresh weight by 4, 6, and 9 % respectively.

It was also determined that exogenous application of *m*T was effective in preventing chlorophyll breakdown during the senescence. There was 54 % total chlorophyll loss in final control leaves which were senesced compared to initial values on 10th day of incubation (Figure 2). Inhibitions of total chlorophyll loss by *m*T was increased with the incubation time: Decrease in chlorophyll a content was 5 % on 3rd day of incubation, but compared to initial value this percentage increased to 41 % compared to final control condition on 10th day (Figure 2). The chlorophyll b content difference between initial and final control leaves was found to be 54 %. Incubation of leaf segments (Figure 3) in 0.25, 0.5 and 1.0 mM *m*T increased the chlorophyll a content by 18, 25, 43 % respectively compared to the final control leaves on 10^{th} day of incubation. In our study *m*T at 1.0 mM concentration has been found to be the most effective in the retardation of senescence. We have established the same trends for chlorophyll b (Figure 3). Total chlorophyll loss was 44 % in final control leaves according to the total chlorophyll content at initial leaves. The loss of total chlorophyll in the final control was greater than that of *m*T-treated

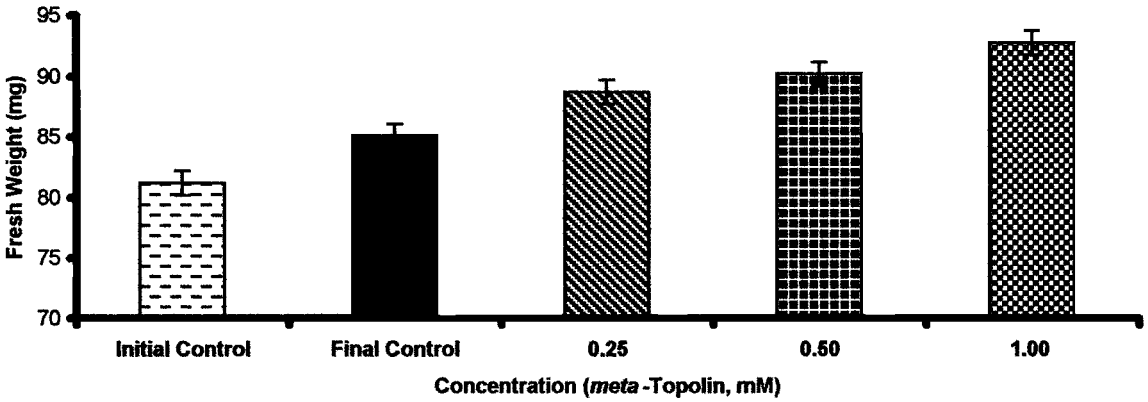

Figure 1: Effects of *meta*-topolin on fresh weight of wheat leaf segments. Vertical bars represent standard errors. Each value are average of 10 experiments.

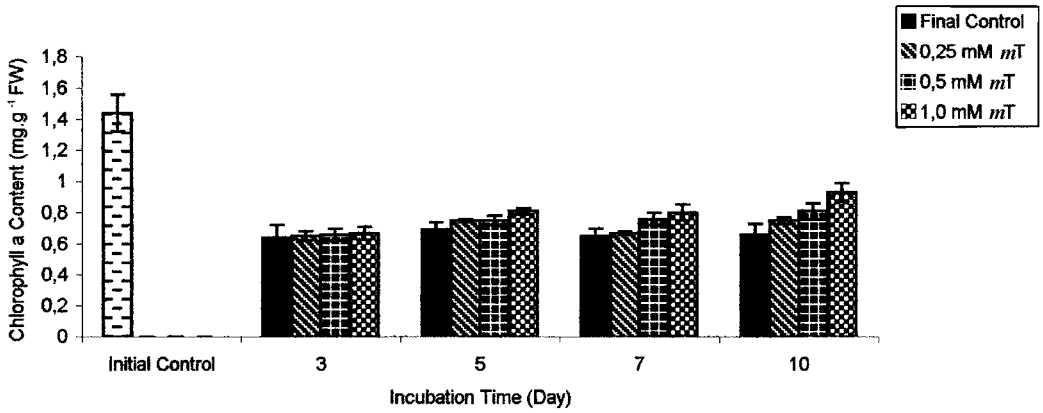

Figure 2: Effects of *meta*-topolin on chlorophyll a content of wheat leaf segments. Vertical bars represent standard errors. Each value are average of 4 experiments.

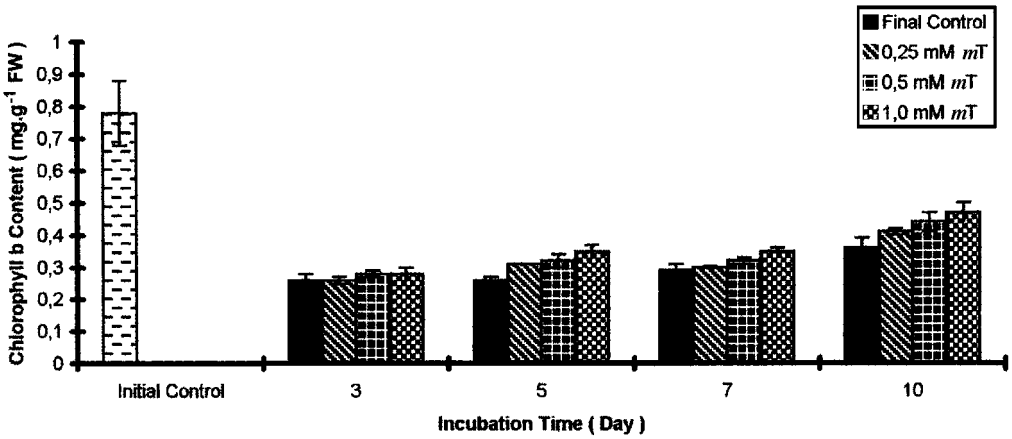

Figure 3: Effects of *meta*-topolin on chlorophyll b content of wheat leaf segments. Vertical bars represent standard errors. Each value are average of 4 experiments.

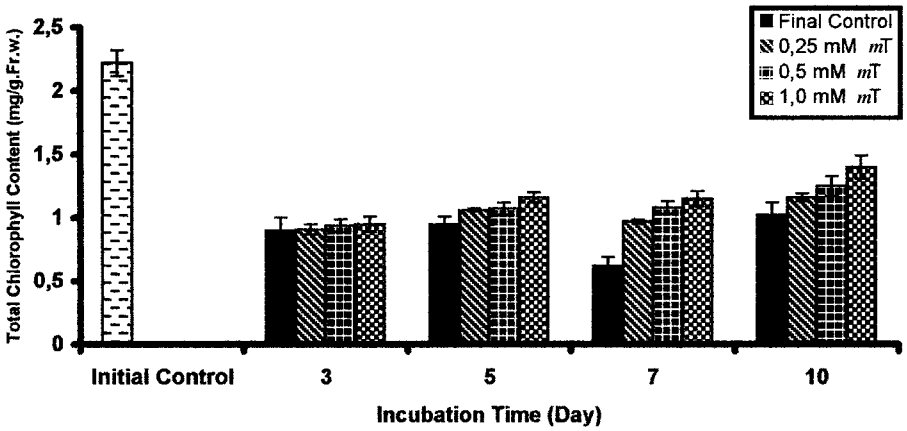

Figure 4: Effects of *meta*-topolin on chlorophyll b content of wheat leaf segments. Vertical bars represent standard errors. Each value are average of 4 experiments.

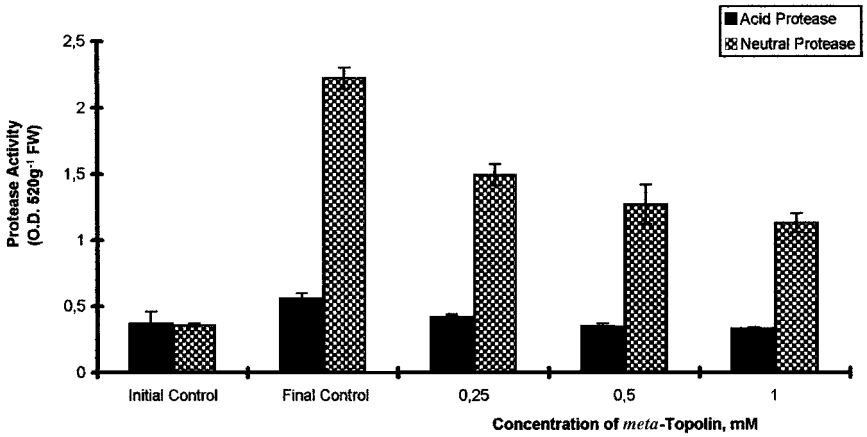

Figure 5: Effects of *meta*-topolin on chlorophyll b content of wheat leaf segments. Vertical bars represent standard errors. Each value are average of 4 experiments.

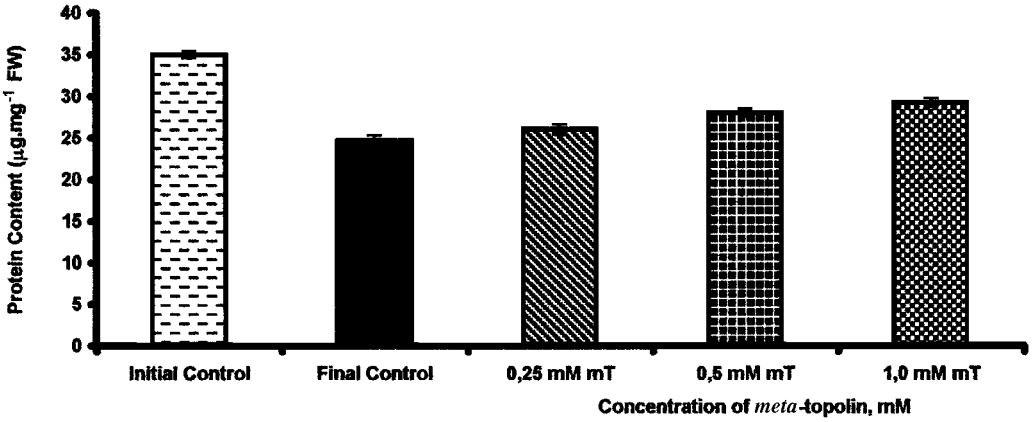

Figure 6: Effects of *meta*-topolin on soluble protein contents of wheat leaf 5 segments. Vertical bars represent standard errors. Each value is average of 4 experiments.

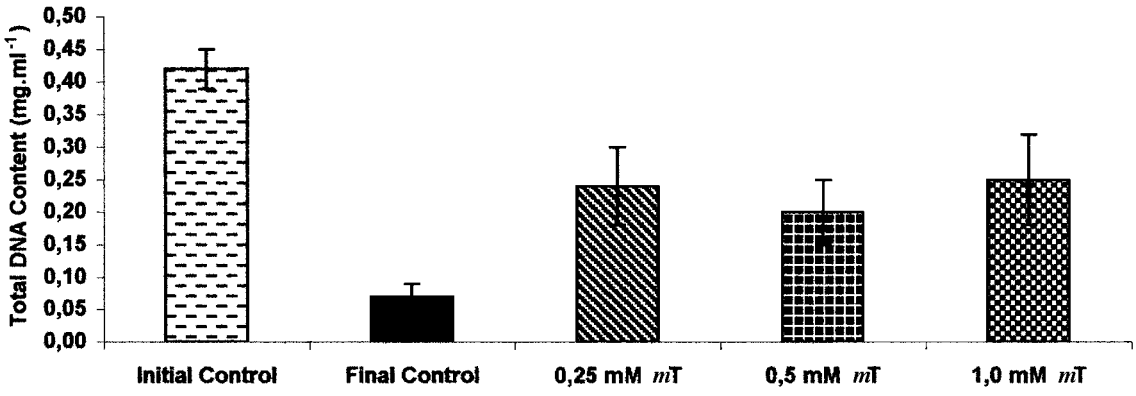

Figure 7: The effect of *meta*-topolin on total genomic DNA content in excised wheat leaf segments. Each value is average of 4 experiments.

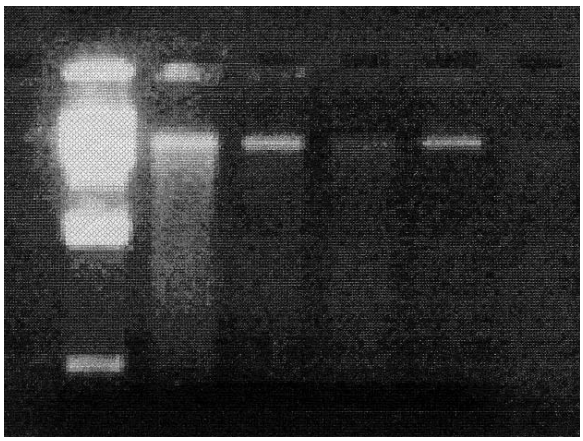

Figure 8: The effect of *meta*-topolin on the pattern of DNA in excised wheat leaf segments. Lane 1: Marker, Lane 2: Initial control, Lane 3: 1 mM *m*T, Lane 4: 0.5 mM *m*T, Lane 5: 0.25 mM *m*T, Lane 6: Final control.

leaf segments (Figure 4). Similarly many researchers have reported that exogenously applied cytokinins retarded the loss of photosynthetic pigments during the senescence of leaves and cotyledons (Thimann, 1980; Stoddart and Thomas, 1982; Chen and Kao, 1991; Jordi et al., 1993; Durmuş and Kadıoğlu, 1998).

When excised wheat leaves are incubated in distilled water their protease activity increases dramatically with the advancement of senescence. Increased activity was observed in two commonly reported proteases having pH optima at 4.2 and 6.6 with Azocoll as substrate. The rise in protease activities was inhibited when leaves were floated on *m*T solutions (Figure 5). Various concentrations of *m*T had inhibitory effect on acid and neutral protease activities, specially neutral protease activity decreased gradually with the increasing concentration of *m*T (Figure 5). The inhibition was greater for neutral than for acid proteases. The acid protease activity was inhibited by 41 % and neutral protease activity was inhibited by 49 % after the treatment of *m*T at 1 mM concentration compared to the final control leaves. These results indicate that protease activity is inhibited by *m*T. Besides these protease activites especially neutral protease activities was very low at initial compared to final control leaf segments which were already senesced.

Similarly Anderson and Rowan (1965) have established the decrease in protein contents during the aging of tobacco leaves. It has also been shown that the synthesis of the proteolytic enzymes synthesis preceded the senescence period (Martin and Thimann, 1972; Drivdahl and Thimann, 1977). Besides these Thayer et al. (1987) have revealed the necessity of protein synthesis to occur senescence signal and also the requirement of the synthesis of proteases for breakdown of proteins during the senescence.

Figure 6 shows the changes of soluble protein contents in wheat leaf segments treated with *m*T compared to the final control leaves. Total protein amounts was 30 % less in final control leaves compared to initial protein amounts. All concentration of *m*T used in this research increased the soluble protein contents during retarding the senescence insignificantly. These results are closely correlated with the protease activities. It has been well established that cytokinins are effective in retarding

the loss of protein (Tavares and Kende, 1970; Lamattina et al., 1987; Kraus et al., 1993).

Total genomic DNA amount was decreased by 83 % in final control samples which were senesced for 10^{th} days compared to initial control samples (Figure 8). Whereas, total DNA content increased by the treatment with *m*T compared to final control samples; 0,25, 0,50 and 1,0 mM *m*T applications decreased the total genomic DNA amounts by 43, 52 and 41 % respectively. All data showed that *m*T treatments prevented the DNA degradation during the senescence, but there was no significant differences depending on concentration of *m*T (Figure 8).

Electrophoretic patterns of DNA showed that there was no detectable amount of DNA in final control samples (Figure 8). However, the level of DNA in 1 mM *m*T treated leaves was almost the same as in initial control. These results also showed that *m*T retarded the senescence by inhibiting DNA destruction in excised wheat leaves.

In conclusion natural aromatic cytokinin *m*T is very active in the retardation of senescence of excised wheat leaves. The results of this research are exhibited *m*T as a promising plant growth regulator in physiological studies.

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