



Cytological evidences of the role of *Meta-topolin* and Benzyladenin in barley root tips

Zekiye Huyluoglu¹, Meral Unal^{1*}
and Narcin Palavan - Unsal²

¹ University of Marmara, Faculty of Arts and Sciences, Department of Biology, Göztepe, Istanbul-Turkey

² Istanbul Kultur University, Faculty of Science and Letters, Atakoy Campus, 34156 Bakirkoy, Istanbul-Turkey

Abstract

The responses of mitotic activity affecting root growth to benzyladenine (BA) and *meta-topolin* (mT) which is thought to be alternative of BA were studied with time and dose dependent experiments in *Hordeum vulgare* Tarm-92. The all concentrations of mT caused an induction on mitotic index. 10⁻⁵ mT was the most effective stimulator, increasing division frequency from 16 to 26 BA treatment exhibited variations; the low concentrations (10⁻⁵ M and 10⁻⁶ M) increased the mitotic index but the higher concentration (10⁻⁴ M) decreased. The values of mitotic index reflected to the elongation of roots, coordinately. BA and mT are both affected chromosomal behavior and caused abnormalities such as unoriented metaphase, chromosome bridges, 2 nucleate cells, micronuclei formation.

Introduction

Cytokinins, N⁶-substituted adenine derivatives, are a class of plant hormones that were first identified as cell division promoter factors (Miller *et al.*, 1955, Miller *et al.*, 1956). Cytokinins have been found in almost all higher plants as well as mosses, fungi, bacteria, and also in tRNA of many prokaryotes and eukaryotes (McGaw, 1987). Today there are more than 200 natural and synthetic cytokinins combined. Cytokinin concentrations

are highest in meristematic regions and areas of continuous growth potential such as roots, young leaves, developing fruits, and seeds (Arteca, 1996; Salisbury and Ross, 1992; Mok, 1994). Application of exogenous cytokinin to some organs that normally lack this hormone has been shown to induce cell division (Riou-Khamlichi *et al.*, 1999).

The cytokinins effect not only cell division but also many other aspects of plant growth and developmental processes including seed germination, shoot initiation and growth, apical dominance, senescence and abscission (Thimann 1980; Nooden and Leopold, 1988; Mok, 1994; Dewitte *et al.*, 1999; Werner *et al.*, 2001). Saha *et al.*, (1984) investigated the changes in cytokinin activity during seed germination in rice and they observed qualitative and quantitative changes in cytokinins in both embryo and endosperm.

Strnad *et al.*, (1997) found a new highly active aromatic cytokinin, N⁶-(meta-hydroxybenzyl) adenine from poplar leaves and proposed the name *meta-topolin* (mT). These researchers recorded that mT is more active than zeatin and benzyladenine (BA) in the promotion of shoot formation in plant tissue cultures (Kubalaková and Strnad, 1992; Werbrück and Strnad, 1996).

Numerous reports assign a stimulatory or inhibitory effect of cytokinins in different development processes such as root growth and branching, control of apical dominance in the shoot, chloroplast development and leaf senescence (Mok, 1994).

Werner *et al.*, (2001) reported that cytokinins are an important regulatory factor of plant meristem activity and morphogenesis, with opposing role in shoots and roots.

They used genetically engineered cytokinin oxidase expression in transgenic tobacco plants to reduce their endogenous cytokinin content and they observed stunted shoots with smaller apical meristems in developed cytokinin-deficient plants. In contrast, they also observed root meristems of transgenic plants were enlarged and gave rise to faster growing and more

*Correspondence Author:

University of Marmara, Faculty of Arts and Sciences,
Department of Biology, Göztepe, İstanbul - Turkey

Received: March 22, 2008; Accepted: April 5, 2008.

branched roots. These results suggested that cytokinins are an important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots.

Therefore, the objective of the present study is to compare the effects of new cytokinin *mT* with BA during seed germination, root elongation and mitotic activity in the root tips of barley.

Material and Methods

The seeds of *Hordeum vulgare* L.cv.Tarm-92 were surface sterilized with 1 % sodium hypochloride for 5 min and washed with tap water. Then they were soaked in water (control) or in BA and *mT* solutions in 10^{-6} , 10^{-5} and 10^{-4} M concentrations for overnight. These concentrations were chosen on the basis of previously reported favorable results (Haber and Luippold, 1960; Reynolds and Thompson, 1973). After that 30 seeds for each group were sown to petri dishes on filter paper moistened with distilled water or experimental solutions at room temperature. 5 replications were performed to estimate percentage of germination. Root lengths were measured with 12 hours intervals. For cytological analyses, root tips were fixed in 3:1 (v/v) ethanol-acetic acid overnight and were kept in 70 % ethyl alcohol in +4 °C. Later they were hydrolyzed with 1 N HCl at 60 °C for 13 min, they were transferred to basic fuchsin for 1.5-2 h in dark. Squash preparations were made in 2% aceto-orcein. 10 well spread slides were chosen and averages of 5000 cells were scored for each experimental group for mitotic index.

Results

Seed germination started at 12 hours after sowing and seeds were considered to be germinated after the radicle emergence from the testa. Seed germination was stimulated by all *mT* treatments used; 10^{-6} M *mT* was the most effective application and 115.4 % seed germination was estimated (Table 1). On the other hand different concentrations (10^{-6} - 10^{-4} M) of BA caused great variations in seed germination of barley. The high concentration (10^{-4} M) of BA markedly (92.3 %) reduced the seed germination compared to the control samples.

The morphology of the roots after treatment with *mT* and BA did not changed significantly. The primary root elongation was monitored up to 60 h in barley seedlings. The root growth was increased by *mT* the

Table 1. The effect of *meta*-topolin and benzyladenine on seed germination in *Hordeum vulgare*. Control: distilled water. *mT*: *Meta*-topolin, BA: Benzyladenine.

Treatment (M)	Seed Germination (%)
Control	100.0
10^{-6} <i>mT</i>	115.4
10^{-5} <i>mT</i>	107.7
10^{-4} <i>mT</i>	103.8
10^{-6} BA	100.0
10^{-5} BA	88.5
10^{-4} BA	7.7

applied all concentrations in this study (Figure 1). 10^{-5} M *mT* increased the root growth significantly associating with the treatment time.

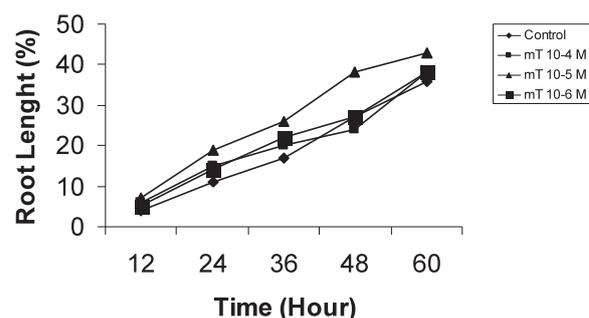


Figure 1. The effect of *meta*-topolin on the root elongation of *Hordeum vulgare*. Control: distilled water, *mT*: *meta*-topolin.

The effects of different concentrations of BA exhibited variations in the root growth of barley; while 10^{-6} M BA did not influence significantly, 10^{-5} M enhanced and, even 10^{-4} M inhibited the root growth of barley (Figure 2).

Mitotic index reveals the frequency of cell division and it is an important parameter to determine the rate of root growth. Mitotic index in control roots was established as 16.3 %. On the other hand, *mT* at all concentrations used in this study increased the cell division rate, and 10^{-5} *mT* was the most effective stimulator in division frequency (Figure 2). The mitotic index was 16.3 in the control group, while it was 26.4 in 10^{-5} *mT*. The low concentrations of BA (10^{-5} , 10^{-6} M) increased the mitotic index but the high concentration (10^{-4} M) decreased the division frequency to some extent (Figure 3). These findings are entirely

associated with the former findings with *mT* and BA in root growth.

The comparative analysis of phase indices were done. The percentages of the cells in interphase and dividing cells were established as 83.7 and 16.3 respectively in control samples. These percentages were determined as 78.1 and 21.9 in 10^{-4} M, 73.6 and 26.4 in 10^{-5} M and 80.4 and 19.6 in 10^{-6} M *mT* treatments (Table 2).

On the other hand, interphase and dividing cell percentages were found 84.8 and 15.2 % in 10^{-4} M BA, 76.1 and 23.9 % in 10^{-5} M BA and 80.7 and 19.3 % in 10^{-6} M BA treated samples respectively.

The chromosome numbers were established as 14

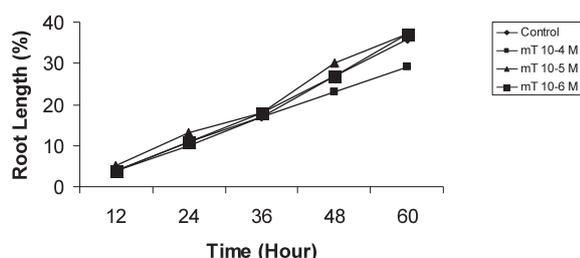


Figure 2. The effect of benzyldenine on root elongation in *Hordeum vulgare*. Control: distilled water, BA: benzyldenine.

in the root tips of *Hordeum vulgare*. Exogenously applied *mT* and BA caused some changes in the cytoplasm and in the nucleus. Vacuolization was very conspicuous in the nucleoplasm. *mT* and BA treatments reasoned damages in cell membrane in the form of breaks at several places. They also affected the chromosomal behavior and caused some mitotic irregularities. It was frequently observed that the chromosomes in prophase were not typically arranged; the arms of chromosomes pointed outwards (Figure 4a). Chromosomes didn't typically aligned in equatorial plate in metaphase (Figure 4b-d), but one or more chromosomes were randomly oriented on the spindle. Chromosome bridges, laggards, randomly distribution of chromosomes on the spindle were observed in anaphase and telophase (Figure 4e-h). Instead of one group of chromatids at a pole 2 or 3 chromatid clusters were also observed in anaphase. Binucleated cells were established in *mT* and BA treated root tips (Figure 5a). In these cells karyokinesis was not followed by cytokinesis that means cell plate formation is prevented by *mT* and BA at 10^{-4} , 10^{-5} , 10^{-6} M concentrations. In

the smaller binucleate cells the fusion of the 2 nuclei were observed and as a consequence, irregular shaped or dumbbell shaped nuclei were produced. On the other hand, most of the binucleate cells underwent synchronous mitosis (bimitosis) but the fusion of two equatorial plates at metaphase (tetraploid plate) was quite frequent in all treated cells (Figure 5 b,c); one of the other reasons of polyploidy was endoreduplication.

In addition to the abnormalities mentioned above,

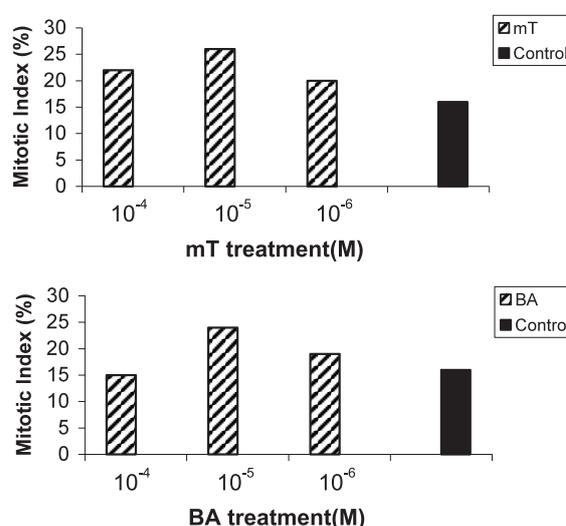


Figure 3. The effects of *mT* and BA on mitotic index in barley root tips. Control: distilled water, *mT*. *meta*-topolin; BA: benzyldenine.

interphase cells with one or two micronuclei including one or more chromosomes were observed in 10^{-4} M BA treated root tips (Figure 5c). It was also recorded that very large nucleus was formed by endoreduplication in the cells growing in length and in width.

Discussion

In the present study, the effects of the *mT* were compared with the BA on seed germination, root growth and mitotic activity in *Hordeum vulgare* L. The effects of *mT* and BA on the germination of barley seeds were dose dependent. *mT* increased seed germination at all concentration applied. The lowest concentration of *mT* (10^{-6} M) was the most effective stimulator. Whereas the percentage of seed germination decreased by increasing concentration of BA. The highest concentration of BA was the most

Table 2. Number of cells examined; interphase, prophase, metaphase, anaphase, telophase indices in the analysed samples. Control: distilled water, *mT*. *meta*-topolin; BA: benzyldene.

Stages	<i>Meta</i> -topolin (mT)				Benzyldene (BA)		
	Control (%)	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M
Interphase	83.7	80.4	73.6	78.1	80.7	76.1	84.8
Prophase	12.2	16.1	20.6	19.5	15.3	19.4	11.6
Metaphase	1.7	1.4	2.1	0.7	1.2	1.5	1.9
Anaphase	1.0	0.7	1.0	0.5	1.0	0.8	0.7
Telophase	1.4	1.4	2.7	1.2	1.8	2.2	1.0

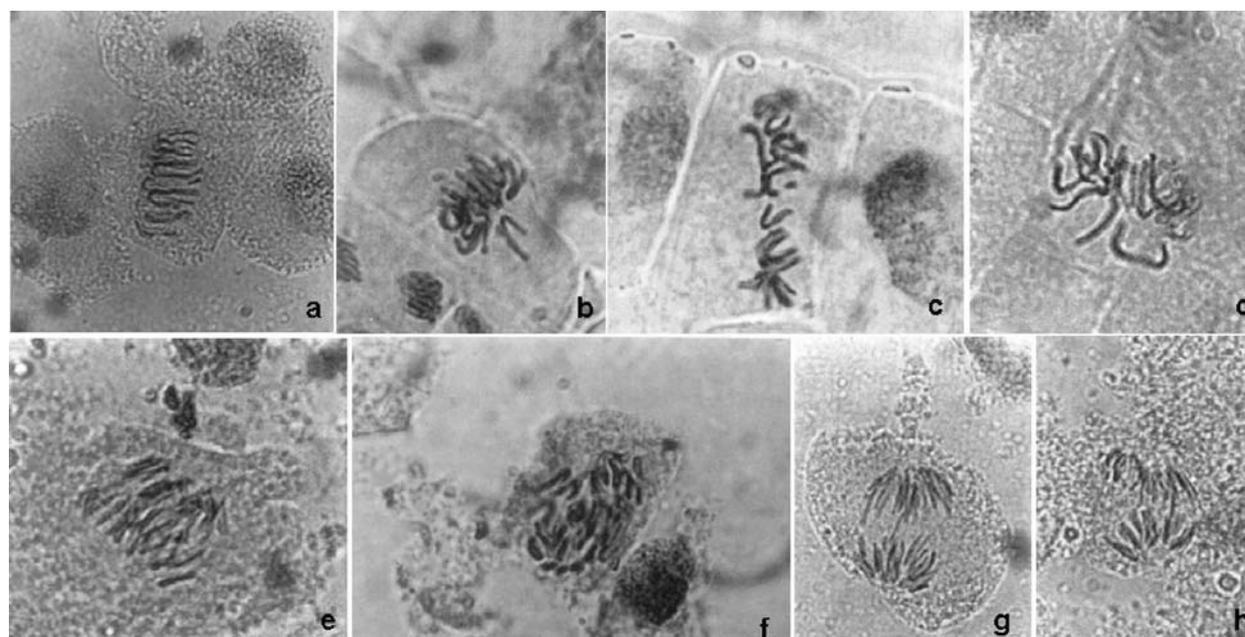


Figure 4. Effect of mT and BA on mitotic division in the root tips of barley. a: prophase; Note the arms of chromosomes pointed outwards. b-d: abnormal metaphase; e-h: abnormal anaphase.

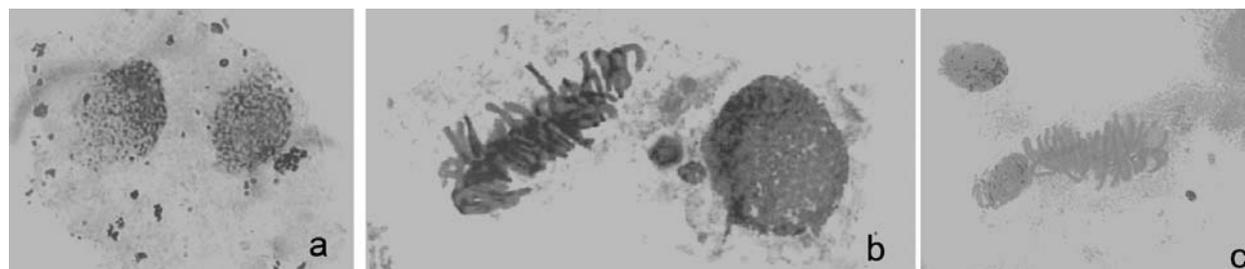


Figure 5. a: Binucleate cell. b: Metaphase in binucleate cell. c: Note 2 micronuclei in interphase cell on left.

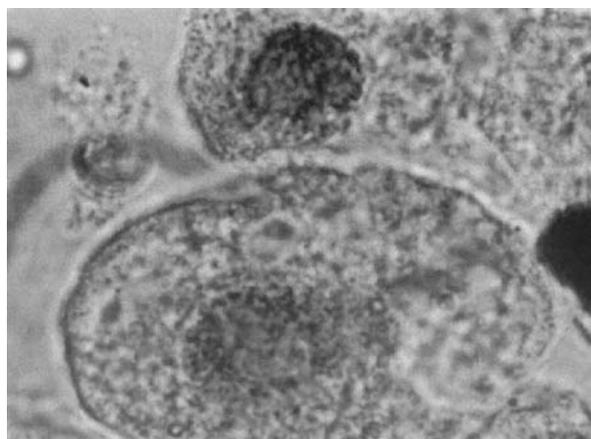


Figure 6. A cell growing in length and width. It is obvious the volume difference between a giant cell and a normal cell.

effective inhibitor. This indicates the necessity of *mT* in seed germination of barley. Promotive role of other cytokinin kinetin in seed germination was also reported before by Haber and Luippold, 1960; Reynolds and Thompson, 1973.

Cytokinins were implicated in many aspects of plant development, including a crucial role in regulating cell proliferation. Cytokinins may elevate cell division rates by induction of expression of CycD3, which encodes a D-type cyclin thought to play a role in the G1-M transition of the cell cycle. However the role of cytokinins in other aspects of the cell cycle remains unclear (D'Agostino and Kieber, 1999). Another cytokinin, kinetin also had stimulatory effect on seed germination and mitotic activity in association with our findings (Das *et al.*, 1956; Haber and Luippold, 1960; Reynolds and Thompson, 1973).

Recently, Werner *et al.*, (2001) also found that cytokinins are an important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots in agreement with our results.

Meta-hydroxybenzyladenines are natural components of plants and belong to a class of natural plant growth substances, the aromatic cytokinins. The functions of the aromatic cytokinins in plants are distinct from those of the isoprenoid cytokinins (zeatin, izopenteniladenin ve dihidrozeatin). It is interesting to note that *mT* is more active than zeatin and BA in the promotion of shoot formation in plant tissue cultures (Kubalakova and Strnad, 1992; Werbrouck *et al.*, 1996).

Werbrouck *et al.*, (1996) compared the effects of BA and *mT* on *in vitro* shoot and root production and reported *mT* was a suitable alternative to BA for the micropropagation of *Spathiphyllum floribundum*.

Cytokinins are now known to promote cell division in many excised tissues (Letham, 1967). Some significant observations were done regarding the ability of cytokinins to influence cell division in excised roots. In the absence of exogenous cytokinin, a diploid callus tissue was formed by pea root segments, but, in the presence of a supplied cytokinin, a predominantly tetraploid callus tissue resulted (Short and Torrey, 1972). Development of this callus tissue appeared to result from cytokinin-induced suppression of diploid divisions in the central cylinder, and from the induction of division of polyploid cortical cells (Torrey, 1961). Excised radish roots depleted of endogenous growth stimulants by subculture require both auxin and cytokinin for induction of vascular cambial activity (Loomis and Torrey, 1964). Tileklioğlu and Algan (1997) investigated the effects of zeatin, zeatin riboside and their combination on cambial activity and found zeatin riboside at 10 mg/l increased cell division.

mT induced the division frequency in the root tips of barley in the present study. BA also increased the mitotic indices at 10^{-5} , 10^{-6} concentrations, but it caused inhibition at highest concentration applied in this study (10^{-4} M).

Soh and Yang (1993) studied the effects of plant growth regulators on mitotic activity and the chromosomal behavior and concluded that they increased the mitotic activity at lower concentration but the cytokinins kinetin and BA were not as effective as auxins to increase the mitotic index. The two cytokinins caused some mitotic abnormalities such as anaphase or telophase bridges, laggards, multipolar spindle formation, C-mitosis, micronuclei and endoreduplication at 50 and 100 ppm concentrations. Twelve hours of auxin treatment enhanced division frequency whereas cytokinins at 10 ppm for 12 hours showed lower mitotic index than the control.

Application of exogenous *mT* and BA induced restitutional mitosis resulted from spindle disturbances at anaphase or less frequently at metaphase. Spindle disturbances may frequently be associated with defective movement of chromosomes and chromosome stickiness which favors chromosome bridges at anaphase or chromosome clumping at metaphase. The two cytokinins used in this study

caused abortion in the formation of phragmoplast and binucleate cells which were produced as a result of acytokinetic mitosis. These results suggest the possible role of *mT* and BA in the prevention of cytoplasmic division of barley root tips.

Ploidy in plants appears to be in the most frequent response to different types of stimuli, both endogenous and exogenous. Restitutive mitosis and endoreduplication lead to the production of giant nuclei in barley root tips treated with *mT* and BA.

Our results revealed that *mT* was more effective stimulator on division frequency than BA and the behavior of chromosomes is sensitive to *mT* and BA.

References

- Arteca RN. Plant Growth Substances: Principles and Applications. Chapman & Hall, New York, 1996.
- D'Agostino IB and Kiebe JJ. Molecular mechanisms of cytokinin action. *Curr Opin Mol Biol.* 2: 359-364, 1999.
- Das NK, Patau K and Skoog F. Initiation of mitosis and cell division by kinetin and indolacetic acid in excised tobacco pith tissue. *Physiol Plant* 9:640-651, 1956.
- Dewitte W, Chiapetta A, Azmi A, Witters E, Strnad M, Rembur J, Noin M, Chriqui D and Onckelen HV. Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. *Plant Physiol.* 119:111-121, 1999.
- Haber AH and Luippold HJ. Effects of gibberellin, kinetin, thiourea and photomorphogenic radiation on mitotic activity in dormant lettuce seed. *Plant Physiol.* 35: 486-494, 1960.
- Kubalaková M and Strnad M. The effects of aromatic cytokinins on micropropagation and regeneration of sugar beet *in vitro*. *Biologia Plantarum Suppl.* 34: 578-579, 1992.
- Latham DS. Chemistry and physiology of kinetin-like compounds. *Plant Physiol.* 18:349-364 1967.
- Loomis RS and Torrey JG. Chemical control of vascular cambium initiation in isolated raddish roots. *Proc Natl Acad Sci USA.* 52: 3-11, 1964.
- McGaw BA. Cytokinin biosynthesis and metabolism In: Plant Hormones and Their Role in Plant Growth and Development. Davies PJ (Ed.), Martinus Nijhoff, Dordrecht, 76-93, 1987.
- Miller CO, Skoog F, Okumura FS, Saltza MH and Strong FM. Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J Am Chem Soc.* 78:1375-1380, 1956.
- Miller CO, Skoog F, von Saltza MH and Strong M. Kinetin, a cell division factor from deoxyribonucleic acid. *J Am Chem Soc.* 77:1329-1334, 1955.
- Mok MC. In Cytokinins: Chemistry, Activity and Function, Mok, D W S & Mok, M. C. (Eds.) CRC, Boca Raton, FL, 155-166, 1994.
- Noodén LD and Leopold AC. Senescence and aging in plants. Academic Press San Diego, 1988.
- Reynolds T and Thompson PA. Effects of kinetin, gibberellins and abscisic acid on the seed germination of lettuce (*Lactuca sativa*). *Physiol Plant.* 28:516-522, 1973.
- Riou-Khamlich C, Huntley R, Jacquemard A and Murray JAH. Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science.* 283:1541-1544, 1999.
- Saha S, Nagar PK and Sircar PK. Changes in cytokinin activity during seed germination in rice (*Oryza sativa* L.). *Ann Bot.* 54: 1-5, 1984.
- Salisbury FB and Ross CW. Plant Physiology, Eds. Belmont, CA 1992.
- Short KC and Torrey JG. Cytokinin production in relation to the growth of pea-root callus tissue. *J Exp Bot.* 23: 1099-1105, 1972.
- Soh YW and Yang WY. Effect of plant growth regulators on mitotic chromosomes in *Allium cepa* L. Nucleus. 36: 109-113, 1993.
- Strnad M, Hanus J, Vanek T, Kaminek M, Ballantine JA, Brynley and F Hanke DE. Meta-topolin, a highly active aromatic cytokinin from polar laves (*Populus x canadensis* Moench., CV Robusta). *Phytochem.* 45:213-218, 1997.
- Thimann K. Senescence in plants. CRC Press. Boca Raton, USA, 1980.
- Tileklioğlu B and Algan G. The effects of zeatin, zeatin riboside and their combinations on cambial activity in *Coleus scutellarioides* Bent. *Bulletin of Pure and Applied Sciences.* 16:35-37, 1997.
- Torrey JG. Kinetin as trigger for mitosis in mature endomitotic plant cells. *Exp Cell Res.* 23:281-299, 1961.

Werbrouk SPO, Strnad M, Van Onckelen HA and Deberg PC. *Meta-topolin* an alternative to benzyladenine in tissue culture. *Physiol Plant.*, 98:291-297,1996.

Werner T, Matykav, Strnad M and Schmülling T. Regulation of plant growth by oytokinin. *Proc Natl Acad Sci USA.* 98:10487-10492, 2001.