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A novel strategy for plant protection: Induced resistance

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Abstract

Most plant protection methods currently applied use toxic chemicals noxious to the environment for pathogen and pest control. Induced resistance exploiting natural defense machinery of plants could be proposed as an alternative, non-conventional and ecologically-friendly approach for plant protection. Its introduction into agricultural practice could minimize the scope of chemical control, thus contributing to the development of sustainable agriculture. Induced resistance can be defined as an increased expression of natural defence mechanisms of plants against various type of pathogens, provoked by a range of factors: pathogens causing hypersensitive necrotic reaction; avirulent or attenuated pathogenic strains; elicitors of pathogenic origin (glucans, proteins, lipids, etc.); abiotic elicitors, including synthetic harmless chemical products, such as 2,6-dichloroisonicotinic acid (INA), b-aminobutyric acid (BABA), benzothiadiazole (BTH), etc. Induced resistance, being based on the expression of latent genetic information present in plants, is not underlied by genome alterations (mutations, introgression of foreign genetic material), this enhancing its biological safety. Molecular bases of induced resistance, involving receptor-elicitor interactions, signal transducing-pathways and SAR gene expression, are discussed.

Key words: localized acquired resistance (LAR), systemic acquired resistance (SAR), induced systemic resistance (ISR), elicitors, SAR genes

Bitki korumada yapay strateji: Direncin arttırımı

Özet

Birçok bitki koruma metodu günümüzde toksik kimyasalların patojen veya böcek koruma amacı ile çevreye uygulanmasıdır. Ancak bunlara alternatif olarak direnç arttırımı ile bitki koruma yapılmakta ve yaygın olmayan ekolojik bir uygulama olarak doğal savunma sağlanmaktadır. Direnç arttırımı veya teşvik edilmesi; bitkilerin değişik patojenlere karşı savunma mekanizmalarında ekspresyonlarını arttırılması olarak tanımlanabilir. Bu patojenler, hipesensitif nekroz reaksiyonları, avirulent veya güçlenmiş patolojik suşlar olabilir. Gecikmiş genetik bilginin bitkilerde ekspresyonunun antması ile genomik değişiklikler altında olmadan (mutasyon, yabancı genin integrasyonu) biyolojik güvenliği arttırmaktadır. Arttırılmış direncin moleküler temeli, reseptör ve kimyasallar arasındaki etkileşimleri, sinyal iletim yolları ve SAR gen ekspresyonu bu derleme yazısında tartışılmaktadır.

Anahtar sözcükler: lokalize gerekli direnç, sistemik gerekli direnç, indüklenmiş sistemik direnç, elisitör, SAR genleri

Introduction

Presently disease control is largely based on the use of fungicides, bactericides and insecticides – chemical compounds toxic to plant invaders, causative agents or vectors of plant diseases. However, the hazardous effect of these chemicals or their degradation products on the environment and human health strongly necessitates the search for new, harmless means of disease control. Since the late 1950s increasing body of evidence on the natural phenomenon of induced resistance has been accumulated, culminating in its successful practical application in the last decade (Kuc, 2001).

The resistance in plants induced by pathogens was first recognized by Ray (1901) and Beauverie (1901). Chester (1930) confirmed those studies, and, by summarizing field observations, supposed that this phenomenon may play an important role in the preservation of plants in nature. Convincing evidences however were obtained only in the 1960s, when reproducible models using tobacco plant were developed (Cruickshank and Mandryk, 1960; Ross, 1961a; Ross, 1961b; Mandryk, 1963). Greenhouse and field experiments in the laboratory of Kuc and co-workers paved the way to the present comprehension of induced resistance as a tool in plant protection (Kuc, 2001), this being supported by numerous authors from around the world (Schönbeck et al., 1993; Kessman et al., 1994; Schneider et al., 1996; Van Loon et al., 1998; Benhamou and Picard, 1999; Tally et al., 1999; Cohen, 2001; Bokshi et al., 2003; Gozzo, 2003; Soylu et al., 2003). Exploiting uniquely the plant potential to combat pathogens, the induced resistance may diminish the use of toxic chemicals for disease control, and thus could be proposed as an alternative, non-conventional, non-biocidal and ecologically-friendly approach for plant protection and hence for sustainable agriculture.

What is induced resistance? Definition, terminology, general models

When a plant is inoculated with a pathogen ("primary inoculation"), and after a time interval is subjected to a secondary ("challenge") inoculation, reduced disease symptoms develop, i.e. the induced plant becomes more resistant than the normal, non-induced plant. Later, stimuli other than pathogens, such as some non-toxic chemicals, were found to be effective at inducing resistance. Thus, the induced resistance can be defined as an **increased expression of natural defense mechanisms of plants** against **different pathogens** provoked by external factors of various type and manifested upon subsequent inoculation. Hence, the low specificity is an inherent character of induced resistance. A very essential trait is that it is based on expression of latent genetic information present in plant, and is not underlied by genome alterations (mutations or introgression of foreign genetic material)-a feature relevant to an important biological safety (Kuc, 1987; Schönbeck et al., 1993; Schneideret al., 1996; Benhamou and Picard, 1999; Kuc, 2001). The induction of resistance in plants is often compared to immunization or vaccination in animals. Although the term "immunization" has been used to denote treatments that enhance the defensive capacity of plants, the correspondence to vaccination in vertebrates is far-fetched: the induced state is by no means specific (absence of antibody formation). Moreover, it is less efficace and durable, with seldom preventing disease from occurring but generally reducing its extent of severity (Hammerschmidt et al., 2001).

The term "induced resistance" (IR) is used synonymously to "acquired resistance" (AR). Depending on the mode of its expression, induced resistance can be systemic (SAR) or local (LAR). As mentioned before, in the early 1960s Ross as a result of his carefully controlled laboratory experiments with tobacco-TMV system coined the terms LAR (Ross, 1961a) and SAR (Ross, 1961b). He inoculated leaves of the cv. Xanthi nc, hypersensitively reacting to TMV, i.e. forming small necrotic lesions following TMV inoculation. The subsequent, "challenge" inoculation of the same leaf after a few days resulted in development of smaller-sized and less numerous lesions, i.e. the disease severity was reduced. In the same system resistance to TMV was also expressed after secondary inoculation of half-leaf, with the opposite half-leaf being previously inoculated with TMV. These phenomena were referred to as LAR (Ross, 1961a). In this series of experiments Ross succeeded also in inducing resistance to TMV in distant upper leaves of tobacco by primary inoculation of lower leaves with the virus, a phenomenon referred to as SAR (Ross, 1961b).

Cruickshank and Mandryk (1960) were the first to report on SAR in tobacco induced by fungi, much more complex and highly structured pathogens than

viruses. Data were presented that SAR against *P.tabacina* was expressed after "challenge" inoculation of upper leaves when the lower leaves or stems of the plants were inoculated 14-21 days ago with this fungus. It is noteworthy that inoculation with *P.tabacina* induced SAR not only to the fungus, but also to TMV (Mandryk, 1963), this pointing to the low specificity of SAR phenomena.

Recently, the term "induced systemic resistance (ISR) was introduced to designate the resistance induced in leaves of plants by inoculation of roots with non-pathogenic rhizobacteria. This novel type of induced resistance was first described in *Arabidopsis* plants, inoculated with the root-colonizing non-pathogenic bacteria *Pseudomonas fluorescens*; leaves of these plant exhibited resistance against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (Pieterse et al., 1998). Rhizobacteria-mediated ISR has also been demonstrated against fungi, bacteria and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco and tomato (Van Loon et al., 1998), this confirming the low specificity proper to IR.

In all cases of IR generation of signals is proposed which transmit information from the site of primary treatment to the adjacent tissues (LAR) or to the distant tissues (SAR, ISR) where IR is expressed upon subsequent, "challenge" inoculation. A time interval (lag period) between the primary and "challenge" inoculation is a prerequisite for effective expression of induced resistance. Span of time is necessary for signals to be translocated to non-inoculated tissues and for triggering and development of defense potential in these tissues (Kuc, 1987; Schneider et al., 1996; Benhamou and Picard, 1999; Kuc, 2001).

Inducers of resistance

A multitude of factors are reported to induce resistance in plants: pathogens (fungi, bacteria, viruses) causing hypersensitive necrotic reaction (HR); avirulent and attenuated pathogenic strains; pests (insects, nematodes); elicitors of biotic origin; abiotic elicitors, i.e. chemical products, such as benzothiadiazole (BTH), β-aminobutyric acid (BABA), 2,6-dichloroisonicotinic acid (INA), salicylic acid, inorganic salts, etc. (Kessman et al., 1994; Lyon et al., 1995; Schneider et al., 1996; Benhamou and Picard, 1999; Cohen, 2001; Kuc, 2001).

Pathogen inducers

Numerous experiments have shown that besides TMV and *P.tabacina*, other pathogens are also able to induce SAR in tobacco, such as TNV, PVY, *Thielaviopsis basicola*, *Pseudomonas syringae*. The SAR response in tobacco gives broad-spectrum disease resistance to fungal, bacterial and viral pathogens, namely *Thielaviopsis basicola*, *Phytophthora parasitica, Peronospora tabacina, Pseudomonas syringae, Pseudomonas tabaci, Erysiphe cichoracearum*, TMV, TNV (Schneider et al., 1996).

The extensive work of Kuc and his coworkers enlarged the knowledge on SAR in tobacco and traced the avenues for its practical application. The importance of inoculation procedure for SAR inoculation was demonstrated. When conidia were injected into the stem cambium, the SAR response was linked to severe dwarfing and premature senescence. However, infection external to the cambium leads to an increase in plant weight and leaf number (Tuzun and Kuc, 1985). Interestingly, regenerant plants obtained via callus from leaves of tobacco plants immunized by stem-injection with *P.tabacina* were highly resistant to this pathogen which was demonstrated by both greenhouse and field tests (Kuc, 1987). Abundant data on other pathosystems are available. Thus, stem inoculation of tomato by an avirulent strain of the bacteria *Clavibacter michiganensis ssp. michiganensis* induced long-lasting, high level-protection against the virulent bacterial strain (Griesbach et al., 2000). An avirulent strain of *Pseudomonas syringae* p.v. *pisi* was shown to induce SAR against the fungus *Mycosphaerella pinodes* in pea (Dann and Deverall, 2000). Successful induction of SAR in cucurbits by *Colletetrichum* sp. was demonstrated by Kuc (1987). The hypersensitive necrotic reaction-causing bacterium *Pseudomonas syringae* pv. *syringae* induced SAR against the fungus *Pyricularia oryzae* in rice (Smith and Metraux, 1991).

Induction of SAR by biotic elicitors

A fascinating area of research is the induction of SAR by biotic elicitors, i.e. by pathogen-derived molecules. Biotic elicitors encompass diverse chemical classes (polysaccharides, lipids, proteins, and complexes between them) and are active on various host plants against different pathogens (Lyon et al., 1995; Benhamou and Picard, 1999; Aziz et al., 2003). In tobacco encouraging results are obtained with biotic elicitors of fungal origin named "elicitins".

Elicitins, a small family of highly conserved 10kD secretory holoproteins bearing this generic name, are the first fully characterized proteinaceous fungal elicitors. They are secreted from various species of *Phytophthora* fungi: acidic a-elicitins (capsicein and parasiticein) - from P. *capsici* and P. *parasitica*, respectively, and basic β-elicitins (cryptogein and cinnamomin) - from *P. cryptogea and P. cinnamomi*, respectively. Elicitins were discovered by the group of Bonnet and Ricci and found to induce resistance in tobacco and other plants (Bonnet et al., 1989; Bonnet et al., 1996). Their structure, biological activity, genetical bases and biochemical mechanisms of action are a subject of intensive research (Blein et al., 1991; Viard et al., 1994; Rusterucci et al., 1996; Simon – Plas et al., 1997; Dahan et al., 2001). β-cryptogein belonging to the group of β-elicitins is secreted from *P. cryptogea*, a non-pathogen on tobacco. It produces necroses on leaves of tobacco when applied on both leaves and stems (systemic effect), and induces resistance in the perinecrotic area which is characterized as follows: may be local or systemic; is not established immediately, i.e. requires a lag period; is more or less durable; is non-specific, i.e. is effective against a range of unrelated pathogens, such as *Phytophthora parasitica* var. *nicotianae*, *Botrytis cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani*, and *Erysiphe cichoracearum* (Bonnet et al., 1996; Blancard et al., 1998). The broad spectrum and long duration of β-cryptogein - induced resistance incited the research on possible exploitment of the phenomenon. Resistant transgenic tobacco plants are developed expressing the gene coding for β-cryptogein (Tepfer et al., 1998). Recently, transgenic plants were obtained harboring a fusion between the pathogen - inducible tobacco hsr203J gene promoter and *P. cryptogea* gene encoding β-cryptogein. In non-induced conditions this transgene is silent, and becomes expressed (β-cryptogein is synthesized) only upon pathogen infection (Keller et al., 1999).

The biochemical mechanisms underlying the induction of protection in β-cryptogein treated tobacco involve early events, such as oxidative burst (Rusterucci et al., 1996; Simon-Plas et al., 1997), K^+ efflux, Ca^{2+} influx, alkalinization and increased conductivity of extracellular medium accompanied by acidification of cytoplasm (Blein et al., 1991; Simon-Plas et al., 1997), lipid peroxidation (Schneider et al., 1996) and protein phosphorylation (Viard et al., 1994). The data point to membranes being a primary target for b-cryptogein interaction with the plant interface (Wendehenne et al., 1995) followed by triggering of signal transducing pathways. Evolution of ethylene and phytoalexin biosynthesis were reported to occur in later stages (Blein et al., 1991; Rusterucci et al., 1996). In our study (Edreva et al., 2002) we established induction of peroxidase, b-1,3-glucanase and PR-proteins in β-cryptogein stem-treated tobacco. Defensive functions could be inferred to these molecules. Thus, PO is implicated in the control of the active oxygen species pool, including H_2O_2 which is thought to have a central role in plant signalling (Overney et al., 1998). Moreover, PO/H_2O_2 system is implicated in the regulation of cell wall plasticity by catalyzing lignin biosynthesis and oxidative polymerisation of ferulate and tyrosine residues in cell wall components; this may contribute to cell wall cross-linking and fortification, i.e. to building up of mechanical barrier at the plant interface against potential pathogens (Gaspar et al., 1986; Overney et al., 1998). Recent findings (Kieffer et al., 2000) lend experimental support to this assumption, showing that β-cryptogein applied to tobacco cell suspension cultures induces lose of digestibility and strengthening of cell walls. β-1,3-glucanase is involved in the hydrolysis of fungal cell wall glucans and the release of active fragments eliciting phytoalexin synthesis in plants (Ham et al., 1991). Thus, the enzyme exerts lytic action on pathogens, and may also conduce to the to the formation of toxic barrier against subsequent fungal attack. An impressive body of evidence points to the importance of PR-proteins in plant protection (Viard et al., 1994; Abad et al., 1996). Taken together, the data imply that peroxidase, b-1,3-glucanase and PR-proteins induced in β-cryptogein treated tobacco, acting cooperatively, could contribute to the development of a hostile plant environment to meet forthcoming pathogen invasion.

Chemical inducers

The use of chemicals as inducers of resistance is an area of extensive work aiming at developing new compounds for disease control meeting the requirements for safe application in greenhouse and fields conditions, namely: no direct toxicity to pathogens; no toxicity to plants and animals; no negative effects on plant growth, development and yield; broad spectrum of defense; low loading amount; long lasting protection; low economical cost for

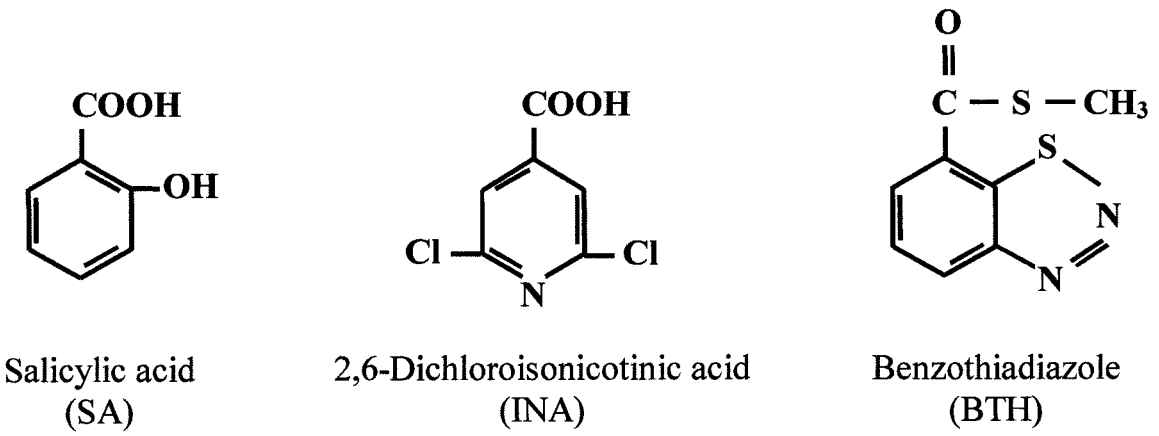

Figure 1: Formulae of commonly used chemical inducers of SAR

farmers; good profit for producers (Kessman et al., 1994; Tally et al., 1999; Kuc, 2001).

Chemical inducers of plant resistance possess quite different mode of action as compared to fungicides and pesticides. The latter products have direct toxic effect on pathogens; are noxious to the environment; have narrow spectrum of defense; ensure shortly lasting protection; are economically costly (Schönbeck et al., 1993; Tally et al., 1999; Kuc, 2001). Thus, the application of chemical inducers of resistance is an exciting new perspective to supplement the classical chemical means of disease control by providing both effective and ecologically-friendly plant protection.

A large array of chemical products are shown to induce SAR in tobacco: salicylic acid, isonicotinic acid (INA), benzothiadiazole (BTH), β-aminobutyric acid (BABA) (Fig. 1), $NaClO_3$, $HgCl_2$, paraquat, polyacrylic acid, SiO_2, etc. Chemically-induced SAR was found to be effective against fungi, bacteria and viruses, namely *Peronospora tabacina, Cercospora nicotianae, Phytophthora parasitica* var. *nicotianae, Pseudomonas syringae* pv. *tabaci*, TMV (Lyon et al., 1995; Strobel and Kuc, 1995; Schneider et al., 1996; Kuc, 2001).

The only commercialized inducer of resistance in tobacco is BTH (full chemical name benzo[1, 2, 3] thiadiazole-7-carbothioic acid S-methyl ester) (Fig. 1). Discovered in 1989 in Ciba-Geigy (Novartis), described by Ryals and coworkers (Friedrich et al., 1996), it was given the trade name BION^{TMV} (in Europe) and Actigard^{TMV} (in USA), and registered and classified as "Reduced Risk Compound" in USA in 1998 (Tally et al., 1999). BTH is the first synthetic non-toxic chemical developed and marketed that functions exclusively by activating the SAR genes. It is supposed to act as a functional analog of salicylic acid entering the signal transducing pathway downstream of it (Friedrich et al., 1996; Wendehenne et al., 1998; Cohen, 2001).

Numerous data are available that BTH is strongly effective against *P. tabacina*, causative agent of blue mold, the most important world-wide distributed tobacco disease. Applied in minimal amounts (around 50 g ha^{-1}), BTH provides field protection lasting until flowering without negative influence on growth, development and yield of tobacco. BTH appears more efficient than metalaxyl, the commonly used blue mold fungicide. It ensures 90% disease reduction on the 17th day after its application versus only 46% for metalaxyl (Tally et al., 1999). It is noteworthy that BTH is an effective inducer of resistance in tobacco not only against fungal pathogens, but also against viruses and bacteria (Tally et al., 1999). Other chemicals, such as BABA and INA, which induced SAR against blue mold, were not commercialized because of side effects, such as low stability and phytotoxicity (Schneider et al., 1996; Tally et al., 1999). BTH was also found to be effective in inducing SAR in wheat (Görlach et al., 1996), pea (Dann and Deverall, 2000), potato (Bokshi et al., 2003), cotton (Colson-Hanks et al., 2000), tomato (Soylu et al., 2003), etc.

BABA was shown to be a unique inducer of plant defense. It is a simple non-protein amino acid which, when sprayed onto the leaf surface or drenched into the soil, induced SAR against various foliar and root pathogens. BABA provided almost complete control

of late blight in tomato plants without being fungitoxic. It has instantaneous action, even when applied post-infection. This feature bears a significant advantage over BTH which has to be applied before the appearance of the disease (Cohen, 2001).

Synergistic effects of BABA and BTH were successfully applied in crop protection. Moreover, synergistic interactions of BABA with fungicides were reported, namely with metalaxyl, controlling blue mold in tobacco, and mancozeb, controlling *Phytophthora infestans* in potato (Baider and Cohen, 2003).

The practical application of chemicals as resistance inducers is mainly based on their systemic effect, i.e. on SAR expression in plants. As mentioned before, an important feature of IR, including SAR, is the low specificity. Thus, SAR is induced by: structurally unrelated compounds (for example, β-aminobutyric acid, isonicotinic acid or phosphates) or unrelated pathogens (fungi, bacteria, viruses); in unrelated plants, i.e. plants belonging to different families; against unrelated pathogens (fungi, bacteria, viruses). The long duration of protection covering the whole vegetation period is a very important feature stimulating the field exploitment of SAR (Kuc, 1987; Schneideret et al., 1996; Kuc, 2001). The successful application of SAR necessitates to determine the exact timing and duration of the lag period between the primary treatment and the secondary ("challenge") inoculation (Schönbeck et al., 1993). In practical terms that assumes a knowledge on the eventual time of invasion of a given pathogen in the concrete situation. Services to warn the appearance of epidemics are a very useful tool to this end applied in many countries; thus, in USA a reliable network for tobacco blue mold warning is functioning (Main et al., 1998).

Mechanisms of SAR

A cascade of molecular and biochemical events underlies the expression of SAR. It is initiated by perception of inducers (pathogens, chemicals) resulting in generation of signal molecules translocated at long distance, and switching on of diverse processes contributing to the development of the defense potential of plants realized upon secondary inoculation. Perception of inducers is effectuated through binding of pathogen-derived molecules (elicitors) or chemical products with receptor sites on plant membranes or cell walls. Generation and nature of signals, the mode of their translocation and interactions are a matter of intensive research. Salicylic acid is commonly recognized as a signal molecule or a prerequisite for signal production in SAR; jasmonic acid and ethylene are involved in signalling upon expression of resistance induced by rhizobacteria (ISR) (Wendehenne et al., 1995, 1998; Schneider et al., 1996; Van Loon et al., 1998; Benhamou and Picard, 1999). Both SAR-mediating signal pathways may act simultaneously, thus providing an additive effect (Van Wees et al., 2000), and enter signal-transducing cascades involving MAP-kinases (Gozzo, 2003). Then interaction with gene promoters or other regulatory factors triggers the expression of the so-called SAR-genes (Ward et al., 1991). The term "SAR-genes" is used to collectively designate this family of nine genes whose expression is correlated with the onset of SAR. For TMV-infected tobacco the SAR-genes code for PR-1 proteins, β-glucanase (PR-2), chitinase (PR-3), hevein-like protein (PR-4), thaumatin-like and osmotin-like proteins (PR-5), PR-1 (basic), basic class III chitinase, acidic class III chitinase, and PR-Q' (Ward et al., 1991). The involvement of PR-proteins in SAR could be related to their characteristic functions. Thus, some PR-proteins exert hydrolytic action (glucanase, chitinase), this suggesting a lytic effect on pathogen cell walls built-up of glucans or chitins (Van Loon et al., 1997; Gozzo, 2003). Members of PR-5 protein family (thaumatin-like, osmotin-like) have membrane-permeabilizing activity due to interaction with membrane components, this leading to conformational changes, dissipation of pH membrane gradient, and formation of pores in membranes (Abad et al., 1996). Systemic induction of lipoxygenase, hydroxyproline rich glycoproteins (HRGP) and callose in non-inoculated leaves may indicate an important role of fatty acid derivatives and cell wall - related structural compounds in SAR. Peroxidase which is also systemically induced is essential for cross-linking and reinforcement of cell walls, the latter being a marker of the induced state. Oxidative burst is proposed to mediate SAR expression (Schneider et al., 1996; Benhamou and Picard, 1999; Kuc, 2001; Gozzo, 2003). It may be assumed that the deployment of SAR-related events allows the plant to respond more rapidly and effectively to a subsequent, "challenge" inoculation.

In addition it is essential to note that application of SAR interferes with the appearance of new strains of pathogens able to overcome the defense of the induced plants. This could be accounted for by the fact that as stated above various components with diverse functions are involved in SAR, i.e. multiple molecular targets for pathogens are available. The situation is opposite to that observed when fungicides or transgenic resistant plants are used. In these cases few targets to be overcome are present which facilitates the arising of new strains pathogenic to plants (Lyon et al., 1995).

Strategies to engineer SAR have been implemented: plants can be manipulated to constitutively express SAR genes (Durand-Tardif and Pelletier, 2003). New concepts however were recently developed (Heil, 2001, 2003), concerning the costs of the constitutive presence of defensive traits in fixed high amounts. Investment in defense is thought to reduce the fitness of plants in enemy-free environment. Phenotypic plasticity, leading to SAR responses, might have evolved mainly to reduce costs, since investments in defense is restricted to situations actually requiring defense. This might have important influences on the evolution of plant defensive traits.

Conclusion

Although not fully understood, induced resistance in plants opens new horizons in plant protection, being a promising tool for ecologically-friendly disease control and sustainable agriculture. It remains a challenge for both fundamental and applied research.

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In vitro pollen germination of some plant species in basic culture medium

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Abstract

In this study *in vitro* pollen germination test was performed on 7 different plant species in basic medium. These were; *Malva sylvestris* L. (*Malvaceae*), *Ecballium elaterium* (L.) A. Rich. (*Cucurbitaceae*), *Lonicera fragrantissima* Lindley (*Caprifoliaceae*), *Cyclamen hederifolium* Aiton (*Primulaceae*), *Linaria vulgaris* Miller (*Scrophulariaceae*), *Antirrhinum majus* L. (*Scrophulariaceae*), *Yucca filamentosa* L. (*Liliaceae*). 10% Sucrose and 0.01% boric acid was used as germination medium and germination was occured in petri dishes. The percentages of *in vitro* germination of pollen tubes among the species was found to be quite different. Percentage of germination; 80% in *Anthirrinum majus*, 70% in *Linaria vulgaris*, 30% in *Cyclamen hederifolium*, 15% in *Lonicera fragrantissima*, 5% in *Ecballium elaterium* and no pollen germination was observed in *Malva sylvestris* and *Yucca filamentosa*.

Key words: Pollen germination, *Ecballium*, *Lonicera*, *Cyclamen, Linaria, Antirrhinum.*

Bazı bitki türlerinin temel besiyerinde *in vitro* polen çimlenmesi

Özet

Bu çalışmada *Malva sylvestris* L. (*Malvaceae*), *Ecballium elaterium* (L.) A. Rich. (*Cucurbitaceae*), *Lonicera fragrantissima* Lindley (*Caprifoliaceae*), *Cyclamen hederifolium* Aiton (Primulaceae), *Linaria vulgaris* Miller (*Scrophulariaceae*), *Antirrhinum majus* L. (*Scrophulariaceae*), *Yucca filamentosa* L. (*Liliaceae*)'ye ait toplam 7 bitki türünün polenlerine temel kültür ortamında *in vitro* koşullarda çimlenme testi uygulanmıştır. Çimlenme ortamı olarak %10'luk sakkaroz ve %0,01'lik borik asit kullanılarak, petrilerde çimlendirilmiştir. Türler arasında *in vitro* çimlenme yüzdeleri oldukça farklı bulunmuştur. Çimlenme yüzdeleri; *Anthirrinum majus*'ta %80, *Linaria vulgaris*'de %70, *Cyclamen hederifolium*'da %30, *Lonicera fragrantissima*'da %15 ve *Ecballium elaterium*'da %5 olarak bulunmuştur. *Malva sylvestris* ve *Yucca filamentosa*'nın polenlerinde ise çimlenme gözlenmemiştir.

Anahtar sözcükler: Polen çimlenmesi, *Malva, Ecballium, Lonicera, Cyclamen, Linaria, Antirrhinum.*

Introduction

Pollen grains are simple structured plant cells. Pollen tube formation is a good and simple model of growth and development (Taylor and Hepler, 1997). Thus, pollen germination and growth of pollen tubes are important research materials for morphological, physiological, biotechnological, ecological, evolutional, biochemical and molecular biological studies (Ottavio et al., 1992).

Pollen tube elongation is a dynamic process in which pollen tubes navigate and respond to female tissues to accomplish their mission of delivering the sperm cells for fertilization. Pollen tubes extend

exclusively at the cell apex via an extreme form of polar growth, known as tip growth, producing uniformly shaped cylindrical cells (Cheung, 2001). Pollen tubes are an excellent system for the study of polarized tip growth, cell movement, cell to cell communication, cell to cell recognition and signalling in plants. In recent years, pollen germination and pollen tube development are used as materials for determining the importance of cytoskeleton in cell growth and differentiation (Ma et al., 2000). Pollens normally germinate in stigmata of plants (Ünal, 1986, 1988), in some plant groups they germinate in anther loculus (Yıldırım and Ünal, 1996; Dane and Meriç, 1999a; Dane and Meriç, 1999b; Dane, 2000). In some species pollens germinate in atmospheric humidity and H_2O, some pollens germinate in basic medium containing 10% sucrose and 0.01% boric acid *in vitro* (Vasil, 1960; Ünal, 1988; Dane and Olgun, 1994). The media used for pollen germination vary according to the plant species (Vasil, 1960; Baker and Baker, 1979). Pollens of some species need more complicated media (Çetin et al., 2000). The required environment for pollen germination *in vitro* is related to genetic composition and also the quality and quantity of nutriment reserves of pollen (Baker and Baker, 1979).

During the past few years pollen tubes grown *in vitro* became a popular model system for cell biology studies in plant cells (Moutinho et al., 2001). Pollen grains are morphologically simple and the process of tube formation is a relatively uncomplicated example of growth and development. For these reasons, and because of the rapid rate of tube formation *in vitro* exhibited by some species, pollen tube formation has become a model system for studying growth and development in plants.

The aim of this study is firstly to find the suitable material for *in vitro* pollen germination tests for further biological studies about the effects of different kinds of media and chemicals on pollen germination. Secondly we want to determine the relation between carbohydrate content of pollens and germination percentages on some plant species from different families.

Materials and methods

In this study the specimens of *Malva sylvestris* L. *(Malvaceae)*, *Ecballium elaterium* (L.) A. Rich. (*Cucurbitaceae*) and *Linaria vulgaris* Miller (*Scrophulariaceae*) were collected from natural habitats in Edirne, *Cyclamen hederifolium* Aiton (*Primulaceae*) from Kaz mountain and cultured in botanic garden of Trakya University, *Lonicera fragrantissima* Lindley (*Caprifoliaceae*), *Antirrhinum majus* L. (*Scrophulariaceae*) and *Yucca filamentosa* L. (*Liliaceae*) from cultural habitats in Edirne. The pollen grains were collected from plants soon after the dehiscence of anthers, which usually takes place early in the morning. In order to avoid discrepancies pollen from a single flower was used. The hanging drop technique was used for culturing the pollen grains in liquid media containing 10% sucrose and 0.01% boric acid (Vasil, 1960). The cultures were stored in room temperature and in diffuse laboratory light. The experiments were run in duplicate and for each germination test, random counts of 100-200 grains (in groups of 25 or more from different fields on the slide) were made as a magnification of 60X to determine the percentage of germination. The nutriment contents of pollens were investigated with basic tests like IKI (Iodine-Potassium-Iodure) (Baker and Baker, 1979). After pollen tube formation, pollen tubes were stained with lactophenol anilin-blue and aceto orcein (Ünal, 1986). Photographs were taken with the help of Olympus photomicroscope.

Results

In vitro pollen germination percentages were varied among plant species. Percentages of pollen germination were; 80% in *Antirrhinum majus* (Fig. 1), 70% in *Linaria vulgaris* (Fig. 2), 30% in *Cyclamen hederifolium* (Fig. 3), 15% in *Lonicera fragrantissima* (Fig. 4) and 5% in *Ecballium elaterium* (Fig. 5). There were almost no pollen germination in *Malva sylvestris* (Fig. 6) and *Yucca filamentosa* (Fig. 7). From IKI tests, its observed that pollens of *Malva sylvestris* contains starch and the others contain dextrine as nutriment reserves (Table. 1).

Discussion

In vitro germination of pollen has been used as a powerful tool for genetical, physiological, biochemical and cytochemical studies for a wide range of plant

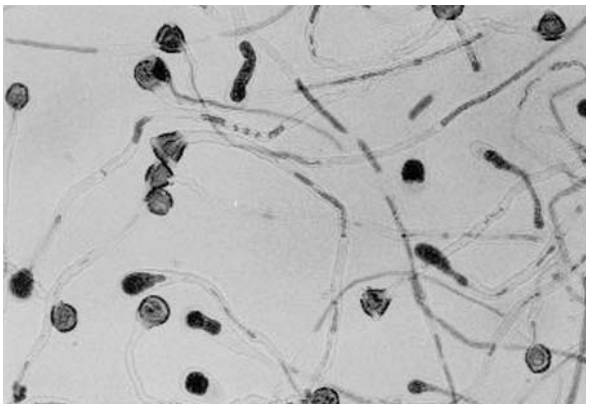

Figure 1: The pollen tubes of *Antirrhinum majus* (x200)

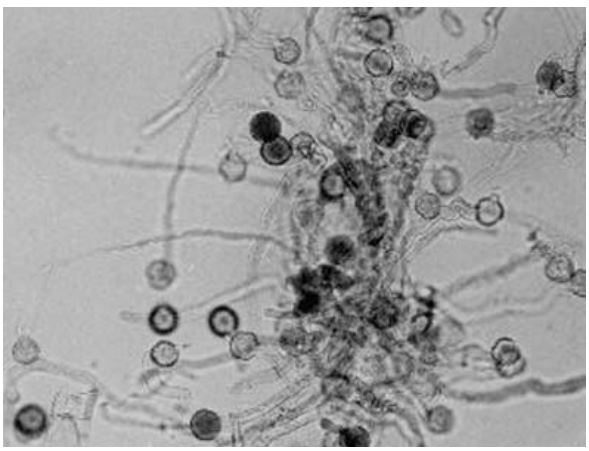

Figure 2: The pollen tubes of *Linaria vulgaris* (x200)

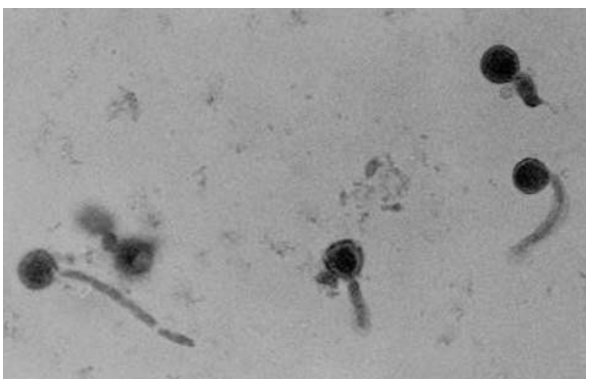

Figure 3: The pollen tubes of *Cyclamen hederifolium* (x400)

Figure 4: The pollen tube of *Lonicera fragrantissima* (x400)

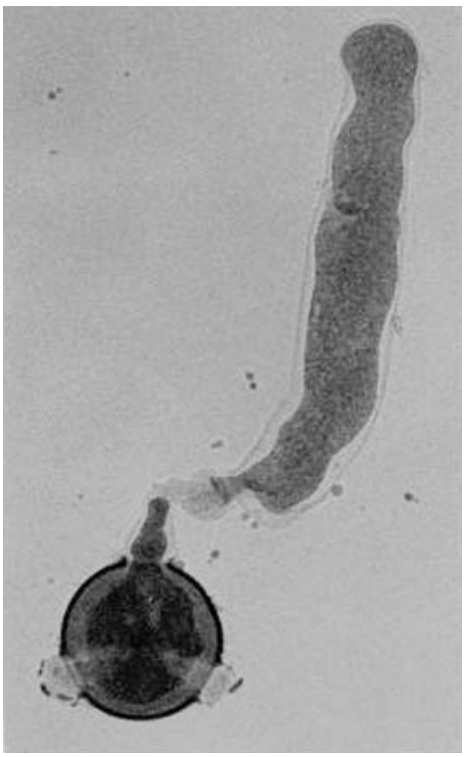

Figure 5: The pollen tube of *Ecballium elaterium* (x200)

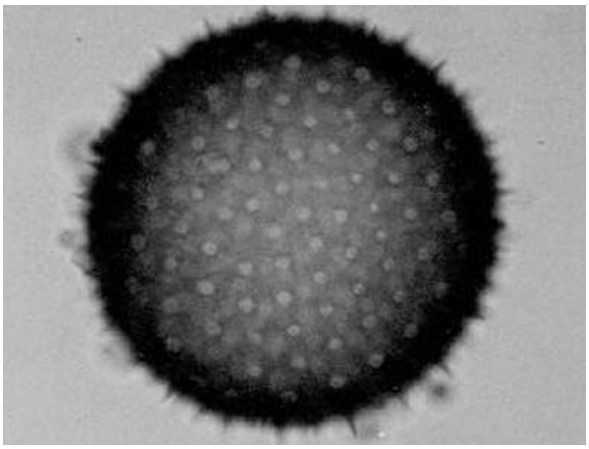

Figure 6: The pollen grain of *Malva sylvestris* (x200)

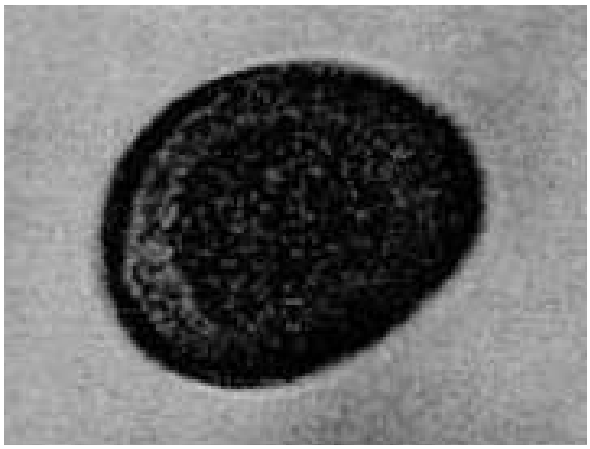

Figure 7: The pollen grain of *Yucca filamentosa* (x400)

Table 1: Family, habitat type, IKI results and pollen germination percentages of 7 plant species.

Plant species	Family	Habitat	IKI results	Pollen germ.
Malva sylvestris	*Malvaceae*	Natural	Starch	-
Ecballium elaterium	*Cucurbitaceae*	Natural	Dextrine	% 5
Linaria vulgaris	*Scrophulariaceae*	Natural	Dextrine	% 70
Cyclamen hederifolium	*Primulaceae*	Natural	Dextrine	% 30
Lonicera fragrantissima	*Caprifoliaceae*	Cultural	Dextrine	% 15
Antirrhinum majus	*Scrophulariaceae*	Cultural	Dextrine	% 80
Yucca filamentosa	*Liliaceae*	Cultural	Dextrine	-

species belonging to different families (Heslop-Harrison, 1992). It might also be possible to select resistant pollen grains in vitro and after it to transfer germinated grains on the style of mother plant for artificial pollination. The possibilities of such an approach are experimentally shown for a number of plants. *Arabidopsis thaliana* is a very favourable object for studies of the type due to its short vegetation and high seed production. But for *in vitro* pollen germination of *Arabidopsis thaliana* complicated media were used (Azarov et al., 1989).

The pollen tube formed in many species is a massive structure relative to the reserve materials stored in the pollen grain, and the reserves often are quickly consumed. According to Baker and Baker, the required energy for the germination of pollen grains, formation of cell wall components and callose in Angiosperms is provided from the nutriment reserves stored in pollen grains (Baker and Baker, 1979). These nutriment reserves are lipids, starches and sugars. Lipids and sugars are generally exist in pollen grains. Thus pollens can be classified in two groups as starchy and starchless. The reserved nutriments in pollen are important for the regulation of sucrose concentration used for *in vitro* pollen germination. From the literature cited, the need of sucrose concentration varies with the nutriment reserve of pollens. The pollens of *Pistacia vera* from *Anacardiaceae* need 50% sucrose concentration (Golan-Goldhirh et al., 1991), Avocado cultivars germinate in 15% sucrose concentration (Sahar and Spiegel, 1984).

In this study, among the investigated 7 plant species, starch granules were observed only in *Malva sylvestris* pollens. The other pollens became red in colour when treatened with IKI, so we thought that all of them contain dextrine. Vasil made some investigations about the pollen germination on 9 taxa from *Cucurbitaceae* and he used different concentrations of sucrose. His results show that pollen germination is optimum between 7.5% and 20% sucrose concentrations. He also investigated the effects of sucrose and boric acid in *Cucumis melo* and

Cucumis melo var. *utilissimus* from *Cucurbitaceae* and in both of these plants, optimal germination and tube lenght were obtained with 20% sucrose concentration and 0.01% boric acid. He found that boric acid concentrations higher than 0.02% were toxic for both taxa (Vasil, 1960). Recently scientists found that pollens of *Helianthus annuus* could germinate in boric acid-deficient conditions (Çetin et al, 2000). In this study *Ecballium elaterium* (*Cucurbitaceae*) pollens germinate in a very small percentage which is about 5% in 10% sucrose concentration. *In vitro* pollen germination of *Antirrhinum majus* and *Linaria vulgaris* from *Scrophulariaceae* gave the best results as in *Paeonia tenufolia* from *Paeoniaceae* (Dane and Olgun, 1994) and *Petunia hybrida* from *Scrophulariaceae* (Ünal, 1986). There were almost no pollen germination in *Malva sylvestris* from *Malvaceae* and *Yucca filamentosa* from *Liliaceae*.

In this study we tested the pollen germination of different families in basic medium and also determined the plant species which can be used for further studies. In conclusion it is found that pollens of *Antirrhinum majus* and *Linaria vulgaris* from *Scrophulariaceae* are suitable for further experimental studies. In recent years the studies on pollen germination of *Antirrhinum majus* support our fındings (Barinova et al., 2002). All the investigated 7 plant species, except *Malva sylvestris*, contain dextrine and among them the best pollen germination rates were in *Antirrhinum majus* and *Linaria vulgaris* (*Scrophulariace*). It is not clear weather the starch or dextrine ingredient of pollen is related with the germination rate or not. The question of whether there is a relation between starch and dextrine ingredient and pollen germination will become clear with the further research.

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Endogenous deoxyribonucleases involved in nuclear DNA degradation of wheat aleurone cells

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Abstract

By using PAAG gel electrophoresis enzyme assay methods and on the basis of experiments in isolated nucleus three types of deoxyribonuclease (A,B,C) of aleurone layer of wheat seeds were revealed. Deoxyribonucleases were differentiated by their sensitivity to divalent cations and gibberellic acid (GA). It has been shown that for activation of B deoxyribonuclease Ca^{2+} and Mg^{2+} are not required, whereas for A forms of enzyme the presence of cations for activity (Ca^{2+} / Mg^{2+} dependent deoxyribonucleas) is necessary. Activation of GA- induced form (C form) was observed only in the presence of Ca^{2+} ions (Ca^{2+} dependent deoxyribonuclease).

Key words: DNA, gibberellic acid, aleurone, endonuclease, nucleus

Buğday alevrone hücrelerinin nuklear DNA'sının yıkımına katılan endogenous deoksiribonukleazlar

Özet

PAAG jel elektroforez enzim metodlarının kullanılmasıyla ve izole edilmiş çekirdeklerle yapılan deneylerle buğday tanelerinin alevrone tabakasının 3 tür deoksiribonükleazı (A, B, C) ortaya çıkarıldı. Deoksiribonükleazlar Ca^{2+}, Mg^{2+} ve gibberellik aside duyarlılıklarına göre ayrıldı. B deoksiribonükleazın aktivasyonu için Ca^{2+} ve Mg^{2+} gerekli değilken, A tür deoksiribonükleaz için varlığının gerekli oldugu görüldü (Ca^{2+} / Mg^{2+}, e bağımlı deoksiribonükleaz). GA'den etkilenen türün (C türü) aktivasyonu sadece Ca^{2+} iyonlarının varlığında gözlendi (Ca^{2+} e bağımlı deoksiribonükleaz).

Anahtar sözcükler: DNA, gibberellik asit, alevrone endonükleaz, nükleus

Introduction

Programmed cell death (PCD) is an energy-dependent physiological process that is genetically programmed with the involvement of regulatory genes, stimulatory events, signalling pathways and morphologically expressed distinctive features. The hallmarks of PCD include early cell membrane blebbing, generalized chromatin condensation, compacting of cytoplasmic organelles and internucleosomal DNA cleavage (Greenberg, 1996).

The aleurone layer of cereal grain secretes hydrolases that mobilize endosperm reserves during germination (Fincher, 1989; Jones and Jacobsen, 1991). The synthesis and secretion of these hydrolases (including α-amylase) is under hormonal regulation. Gibberellic acid (GA) stimulates the synthesis and secretion of hydrolases and abscisic acid reverses this effect (Fincher, 1989; Bissenbaev et al., 1992).

Kuo et al., (1996) showed that gibberellic acid treatment accelerated cell death in wheat aleurone

layer. It has been shown that GA induced cell death in wheat was prevented by the protein phosphatase inhibitor - okadaik acid. However, these experiments were carried out on the level of detection viable cells, without reveal any morphobiochemical PCD events.

Our preliminary experiments showed that the gibberellic acid stimulated wheat aleurone cell death accompanied by the breakage of DNA into oligonucleosomal fragments. When isolated wheat aleurone layer were incubated in the presence of RNA synthesis inhibitor – actinomycin D (Act. D) or abscisic acid (ABA), GA stimulated DNA ladding were prevented (Bissenbaev et al., 2001). It is shown that these cleavages are specific to death stimulated by GA and genetically determined.

This might suggest that the activation of nuclear DNase (including induction of protein synthesis) is necessary link in stimulatory action of GA on the PCD of wheat aleurone cells. The goal of the present work was to investigate the participation of nuclear DNase in ontogenetically programmed cell death of aleurone layer of wheat seeds.

Material and methods

Plant material

Wheat (*Triticum aestivum*, variety Kazakhstanskaya 4) seeds were used in this study. Aleurone layers were obtained from de-embryonated seeds. De-embryonated seeds were sterilized and allowed to imbibe in sterile water at room temperature for two days. Aleurone layers were isolated from the imbibed grain by removing the starchy endosperm under sterile conditions. Isolated aleurone layer (20-30 layer) incubated in a medium contained $CaCl_2$ (10 mM), GA and/or Act. D (at concentrations indicated in the text and figures).

Isolation of nuclei

The freshly isolated aleurone layer (500-1000 layer) was homogenized in 15-20 ml buffer A (15 mM Tris-HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 15 mM β-mercaptoethanol) in the cold. Homogenate was filtered through a nylon filter and the nuclei were pelleted by centrifugation at 2000 g for 10 min. The prepared pellets were washed twice in the buffer B (Buffer A + 0.5% Triton X-100). For the preparation of highly-purified nuclei, pellets were centrifuged in the gradient of glycerin (prepared by mixing 50% glycerin in buffer A with 80% glycerin in Tris-HCl, pH 7.4). Fraction of nuclei was collected on the border of two gradients. The obtained nucleus was washed and resuspended in the buffer C (buffer A without β-mercaptoethanol) and used for experiments.

DNA extraction and electrophoresis

Isolated nuclei were lysed in a 0.7 ml ice cold buffer (5 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.5% (w/v) Triton X-100, 2% (w/v) sodium dodecyl sulphate (SDS), 1.4 M NaCl, 0.2% (w/v) β-mercaptoethanol) and incubated for 30 min at 60^0 C. The DNA was extracted with equal volumes of chloroform and centrifuged for 10 minutes at 10 000 g. The aqueous phase was transferred to a new tube and 2/3 volumes of ice-cold isopropanol (-20^0 C) was added and centrifuged for 10 min at 5000 g. The supernatant was discarded, and the pellet containing DNA was dried and resuspended in 500 μl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5). To the obtained suspension, equal volume of phenol-chlonoform (1:1) mixture was added. After 15 min. centrifugation at 5000 g for 10 min. 1/10 volume of sodium acetate (pH 7.0) and 2/5 volume of ice-cold ethanol were added to the aqueous phases. DNA was allowed to precipitate at –20^0 C, pellet-containing DNA dried and resuspended in 50 μl of TE buffer. DNA electrophoresis was carried for 2 h at 70 V on 1.8% agarose gels. DNA was visualised by ultraviolet fluorescence after staining with 10 μg/ml (w/v) ethidium bromide.

Nuclease activity gels

Aleurone layers were ground as a fine powder in liquid nitrogen and extracted in buffer (150 mM Tris-HCl (pH 6.8), 0.5 mM PMSF). The homogenate was centrifuged and the supernatant was used as source of nucleases. Protein electrophoresis were carried on 12.5% SDS-PAGE co-polymerized with 0.5% (w/v) DNA (extracted from leaves of wheat grains germinated for 4 day), 0.5% (w/v) bovine albumin, as previously described (Fath et al., 1999) with some modifications. After electrophoresis gels were washed twice in the medium containing 10 mM Tris-HCl (pH 6.0), 25% isopropanol, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1 mM DTT and washed for 30 min with same buffer but without isopropanol. Gels were then incubated in

the buffer containing 10 mM Tris-HCl (pH 6.0), 1mM $CaCl_2$, 1mM $MgCl_2$ for 24 h at 37^0C. EDTA and EGTA were added to incubation medium at concentrations indicated in the text and figures. Nuclease activity was detected by staining the gel with 10 μg/ml (w/v) ethidium bromide for 15 min. Nuclease activities were visualized under ultraviolet lights as clear DNA-depleted bands from the fluorescent background.

Results and discussion

To examine the role of deoxyribonucleases in PCD of wheat aleurone cells we have used gel enzyme assay method. In the control conditions (in absence of GA in incubation medium) histochemical staining revealed two distinct protein bands with nuclease activity (A- and B -forms) on electrophoregrams (Figure 1.1). Addition of 1 μM GA to the incubation medium was accompanied with change in the spectrum of nucleases: one additional electronegative electrophoretic band of deoxyribonuclease (C-form) was found in addition to A- and B-forms of nucleases (Figure 1.2). Addition of RNA synthesis inhibitor – Act D (25 μg/ml) to incubation medium blocked

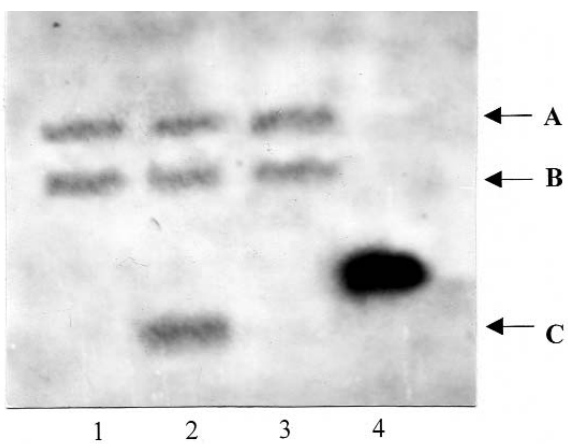

Figure 1: Effects of GA and Act. D to activity of wheat aleurone deoxyribonucleases.
1. Control; 2. GA; 3. Act. D; 4. Commercial deoxyribonucleases. A, B and C – protein bands with deoxyribonucleases activity.

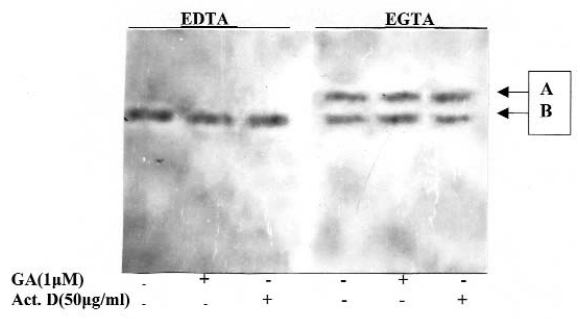

Figure 2: Effects of EGTA and EDTA to activity of wheat aleurone deoxyribonucleases.

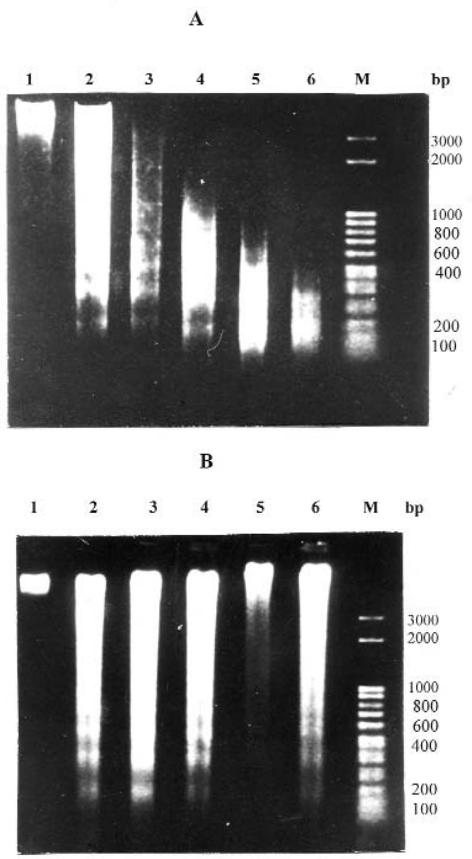

Figure 3: Resolution of DNA fragments from wheat aleurone nuclei by agarose gel electrophoresis. (A) Time course of DNA fragmentation by isolated nuclei incubated in 1 mM $CaCl_2$ and 5 mM $MgCl_2$. 1. Freshly isolated nuclei; 2. 15 min; 3. 30 min; 4. 45 min; 5. 60 min; 6. 75 min. (B) Inhibition of Ca^{2+}/Mg^{2+} dependent fragmentation of DNA by EGTA and EDTA. 1. Ca^{2+} and Mg^{2+} free medium; 2. 1 mM Ca^{2+}; 3. 1 mM Ca^{2+} + 5 mM Mg^{2+}; 4. 5 mM Mg^{2+}; 5. 1 mM Ca^{2+} + 5 mM Mg^{2+} + 25 mM EGTA; 6. 1 mM Ca^{2+} + 5 mM Mg^{2+} + 50 mM EDTA; M. Marker.

activation of GA –inducing form, whereas activities of A- and B- forms deoxyribonucleases were not influenced by this inhibitor (Figure 1.3).

In subsequent experiments the effects of bivalent cations to the activity of wheat aleurone nucleases were studied. For this reason polyacrylamide gel was incubated in the medium containing bivalent cation's chelators – EDTA or EGTA after electrophoresis. Figure 2 shows that the presence of 20 mM EGTA completely blocked the appearance of C-form of nuclease (GA-inducible form) on the gel. The presence of EDTA (50 mM) inhibited the activation A and C forms of this enzyme. At the same time EGTA and EDTA had no detectable effect on activity of B-nuclease.

Therefore, results of these series experiments revealed three types of wheat aleurone nucleases (A, B, C) with different sensitivity to divalent cations, GA and Act D. The effect of GA on the C-nuclease activity depends on RNA synthesis, whereas activity of other forms of this enzymes is not required the GA-depended activation of transcription process.

Wide variety of lytic enzymes have been shown to be associated with PCD in animal systems. From the studies of apoptosis, two different DNase enzyme have been suggested to be responsible for DNA degradation at the end of apoptic cell death. These nucleases differ in their cation requirement and their intracellular location (Hess and Fitzgerald, 1996).

Various nucleases have been identified in plant undergoing PCD. Two prominent and several minor nuclease activities were detected in dying pericarp and nucellar cells of maize. Nuclease activity was also detected in dying endosperm cells (Young and Gallie, 2000). It remains to be determined whether one or more of the observed nuclease activities is responsible for the internucleosomal degradation of endosperm DNA. There is no conclusive evidence that any of these nucleases are responsible for the internucleosomal fragmentation associated with PCD in plant.

For detailed analysis of involvement of nuclear endonucleases in GA induced PCD of wheat aleurone cells, we studied DNA fragmentation in isolated nuclei. Figure 3.1 shows that DNA remains intact in freshly isolated nuclei. Incubation of nuclei with Ca^{2+}(1 mM) and Mg^{2+}(5 mM) ions for 15 min significantly increased the DNA fragmentation. Under these conditions different sized fragments formed characteristic internucleosomal ladders on agarose gel electrophoresis (Figure 3.2). Following incubation of isolated nuclei accompanied by enhancement of low molecular DNA fragments and much DNA is fragmented in 75 min (Figure 3.6).

These data indicate that there is a Ca^{2+} and Mg^{2+} dependent endonuclease endogenously situated in the nuclei of wheat aleurone cells.

To study the effects of divalent cations on DNA fragmentation, nuclei were incubated for 30 min with Ca^{2+} and Mg^{2+} alone and also in the conditions when both ions are presents. In the presence of 1 mM $CaCl_2$ DNA fragmentation increased (Figure 3.B). Combined addition of $CaCl_2$ (1 mM) and $MgCl_2$ (5 mM) to the incubation medium significantly increased the DNA fragmentation compared with control (Ca^{2+} free medium) and Ca^{2+} alone. Incubation of isolated nuclei in the presence of EGTA decreased level of fragmented DNA. Addition of EDTA in the dose 50 mM to the incubation medium fully prevented nuclear DNA fragmentation.

Our results suggest that the stimulatory effect of GA on PCD of wheat aleurone cells may involve activation of several form of nucleases. Different forms of nucleases revealed histochemically in the present work differ by the sensitivity to Ca^{2+} , Mg^{2+} , GA and Act D. Particularly, for activation of B-deoxyribonuclease, Ca^{2+} and Mg^{2+} ions are not required, whereas A-form of enzyme is activated in the presence of these cations.

Activation of GA-induced form (C-form) was prevented if RNA synthesis was inhibited by Act D and this activity was observed in the presence of Ca^{2+} ions (Ca^{2+}-dependent deoxyribonuclease). The synthesis of RNA is not required for *de novo* synthesis of the endonuclease itself, because when freshly isolated nuclei were incubated in the presence of Ca^{2+} and/or Mg^{2+}, the DNA was rapidly fragmented into nucleosomal-sized fragments.

It seems probable that RNA synthesis is required for activation of pre-existed forms of endonucleases by Ca^{2+} dependent mechanism. Presumably this enzyme is inactive when cells are not exposed to GA, because the intranuclear availability of calcium is low. The GA then might induce the mechanism, which transports calcium into the nucleus in the form that is able to activate the endogenous endonuclease. Indeed it has been shown that GA stimulates aleurone cell death by a calcium-dependent event (Kuo et al.,1996). This

makes Ca^{2+} ion a most likely component of GA signalling pathway that leads to the cell death in wheat aleurone cells.

From all these results it is possible to conclude that activation of nuclear Ca^{2+} dependent endonucleases may be one of the final steps in GA-stimulating wheat aleurone cell death program.

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Effects of spermidine, spermine and cyclohexylamine on mitotic activity of 2X, 4X and 6X wheats

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Abstract

The effects of spermidine (Spd), spermine (Spm) and cyclohexylamine (CHA) on seed germination, root elongation, mitotic index and chromosomal behavior were studied in diploid (*Triticum monococcum L.*), tetraploid (*T. durum* Desf.) and hexaploid (*T. aestivum* L.) wheats. The polyamines (PA) inhibited seed the germination in three species. CHA inhibited seed germination in diploid and tetraploid species, but it showed a stimulation in hexaploid seeds.

Spd, Spm and CHA reduced root elongation in three species. Spd and Spm decreased mitotic index in diploids and tetraploids. In hexaploids, Spd slightly increased the division frequency whereas Spm decreased it. The root tips of the three species treated with CHA showed maximum inhibition in mitotic index. Some mitotic abnormalities like unoriented metaphase, anaphase bridges, unequal distribution, laggards, multipolar spindle fibers were observed in the control groups of diploid, tetraploid and hexaploid. Spd, Spm and CHA affected the percentages of aberrations. Our results suggested that the reason of the inhibition in root elongation was due to the reduction in the mitotic index.

Key words: Spermidine, spermine, cyclohexylamine, mitotic index, chromosomal abnormalities

Spermidin, spermin ve sikloheksilaminin 2X, 4X ve 6X buğdaylarda mitotik aktiviteye etkileri

Özet

Bu çalışmada, spermidin (Spd), spermin (Spm) ve sikloheksilamin'nin (CHA), diploid (*Triticum monococcum* L.), tetraploid (*T. durum* Desf.) ve heksaploid (*T. aestivum* L.) buğdaylarda tohum çimlenmesi, kök uzaması, mitotik indeks ve kromozomların davranışına etkileri incelendi. Poliaminler üç türde de tohum çimlenmesini inhibe etti. CHA diploidlerde ve tetraploidlerde tohum çimlenmesini inhibe ederken, heksaploidlerde çimlenmeyi teşvik etti.

Spd, Spm ve CHA üç türün kök uzamasını inhibe etti. Spd ve Spm diploidlerde ve tetraploidlerde mitotik indekste inhibisyona neden olurken, heksaploidlerde Spd artışa, Spm ise düşüşe neden oldu. CHA üç türün kök uçlarında da mitoz bölünme frekansını düşürdü. Her üç türün kontrol grubunda da mitotik anormallikler gözlendi. Bu anormallikler bozuk metafaz tablası, anafazda kromozom köprüleri, eşit olmayan dağılım, geç kalan kromozomlar, çok kutuplu iğ ipliği şeklinde özetlenebilir. Spd, Spm ve CHA üç türde de anormallik yüzdesini etkiledi. Sonuçlar, kök uzamasındaki inhibisyonun bu maddelerin mitotik indeksi düşürmelerinden kaynaklandığını gösterdi.

Anahtar sözcükler: Spermidin, spermin, sikloheksilamin, mitotik indeks, kromozomal anormallikler

Introduction

Polyamines, (PA) putrescine (Put), spermidine (Spd) and spermine (Spm) are found ubiquitous in higher plants. The exact function of these polycations is unknown, but many studies indicate their involvement in various events such as cell division, DNA and protein synthesis, growth and differentiation, senescence inhibition, fruit ripening (Slocum and Flores., 1991; Martin-Tanguy, 2001).

PAs were found as powerful inhibitors of seed germination in *Arabidopsis* (Mirza and Bagni, 1991). On the other hand cyclohexlyamine (CHA), an inhibitor of spermidine synthase, accelerated radicle emergence in *Cicer arietinum* seeds (Gallardo et al., 1992).

Although high level of PAs is generally related to cell division some inhibitory effects of exogenously applied PA have been reported (Gatta et al., 1992; De Agazio et al., 1992). The inhibition of root growth observed during the Spd treatment of maize seedlings is due to reduction of both mitotic index and cell elongation (De Agazio et al., 1995).

PAs can bind to DNA and associate with chromosomes (Hougaard, 1992). Direct binding of PAs to DNA and their ability to modulate DNA-protein interactions appear to be important in mitotic activity and chromosomal behavior. Recently, Ünal et al. (2002) observed mitotic abnormalities in barley seedlings with Put treatment however little is known about the effect of PAs and their biosynthetic inhibitors on chromosome behavior.

Presence of ploidy series is well known since long years in wheat and the basic number is seven in this genus. It is also known diploid wheat species having 2n=2X=14, tetraploid 2n=4X=28, and hexaploid species 2n=6X=42 chromosomes. Plants at different ploidy level giving different responses are also well documented (Kerby and Kuspira, 1988).

The aim of this research is to investigate the effect of PAs on seed germination, root elongation, mitotic index and chromosome behavior and also to reveal the response of plants at different ploidy levels to exogenous PAs.

Material and methods

The seeds of diploid *Triticum monococcum* L. cv. TUR O2343, tetraploid *Triticum durum* Desf. cv. *Ankara 09/96* and hexaploid *Triticum aestivum* L. *cv. Kutluk-94* were used as experimental materials. The seeds were surface sterilized with 1% sodium hypochloride for 5 min and washed with tap water. They were soaked in water (control) or in experimental solutions (1 mM Spd, 1 mM Spm, 10 mM CHA) overnight. These concentrations were chosen, based on previously reported favorable results (De Agazio et al., 1992). After that, the seeds were placed to petri dishes containing filter paper moistened with distilled water or experimental solution. 30 seeds were used to estimate germination percentages and each experiment has 5 replications. 2 days after sowing, root tips were cut 0.5 cm and hydrolyzed with 1 N HCl at 60^0 C for 10 min. Then they were transferred in to basic fuchsin for 1.5-2 h in dark. Squash preparations were made in 2% aseto-orsein. Cytological analysis included mitotic index and scoring of aberrant cells. Ten well spread slides were chosen and more than 5000 cells were scored for each treatment. Mitotic index was calculated as the percent ratio of dividing cells and total number of cells observed.

Results

In control, the seeds of diploid (2X) and hexaploid (6X) species started to germinate at 12^{th} hours after sowing but the germination started at 18^{th} hours in tetraploids (4X). No difference was established in Spd and Spm treated seeds in respect to the starting time of germination. Seed germination was started at 12^{th} hours in 2X, 4X and 6X seeds treated with CHA. Spd and Spm inhibited seed germination at three species (Table 1). The seeds of T. *monococcum* were the most sensitive to PAs.

Table 1: The effect of spermidine, spermine and cyclohexylamine on seed germination in diploid, tetraploid and hexaploid wheats. Control: Distilled water, Spd: Spermidine (1mM), Spm: Spermine (1mM), CHA: Cyclohexylamine (10mM).

	Seed Germination (%)		
Treatment	*T. monococcum* (2X)	*T. durum* (4X)	*T. aestivum* (6X)
Control	100.0	100.0	100.0
Spd	75.1	80.6	85.0
Spm	18.8	60.4	72.7
CHA	89.1	98.5	107.2

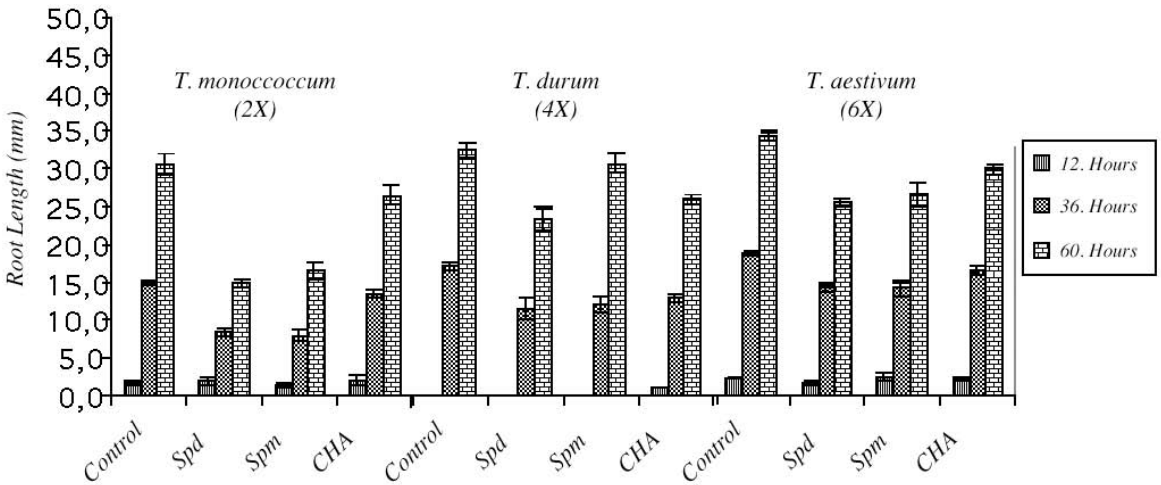

Figure 1: Effect of spermidine, spermine and cyclohexylamine on the primary root growth of 2X, 4X and 6X wheats. Control: Distilled water, Spd: Spermidine (1mM), Spm: Spermine (1mM), CHA: Cyclohexylamine (10mM).

Table 2: Mitotic index and frequency of chromosome aberration in the root tips of 2X, 4X and 6X wheats treated by Spd, Spm and CHA. Control: Distilled water, Spd: Spermidine (1mM), Spm: Spermine (1mM), CHA: Cyclohexylamine (10mM).

Species	Treatment	Total cell scored	Mitotic index (%)	Abnormal metaphase	Abnormal anaphase	Total aberration frequency (%)
T. monococcum	Control	4608	18.6±0.009	8/114*	11/84**	9.6
(2X)	Spd	4604	13.4±0.009	31/104	10/52	26.3
	Spm	2951	12.3±0.003	6/52	4/49	9.9
	CHA	2988	9.1±0.006	3/36	14/28	26.6
T. durum	Control	5563	19.2±0.006	21/142	22/75	19.8
(4X)	Spd	2045	18.8±0.006	7/40	9/38	20.5
	Spm	1634	17.8±0.009	8/35	3/23	19.0
	CHA	6851	10.6±0.003	40/124	34/79	36.5
T. aestivum	Control	8722	18.5±0.006	35/173	40/116	26.0
(6X)	Spd	6709	18.8±0.006	24/131	26/136	18.7
	Spm	5784	17.8±0.009	15/115	21/121	15.3
	CHA	5295	7.1±0.003	5/38	10/24	24.2

** Ratio of cells at abnormal metaphase to total metaphase*
*** Ratio of cells at abnormal anaphase to total anaphase*

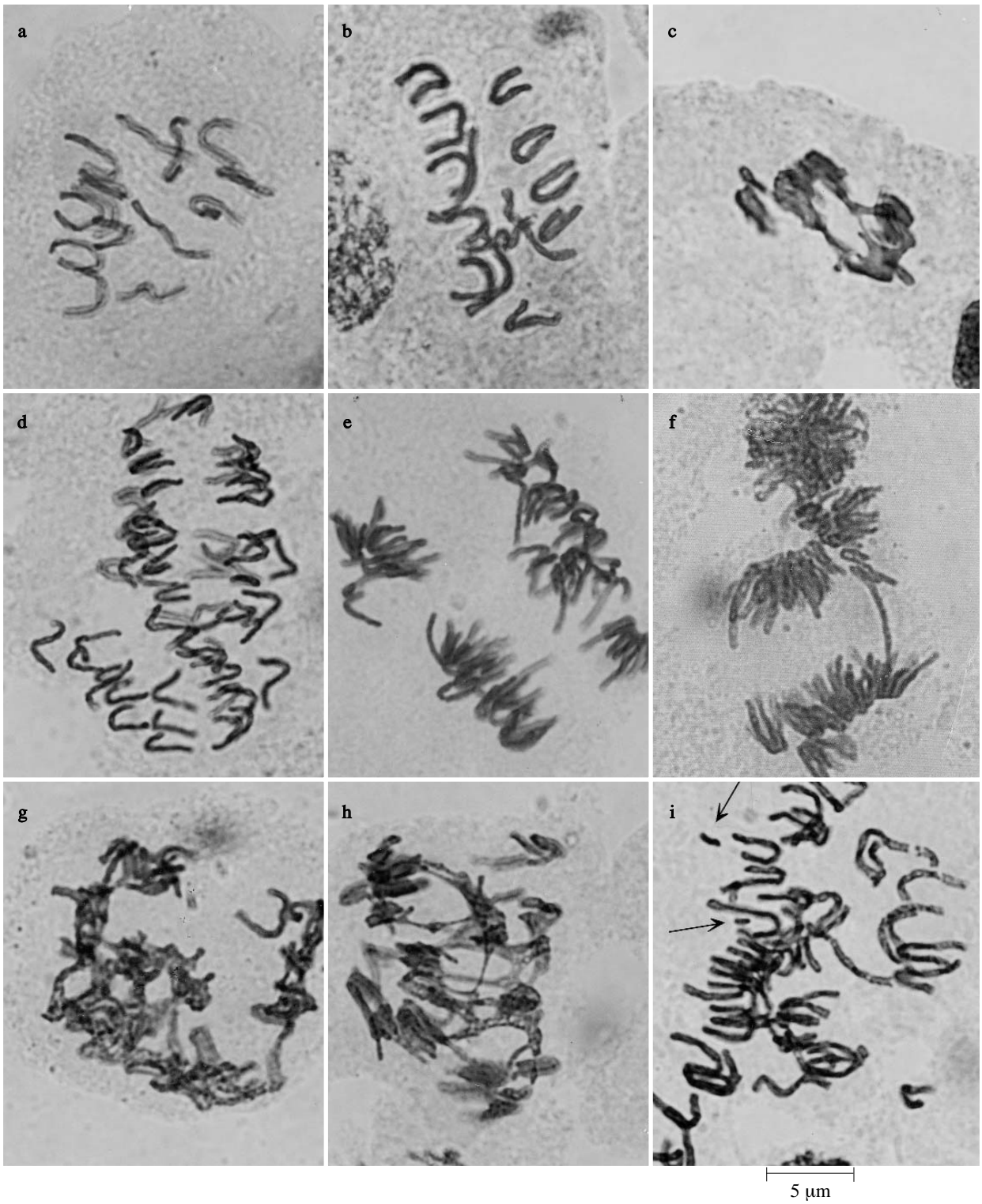

Figure 2: Mitotic abnormalities in the root tip cells treated with Spd, Spm and CHA of diploid tetraploid and hexaploid wheats. a,b: Abnormal metaphase in diploids; c: Abnormal anaphase in diploids; d-f: Abnormal anaphase in tetraploids; g-h: Stickness of chromosomas in hexaploids; i: Abnormal metaphase in hexaploids. Arrow points B chromosomes.

Any morphological change was not observed by Spd, Spm and CHA treatments. Primary root lengths were measured after Spd, Spm and CHA inhibited seed germination up to 60 h. Root elongation in diploid, tetraploid and hexaploid wheats (Fig 1). Root lengths were reduced by 51.6% and 46.1% with Spd and Spm treatments respectively in diploids after 60 hours. Spd and Spm applications also caused by 28.6% and 5.5% decrements in tetraploids and by 25.7% and 22.4% in hexaploids respectively.

Mitotic index was summarized in Table 2. Mitotic index reflects frequency of cell divisions and it is an important parameter to determine the rate of root growth. Mitotic index was almost the same in the control groups of wheats at different ploidy levels and it was determined to be 18.6% in *T.monococcum*, 19.2% and 18.5% in *T.aestivum* (Table 2). Spd decreased the division frequency from 18.6 to 13.4 in the root tips of diploid wheat but no significant difference was observed in mitotic index at Spd treated root tips of tetraploid and hexaploid wheats. Spm decreased the division frequency from 18.6 to 12.3 in the root tips of diploid wheat. This value was also reduced to 17.8 in tetraploids and to 17.6 in hexaploids. CHA, a biosynthetic inhibitor of Spd and Spm, showed a drastic reduction in the mitotic index of the three species studied; to 9.1 in diploids, to 10.6 in tetraploids and to 7.1 in hexaploids. The results show that the root tip meristems of diploid wheats are more sensitive to PAs and to their inhibitor in respect to division frequency.

Mitotic abnormalities like unoriented metaphase, anaphase or telophase bridges, unequal anaphase separation, laggards, fragments, stickiness, multipolar spindle were observed in the control groups of three species. Spd, Spm and their biosynthetic inhibitor, CHA did not cause a considerable change on the shape of the cells and also in their cytoplasm but they affected the percentage of mitotic abnormality (Fig. 2) results were presented in the Table 2. The lowest percentage of chromosome aberration in the control existed in diploids, and the highest percentage in hexaploids. Although the percentage of aberration was highly increased in diploids by Spd and CHA, in tetraploids by CHA, whereas it was reduced in hexaploids by all treatments.

Discussion

PAs are activators of protein synthesis occurring in early phase of germination (Villanueva and Huang, 1993; Anguillesi et al., 1982). Spd and Spm inhibited seed germination in *Arabidopsis thaliana* but they had no effect on root growth whereas Put at low concentration stimulated root growth. This suggested that endogenous concentration of Put in the roots of *A. thaliana* could be growth limiting (De Agazio et al., 1995). In this paper, Spd and Spm inhibited seed germination in three wheat species. CHA also inhibited seed germination in 2X and 4X species but slightly increased in 6X species. Spd, Spm, inhibited Root growth and CHA in three species studied. Comparison of root length with mitotic index revealed close correlation for the investigated species.

Some inhibitory effect of exogenously applied PA in various cell functions has been described (Gatta et al., 1992; De Agazio et al., 1995). De Agazio et al. (1992) reported that Spd pretreatment induced 50% inhibition of root extension in intact maize seedlings after 24 h. They concluded the inhibition of root growth of maize seedlings is a complex phenomenon due to reduction of both the mitotic index and cell elongation accompanied by stiffening and lignification of cell wall (De Agazio et al., 1995). Although much evidence supports the involvement of PA in various growth and developmental processes in higher plants it is not completely clear whether PA per se, compounds made from PA, or their catabolites are responsible for some of the observed effects. Therefore, the possibility exists that its degradation products mediate the inhibitory effects of Spd in the inhibition of maize root growth (de Agazio et al., 1995).

Bharti and Rajam (1995) investigated the effect of DFMO on growth, PA levels and chromosome behavior in *Triticum aestivum* L cv. Agra. They sprayed 5 days old wheat seedlings with DFMO (0.1, 1.5 and 10 mM) and they calculated the mitotic index. They established no significant effect on seedling height, root length, number of roots in seedlings treated with DFMO at any concentration tested (0.1-5 mM). 10 mM DFMO treatment resulted in a marked decrease in plant height, root length and number of roots. They observed some mitotic alterations like unoriented chromosomes at metaphase and anaphase upon treatment with 10 mM DFMO, however they established no significant difference in mitotic index.

These researchers suggested that DFMO could be safely used as a protecting agent against the fungal diseases without affecting the host plant.

In our previous study it was established an inhibition in mitotic index by applying 10 mM DFMO in three wheat species differing from the results of Bharti and Rajam (1995).

Sauve et al. (1999) determined that PAs play some role in chromosome condensation in human breast cancer cells (MCF-7) and they also found more Spd content in interphase cells than in mitotic cells. These findings are agreed with the studies that PAs are almost entirely cytoplasmic during interphase but associate with chromosomes in mitosis (Hougaard et al., 1987; Hougaard, 1992). The close relation between PAs and chromosomes elucidates the chromosomal abnormality observed in PAs treated root tip cells of wheat.

In conclusion, The PAs, Spd and Spm and their biosynthetic inhibitor, CHA caused a reduction in root growth resulted from the reduction of mitotic index and mitotic irregularities in the root tips of diploid, tetraploid and hexaploid wheats and diploid species is more sensitive to Spd and Spm and CHA than the others.

Acknowledgement

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E-cadherin immunohistochemistry for prostate cancer early diagnosis and monitoring of illness

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Abstract

Prostate cancer (pCA) is the most commonly diagnosed noncutaneous malignancy in men world wide. The natural history of prognostic factors involved in prostate cancer are not clearly defined. Hence, molecular parameters able to accurately assess the aggressiveness and the metastatic potential of the cancer are urgently needed. The E-cadherin gene, on chromosome 16q22 encodes for transmembran 120 kDa glycoprotein belonging to the group of Ca^{2+} depending homophilic cell adhesion molecules (CAMs). Loss of E-cadherin function in cancer cells likely plays at least two different roles in tumor development and progression. In this study after prostatectomy procedure, the prostate specimens were analyzed for E-cadherin activity and compare with Gleason scores. It is aimed to reveal the prognostic importance of E-cadherin molecules in pCA.

Keywords: e-cadherin, prostate cancer, metastasis

Prostat kanseri erken tanı ve hastalığın izlenmesinde e-cadherin immunohistokimya uygulanması

Özet

Prostat kanseri, hemen hemen en sık rastlanılan erkek kanserlerinden bir tanesi olup, çok yaygındır. Halen doğal prognostik faktörleri prostat kanseri için aydınlatılamamıştır. Bu yüzden acilen agresivite ve prognoz ile moleküler mekanizmaların bulunmasına ihtiyaç vardır. E-cadherin geni 16q22 kromozomu üzerinde 120kDa büyüklüğünde ve Ca^{2+} bağlı adezyon molekülleri ailesindendir. E-cadherinin fonksiyonel kaybı kanser hücrelerinde tumor gelişimi ve progresyonuna neden olur. Bu çalışmada prostatectomy sonrasında Gleason skorlamaları ve e-cadherin aktivitesi karşılaştırılmış ve prostat kanseri için E-cadherin moleküllerinin önemi ortaya konmaya çalışılmıştır.

Anahtar sözcükler: e-cadherin, prostat kanseri, metastaz

Introduction:

Prostate cancer (pCA) is the most commonly diagnosed noncutaneous malignancy in men in USA and also world wide (American Cancer Society, 2002; DeMarzo et al., 1999). Mortality in prostate carcinoma is associated with metastasis and metastasis in prostate carcinoma is usually associated with perineural invasion, for it is the preferred mechanism of prostate cancer metastasis. One of the current theory states this form of metastasis follows the path of least resistance offered by the perineural sheath. Understanding

specific mechanisms of this carcinoma/nerve interaction is key to potential therapeutics targeted to this process. Death from prostate cancer is the result of metastasis and local spread, not from organ-confined disease. Hence, understanding the mechanism of prostate cancer spread and metastasis is the key to treating this disease successfully and increasing survivability (Umbas et al., 1997; Yagi and Takeichi, 2000).

Currently, however, prostate spesific antigen (PSA) in combination with digital rectal examination (DRE) are undergoing evaluation as screening modalities for prostate cancer. Because of a lack of sensitivity and spesificity markers are rarely of use in early diagnosis of cancer. Also they can be used as monitoring disease evaluation with therapy. The goal of future research should be that development of the most specific, cheap and easy markers for common cancer types as prostate cancer (Gao et al., 1997; Buyuktuncer et al., 2003).

The natural history of prognostic factors involved in prostate cancer are not clearly defined. Hence, molecular parameters able to accurately assess the aggressiveness and the metastatic potential of the cancer are urgently needed (Arisan, 2003). Since vascular invasion and the spread of prostate tumour cells to the blood represent preliminary steps in the metastatic process, blood-borne detection of circulating prostate epithelial cells (CPC) could be an early marker of invasiveness (Tomita et al., 2000; Mason et al., 2002). One of the prominent features of the development and progression of prostate cancer is the development of abnormalities in cell adhesion in prostate epithelium and prostate cancer cells. These abnormalities extend to inter-cellular adhesion structures and cell–matrix adhesion molecules.

The E-cadherin gene, on chromosome 16q22 encodes for transmembran 120 kDa glycoprotein belonging to the group of Ca^{2+} depending homophilic cell adhesion molecules (CAMs). These molecules involved in developmental morphogenesis and maintenance of the epithelial phenotype and they are almost exclusively expressed in (adult) epithelial tissues. E-cadherin is bound *via* a series of undercoat proteins, the catenins to the actin cytoskeleton. This linkage between transmembranous cadherins and actin filaments of the cytoskeleton is necessary to form strong cell-cell adhesion. Furthermore, deletion of the a-catenin gene in *vitro* results in the inactivation of E-cadherin-mediated cell–cell adhesion in prostate cancer cells (Nathke et al., 1994). Dysfunction or mutation of β-catenin may also result in cell–cell disengagement, and in subsequent invasion (Vleminckx et al., 2001).

Loss of E-cadherin function in cancer cells likely plays at least two different roles in tumor development and progression. Most of the studies on E-cadherin and cancer have focussed on its role in suppression of metastasis. Loss of E-cadherin expression was demonstrated to be associated withincreased invasiveness in many human tumor types. Furthermore, re-expression of E-cadherin in cell culture results in reversion of the invasive phenotype (Poser et al., 2001; Chen CL et al., 2003). These results strongly suggest that E-cadherin plays a major role as a suppressor of invasion in human tumors.

Immunohistochemical studies in human cancers, including prostate cancer have requently shown that a proportion of invasive carcinomas and carcinomas *in situ* show aberrant levels of E-cadherin and/or catenin expression in comparison to their related normal tissue (El Hariry et al., 2001). In this study after prostatectomy procedure, the prostate specimens were analyzed for E-cadherin activity and compare with Gleason scores. It is aimed to reveal the prognostic importance of E-cadherin molecules in pCA.

Materials and methods

The study comprised 44 selected patients (median age= 68,9, range 48-74) treated in the Urology Department of Sisli Etfal Research and Training Hospital from 1995 to 2001. They were diagnosed as having clinically localized pCA. Before radical retropubic prostatectomy (RRP), distant metastases were excluded by abdominal CT, X-rays of the lungs and bone scans. The patients also were followed detailed anammes, physical examination, PSA amount, TRUS biopsy. None of the patients were received preoperatively *hormon* therapy or radiotherapy. RRP were followed up according to Walsh tecnique.

Tumor specimens were pathologically classified according to TNM system by the same pathologist. All specimens were classified 2 groups for Gleason score <7 and Gleason score>7 and followup ranged 6 to 84 months and included at 3 months postoperatively and over 0,4ng/ml PSA was accepted as nux. Specimens were selected to represent a spectrum of Gleason scores ranging from 2 to 10 with a wide spectrum of pathologic stages.

Pathological determination and immunohistochemistry

Formalin fixed, paraffin embedded archival tissues containing RRP specimens were obtained from Pathology Department of Sisli Etfal Research and Training Hospital.

After dewaxing, paraffin embedded sections were cut serially at 2-5 mm thick, respectively, and stained for E-cadherin. Endogeneous peroxidase activity was blocked by 10% hydrogen peroxide solution in methanol in 20 min. After rinsing in distiled water tumour bearind slides were kept in 10 mM citrate buffer (pH 6) four times for 5 min. They treated with blocking serum (Horse serum, NHS-Novacastra) for prevent of non-spesific binding. The spesific primer antibody (Novacastra 36B5) for the detection of E-cadherin was then added. The anti E-cadherin antibody was applied for 10 min. After rinsing with PBS for three times, standart streptavidin-peroxidase complex was used according to the manufacturer's instructions.

Classification of immunohistochemical responses: Depending on the percentage of cells showing membrane-bound positive immunohistochemical staining for E-cadherin, the tumours were classified into three groups, i.e. no staining <5% (negative distribution), 5-95% heteregenous distribution and over 95% is called as positive distribution.

Positive distribution was accepted as normal E-cadherin distribution, negative and heteregenous distribution called as abberant E-cadherin distribution.

Statistically all data were analyzed with SPSS statistical software. Fischer exact test and probability analyses were done in this study.

Results

In this study RRP operated 44 prostate cancer patiens were investigated pathologically and they were classified as pT2 (14 patients), pT2b (13 patients), pT3a (8 patients) and/or pT3b (9 patients). These classes were investigated for E-cadherin activity. 27 patients (61%) had lymph, vesicle seminalis and organ confined disease (pT2). 8 of the total patients (18%) had no lymph and vesicle seminalis invasion and they were classified as pT3a. 9 patients (21%) have no lymph and have seminal vesicle invasion (pT3b) (Table1).

Table 1: Patient profile

Patient Characteristics	
Patient number	44
Age	
Average	63 years
Range	48-78 years
Pre-op PSA levels	
Average	17.28 ng\ml
Range	4.14-41.4 ng\ml
PIN presented case number with adenocarcinoma	33 patients (73%)
Gleason score	
>7	6 patients (13%)
7	17 patients (39%)
<7	21 patients (48%)
Stages	
pT2a	14
pT2b	13
pT3a	8
pT3b	9
Followup period	
Average	31.6 months
Range	9-84 months
Recurrence according to post op PSA value	5 patients

The average age of the patients were 63.35, median was 64 and range is 48 to 78. The average of PSA levels of patients before operation was 17.28 ng/ml and median was 16.42 ng/ml and range was 4.14-41.4 ng/ml. Reinvestigated 33 of 44 specimens (73%) had adenocarcinoma and PIN. 6 patients (13%) Gleason score was higher than 7, 17 patients (39%) Gleason score was 7 and 21 patients (48%) was smaller than 7. The average survey was 31.6 months, median was 35 months and range was 9 to 84 months. 5 patients (10%) showed biochemical (PSA levels) nux in average 19.11 months, median 18 months and range 6 to 35 months. One of the patient died from lung metastasis at 38th month (Table 1).

pT2 classified 11 of 27 patients (41%) had normal E-cadherin distribution, 16 had (59%) aberrant distribution. pT3 classified 2 of 17 patients (11%) E-cadherin distributed normal and 15 patients (89%) had distribution aberrant. The results of pT2 and pT3 classified groups were not determined significant as statistically (p>0,05).

E-cadherin was distributed in 2 of 23 patients (8%) whose Gleason score was higher than 7 normally, 21 of

them showed aberrant distribution. Beside of these 7 of 21 patients (34%) whose Gleason score was smaller than 7 had normal distribution and 14 of them (66%) aberrant distribution. The results of the E-cadherin distribution due to Gleason Score classification were not significant as statistically (p>0,05).

6 of 17 patients (35%) which was classified as pT3 had Gleason Score smaller than 7 and 1 of them (17%) had normal E-cadherin distribution. 6 patients (83%) had aberrant E-cadherin distribution. 12 patients showed Gleason Score higher than 7 and one of them (8%) had normal E-cadherin distribution and 11 of the group (92%) had aberrant E-cadherin distribution. The results of the E-cadherin distribution due to Gleason Score classification were not significant as statistically (p>0,05).

The Gleason Score of pT2 classified 23 of 27 patients (82%) was smaller than 7 and 9 of them (39%) had normal E-cadherin distribution and 13 of the group (61%) had aberrant distribution. In the same classification 5 patients (18%) had higher Gleason Score than 7 and one of them (20%) had normal E-cadherin distribution and remain part of the group (80%) had aberrant E-cadherin distribution. In this comparion between E-cadherin distribution and Gleason Score level in pT2 was not significant as statistically (p>0,05) (Table 2).

Table 2: E-cadherin distribution according to stage and Gleason score rates.

Stage	E-cadherin distribution
pT2	11\27 patients (41 %)
7 and higher values of Gleason score	1\4 patients (25 %)
Gleason score smaller than 7	9\23 patients (39 %)
pT3	2\17 patients (11 %)
7 and higher values of Gleason score	1\11 patients (9 %)
Gleason score smaller than 7	1\17 patients (17 %)
E-cadherin aberrant distribution	
pT2	16\27 patients (59 %)
7 and higher values of Gleason score	3\4 patiens (75 %)
Gleason score smaller than 7	13\23 patients (61 %)
pT3	15\17 patiens (89 %)
7 and higher values of Gleason score	10\11 patients (91 %)
Gleason score smaller than 7	5\17 patients (83 %)
p>0.05	

After the RRP operation 5 patients (10%) had biochemically (PSA level) nux and all of these patients had aberrant E-cadherin distribution. One of the patients who showed recurrence had positive E-cadherin distribution but, it is accepted as aberrant for intracytoplasmic distribution (Figure 1).

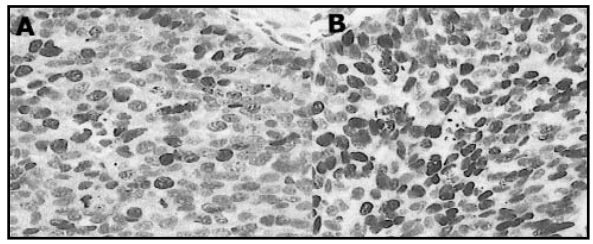

Figure 1: E-cadherin immunostaining in normal (A) and adenocarcinoma prostate (B) tissue.

Discussion

E-cadherin expression is a promising marker of prostate cancer progression that a large, controlled clinical trial or the marker is warranted. Abnormal expression of E-cadherin has been correlated in several human carcinomas with the pathological characteristics of the tumor, such as tumor stage, grade of differentiation, invasiveness, lymph node involvement and distant metastases (Yagi and Takechi, 2000). E-cadherin expression in the primary tumor might be a powerful tool in clinical practice as a marker to predict the metastatic potential in individual patients with prostate. Of course, other factors like histological grade of disease can be detected by E-cadherins (Hirohashi, 1998).

E-cadherin expression correlates with epithelial differentiation whereas loss of E-cadherin expression promotes epithelial dedifferentiation and invasiveness of human carcinoma cells (Jiang et al., 1994). Restoration of wild-type E-cadherin function prevents invasiveness of epithelial tumour cells (Vleminckx et al., 1991). Loss of expression of E-cadherin at chromosome band 16q22 may be associated with a biallelic mutation mechanism characteristic of tumor suppressor gene inactivation. Downregulation of E-cadherin expression is often accompanied by methylation of 5' CpG island of E-cadherin in lung, liver, bladder and gastric carcinoma cell lines, further suggesting that the mechanisms by which E-cadherin

is inactivated in cancer are analogous to those observed for classical tumour suppressor genes (Buyuktuncer et al., 2002). Some studies showed that in human prostate cancer, E-cadherin immunostaining was reduced or absent in 46 of 92 primary and metastatic cases whereas benign non-malignant tissue stained uniformily positive (De Marzo et al., 1999).

In another study decreased E-cadherin immunostaining was correlated with increased invasive potential: a spectrum of staining was found between well-differentiated tumours (Gleason score 4) and undifferentiated tumours (Gleason score > 10) which stained positive and negative, respectively. Between these two extremes, mixed populations of E-cadherin positive and E-cadherin negative cells were associated with moderately differentiated (Gleason score 5–7) and poorly differentiated (Gleason 8–9) tumours (De Marzo et al., 1999; Umbas et al., 1997).

In conclusion, classical prognostic factors are not enough for true determination in prostate cancer studies. Therefore more reliable markers are needed for clinical usage. E-cadherin is one of the most studied molecules for this reason. Distribution of e-cadherin status of patients can show a new approach for prognosis of illness.

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Final version chemical signaling during organism's growth and development

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Abstract

Scientific research on chemical signaling is based on messages send by genetic, ionic, and hormonal integrative systems. Different living organisms such as plants, microorganisms and certainly animals and humans are participants in this signaling exchange. The concept of correlations between hormones and anti-hormones in plant tissue was chosen for this study. Even though it is not a new concept, but it is still yielding a great potential, and though was chosen by authors for detailed investigation.
Scientific idea of signaling is mostly based on the early interrelations between biosynthesis of phenolic and indolic substances, rather than on the quantity of these naturally occurring plant chemicals. Molecular interactions between hormones and antihormones could be not only bases for morphological correlations, but also for the intermediate messages under the effect of the external factors.

Key words: hormones, correlations, stress factors, rooting regeneration,chemical signaling

Organizma büyüme ve gelişimi sırasında kimyasal sinyalleşmenin son durumu

Özet

Kimyasal sinyallesme ile ilgili yapılan bilimsel çalışmalar genetic, iyonik ve hormonal iç sistemlerin mesajları üzerine kuruludur. Bitkiler, mikroorganizmalar, bazı hayvanlar ve insanlar gibi değişik organizmalar bu sinyal değişiminin birer üyesidirler. Bu çalışmada içerik bitkilerde hormonlar ve antihormonların arasındaki korelasyonlar olarak seçildi. Yeni bir içerik olmamasına rağmen, artan potansiyeli nedeni ile araştırmacılar için detaylı bir araştırma konusu olarak halen seçilmektedir.
Bilimsel yargılara göre, sinyalleşme fenolik ve indolik maddelerin miktarından daha çok aralarındaki ilişkiler yönünde temellendirilmektedir. Hormonlar ile antihormonlar arasındaki moleküler etkileşimler sadece morfolojik korelasyonlar üzerinde olmayıp, ara mesajların dış faktörler altındaki etkilerinden ötürü de gerçekleşebilmektedir.

Anahtar sözcükler: Hormonlar, korelasyonlar, stres faktörü, kök rejenerasyonu, kimyasal sinyal.

Introduction

Only few researchers studied correlative processes in the multicellular organisms. However practical applications of scientific research and holistic approach itself are tightly connected with correlative messages based on genetic, ionic, and hormonal integrative systems. These systems do determine growth and development of the organisms. All correlative signals could be divided into internal and external. Among external signals one can find: physical signals such as temperature, light, gravity,

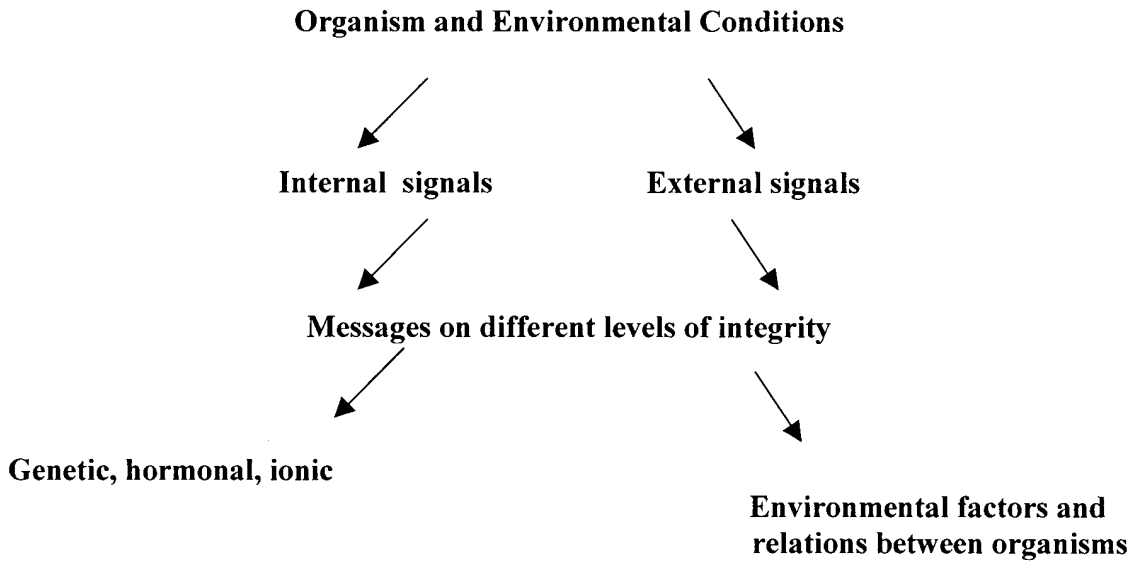

Figure 1: Correlations and signals in living entity.

magnetic fields, etc. Internal signals are chemical signals, for example mineral elements, organic substances, food components, phytohormones and others (Fig 1).

Different living organisms such as plants, microorganisms and certainly animals and humans are participants in this signaling exchange. Environmental effects are also taken in consideration. (Kalevitch et al. 2004; Palavan–Unsal, 2003, and Ozalpan, 2001). Correlations could be connected and disconnected, as it was previously shown on the models of plant growth and organogenesis (Kefeli et al., 2004; Kefeli et al., 2003).

The concept of correlations between hormones and anti-hormones in plant tissue was chosen for this study. Even though it is not a new concept, but it is still yielding a great potential, and though was chosen by authors for detailed investigation.

Previously this concept was discussed by Valentine Kefeli and Cingiz Kadyrov (1971) in Annual Review of Plant Physiology. Few years later Carl Leopold and Paul Kriedemann (1975) suggested that these scientific idea of signaling is mostly based on the early interrelations between biosynthesis of phenolic and indolic substances, rather than on the quantity of these naturally occurring plant chemicals. Molecular interactions between hormones and antihormones could be not only bases for morphological correlations, but also for the intermediate messages under the effect of the external factors.

Thus Çotuk (2002) in his review of the book by Ozalpan–Basic Radiobiology wrote that for understanding the biological effects of radiation on the organism it is necessary to summarize the effects on biomolecules, subcellular structures, cells, tissues, and organs respectively. Earlier N.Tretjakov and co-authors while continuing the traditions of N. Maximov (Honorary Member of the American Society of Plant Physiologists) also discussed the similar concept of phytohormones and inhibitors interactions during plant growth. He confirmed our idea that the growth process is a balance between plant hormones and natural growth inhibitors. These relations are modified during the life cycle of the plant. Our latest data helped us to contribute into the development of the concept that deals with the role of plant hormones and inhibitors in the life of single plant and plant cenosis.

Some chemicals found in abundance in plant tissue are phenolics. Their main properties include: presence in tissue could easily determined, they participate in oxidative reactions in the cells and are not transported to the long distances from the point of formation as hormones do.

This internal system of chemical signals is the only part of endogenous systems of regulation. In this article we continue to develop this concept and research further the effects of ecological factors on plants and their internal signaling processes.

Materials and methods

Leaves from different trees species were used for this study. At first, different plant material, for example willow leaves of three species- *Salix viminalis*; *Salix purpurea* and *Salix glaucophylla* were dried up prior to investigation and fixed on the herbarium sheet. Others were used as well; see below in results and discussion section.

Later water extraction took place- 30 g leaves / 200 ml water. Water extracts were subjected to the paper chromatography in the 5% acetic acid. Chromatograms were dried up and then investigated under UV-B light, and treated with 10% baking soda solution for further investigation. Determination of pH of tissue extracts was done by pH meter model 2000, VWR Scientific. For the comparison we also used another method of pH determination – color reaction of La Motte field detector.

Standard was used every time. Each experiment had 3-5 repetitions, standard deviation was considered in experiments.

Results and discussion

Role of phenolics in plants

Willow leaves contained coumarins and phenolic acids, less flavonoids and no anthocyanins. Hydroxy-coumarins like umbeliferone (UF) could be used as a standard for the determination of the quantity of phenolics in water extracts. The different concentrations of UF on chromatograms formed pigments or colorless stains with different square area. The higher dosage of UF apparently made a larger stain or pigmentation. With the help of diazo-sulfanilic acid as a color reagent it was possible to observe UF pigmentation and then create a calibration curve.

The more specific substance for the Willow species that were under investigation was flavonol with the RF not higher than 0.2. The higher amount of flavonol possessed *Salix viminalis*, the less – *Salix glaucophylla*. All 3 investigated species contained coumarins with the RF 0.8- bright blue color reaction. Paper chromatography and color reactions could be helpful tools in identification of chemical markers. Thus this method of chemo- taxonomy could be a sufficient supplement for the establishing real identification of some species with proper morphological features.

Other tree species such as sumac leaves for example contained flavonoids which had low mobility and were yellow in color under UV-light. Application of baking soda spray increased its intensity, pink color in day light- anthocyanins, coumarins- blue in the UV, changed color to green with baking soda, Rf-0.58. Our previous research also supported these data.

Interactions between plants and environment

Some substances could be retained by willow roots and some are excreted in the external water medium. Chromatography of these water exudates and the subsequent investigation of chromatograms in UV-B light showed that most of these substances are polyphenols; coumarin or phenolic acids. The phenolic substances which are retained by the cells have different properties than the ones that are located in the root exudates. These data confirm the idea about excreted substances being allelopathogens in nature and their involvement into ecological interactions between different plant species.

Environmental factors and plant reactions

Effects of UV-B (280-320 nm) and UV-C (300-354 nm) on the seedlings of some crops were also studied. There are some plants that are resistant to both types of UV-light (30, 000 J/sq.cm) like pea, bean or wheat. Lettuce and *Arabidopsis* seedlings are very sensitive: the growth of roots of irradiated arabidopsis seedlings was inhibited 50%. 3000 J/sq.cm was applied. As a rule roots were more sensitive to UV effect, than shoots. One of the primary effects of UV-C on the seedlings was activation of potassium and sodium secretions. Calcium was more stable and was not secreted actively. Dry seeds were more stable to UV stress, than germinated seeds or seedlings. *Arabidopsis* mutants with different sensitivity to UV-C were also tested. Level of ethylene and ABA was the highest in the UV-B resistant mutants. These data corresponds to the ideas presented by A. Ozalpan in his book, 2001.

Phenolics in the leaves of different age

The leaves of scarlet maple *Acer rubrum* L. were collected in July (green), September (abscised, yellow) and in February (brown, composted). Water extracts of these leaves were subjected to paper chromatography in 5% acetic acid. Chromatograms were investigated under UV-B light and sprayed by baking soda and Pauli reagent (diazo-sulfanilic acid). This complex of color reactions allowed identifying monophenols-coumarins and phenol-carbonic acids as well as flavonoids and antocyanins. During the composting process the pattern of phenolics in leaves had changed remarkably. The most dramatic changes were observed in the flavonoid complex. Incubation of water extracts with fungi imperfecti changed phenolic complex-the amount of anthocyanin was decreased. The concept of the interaction between the active molecules during ontogenesis is accepted by Palavan-Unsal (2003).

Transformation of phenolic compounds in maple leaves during the composting

Leaves are the good source of carbon in the composting process. During the microbial decay of leaves, some chemicals in the soil split very quickly, and some are more stable. Among stable substances are phenolics with some inhibitory activity. To determine pH of leaves (water extracts) and biological activity of these extracts, the following experiments were done and results obtained:

1. the most specific substance for the Willow species investigation was flavonol with the RF not higher than 0.2.
2. The higher amount of flavonol possessed *Salix viminalis,* the less – *Salix glaucophylla*
3. All 3 investigated species contain coumarins with the RF 0.8- bright blue color reaction.

It is interesting to mention that leaves during composting lost their acid reaction and their inhibitory activity. These effects made maple leaves and sumac leaves more acceptable in ecological sense. Leaves during 1-4 months also lost their allelopathic activity and became just a carbon substrate for the composting process.

Age and regeneration

Willow (*Salix discolor Muhl*) and poplar (*Populus nigra* L) cuttings were obtained from one year shoots of original plants. Cuttings were equal in their length-18 cm but differed in their weight remarkably-cuttings from lower part of the shoot were 3 times heavier for both plants than cuttings from upper part of the shoot. The weight of square of leaves on the corresponding parts of the willow mother shoots were higher in the central part of the shoot and less in the upper and basal parts of the shoot. Poplar cuttings from the upper part of the shoot formed less roots and shorter roots than cuttings from the lower part. These roots were also shorter. We suppose that the age of the shoot influenced on the rooting process not only via hormonal balance but also via the biosynthesis of carbon polymers such as lignin and cellulose.

Bioassays in determination of plant growth activity

Biological activity of polluted water, contaminated or healthy soil, water extracts and root exudates could be analyzed by proposed complex of seed bioassays. Lettuce, wheat, mustard and clover are plants from different families, which can easily germinate and have no effect on the growth of each other. Germination of seeds of these test plants as well as growth of their stems was important criteria in determination of toxic and inhibitory activity of the substrates. Because of that seed property it was possible to investigate the substrate in one Petri dish separated into quadrants. Test itself is very rapid and data could be obtained after one week. This test is successfully applied both for the investigation of fabricated soils (artificial soils created for revitalizing eroded soils) and exudates from chromatograms. Roots and leaves of willow and poplar contained salicilate, coumarins and some phenolic acids. Part of these substances could secrete from roots and subjected to microbial transformation.

The cuttings of willow (*Salix excelsior*) and Black poplar (*Populus nigra*) were rooted in water and the rooting process of cuttings was investigated. The upper part of the mother shoot is usually less active for rooting in comparison with basal part. After rooting the cuttings were planted into fabricated soil plots.

Phenolic secretions and leaves-inhibitory properties

Natural plant exudates may often inhibit seed germination and seedling development thus triggering allelopathy within soil systems and other growing media. The purpose was to determine the ability of

mustard seeds to recover from an extended exposure to various extracts from sumac leaves (*Rhus typhina*), as sumac excretes allelopathic compounds, which inhibit germination and growth of some types of plants when these accumulate in adjacent rhizospheres. Four different types of seeds of common cultivated agronomic crops were placed into Petri dishes lined with filter paper, which was divided into quadrants. The filter papers were imbibed with 2ml each of sumac leaf and root extracts, while tap water served as a control. Tested in each of the quadrants were mustard, red clover, lettuce and wheat, of which mustard appeared, affected with 0% germination? The seed were then removed from testing conditions, rinsed in tap water, and placed back into control conditions in order to study their capability to grow. After 48 hours under these conditions, the 80% of mustard seeds germinated without apparent inhibition. (Tables 1-4). These initial observations indicate that the allelopathic exudates of sumac, most particularly those extracted from red, fall leaves, only temporarily stunt the growth of mustard. This indicates that the inhibiting substances do not have long term effects on the process of seeds germination; that the search for botanical herbicides amongst natural sources, like leaves and roots of sumac are less harmful than synthetic herbicides. Over time, as the chemical compounds are leached out of the soil, the seeds, germinate. Paper chromatography showed that sumac leaves contain at least four different groups of phenolics, which were identified by RF position, UV light, and soda. Thus, water extracts of leaves can yield at least four classes of phenolic substances such as anthocyanins (red pigment), flavonoids (yellow pigment), coumarins (colorless, bright blue under UV light), and phenolic acids. These substances have different Rfs' on chromatograms. The phenolic complexes of the leaves are more toxic than the phenolic complexes of the roots. The phenolic complexes of sumac leaves (collected in autumn) inhibit mustard, clover and also exert a potent inhibiting effect on lettuce, whereas wheat is most resistant. Therefore, the phenolic complex of leaves may be used as a source for botanical herbicide with inhibiting effects on the germination of weeds that belong to the *Cruciferae* family. Although this inhibition is not permanent, but temporary yet it may suffice, if the treatment is properly timed, to compete effectively against unwanted vegetation in the cultivated field or nursery and with foreseeable environmental benefits. (Boulos et al., 2004). Table 1 showed selective inhibiting effect of leaves extracts on

Table 1: Effect of water extracts from sumac leaves and roots on the germination of seeds of 4 crops (in %% to control). Extracts -1 part of dry material-7 parts of water.

Trial/Extract	Wheat	Clover	Mustard	Lettuce
Control, water	46	74	78	86
Leaves, green	51	40	0	68
Leaves, red, abscised	63	22	1	23
Roots	53	93	92	90

Table 2: Effect of water extracts from sumac leaves and roots* on the seedlings of the 4 crop tests (length of seedlings in mm and in %% to the control).

Trial	Length of the seedlings (mm)							
	Wheat		Clover		Mustard		Lettuce	
	Average	%%	Average	%	Average	%%	Average	%
Control, water	99	100	34	100	35	100	34	100
Sumac abscised leaves	29	29	15	44	14	40	20	59
Sumac roots	75	75	28	82	28	82	23	68

* *10 g of dry matter was extracted 24 h by 70 ml of water at 18°C.*

Table 3: PH of sumac leaves and roots extracts.

Trial	Control	Leaves Extract	Root Extract
pH	7.66	3.63	5.89

Table 4: Effect of water extracts of sumac leaves and roots on the rooting processes of bean plants.

Trial	Number of Roots on 1 Cutting		Length in mm and %% of the Longest Root			
					Shoot	
	Average	%	Average	%%	Average	%
Control, water	18.6	100	90	100	63	100
Standard rooting Hormone, Indolyl-3 acetic acid, IAA, 70 mg/l	32	170	310	340	193	300
Sumac leaves	0	0	0	0	0	0
Sumac roots	26.6	142	302	143	195	300

Table 5: Effect of water extracts of sumac leaves and roots on rooting processes in willow (*Salix discolor* Muhl) stem cuttings (amount of roots per cutting R/C and %% to control)

Trial	Willow Rooting After					
	2 weeks		3 weeks		4 weeks	
	R/C	% to con	R/C	%%	R/C	% to con
Control	3.5	100	11.8	100	17.0	100
Leaves	1.6	45	6.4	54	15.0	88
Roots	3.8	111	9.8	83	15	88

the seeds germination of the 4 crops. More sensitive to the extracts were mustard seeds. Extracts from the roots of sumac were inert.

These data show that mustard seeds are mostly sensitive to the inhibitory effect of water extracts from the green leaves of sumac. Such selectivity in the reactions reminds the selectivity reactions of the herbicides. This type of Botanical herbicides has a privilege- short term effects, see table 2. After washing of seeds they restore the capability to growth.

Table 3 demonstrated that extracts from sumac leaves are acidic, probably because of the presence of weak phenolic acids such as p- coumaric, caffeic and other C-6-C-3 products. These substances are not present in the roots in significant amounts.

Previous data showed effects of sumac leaves extracts on the processes of germination and growth. Table 4 demonstrated strong inhibitory effect of the extract on the rooting of bean cuttings.

Similar inhibitory effect but not as strong as before was observed in case of stem wood cuttings of the willow trees.

Indolic auxins as chemical signals

The natural auxin Indolyl-3-acetic acid (IAA) in the concentration 70 mg/l induced formation of the roots on the stem cuttings of bean plants (*Phaseolus vulgaris* L.) after12 hours of incubation in the auxin solution and successful rooting in water during 7 days, 22^0C. Control- water- 4 roots, IAA-60 roots. If IAA was oxidized preliminary by bean root enzyme-peroxydase the auxin effect disappeared. (Tables 6-8). If auxin was applied to the cuttings not at the

Table 6: Effect of indol-3 acetic acid, IAA, 70 mg/l on the rooting of bean cuttings.

	Results for 1 Cutting					
	Number of Roots		Length of the Longest Root (mm)		Length of Shoot (mm)	
	3 experiments (1 and 2 are week exposure)					
Trial	1	2	1	2	1	2
Control, water	2.9	15	7.4	3.3	7.7	15
IAA, penetration Via stem cut	5.3	40	7.6	35	7.6	19
IAA, penetration Via roots	1.1	32	3.0	23	9.6	14

Table 7: Effect of oxidized IAA on rooting of bean cuttings.

	Results for per Cutting					
	Number of Roots		Length of the Longest Root (mm)		Length of Shoot (mm)	
	3 experiments (2 week exposure of each)					
Trial	1	2	1	2	1	2
Control, water	2.4	20.3	8.2	79.8	2.9	31.3
IAA, 75 mg/I	12	39.6	10.4	56.8	6.8	32.1
IAA preliminary passed through Roots*	8.6	35.2	13.6	49.0	11.8	26.8
Oxidized IAA (OIAA)**	1.2	19.0	2.3	3	4.1	5.0

** Roots as centers of auxin-oxidase, peroxidase (OIAA)*
*** OIAA-oxidized IAA.*

beginning of the experiment but after root primordial formation (3rd day) the effect of auxin was lost .These data showed that auxin induced rooting via some intermediate processes. The pretreatment of the cuttings with the low dosages appreciably attenuated the stimulating effect of IAA on the root formation process of metabolic inhibitors actinomycin D, 8-azaguanine and chloramphenicol. It was shown that root forming cuttings in which cell division occurs 500-1000 times higher are more sensitive to the inhibitors of nucleic acid-protein metabolism, compared to coleoptile segment growth. This coleoptile growth is based on cell elongation, and is also sensitive to IAA (7 mg/l). Willow (*Salix discolor Muhl*) stem cuttings after the treatment by IAA, 150 mg/l formed roots after 14 days of incubation, at 22^0C (Davis, et al., 2004).

If preliminary bark and cambium of the stem cuttings were separated from wood the rooting process stopped. If only strip of bark/ cambium was separated-rooting proceeded on the intact part of the cutting thus rooting process is connected with the bark-cambium complex. This complex is inactive during the autumn dormancy period (1.5-2 months). During this time buds did not open and roots did not form even in the lab conditions. Rooting process of dormant cutting is suppressed, however not as long as bud dormancy. Usually buds attached to the rooting zone open earlier than upper buds of the stem cuttings. These data showed the effect of local break of dormancy and probably local activation of native auxin biosynthesis in the zone of the stem cambium. We checked the possibility of auxin biosynthesis in the isolated cells of *Dioscorea* and confirmed its possibility. It is necessary to mention that tagged cells sensitivity of cambium is very important for the root initiation.

Table 6 showed the strongest effect of IAA while penetrating via stem cut, but not via roots of bean plants.

IAA stimulated rooting of cuttings only if it penetrated into the cutting via stem cuts. Preliminary oxidized IAA became inactive (Table 7). These data proof that root enzymes-peroxidases are compartmentalized and separated from xylem pathways.

Conclusion

Chemical signaling in plants proceeded on metabolic, hormonal and ionic levels. Some of the regulating processes are associated with the cell structures and some with tissues or organs development. Plants as any other living organism accept signals from the environment and sent signals outside in forms of ions and organic substrates. Among these substances could be relatively stable products like phenolics which can regulate growth and development activity of other species and participate in the construction of the natural cenosis. However unstable chemicals are also present.

Disruption of signaling system led to the development of abnormalities, such as interruption in processes of tissue differentiation and tumor formation. Reconstruction of plant integrity proceeds with the help of phytohormones, mostly indolic auxins. It is essential to consider that after plant death, its organic matter is subjected to humus transformation. Primary stages of this transformation could be connected with the elaboration of phenolic substances from the cells, and they could play a role of allelopathogens, thus becoming crucially important in signaling system. This article is just an attempt to discuss the issues of chemical signaling in the plant or plant community. Ion regulation of plant organism under normal and stress conditions is the next step in deciphering the processes of endogenous signaling.

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Apoptosis detection by TUNEL assay in BPH patients

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Abstract

This study presents whether benign prostate growth in aging men correlates with a decrease in apoptotic rate, which were determined with terminal deoxnucleotidyl transferase mediated dUTP biotin nick end labelling (TUNEL). Thirty-eight prostate adenoma were taken from BPH patient who underwent TUR-P.The patient were devided two groups according to their age, 14 of them were under 65 years-old, the other 24 were over 65. Apoptotic indeces, PSA levels, IPSS-LQ, prostate tissue weight were evaluated in these two groups of patients. A significant higher apoptotic rate was observed in second group which contain prostatectomy speciment from patients over 65 age as compared under 65. There were positive correlation between the age and apoptotic rate in BPH patients.

Key words: Apoptosis, prostate, prostatic hyperplasia.

TUNEL yöntemi ile BPH hastalarında apoptoz tespiti

Özet

Bu çalışmada benign prostat hiperplazisine sahip olan yaşlı erkeklerde apoptotik indeks hızındaki azalmayı göstermek amacı ile TUNEL yöntemi kullanılmıştır. Otuz sekiz prostat adenom kitlesi TUR-P sonrasında hastalarda alınmış ve hastaların 65 yaş veya üstü olmalarına göre gruplandırılmıştır. Apoptotik indeks, PSA seviyesi, IPSS ve yaşam kalitesi değerleri, her bir hasta için incelenmiştir. Hasta yaşı ve apoptoz arasında pozitif korelasyon saptanmıştır.

Anahtar sözcükler: Apoptoz, prostat, prostat hiperplazisi

Introduction

Benign prostate hyperplasia (BPH) is one of the most common causes of hospitalization in men more than 50 years old. However, many of the characteristics of these conditions, such as their etiology, biology, behavior, prognosis have not fully investigated (Coffey et al., 1999; Kyprianou et al., 1996). The development of BPH is both androgens control and associated with aging. Although epidemiological facts underscore the importance of BPH as a major health problem, suprisingly little is known about or agreed on the cellular and molecular processes involved in the development of disease (Deng et al., 1996; Tahmatzopoulos and Kyprianou, 2004).

The intensive research into study of tissue homeostatis over the past 10 years has confirmed that the kinetics of tissue growth in benign or malign conditions, is contingent on two independent parameters, the rate of cell proliferation and the rate of

cell death. While uncontrolled prostate cancer cell proliferation death comment is very important to control confused events. Generally when cells decides death some molecular pathways changes their products and this situtaion evaluated many times to get true decision. Therefore, there are many important proteins which prevents apoptosis or induces this pathway (Isaacs and Coffey, 1989). After death stimulus, biochemical and morphological great changes occur. In contrast, during necrosis, the cell has a passive role in initiating cell death in responses to pathological changes, hypoxia, extreme temperature, toxins and ionizing radiation, outside the cell (Kyprianou et al., 1996). Apoptosis was originally defined by ultrastructural characteristic features diffentiating it from necrosis. Cells undergoing apoptosis all show similar characteristics. Several morphology identifiable stages have been reported, including nuclear apparent changes, exuberant cell surface protrusion and disintegration of the nucleus to form multiple compacted fragments in chromatin material. The nuclear collapse that is the clue of apoptosis has as its biochemical correlate the fragmentation of DNA by endonucleases, producing fragments of 300-5000bp (Claus et al., 1997; Colombel et al., 1998).

Apoptosis refers to programmed cell death different from necrosis, in which individual cells participate in own fragmentation and deletion from living tissue. This process is under genetic control and a defect in apoptosis may result in the development of neoplasms (Berges et al., 1995). This death type of cells are generally seen eucaryotic cells. In general, cells undergoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal lenght DNA fragments after activation of a calcium-dependent endoeneous endonuclease. Apoptosis can be induced in a variety of cell types by a wide variety of agents, including hormones, growth factors, chemotherapeutic agents and ionizing radiation. In particular, androgens appear to be important factors influencing apoptosis. Apoptosis was originally defined by ultrastructural characteristic features differentiating it from necrosis (Berry et al., 1984; Arrends and Wyllie, 1991).

Identification of apoptotic structures are highly difficult, but they are more reliable than histopathological examinations. Especially, molecular identification and probing target structures in apoptotic bodies with floresence provides high sensitivity in approaches. One of the apoptosis determination method is *in situ* cell death detection. This method is highly fast, simple, non-radioactive technique and suitable for routine analysis (Arrends and Wyllie, 1991).

In this study we try to show whether the age-related growth of the prostate may be based on decrease in apoptosis, in patients of different ages with BPH.

Material and methods

Tissues

Thirty-eight prostate adenoma were sampled from BPH patients who underwent prostatectomy (transvesical or transuretral prostatectomy). The patients were divided into two groups according to their age, 14 of them were under 65 years old (age range=46-65, mean age=57,8), the other 24 were over 65 (age range=66-83, mean age=72,3).

Apoptotis determination

Formalin fixed, paraffin embedded pathological specimens were obtained from above patient populations and histological sections (3 μm) were subjected to the following analysis. Tissue sections were analyzed for *in situ* apoptotic DNA fragmentation using the terminal transferase TdT-mediated dUTP-biotin end-labeling (TUNEL) assay using the *in situ cell* detection kit. Negative controls consisted of sections in which the terminal transferase was omitted. To strip nuclei of tissue sections from proteins an incubation in 20 μg. Proteinase K (Sigma) per ml 0.01 M phosphate-buffered saline (PBS), pH 7.4 for 15 min. Endogenous peroxidase was inactivated by covering the sections with 0.3% hydrogen peroxide in 50% methanol for 20 min at room temperature. The sections were rinsed three times with PBS for 3 min. Each and immersed in TDT buffer (30 mm Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). Terminal desoxynucleotidyl transferase (TDT, 0.3 U/μl) and biotinylated uridine triphosphate (6.25 μM), in TDT buffer were then added to cover the sections, and then incubated in a humidified chamber at 370C for 60 min. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium

chloride, 30 mM sodium citrate) for 15 min at room temperature. The sections were rinsed with PBS, and covered with 2% bovine serum albumin (BSA) in PBS for 10 min. Then all of them rinsed in PBS three times for 5 min. Labelled-peroxidase complex (Roche, Istanbul, Turkey), prepared according to the instructions supplied by the manufacturer, was applied to the sections for 30 min, then rinsed in PBS three times for 5 min. Sections were flooded with diaminobenzidine (0.5 mg/ml in PBS) plus H_2O_2 (0.01%) and stored in the dark for five to ten min. After washing in PBS for 3 min. The sections were counterstained with ethyl green dehydrated with alcohol and cleared in Histoclear Coverslips were mounted using Histomount (Shandon).

Statistical evaluation

All prostate specimens were evaluated by a pathologist to confirm the histological precence of BPH. Pearson corelation test was used to compare these two groups. Age and other parameters for each patient were analyzed for apoptotic index results by SPSS programme. TUNEL-positive, darkly stained nuclei or nuclear fragments with a cytoplasmic halo were recognized as positive apoptotic cells. The apoptotic index was evaluated by counting the number of cells exhibiting TUNEL positivity over the total number of cells in the prostate epithelium (300 to 500), and the apoptotic index was expressed based on this percentage. For all immunostaining procedures, positive staining was evaluated in 3 random fields for each lesion, and the mean values were determined.

Results

The patients were divided two groups according to their age. At the first group there were 14 patient ≤65 years old (mean 60). At the second group there were 24 patient over 65 (mean 72) years old. At the first group; mean prostatic tissue weight was 46.63 cc, prostatic spesific antigen (PSA) level was 3.65 ng/dl , International Prostatic Symptom Score (IPSS) was 20.0 and Life Qualty was 4.1. Statistical comparison between two groups showed no significant difference in prostatic tissue weight, PSA levels, IPSS and life quality assay significant higher apoptotic index was observed in second group which contain prostatectomy speciment from patient over 65 years old (Table 1) as compared under 65 years old. There were negative correlation between the age and apoptotoic rate in BPH patient.

Table 1: Patient characteristics.

	≤65 years old	>65 years old
Mean age	60	72
Prostate volume	46.63 cc	53.37 cc
PSA (ng/ml)	3,65ng/ml	4,43ng/ml
Life Quality	4,1	3,94
IPSS	20	20,66

p<0,05 is considered as significant

TUNEL-positive cells were detected in the benign prostate epithelial cells (Fig 1). The apoptotic index was about 25.6% in BPH for >65 years old patients, but was significantly lower in ≤65 years old patients samples 20.3% (p<0.001). A significant difference in the incidence of apoptosis was detected among prostate epithelial cells in >65 years old group compared with ≤65 years old patients samples (p<0.001) (Table 2).

Table 2: Apoptotic rates of BPH patients.

	≤65 years old (%)	>65 years old (%)
Apoptosis rate	20.3 (p<0.001)	25.6 (p<0.001)

p<0,05 is considered as significant

TUNEL-positive slides were evaluated for age, PSA and other factors for each patient. However, when apoptotic index compared with IPSS, PSA and Life quality assays, found not statistically significant (p>0.05). The apoptotic index of the secretory and basal cells of the prostate epithelium was higher in the over 65 aged patients compared with others, whereas there was a significant increase in the proliferative index of the respective cell populations in the hyperplastic prostate. Balancing the apoptotic versus the proliferative activities revealed a substantial net decrease (fourfold) in the total number of cells dying via apoptosis in both the glandular and basal epithelial cell compartments of the hypertrophic prostate (BPH) when compared with the normal gland.

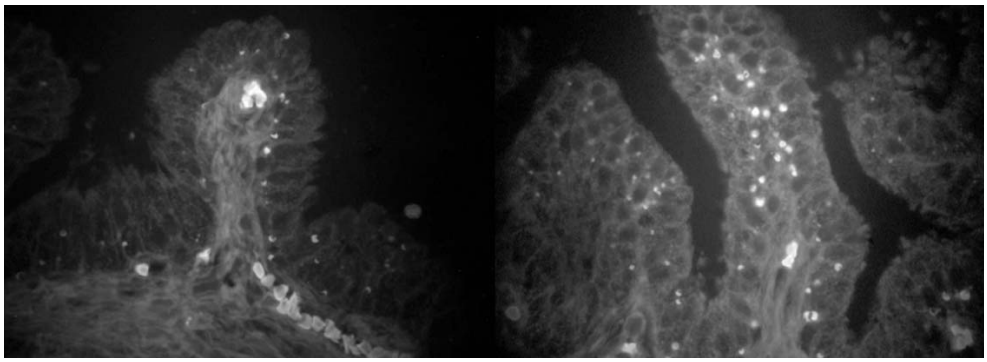

Figure 1: Apoptotic profile of BPH patients. Left side is ≤65 years old and right side is >65 years old.

Discussion

BPH is a phenomenon followed by an age-dependent increase in volume of the prostate throughout the entire life of a man.The growth and involution of the prostate depend on the quantitative relationship between the rate of cell proliferation and cell death. Most previous studies have focused on proliferation and apoptotic rates in benign hyperplastic human prostates (Isaacs and Coffey, 1989; Walsh, 1986)

Siegfried et al., (1993) reported that there is an increase in proliferation rate and decrease in apoptotic rate in benign hyperplastic prostate tissue. Therefore they suggest that the growth of the aging prostate results from this disturbance in the balance between cell proliferation and apoptosis (McNeal, 1983).

Claus et al., (1997) studied the apoptotic and proliferative rates in epitelium and in stroma of BPH. They demonstrated the apoptotic and proliferative rate in epitelium of normal prostate tissue. On the other hand the results of this study indicate that the apoptotic rate in stroma of BPH decrease. Because of this results they suggest the decrease of the apoptotic rate in stroma of BPH maybe a reason for growth of the prostate tissue (Berges et al., 1995).

In this study, we found a significant increase in apoptosis in the epithelium and stroma of benign hyperplastic human prostates age dependent. Increase of apoptosis is thought to be due to the induction by the following factors which are the enfection caused by the residual urine due to BPH, the bladder neck pressure by the retention and the cateterisation. These results suggest a potential involvement of enhanced expression of some antiapoptotic or apoptotic proteins in deregulation of the normal apoptotic cell death mechanisms in the human prostate with age dependency, thus resulting in a growth imbalance in favor of cell proliferation that might ultimately promote prostatic hyperplasia.

In conclusion, there is a good evidence for a wide range of proliferative diseases that some disturbance of proliferation/apoptosis rate is a factor in tissue growth. Obviously the induction of BPH from normal prostate tissue may be associated with a distinct increase in proliferation rate or a decrease in apoptotoic rate. However, our data indicate that the further increase of BPH volume in aging men is not correlated with a decrease in apoptotic rate.

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Letters to editor

Tumor markers for leukemia diagnosis

Lösemi teşhisinde tümor belirteçleri

In the United States, more than 30,000 new cases of leukemia will be diagnosed in the coming year, and adult onset of the disease will account for 90% of these cases. Leukemia is not a single disease but a group of related diseases. There are no specific symptoms for leukemias; instead, symptoms are more generalized and include fatigue, weakness, unexplained weight loss, and pain. Most cases of leukemia are found during routine laboratory tests such as a complete blood count (CBC with differential). Once the initial diagnosis of leukemia is made, further testing includes bone marrow aspiration, lumbar puncture, and excisional biopsies to determine the specific type of leukemia. When leukemias are detected, they are not classified by stages because they are systemic diseases and other organs such as the spleen, lymph nodes, liver, and central nervous system are already involved.

Leukemias are classified into acute and chronic forms. Cancerous cells rapidly reproduce and accumulate in both forms of the disease, crowding out normal white blood cells. The difference between the two forms of leukemia is that, in the acute form, bone marrow cells do not reach maturity and immature cells accumulate. In the chronic form, the cells appear mature but are abnormal and live longer than normal white cells. If left untreated, the majority of patients with an acute form of the disease have a life expectancy of 1 year.

Leukemias are further classified according to the type of affected bone marrow cells. The cancer is myelogenous if the involved blood cells are granulocytes or monocytes. The cancer is lymphocytic if the affected cells are lymphocytes. Leukemias are divided into four main types: acute myelogenous (AML), chronic myelogenous (CML), acute lymphocytic (ALL), and chronic lymphocytic (CLL). There are also several subtypes of these diseases based upon the French-American-British (FAB) classification system for acute leukemias. Prognosis and treatment are based on the diagnosis of the type and subtype of the disease.

Leukemias respond well to chemotherapy and radiation therapy, and these treatment methods are often used in combination. The treatment of leukemia involves the use of a combination of cancer medications given over a period of time. As a general rule, AML will be treated with high doses of chemotherapy agents over a short period of time, whereas ALL is treated with lower doses of chemotherapy over a longer period of time. Chemotherapy agents attack rapidly dividing cells; however, they also interfere with the production of white blood cells, thereby exposing the patient to the risk of infection. Medications known as growth factors increase white blood cell counts and are often given in combination with chemotherapy. Interferons (IFN) are a group of naturally occurring biologic response modifiers that are sometimes used in the treatment of chronic leukemias. The most commonly used of these substances is interferon-alpha.

Interferon reduces the growth of cancerous cells, inhibits their replication, and enhances the immune system's response to the cancer. Interferon appears to be particularly useful when it is used as a maintenance therapy in patients with minimal residual disease (postremission) or complete remission. In addition, all-trans retinoic acid (a vitamin analogue), when used in combination with interferon, may be useful in prolonging the lives of patients with promyelocytic leukemia and other forms of the disease. A cautionary note to the use of this therapy is that the patient may be at risk for thrombosis (blood clots). However, heparin therapy or the use of certain nutrients may reduce this risk.

Other therapies for the treatment of leukemias include stem-cell therapy. Stem-cell therapy involves removing stem cells from the patient either by bone marrow aspiration or by a procedure called *apheresis* (also called peripheral blood stem-cell (PBSC) transplant), when the cells are removed from the peripheral blood system. Stem cells may be obtained

from the patient or from a donor who is a close tissue match to the patient. In this therapy, high doses of chemotherapy and radiation therapy destroy the patient's bone marrow, and the collected stem cells are then transplanted into the patient to restore normal blood cell production. This type of therapy is still in the experimental stage. As a result, it is very expensive and may not be covered by insurance.

Therefore some exact indicators for leukemic diseases should be found for clinicians to understand disease and progression. Tumor markers can be good indicators to examine and monitor the illness.

Tumor markers are substances that can often be detected in higher-than-normal amounts in the blood, urine, or body tissues of some patients with certain types of cancer. Tumor markers are produced either by the tumor itself or by the body in response to the presence of cancer or certain benign (noncancerous) conditions. This fact sheet describes some tumor markers found in the blood.

Measurements of tumor marker levels can be useful -when used along with x-rays or other tests- in the detection and diagnosis of some types of cancer. However, measurements of tumor marker levels alone are not sufficient to diagnose cancer for the following reasons:

- Tumor marker levels can be elevated in people with benign conditions.
- Tumor marker levels are not elevated in every person with cancer-especially in the early stages of the disease.
- Many tumor markers are not specific to a particular type of cancer; the level of a tumor marker can be raised by more than one type of cancer.

In addition to their role in cancer diagnosis, some tumor marker levels are measured before treatment to help doctors plan appropriate therapy. In some types of cancer, tumor marker levels reflect the extent (stage) of the disease and can be useful in predicting how well the disease will respond to treatment. Tumor marker levels may also be measured during treatment to monitor a patient's response to treatment. A decrease or return to normal in the level of a tumor marker may indicate that the cancer has responded favorably to therapy. If the tumor marker level rises, it may indicate that the cancer is growing. Finally, measurements of tumor marker levels may be used after treatment has ended as a part of followup care to check for recurrence.

Currently, the main use of tumor markers is to assess a cancer's response to treatment and to check for recurrence. Scientists continue to study these uses of tumor markers as well as their potential role in the early detection and diagnosis of cancer. The patient's doctor can explain the role of tumor markers in detection, diagnosis, or treatment for that person. Described below are some of the most commonly measured tumor markers.

Tumor markers are measurable biochemicals that are associated with a malignancy. They are either produced by tumor cells (tumor-derived) or by the body in response to tumor cells (tumor-associated). They are typically substances that are released into the circulation and thus measured in the blood. There are a few exceptions to this, such as tissue-bound receptors that must be measured in a biopsy from the solid tumor or proteins that are secreted into the urine.

As tumor cells grow and multiply, some of their substances can increase and leak into the bloodstream or other fluids. Depending upon the tumor marker, it can be measured in blood, urine, stool or tissue. Some widely used tumor markers include: AFP, beta-HCG, CA 15-3, CA 19-9, CA 27.29, CA 125, CEA, and PSA. Some tumor markers are associated with many types of cancer; others, with as few as one. Some tumor markers are always elevated in specific cancers; most are less predictable. However, no tumor marker is specific for cancer and most are found in low levels in healthy persons, or can be associated with non-neoplastic diseases as well as cancer. Also, no tumor marker test is free of false negatives or false positives.

Once cancer is diagnosed, tumor marker levels sometimes help to determine the extent of cancer. Higher levels can indicate more advanced cancer and a worse prognosis in some cases. The patient and their physician may use this information to choose between more or less aggressive treatments.

Monitoring cancer treatment is the most common use of tumor markers. As cancer is reduced, levels often decrease. Stable or increasing levels often indicate that the cancer is not responding to treatment. The choice of tumor marker to use for monitoring is important. Only a marker elevated before treatment should be used to monitor a person during or after treatment. Timing of the tests is also important. Each tumor marker has a unique life span in the blood. To monitor a treatment's success, enough time must have passed for the initial marker to be cleared from the blood. Tests done too soon may be falsely elevated

because the marker produced by the untreated cancer is still present.

Watching for cancer recurrence after treatment is another reason for tumor marker testing. Periodic testing can sometimes detect a recurrence often months earlier than could an ultrasound, x ray, or physical examination.

Tumor marker tests are performed in a lab using immunological techniques. A sample of blood or other tissue is mixed with a substance containing specific antibodies to each tumor marker. If that tumor marker is present, these very specific antibodies bind to the markers. Some type of label, often a radioactive substance, is then used to measure the amount of bound marker and antibody. From this measurement, the amount of tumor marker is calculated. The results are usually available within a few days.

Conclusions based on tumor marker tests are seldom based on one test result but on a series of test results, called serial measurements. A series of increasing or decreasing values is more significant than a single value.

Tumor marker testing is currently the object of much research and attention. Their use is directed by approval from the Food and Drug Administration (FDA) and guidelines established by organizations such as the American Society of Clinical Oncology and the American Cancer Society. Not all tumor receptor marker tests are widely available nor are they widely accepted.

Hormone assays: Tumors of the endocrine glands oversecrete their corresponding hormones. By measuring particular hormones, clues can be obtained regarding certain cancers. For instance, breast cancer cells may secrete prolactin and estrogen. Medullary carcinoma can secrete calcitonin. Pheochromocytomas secrete catecholamines. Tumors of the pituitary gland may secrete growth hormone or cortisol. Carcinoid tumors secrete serotonin. Some tumors of the pancreas secrete insulin. Serial measurements can also monitor treatment for these tumors.

Enzymes: Several serum enzymes can be measured to help detect metastases in cancer patients. Tumors that metastasize to the liver cause increases in serum alkaline phosphatase, gamma-glutamyltransferase, and transaminases. Although these are not necessarily tumor markers, they indicate liver damage that may be caused by metastatic cancer. Tumors that metastasize to the bone sometimes secrete elevated alkaline phosphatase. Lactate dehydrogenase is an enzyme found throughout the body. Because of this it cannot be used as a marker for cancer. It can, however, be used to monitor the treatment of some types of cancer including germ cell tumors, testicular cancer, Ewing's sarcoma, non-Hodgkins lymphoma and some types of leukemia.

Precautions: There is not a good consensus in the medical community about the value of most tumor markers. Because they lack specificity and accuracy, their use is limited. False positives can cause emotional distress and fear. It is not yet determined if there is a savings of life or money with testing. Currently, much controversy surrounds the issue of mass screening for cancer using tumor markers.

Preparation: Tumor marker tests usually require 5-10 mL of blood. A healthcare worker ties a tourniquet on the patient's upper arm, locates a vein in the inner elbow region, and inserts a needle into that vein. Vacuum action draws the blood through the needle into an attached tube. Collection of the sample takes a few minutes and results are available within a few days.

Some markers, such as those for bladder cancer, multiple myeloma, and plasmacytomas, are measured in the urine. Typically this requires a 24-hour urine sample, which means that the individual must collect all of his or her urine for 24 hours. This is usually about 1.5 quarts or more. These results are then available within a few days.

Other tumor markers require tissue samples for analysis. These include receptor analysis such as estrogen receptor and Her-2/neu. Tissue samples are obtained by biopsy. This is usually done by inserting a needle through the skin and into the tumor. The area is typically numbed prior to the procedure. These results are also available within two to three days.

After human genom project, investigators are tried to understand which genes are different for neoplasms and they make lots of comparisons between benign type tissues and neoplasms. These findings are especially done with microarrays. They are also determining populational differences for cancer occurence. However there are still many questions for establihed tumor markers for their specificity and sensitivity. More detailed studies should be tried on gene expressions and their usage in clinics as promising tumor markers.

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Age dependent response under cadmium toxicity: chlorophyll fluorescence and phytochelatin accumulation

Kadmiyum toksisitesine yaşa bağlı cevaplar: klorofil, floresans ve fitokelatin birikimi

Recently, it is investigated that changes in the efficiency of PSII photochemistry, activity of photosynthetic electron transport chain, and growth parameters, as well as in the morphology of leaves, chloroplast composition and ultrastructure depends on the age of Cadmium (Cd)- and excess Copper (Cu)-treated plants. Results of these studies support the opinion that the growth stage of the primary leaves affects the susceptibility of the photosynthetic apparatus to metal toxicity. However, there are only a few studies including the growth stages of the photosynthetic organs during heavy metal treatment. Differentiated sensitivity of the photosynthetic apparatus to heavy metals, depending on the growth stage of primary leaves can be explained by examining the relationship between the rates of leaf photosynthesis and changes in phytochelatin accumulation in Cd treated plants. Maize, as a monocotyledon characterised by tissue of different age along the main leaf axis, is useful to study the coordination of cellular sequestration of Cd by phytochelatins (PCs) with adaptation of photosynthesis under identical conditions for the same plant material. The studies about the effects of Cd on chlorophyll (Chl) fluorescence and PC accumulation at different growth stages in maize leaf segments still very common debate topics.

Drazkiewicz et al. (2003), determined that after growing maize plants in nutrient solutions containing different Cd concentrations in appropriate conditions, the third leaf cut along the main axis into 3 sections of equal length from the youngest (basal) through the mature (middle) to the oldest part (apical) ones was used for all subsequent analyses. *In vivo* kinetic measurements of fast Chl *a* fluorescence induction and the slow phase of the Chl fluorescence induction curve were performed using the Plant Stress Meter. Segments of maize leaf were cut and extracted for determination of glutathione (GSH) and PCs by HPLC = high performance liquid chromatography method. The Cd content in the leaf segments was determined by using atomic absorption spectrophotometer (Unicam 939AA spectrometer). The results were examined statistically by using the one-way analysis of variance (ANOVA) at a significance level $P < 0.05$.

It is shown that Cd taken up from the growth medium and accumulated in roots more than leaves. Distribution of the Cd in leaf segments changed due to age and concentration in the growth medium, so the oldest leaf segments accumulated more Cd than younger ones. Cd at the highest (200 µmol/L) concentration affected both dark and light reactions of photosynthesis to various degrees, depending on the age of the leaf segment. It caused a greater decrease in fluorescence half rise time ($t_{1/2}$) values in the older leaves than in the younger leaves. It also decreased Chl fluorescence ratios (F_v/F_0) more in the oldest and mature leaf segments. Moreover, GSH content, both in control and Cd treated plants increased with the age of leaf segments. Cd treatment enhanced GSH content in comparison to control. In response to Cd treatment, phytochelatin (PC) accumulation was also observed. It enhanced with increasing Cd concentration in the growth medium.

Therefore, it can be concluded that the much greater reduction of fast Chl fluorescence induction kinetics and lower content of phytochelatins in the older as opposed to the younger leaf segments under Cd treatment allows us to accept that PCs protect the light reactions against toxic Cd effect. Chl fluorescence decrease ratio Rfd in maize by 200µmol/L Cd, was greater than the decrease of Chl fluorescence parameters pointing to light reactions of photosynthesis. It was particularly distinct in the youngest leaf segments. This data supports the idea that the main targets of a Cd effect on photosynthetic apparatus are localised within the carbon reduction cycle.

It is known that accumulation of PCs in the leaves of Cd treated plants in considerable amounts, limits the level of Cd ion in cells. Yet, the large decrease of Chl fluorescence parameters can not be explained by that. However, in this study a high positive correlation between R_{fd} values and (acid soluble thiols) AST content in maize leaf segments suggest that these Cd

complexing compounds can play a special role in protection of the photosynthesis dark phase. On the other hand, AST did not prevent the decrease in R_{fd} values completely. Whatsmore, Cd molar ratio (PC-SH:Cd) for phytochelatins in in leaf segments at 200 µmol/L Cd in the growth medium, was lower in older leaf segments. This result indicates a lower efficiency in Cd binding by PCs. So, it will result in a stronger reduction of photochemical processes by Cd in older leaf segments.

The protective role of PCs in Cd toxicity is extensively studied and proved. Although results of this study display the importance PC-SH : Cd molar ratio for the protective role of phytochelatins in the light phase of photosynthesis, there are contradictory results showing that Cd detoxification by PCs in adult plants fully protected both phases of photosynthesis. Mechanisms underlying these different responses should be studied in the future.

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Protecting effects of Bcl2 against oxidative stress

Bcl2'nin oksidatif strese karşı koruma etkileri

American Urological Assoc. Congress including many sub-society congresses held on May 8-13, 2004 in San Francisco. Society of Basic Urological Research Meeting was in Argent Hotel. This meeting was the 10th Donald Coffey Meeting in this congress. Donald Coffey (John Hopkins University) is one of the most important man in molecular researches in urogenital cancers. The meeting were built on three part. First part was general sesion. Its topic was 'Apoptosis: Mechanisms and Significance in Urology'. In this part John Reed who describes major apoptotic pathway with Bcl2 from San Diego University gave a lecture. He told Bcl2, c-myc and cancer relations. After him, Gerard Ewans from San Francisco University gave a lecture and told estrogen and apoptosis relation in cancer cases. After this lecture, bioinformatics, proteomics and apoptosis pathways were discussed. Second section started after lunch break. First part on Mechanistic Insights of Apoptosis Regulation and all mechanish discussed in details.

In summary Bcl2, c-myc and other genes were disscussed for different solutions for cancer. One of the most important strategy was Bcl2 affects on antioxidative stress elements. Oxidative stress refers to the mismatched redox equilibrium between the production of reactive oxygen intermediates (ROIs) and ability of the cells to defend against them. When ROIs increases, elimination of ROIs or repair of oxidatively damaged macromolecules decreases, or both. ROIs, such as superoxide anion, hydroxyl radicals, and hydrogen peroxide, are unwanted and toxic by-products formed during aerobic metabolism. Also they can be produced under X-Ray exposure, toxic elements and many reasons. Nowadays many researches on ROI has been linked many illnesses including all cancer types, diabetes, neurological disoorders.

ROIs can cause cell death via apoptosis and/or necrosis in many cell types, which can be blocked or delayed by various antioxidants and antioxidative proteins/enzymes. The concentration of ROIs and the cellular microenvironment appear to be important in determining the mode of cell death. Cells undergoing apoptosis exhibit mitochondrial depolarization, membrane blebbing, shrinkage of the nucleus, condensation of chromatin, and DNA degradation by endonucleases into fragments in multiples of 180–200 base pairs and propagated through autocatalytic chain reactions. One of the major genes responsible for regulating apoptotic cell death is the protooncogene bcl-2 that encodes a 26-kDa integral membrane protein found in the nuclear envelope, parts of the endoplasmic reticulum, and the outer mitochondrial membrane. The bcl-2 gene product has been shown to prolong the cell survival by blocking the cell death induced by a wide array of stimuli and treatment, including chemotherapeutic agents, radiation, hydrogen peroxide, growth factor withdrawal, neurotoxins, etc. damage by direct chemical interactions and also by indirect interference with enzymes that can repair DNA damage. Bcl-2 prevents cells or facilitates their recovery from hydrogen peroxide (H_2O_2)-induced oxidative DNA damage, such as base modifications or single-strand DNA breaks.

In recent studies showed that Bcl2 over expression induced antioxidant enzymes such as, gluthation (GSH), catalase (CAT), superoxide dismutase (SOD) and some peroxidase isoforms. Especially GSH, a ubiquitous tripeptide thiol, It is suggested that GSH is important in cell proliferation, apoptosis, immune modulation, detoxification, and scavenging of free radicals death by stimulating apoptosis. Bcl-2-overexpressing cells have elevated pools of GSH, and conversely, downregulation of Bcl-2 expression is associated with GSH depletion. Therefore, Bcl-2 may block apoptosis through modulation of GSH metabolism. Other gene interestingly, the DNA binding activity of NF-kB and its transcriptional activity are constitutively elevated in Bcl-2-overexpressing cells, compared with those in vectortransfected counterparts. Thus, overexpression of Bcl-2 in human embryonic kidney 293 cells and myocytes resulted in enhanced constitutive NF-kB DNA binding activities.

The antiapoptotic functions of Bcl-2 are mediated by antioxidative mechanisms that involve constitutive induction of NF-kB and subsequent upregulation of antioxidative genes. However, the complete molecular events involved in potentiation of cellular antioxidant

defense capacity through sustained constitutive activation of NF-kB remain to be elucidated. Bcl-2 has been reported to counteract apoptotic cell death through multiple mechanisms, which mainly target mitochondrial events, but attention has been recently focused on its role inmaintaining or augmenting cellular antioxidant defense capacity that involve antioxidant enzymes (e.g. CAT, SOD, GPx, and GR) and an antioxidant molecule GSH. Understanding of cellular and molecular regulatory mechanisms of antiapoptotic functions of Bcl-2 may provide a new antioxidant therapeutic strategy for the management of a wide array of human diseases that are caused by oxidative stress.. It seems there are many studies should be done to identify all mechanism under oxidative stress. Because Bcl2 is a very useful tool to understand caspase mediated apoptosis.

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Industrial application fields in microbiology and biotechnology

Mikrobiyoloji ve biyoteknolojide endüstriyel uygulama alanları

During 21st century, microbial resources for industrial application seem to increase depending on development of the genetic engineering technology. The industrial microorganisms utilized in industrial area may be natural isolates, laboratory selected mutants or microbes that have been genetically engineered using recombinant DNA methods. All those microorganisms in microbial technology and biotechnology involves in many industrial area around the world. The various ways in which microorganisms have been used over the years to advance medical technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection and agricultural biotechnology. Microbial biotechnology is also promises to offer less expensive solutions for degradation and remediation of waste through natural microorganism and enzyme products.

Production of antibiotics and antimicrobials is one of the application fields in medical industry. Both natural and chemically enhanced microbial products can be used to control human, animal and plant diseases. Using traditional genetics or recombinant DNA techniques, the microorganism can be modified to improve the yield or action of antibiotics and other antimicrobial agents. New research directions are aimed at discovering microbial metabolites with pharmacological activities useful in the treatment of health problems such as hypertension, coronary heart disease and cancer. Another area related to medical technology is production of vaccines. Vaccines are essential to protect humans and animals from microbial diseases. Recombinant DNA technology has allowed the production of novel vaccines that offer protection without the risk of infection (e.g. hepatitis B vaccine).

Utilizing microorganisms in health-care products is also possible. The development and production of diagnostic assays that use monoclonal antibody or DNA probe technology are essential in the manufacture of health-care products such as rapid tests for strep throat, pregnancy or AIDS. Other examples include the use of microbial cells to produce human or animal biologicals such as insulin, growth hormone and antibodies.

Very wide used application field for microbial activity is placed in food and beverage industry. Cheese, yoghurt, butter, pickles, chocolate, soy sauce, food supplements (such as vitamins and amino acids), food thickeners (produced from microbial polysaccharides), alcohol (beer, whiskeys, wines) and silage for animals are all products of microbial activity. Production of coffee, tea, cocoa, summer sausage, vanilla, cheese, olives and tobacco also require cured or microbial activity. Organic acids such as citric, malic and ascorbic acids and monosodium glutamate are microbial products commonly used in foods as food flavouring agents and preservatives. Mushrooms, truffles and some red and green algae are consumed directly. Yeasts are used as food supplements for humans and animals.

In agriculture field, conventional, recombinant DNA and monoclonal antibody techniques are used to improve microbial inoculants, which serve as fertilizer supplements by fixing atmospheric nitrogen and as plant pest controls. Since microorganisms are useful in eliminating problems associated with the use of chemical fertilizers and pesticides, they are now widely applied in farming and organic agriculture.

Industrial applications of enzymes from microorganisms include many processes such as the production of cheese (vegetarian cheese), the clarification of apple juice, the development of more efficient laundry detergents, pulp and paper production, leather production and the treatment of sewage. These processes have been increased by the use of recombinant DNA techniques to design enzymes of increased activity, stability and specificity. Microbial carbohydrates are used as molecular sieves (e.g., dextran) for purification and separation processes. Another application field is to use microbial carbohydrates as thickening agents (e.g., xanthan) that are stable at high temperature. Thickening agents are used for secondary oil recovery in oil fields, lubricants in drilling oil wells, gelling agents in foods and thickeners in both paints and foods. Microorganisms are also used to obtain lipids or fatty acids, for example production of PUFAs (polyunsaturated fatty acids) that are used for enrichment of babies feeding.

Organic compounds such as acetone, methanol, butanol and ethanol all have multiple applications in industrial settings. Microbes will increasingly be used to supplant or replace those processes, which rely on petroleum and natural gas for the production of these compounds.

Oil recovery may be facilitated by the development of unique bacteria, which produce a surfactant that forces trapped oil out of rocks. In mining industry, extraction of minerals from low-grade ores is enhanced by some bacteria (microbial leaching). In addition, selective binding of metals by biohydrometallurgical processes is important in recycling of metals such as silver and uranium.

Contamination control has an importance at industrial processes. Development of assays to detect microbial contaminants in food; evaluation of natural or synthetic agents for the prevention of disease, deterioration or spoilage; determination of minute quantities of vitamins or amino acids in food samples; development of preservatives for control of food spoilage; and development of procedures for control of deterioration in cosmetics, steel, rubber, textiles, paint and petroleum products rely on extensive knowledge of industrial microbiology and biotechnology.

Waste and waste water management is another important area for microbial application since the world faces problems of pollution from different sources. One technology that holds promise for eventually reducing the toxicity and amount of radioactive waste is bioremediation, using live bacteria. This technology makes use of the ability of live cells or enzymes to clean and reduce the volume of waste. Many microorganisms, including fungi, bacteria, and protists, can break down organic toxins, transforming them into harmless products such as water and carbondioxide. As viable remediation tools for environmental pollution, amongst the microbial products, solutions of effective microorganisms offer significant scope to combat different types of pollution problems. For example, soluble UraniumVI is reduced to insoluble UraniumIV which precipitates as solid uraninite, UO2. These precipitates can be recovered easily, then isolated and contained. Studies have illustrated the benefits of effective microorganisms and its metabolites in overcoming problems of both solid and liquid waste management. Isolating or developing microbial strains capable of degrading and detoxifying hydrocarbon and halogenated hydrocarbon waste (for example organo phosphates, acetyl cholin esterase inhibitors) of industrial, agricultural or military origin is essential in waste management.

Microbiologists, molecular biologist and biotechnologists will be responsible for the discovery, development or implementation of certain processes and the quality of products in those wide industrial areas. The product development and application of developed products to industrial processes will need a much wider range of personnel. Therefore, academia, industry and government should share the responsibility for improvement of industrial microbiology to add value in economic way. Industry will require those who can discover new products and develop methods for productions. Government agencies will employ microbiologists, molecular biologist and biotechnologists in research and regulatory positions and may support researches financially. Academia will require educators to prepare the next generation of industrial microbiologists, molecular biologists and biotechnologists and will need to search more in microbiology and biotechnology for industrial applications.

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Instructions for authors

General

1. **Journal of Cell and Molecular Biology** is published biannually (January and July) and covers all aspects relating to cell biology, molecular biology, genetics, microbiology and related topics.
2. Manuscripts must be written in English. Particular attention should be given to consistency in the use of technical terms and abbreviations.
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4. The title should be as short as possible, but should contain adequate information regarding the contents.
5. A brief, informative abstract not exceeding 200 words should be provided in English and in Turkish.
6. The following sections cover the usual contents: Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References (see below), Tables (see below), Figure legends (see below).
7. Results of experiments should be provided in either tabular or diagrammatic form, but not in both.
8. Acknowledgements should follow the text and precede the reference.
9. All pages must be numbered.

Disk submission

Authors are invited to join to the final version of their article in diskette. Preferably send as a $3^{1/2}$ disk in Word Processing software.

Tables and figures

1. Each table should be typed on a separate sheet, numbered with Arabic numerals and accompanied by a short instructive title line plus an explanatory caption at the top. Indicate footnotes within tables

by superscript small letters and type footnotes below the table. Each table must be referred to in the text.
2. Fine drawings can either submitted as original drawings ready for print or as clean and high contrast glossy black and white photographs.
3. Photographs must be supplied as black and white, high contrast, glossy prints, trimmed at right angles.
4. Captions for figures should be typed double-spaced, on a separate sheet. Each caption should be identified as Figure 1 etc. and be complete, clean and concise, so that each figure and its caption could be understood without reference to the text. Do not give magnification on scales in the figure titles; instead draw bar scales directly on the figures.
5. Each illustration should have the title of the paper and the figure number written on the back in soft pencil. The top of the figure should also be indicated on the back.
6. The approximate position of the tables and figures should be indicated in the margin of the manuscript.

Units, abbreviations and scientific names

1. Only SI units should be used. Current abbreviations can be used without explanation. Other must be explained. In case of doubt always give an explanation.
2. Latin names should be underlined or typed in *italics.*

References:

1. Citation in the text should take the form: Smith and Robinson (1990) or (Smith and Robinson, 1990). If several papers by the same author in the same year are cited, they should be lettered in sequence (1990a), (1990b), etc. When papers are by more then two authors they should be cited as Smith et al. (1990) or (Smith et al., 1990).
2. In the list, references must be placed in alphabetical order. The following models for the reference list cover all situations. The punctuation given must be exactly followed.

Redford IR. Evidence for a general relationship between the induced level of DNA double-strand breakage and cell killing after X-irradiation of mammalian cells. *Int J Radiat Biol.* 49: 611- 620, 1986.

Taccioli CE, Cottlieb TM and Blund T. *Ku 80*: Product of the *XRCCS* gene and its role in DNA repair and V (D) J recombination. *Science.* 265: 1442-1445, 1994.

Ohlrogge JB. Biochemistry of plant acyl carrier proteins. In: *The Biochemistry of Plants: A Comprehensive Treatise.* Stumpf PK and Conn EE (Ed). Academic Press, New York. 137-157, 1987.

Weaver RF. *Molecular Biology.* WCB/Mc Graw-Hill.1999.

2. Only papers published or in press should be cited in the literature list. Unpublished results, including submitted manuscripts and those in preparation, should be cited as unpublished in the text.
3. The list of literature must be typed double space throughout and checked thoroughly before submission.

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