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The role of *meta*-topolin in senescence of wheat leaf segments

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Abstract

Numerous reports ascribe a stimulatory or inhibitory function of cytokinins in different developmental processes such as root growth and branching, control of apical dominance in shoot, chloroplast development and leaf senescence. Recently Strnad et al. (1997) discovered a new aromatic cytokinin, namely *meta*-topolin and they suggested this substance as a potential alternative of benzyladenin.

In our previous study we have determined that a rapid breakdown of chlorophyll and proteins in excised wheat leaf segments during the senescence period prevented by aromatic cytokinin *meta*-topolin . In this study, peroxidase activity was increased and senescence delayed compared to control leaves in *meta*-topolin treated leaf segments, and total chlorophyll content also exhibited similar results. Application of *m*T at high concentrations (0.5-1 mM) caused a decrement in polyamine content, but at low concentration (0.25 mM) it reasoned increment. Besides these, *meta*-topolin decreased the loss of total chlorophyll and it was estimated similar trends for total nitrogen content. In summary, according to the results of our study it can be concluded that *meta*-topolin has a contributing mechanism of the antisenescence action.

Key words: *meta*-topolin, nitrogen, peroxidase, polyamine, senescence

Meta-topolinin buğday yaprak segmentlerinin senesensindeki rolü

Özet

Birçok araştırma sitokininlerin farklı gelişim olaylarında örneğin kök büyümesi ve dallanmasında, gövdede apikal dominansinin kontrolünde, kloroplast gelişiminde ve yaprak senesensinde teşvik edici veya ket vurucu işlevlere sahip olduğunu ortaya koymuştur. Son yıllarda Strnad ve ark. (1997) *meta*-topolin adı verilen yeni bir aromatik sitokinin keşfetmişler ve bu maddenin benziladenine potansiyel bir alternatif olduğunu ileri sürmüşlerdir.

Biz daha önce buğday yaprak segmentlerinde senesens sırasında klorofil ve proteinin hızlı yıkımının aromatik sitokinin *meta*-topolin tarafından önlendiğini gösterdik. Bu araştırmada *meta*-topolin uygulanan yaprak segmentlerinde peroksidaz aktivitesi kontrol yapraklarına kıyasla arttı, senesens gecikti ve total klorofil içeriğinde de benzer bulgular elde edildi. Ayrıca, *m*T in yüksek konsantrasyonda (0.5-1 mM) uygulanması poliamin içeriğinde azalışa, düşük konsantrasyonda ise (0.25 mM) artışa sebep oldu. Bundan başka, *meta*-topolin total klorofil kaybını azalttı ve benzer eğilim total azot içeriğinde de saptandı. Özetle *meta*-topolin'in antisenesens aktivitede yönlendirici bir mekanizmaya sahip olduğu sonucuna varılabilinir.

Anahtar sözcükler: *meta*-topolin, azot, peroksidaz, poliamin, senesens

Introduction

Plant senescence is initiated and accompanied by a series of derivative events. In leaves senescence is correlated with a sharp decrease in nucleic acid and protein content, followed by disintegration of chloroplast structure and ultimately chlorophyll loss (Thimann, 1980; Stoddart and Thomas, 1982).

Cytokinins have been regarded as the most potent senescence-retarding hormones in plants and play a significant role in the regulation of leaf senescence (Richmond and Lang, 1957; Thimann, 1980). Retardation of senescence by cytokinin, including benzyladenin (BA) in excised leaves and cotyledons have been reported in several plant species and this synthetic growth regulator has little or no effect as a retardant of senescence in attached organs (Kraus et al., 1993; Gilbert et al., 1980).

A new family of endogenous aromatic cytokinin have been discovered by Strnad et al. (1997) and named as *meta*-topolin (6-[3-hydroxylbenzyl-amino]purine) (*m*T). *m*T was put forwarded by these researchers as an alternative of BA.

The activities of protease and peroxidase (POD) have been reported to show an increase with the advancement of senescence (Grover and Sinha, 1985). There are several studies showing that POD activity increases during senescence of detached leaves or leaf discs (Parish, 1968; Mukherjee and Rao, 1993). Increases in the activity of POD isoenzymes with the physiological age of the leaves have been reported (Parish, 1968; Ford and Simon, 1972). Parish (1968) also suggested that the increase in the activity of POD is one of the most reliable indicators of maturity and senescence. However Srivastava et al. (1983) found no difference in POD activity between young and mature leaves of barley.

The polyamines (PAs), spermidine (Spd) and spermine (Spm) and the related diamines putrescine (Put) and cadaverine (Cad) are polycations synthesizing in most living cells (Bachrach, 1973) and have been implicated as an essential growth factors for plants (Galston and Kaur-Sawhney, 1990). PAs applied exogenously are potent inhibitors of senescence of oat leaf protoplasts (Altman et al., 1977; Kaur-Sawhney et al., 1980) and of leaves and storage tissue from several plants (Kaur-Sawhney and Galston, 1979). PA-biosynthetic enzyme activities and titers decreased in senescing attached and detached oat leaves incubated in dark (Kaur-Sawhney et al., 1982), therefore these observations suggest that PAs are involved in the control of plant growth and senescence.

One of the early events in leaf senescence is the well-documented rise in protease activity (Thimann, 1980). Exogenous application of PAs retard senescence and the increment in protease activity is one of the early occurrences of senescence (Kaur-Sawhney et al., 1982).

It was established in previous study that application of *m*T to wheat leaf segments retarded senescence by decreasing protease activity and chlorophyll loss by resembling to PAs (Palavan-Ünsal et al., 2002b). The aim of this study was to establish the effect of *m*T on POD activity and nitrogen content and their relation with PA metabolism during the senescence of excised wheat leaf segments.

Materials 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 *m*M) of *m*T for 10 days in plant growth chamber (12 h light, 12 h dark photoperiod and 25±2°C). Distilled water was used for control.

Measurement of chlorophyll content

For chlorophyll determination, six leaf segments were homogenized in 80 % acetone. The samples were centrifuged at 3000 rpm for 5 min and the optical density of the supernatant was read at 663 and 645 nm with a spectrophotometer according to the Arnon (1949).

Peroxidase activity

POD activity was analysed in a reaction volume of 3 ml containing 0.1M K-phosphate buffer, pH 5.8, 15 mM guaiacol and 5 mM H_2O_2. The reaction was started by the addition of an appropriate volume of the crude homogenate and the formation of tetraguaiacol (at 470 nm) was followed continuously for 2 min in spectrophotometer (Shimadzu UV 160) (Birecka et al., 1973).

Electrophoresis for isoperoxidases

Polyacrylamide gel electrophoresis was conducted as described by Liu (1973). A separating gel of 8% acrylamide was used. The enzyme extract in 50% glycerol with 1 % bromophenol blue was applied to the gel. Electrophoresis was conducted in a cold at 4°C using reservoir buffer (14.1 g glycine and 3 g tris per liter, pH 8.3) at 10 mA. For analysis of peroxidase isoenzymes, the gel incubated for 20 min in the solution containing 0.1 M sodium phosphate buffer, pH 6.5 mM guaiacol, 5 mM H_2O_2 and then it was stored in 50% methanol.

Polyamine determinations

The PAs were extracted with 5% $HClO_4$, separated and detected after dansylation as described by Seiler and Wiechman (1967) using silica gel G plates with cyclohexane ethylacetate (3:2, v/v) as the solvent. Fluorescence was measured with spectrofluorimeter (Shimadzu RF 5000) (emission 500 nm, excitation 360 nm) and the results were compared with dansylated standards.

Nitrogen determination

The nitrogen content in the digested plant samples was determined by the procedure modified from Middleton (1960). Nitrogen content was determined by digest with Nessler's reagent. 1.0 ml of diluted digest solution, 4.0 ml H_2O, 1.0 ml of 10 N NaOH, 1.0 ml of 0.6 N sodium tartrate, 1.0 ml 2% (w/v) gum arabic and 2.0 ml Nessler's solution were added. The arabic gum and Nessler's solution were filtered through the filter paper before use. The intensity of the resulting brownish-yellow colour was measured in spectrophotometer. Standard nitrogen samples containing glycine were digested and determined with each analysis.

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

Results and discussion

In the present study it was determined that fresh weight of wheat leaf segments increased gradually with increasing concentrations of *m*T when compared to final control leaves (Table 1). There was no significant difference between initial and final control fresh weight values. 0.25, 0.5 and 1.0 mM *m*T increased the fresh weight by 4, 6 and 9 % respectively. We previously reported that natural aromatic cytokinin *m*T is active in moderating radish cotyledon growth (Palavan-Ünsal et al., 2002a).

Table 1: Fresh weight changes in excised wheat leaf segments after 10 days incubation in *meta*-topolin. Values are average of 10 replicates.

Treatment	Fresh Weight (mg)
Initial control	81.20 ± 1.7
Final control	85.09 ± 1.7
0.25 mM *m*T	88.72 ± 1.3
0.5 mM *m*T	90.22 ± 1.0
1.0 mM *m*T	92.80 ± 1.2

When chlorophyll losses were compared between initial and final control leaf segments, 46, 47 and 55 % losses in chlorophyll a, chlorophyll b and total chlorophyll contents were found respectively. Figure 1 shows the loss of chlorophyll decrement by the application of *m*T in wheat leaf segments and the loss of total chlorophyll, chlorophyll a and chlorophyll b in the senesced final control leaves were greater than that of *m*T treated leaf segments. It was found that the most effective concentration of *m*T was 1 mM, which inhibited the loss of chlorophyll. Total chlorophyll content was 36 % more in 1 mM *m*T treated leaves compared to final control leaves on 10^{th} day of incubation. As seen in Figure 1 there were similar establishments in chlorophyll a content but these differences were not so remarkable for chlorophyll b. Many researchers (Thimann, 1980; Stoddart and Thomas, 1982; Chen and Kao, 1991) have reported that exogenously applied cytokinins retarded the loss of photosynthetic pigments during the senescence of leaves and cotyledons.

The changes of POD activity in excised wheat leaf segments treated with *m*T or distilled water were presented in Figure 2. POD activity increased 48 % in final control leaf segments compared to initial control. It was also increased gradually in *m*T treated leaf segments compared to final control condition on 10^{th} day of incubation. POD activities were estimated as 17, 72, 90 % higher in 0.25, 0.5 and 1 mM *m*T applied leaf segments, respectively. These results showed that

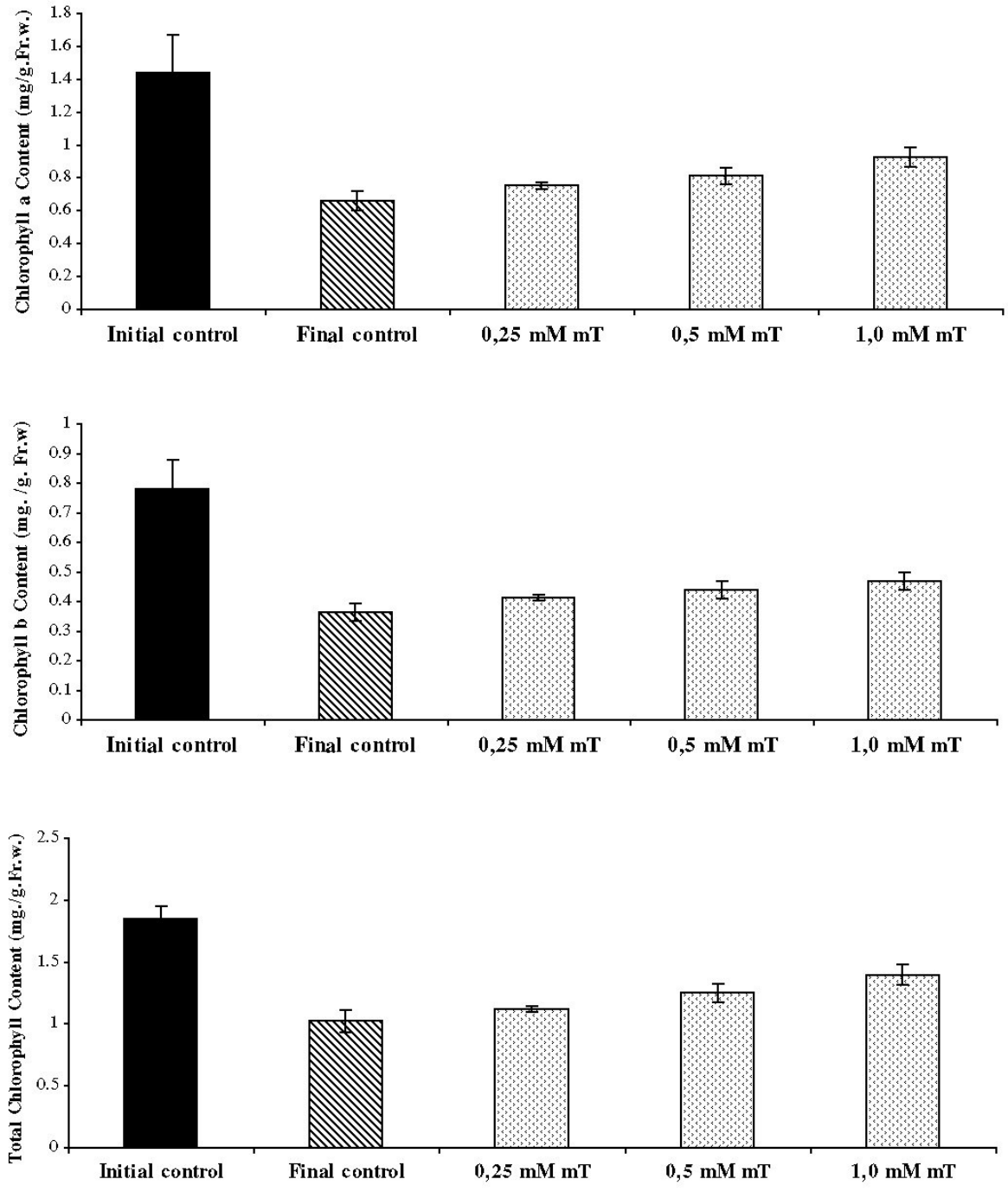

Figure 1: Effect of *meta*-topolin on chlorophyll content of wheat leaf segments on 10th day of incubation. Vertical bars represent standard errors. Each value is average of 4 experiments.

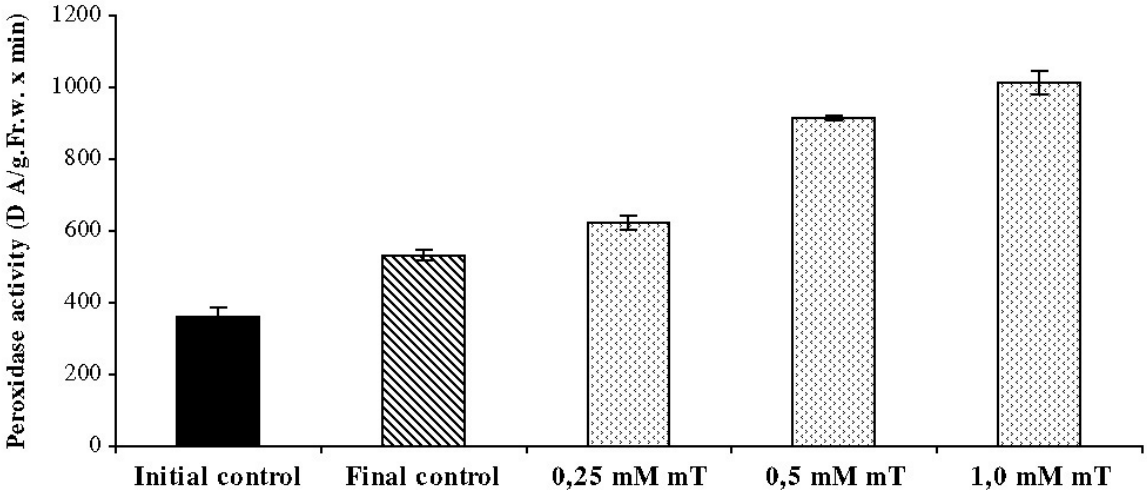

Figure 2: Effects of *meta*-topolin on peroxidase activity of wheat leaf segments on 10th day of incubation. Vertical bars represent standard errors. Each value is average of 5 experiments.

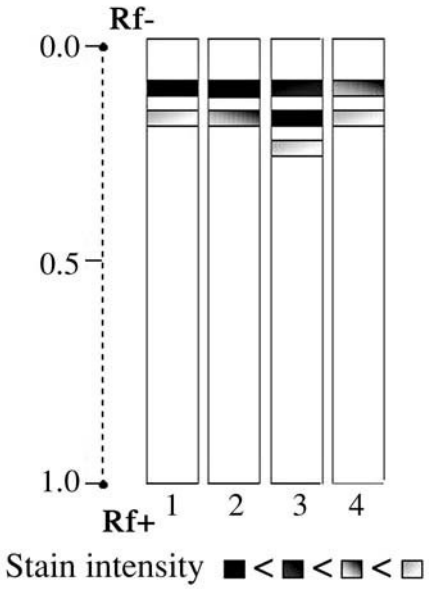

Figure 3: Effects of *meta*-topolin on the number of peroxidase isoenzymes of excised wheat leaf segments on 10th day of incubation. The gel was stained for POD activity using 0.1 M sodium phosphate buffer, pH 6, 5 mM guaiacol, and 5 mM H_2O_2. The direction of electrophoretic migration was from top (-) to bottom (+). Lane 1: Control; Lane 2: 0.25 mM *m*T; Lane 3: 0.5 mM *m*T, Lane 4: 1 mM *m*T.

there was a correlation between chlorophyll contents and POD activity; the increasing ratio in POD activity was in a larger scale than that of the chlorophyll content depending on increasing concentration of *m*T.

Changes in the contents of POD isoenzymes in excised wheat leaf segments that were treated with *m*T and distilled water were also determined. In gel analyses a difference in the number of POD isoenzymes were observed in leaves treated with 0.5 mM *m*T compared to final control and 0.25 and 1.0 mM *m*T treatments (Figure 3). In addition, the effects of 0.25 and 0.5 mM *m*T on the activity of POD were found spectrophotometrically higher than that of final control and of 1 mM *m*T treatments.

There are several reports showing that POD activity increases during the senescence of excised leaves. Increase in the activity of this enzyme with physiological age of the leaves has also been reported (Ford and Simon, 1972). Parish (1968) suggested that the increase in the activity of POD is one of the most reliable indicators of maturity and senescence. Whereas, Ford and Simon (1972) established several fold increment in POD activity when senescence was delayed in cucumber cotyledons confirming our results. It seems possible that POD has no functional significance during the senescence process of leaf segments of wheat.

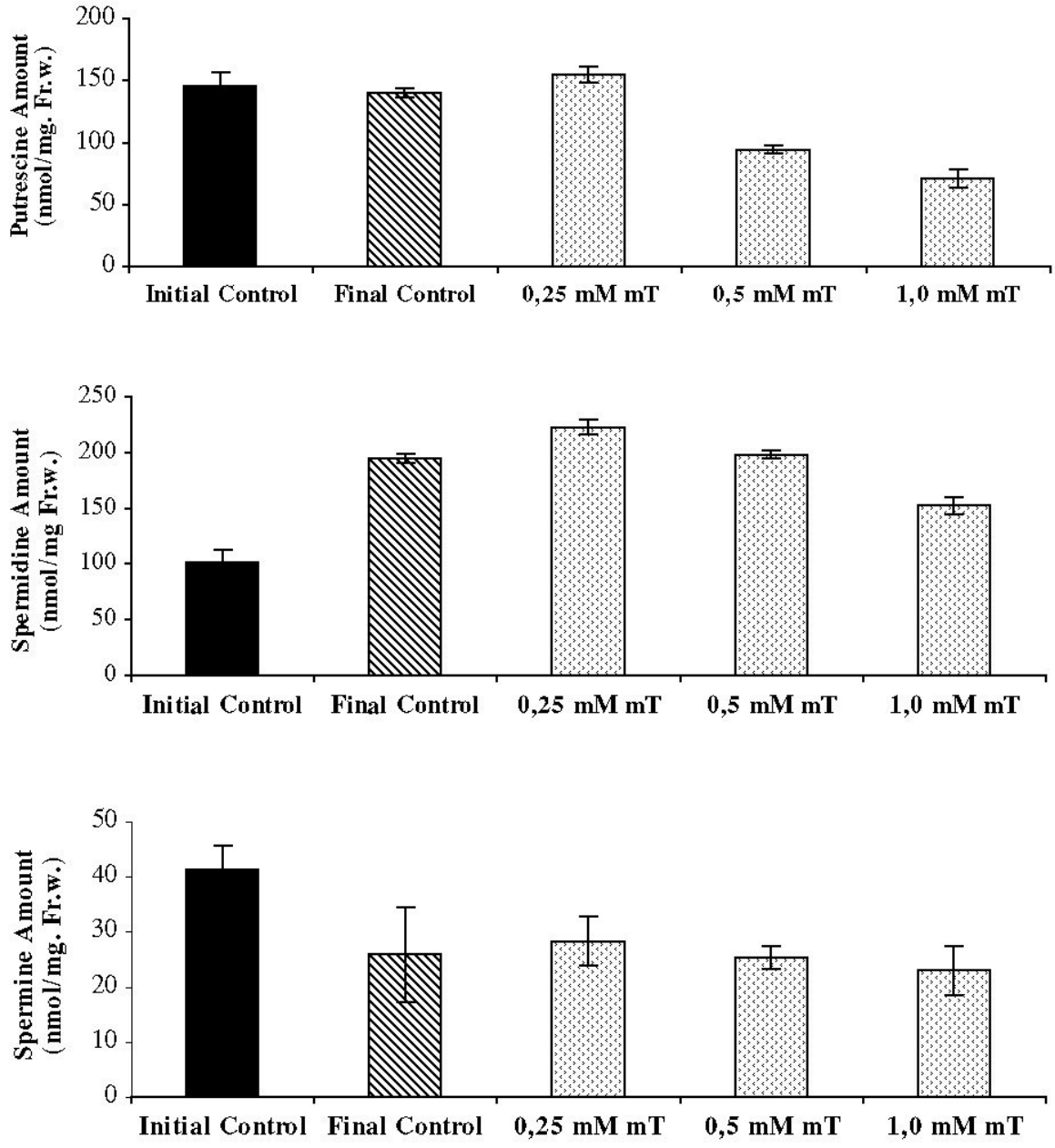

Figure 4: Effects of *meta*-topolin on polyamine content of wheat leaf segments on 10th day of incubation. Vertical bars represent standard errors. The data presented here represent of 5 experiments and each measurement was done in duplicate.

Figure 4 shows the effect of *m*T on endogenous PA contents after 10 days of incubation of wheat leaf segments. Decline in Spm contents in final control comparing to initial control leaves were established. It

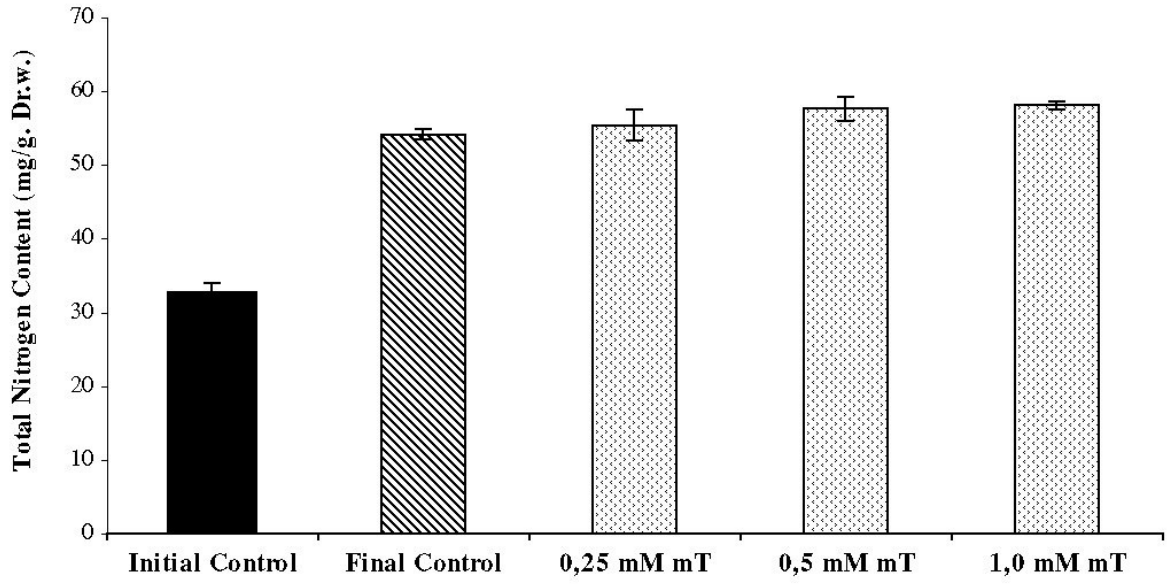

Figure 5: Effects of *meta*-topolin on total nitrogen content of wheat leaf segments on 10th day of incubation. Vertical bars represent standard errors. Each value is average of 4 experiments.

was also estimated about 10 % increments in Put, Spd an Spm contents only with 0.25 mM treatment compared to final control. Application of higher concentrations (0.5-1.0 mM) of *m*T resulted in inhibition of PA levels during the senescence of wheat leaf segments.

Among their other physiological effects, PAs are involved in the control of several stress-related phenomena such as senescence, wounding, heat and salinity both in plant organs and isolated cells and tissues. Thus exogenous application of PAs and related precursors retarded leaf senescence, stabilizing them against lysis. It was demonstrated that PAs retard chlorophyll loss and prevent the rise of RNase and proteases (Altman, 1982; Kaur-Sawhney and Galston, 1979).

It has also been reported that BA and kinetin (K) promote cotyledon growth and this response is accompanied by an increase in arginine decarboxylase activity and Put titer (Cho, 1983). Besides, Walker et al. (1988) reported that application of cytokinin to excised cotyledon resulted in a large increase in chlorophyll and Put levels. In addition, Srivastava et al. (1981) established that K delayed senescence and retarded the decrease in the activity of diamino oxidase and Spd and Spm levels. POD isoenzymes and PAs may play an important role in protecting plants against injury of oxidative factors. These findings are in agreement with only 0.25 mM *m*T treatment in this study.

In addition, the changes in the contents of total nitrogen in leaf segments, which were treated with water or *m*T on 10th day of incubation, were analysed. There was no significant difference in nitrogen content between final control and 0.25 mM *m*T treatments (Figure 5). However, *m*T at 0.5 and 1.0 mM concentrations have been found to be effective in increasing total nitrogen content while retarding the senescence.

Feller et al. (1977) have established the role of leaf proteases in the nitrogen economy of developing cereals and have determined a close relationship between depletion of protein and the built up of certain proteolytic activities in the leaf. Decreasing protease activity and increasing soluble protein content in senescing leaf segments on 10th day of incubation in *m*T solutions were established in a former study (Palavan-Ünsal et al. 2002b). In the same experimental

system, increased total nitrogen content with the application of a new aromatic cytokinin *m*T was also found. Total nitrogen content was lower in senesced leaf segments than that of *m*T treated ones. Generally, decreased total nitrogen content during the senescence was shown in attached leaf or cotyledons (Storey and Beevers, 1977). In our instance no significant differences were determined, but there were some trends in the same direction.

Actually, some authors suggest that PA increment may represent only the tip of huge nitrogen metabolism that is affected all stress conditions. In agreement with this suggestion, it was established in this study that both nitrogen and PAs which are also nitrogenous substances increases by the application of *m*T which is a new antisenescence agent. From all these results it is possible to conclude that increased POD activity, PA and nitrogen contents in *m*T treated excised leaf segments was a contributing mechanism to the overall antisenescence action of this new aromatic cytokinin.

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References

Altman A. Retardation of leaf senescence by polyamines. *Physiol Plant.* 54: 189-193, 1982.

Altman A, Kaur-Sawhney R and Galston AW. Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. *Plant Physiol.* 60: 570-574, 1977.

Arnon DI. Copper enzymes in chloroplasts, polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24: 1-15, 1949.

Bachrach U. Involvement of polyamines in plant growth and senescence. In: *Adv Polyamine Res.* Caldarera CM, Zappia V and Bachrach U (Ed). 3: 365-375, 1973.

Birecka H, Briber A and Catalfamo JL. Comparative studies on tobacco pith and sweet potato root isoperoxidases in relation to injury, indolacetic acid and ethylene effects. *Plant Physiol.* 52: 43-49, 1973.

Chen CT and Kao CH Senescence of rice leaves XXX, levels of endogenous polyamines and dark-induced senescence of rice leaves. *Plant Cell Physiol.* 32: 935-941, 1991.

Cho SC. Effects of cytokinin and several inorganic cations on the polyamine content of lettuce cotyledons. *Plant Cell Physiol.* 24: 27-32, 1983.

Feller UK, Soong T and Haeman RH. Leaf proteolytic activities and senescence during grain development of field-grown corn (*Zea mays* L.). *Plant Physiol.* 59: 290-294, 1977.

Ford TW and Simon EW. Peroxidase and glucose-6 phosphate dehydrogenase levels in cotyledons of *Cucumis sativus* (L.). *J Exp Bot.* 23: 423-431, 1972.

Galston AW and Kaur-Sawhney R. Polyamines in plant physiology. *Plant Physiol.* 94: 406-410, 1990.

Gilbert ML, Thompson JE and Dumbroff EB. Delayed cotyledon senescence following treatment with cytokinin, an effect at the level of membranes. *Can J Bot.* 58: 1797-1803, 1980.

Grover A and Sinha SK. Senescence of detached leaves in pigeon pea and chick pea: Regulation by developing pods. *Physiol Plant.* 65: 368-507, 1985.

Kaur-Sawhney R, Flores HE and Galston AW. Polyamine-induced DNA synthesis and mitosis in oat leaf protoplasts. *Plant Physiol.* 65: 368-371, 1980.

Kaur-Sawhney R and Galston AW. Interaction of polyamines and light biochemical processes involved in leaf senescence. *Plant Cell Environ.* 2: 189-196, 1979.

Kaur-Sawhney R, Shih L, Flores HE and Galston AW. Relation of polyamine synthesis and titer to aging and senescence in oat leaves. *Plant Physiol.* 69: 405-410, 1982.

Kraus TE, Hofstra G and Fletcher RA. Regulation of senescence by benzylaminopurine and uniconazole in intact and excised soybean cotyledons. *Plant Physiol Biochem.* 31: 827-834, 1993.

Liu H. A simple method for determining the relative activities of individual peroxidase isoenzymes in a tissue extract. *Anal Biochem.* 56: 149-154, 1973.

Middleton KR. New Nessler reagent and its use in the direct Nesslerization of Kjeldahl digests. *J Appl Chem.* 10: 281-286, 1960.

Mukherjee D and Rao KVM. Alteration patterns of hill activity, peroxidase activity and sugars of pigeon pea during maturation maturation and senescence. *Indian J Plant Physiol.* 36: 13-16, 1993.

Palavan-Ünsal N, Çağ S and Çetin E. Growth responses of excised radish cotyledons to *meta*-topolin. *Canadian Journal of Plant Sciences.* 82: 191-194, 2002a.

Palavan-Ünsal N, Çağ S, Çetin E and Büyüktuncer D. Retardation of senescence by *meta*-topolin in wheat leaves. *J Cell and Mol Biol.* 1: 101-108, 2002b.

Parish RW. Studies on senescing tobacco leaf discs with special reference to peroxidase. I. The effects of cutting and of inhibition of nucleic acid and protein synthesis. *Planta.* 82: 1-13, 1968.

Richmond AE and Lang A. Effect of kinetin on protein content and survival of detached *Xanthium* leaves.

Science. 125: 650- 1957.

Seiler N and Wiechmann M. Die Mikrobestimmung Von Spermin Und Spermidin als I-dimethylamino-naphtalin 5 sulfon saure Derivative. *Hoppe Seyler's Z Physiol Chem*. 348: 1285, 1967.

Srivastava SK, Raj ADS and Naik BI. Polyamine metabolism during aging and senescence of pea leaves. *Indian J Exp Biol*. 19: 440-444, 1981.

Srivastava SK, Washi DJ and Naik BL. Control of senescence by polyamines and guanidines in young and mature barley leaves. *Phytochem*. 22: 2151-2154, 1983.

Stoddart JL and Thomas H. Nucleic acids and proteins in plants. In: *Encyclopedia of Plant Physiology*. Boulter D and Parthier B (Ed). Springer-Verlag, Berlin and New York. 14A: 592-636, 1982.

Storey R and Beevers L. Proteolytic activity in relationship to senescence and cotyledonary development in *Pisum sativum* L. *Planta*. 133: 37-44, 1977.

Strnad M, Hanus J, Vanek T, Kaminek M, Ballantine JA, Fussell B and Hanke DE. *Meta*-topolin, a highly active aromatic cytokinin from poplar leaves (*Populus x canadensis* Moench., cv. *Robusta*). *Phytochemistry*. 45: 213-218, 1997.

Thimann KV. The senescence of leaves. In: *Senescence in Plants*, Thimann KV (Ed). CRC Press, Boca Raton, 88-115, 1980.

Walker MA, Roberts DR and Dumbroff EB. Effects of cytokinin and light on polyamines during the greening response of cucumber cotyledons. *Plant Cell Physiol*. 29: 201-205, 1988.