TÜBİTAK

Turk J Biol
35 (2011) 137-144
© TÜBİTAK
doi:10.3906/biy-0901-5

UV-B induces cell death in the lichen *Physcia semipinnata* (J.F.Gmel)

Dilek ÜNAL[1], Yiğit UYANIKGİL[2]

[1]Department of Molecular Biology and Genetic, Faculty of Science and Letter, Bilecik University, Bilecik - TURKEY

[2]Department of Histology and Embryology, School of Medicine, Ege University, 35100 İzmir - TURKEY

Received: 06.01.2009

Abstract: We have examined the consequences of Ultraviolet-B (UV-B) irradiation in the thallus of the lichen *Physcia semipinnata* (J.F.Gmel) in terms of cell viability and apoptotic-like formation. UV-B induced oligonucleosomal DNA fragmentation was detected by TUNEL assay and this is the first study showing DNA fragmentation in thalli. The intensity of TUNEL-positive cells after exposure to UV-B at doses up to 95.9 J/cm^2 was higher in the photobiont layer than the mycobiont layer.

Key words: TUNEL reaction, acridine orange staining, UV-B radiation, lichen

Physcia semipinnata (J.F.Gmel)' de UV-B radyasyonunun hücre ölümü üzerine etkisi

Özet: Ultraviolet-B (UV-B) radyasyonunun liken *Physcia semipinnata* thallusunda hücre canlılığına etkisi ve olası apoptozis-benzeri değişiklikler araştırılmıştır. Bu çalışmada, likenlerde UV-B radyasyonunun apoptozise benzer hücre ölümünü tetiklediği ilk defa TUNEL yöntemi ile saptanmıştır. 95,9 J/cm^2 dozajda UV-B'ye maruz kalan örneklerde DNA parçalanmasının özellikle fotobiont tabakasında fazla olduğu, ancak mikobiyont tabakasında da gözlendiği tespit edilmiştir.

Anahtar sözcükler: TUNEL reaksiyonu, acridine orange boyama, UV-B radyasyonu, liken

Introduction

In multicellular organisms, cells are normally eliminated during the developmental stages or when they are damaged. Cell death is an important part of the life cycle which is managed through a genetically defined program leading to cell death in response to developmental signals, as well as biotic and abiotic environmental signals (1). There are 2 forms of cell death: programmed cell death (PCD) and necrosis (2-4). Necrosis is a non-physiological process, which involves cell swelling, lysis and the leakage of the cell contents, and is not genetically controlled (5). PCD is an active cell death process regulated physiologically by a complex genetic mechanism (5).

Some morphological features of PCD are common in fungi, plants, and animals: namely cytoplasmic shrinkage, nuclear condensation, activation of specific proteases, and formation of apoptotic bodies (1,2,6-

11). Additionally, a well accepted biochemical criterion is the detection of multiples of 180 bp due to internucleosomal excision of chromatin fragments by an endonuclease (12).

Ultraviolet (UV) radiation can damage many aspects of plant processes at the physiological level and at the nuclear level in DNA (10). The most important toxic and mutagenic photo-products produced as a result of UV-induced DNA damage are the cyclobutane dimers and photo-products. (4-6,13). Pyrimidine dimers can act as a blocker of transcription and replication of DNA, and thus their persistence is highly toxic (14). In response to such damage, the cells have several strategies to remove the photo-products such as photoreactivation, nucleotide excision repair and recombination repair (15). A failure to repair the damage could lead to mutations or general deterioration of cell function. In mammalian systems, it has been shown that UV radiation leads to the destruction of cellular integrity and to programmed cell death (10).

Although there are numerous reports on different stress conditions inducing cell death in many organisms, such as osmotic or salt stress, exposure to pathogen toxins and UV radiation, studies on the UV-B radiation-induced cell death in lichen are limited. Therefore, the current study focused on the characterization of cell death in the lichen *P. semipinnata* in response to UV-B radiation-induced stress.

Materials and methods

Material and experiment design

P. semipinnata (J.F.Gmel.) samples were collected from tree branches in Karagöl, İzmir (38°33′N 27°13′E, 840 m) in September 2007. *P. semipinnata* has green alga in the genus *Trebouxia* as a photobiont. The mycobiont of *P. semipinnata* belongs to the ascomycete family *Physciaceae*. Lichens were transferred to the laboratory, rinsed 3 times (10 s each) to minimize dust contamination and divided into groups. Experiments were conducted within 3-4 days of collection.

Thalli were exposed to UV-B (314 nm) with doses of 5.9, 47.9, and 95.9 J/cm^2 in petri dishes. UV-B lamps (FSX24T12-UVB-HO, Philips) were fixed 15 cm above the samples without UVA and UVC filter. After exposure to UV-B, all experimental groups were kept in laboratory conditions for 24 h. Temperature measurements confirmed that heat effects were avoided as the temperature near the samples was maintained at 24 °C. Some of the thalli were not exposed to UV-B to serve as controls.

Tissue section preparation

The thallus was cut into small pieces and fixed with 4% paraformaldehyde overnight. The fixed samples were dehydrated through ethanol series (50%, 70%, 80%, 95%, 100% for 15 min at each concentration) and the dehydrated samples were immersed in ethanol-xylene (1:1) and then in 100% xylene for 10 min each. The samples were embedded in paraffin and cut into 5 µm sections in a Leica RM 2145 microtome.

Evan's blue staining

The loss of cell viability was evaluated using Evan's blue staining method, which has been recently modified (16). Sections were stained with 0.05% (w/v) Evan's blue in 10 mM phosphate-buffered saline (PBS, pH 7.0) for 30 min. Slides were examined using an Olympus BX-51 light microscope and photographed by an Olympus C-5050 digital camera. Experiments were repeated 5 times and hundreds of tissue sections were analyzed.

Determination of cell viability by acridine orange staining

Cells were assessed by acridine orange staining. Sections were stained with 0.01% (w/v) acridine orange in 10 mM PBS (pH 7.0) for 30 min. Slides were examined using the Olympus BX-51 and photographed by the Olympus C-5050 digital camera. Brilliant red-orange staining is indicative of RNA. Strongly brilliant green or yellow is indicative of DNA. Experiments were repeated 5 times and hundreds of tissue sections were analyzed.

In situ detection of nuclear DNA fragmentation

Paraffin sections were deparaffinized in xylene for 1 h, and then incubated with 95%, 80%, 70% alcohol series for 3 min. TUNEL staining was performed using Promega In situ apoptosis Detection Kit (Cat # G7130) according to the manufacturer's instructions. Sections were washed with PBS 3 times and then fixed

in 4% paraformaldehyde in PBS for 15 min. After the fixation, sections were incubated in proteinase K (20 mg/mL) for 10 min at room temperature, washed with PBS for 5 min, and then incubated with 4% paraformaldehyde for 5 min. Sections were kept in equilibration buffer for 5 min at room temperature and incubated in 100 µL TdT inside a humidified chamber at 37 °C for 1 h. Sections were then washed with PBS and incubated in 0.3% H_2O_2 for 5 min at room temperature. Section slides were stained with 3,3'-diaminobenzidine (DAB) in the dark. Slides were examined using an Olympus BX-51 light microscope and photographed by an Olympus C-5050 digital camera. Dark brown is indicative of TUNEL (+) reaction and TUNEL (+) cells were counted in the sections. Experiments were repeated 5 times and hundreds of tissue sections were analyzed.

Statistical analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) or Student's t-test followed by the post hoc Tukey test as appropriate (SPSS for Windows version 11.0)

Results and discussion

Due to the current interest in possible apoptotic-like phenomena existing in plant and fungi, recent scientific papers mostly focus on the formation of DNA fragmentation during plant and fungus PCD. The detection of the fragmentation in the DNA ladder is commonly used to distinguish PCD from necrosis at the molecular level (12). Genomic DNA degradation occurs randomly and results in a smear detected when the DNA is separated on agarose gel (17). Alternatively, digested DNA in nuclei can be detected using an in situ method called the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction, which detects free 3'-OH DNA breaks (18). The TUNEL assay has been reported to give variable results due to fixation, embedding and sectioning processes (12,19-21). Moreover, 4% paraformaldehyde in PBS, pH 7.4, is generally used as a fixative in the TUNEL assay, because it does not use acetic acid, which supposedly hydrolyses DNA. However, Liljeroth et al. (19,22) and Liu et al. (1) have successfully adapted the TUNEL reaction protocols to plant cells using FAA as a fixative solution to eliminate artifacts, thus allowing the detection of nuclei with fragmented DNA occurred in vivo. A similar protocol has been adapted to lichen sections in the present study and TUNEL (+) cells have been successfully visualized (Figure 1).

In plants, fungi and algae, fragmentation of the nucleus coupled with positive TUNEL labeling has been reported in cell death induced by UV irradiation. Moharikar et al. showed that when *Chlamydomonas reinhardtii* were exposed to higher UV-C radiation, DNA fragmentation occurred but the characteristic 180 bp ladder pattern, which is indicative of an apoptotic effect of UV-C, has not been detected (23). The treatment of *Arabidopsis* protoplasts with UV-C radiation resulted in the appearance of TUNEL-positive nuclei within 6 h of treatment and DNA laddering 6-24 h after treatment, and the cells appeared to have nuclear fragments at the cell periphery (10). In addition, Del Carratore et al (24) have also found that 40%-70% of *Sacchoromyces cerevisiae* cells were typically TUNEL positive after exposure to UV irradiation at doses of 90-120 J/cm^2. In the current study, TUNEL-positive nuclei were not found frequently in the mycobiont layer in the UV-B-exposed group (as seen in Figure 2). In contrast, the number of TUNEL-positive nuclei was high in the photobiont layer of thallus sections treated with UV-B at dose 47.9 and 95.9 J/cm^2 (percentage of the TUNEL-positive nuclei were 33.6% and 85.7% , respectively as seen in Figure 2).

These results indicate that the UV-B irradiation used in the experiment did not induce a direct physical effect on DNA fragmentation, whereas it increased the TUNEL positive cells in the mycobiont and photobiont layers. If DNA fragmentation was a direct physical effect of UV-B irradiation, we would expect to find the percentage of TUNEL-positive cells reaching a maximum level immediately after irradiation. This suggests that DNA fragmentation is a physiological response to UV-B radiation rather than its direct physical effect on the cell.

Acridine orange is a nucleic acid-specific ultraviolet fluorochrome which stains DNA bright yellowish-green and RNA orange/reddish on a typically green cytoplasmic background, and thus it is an acceptable way to detect chromosome structure. In this study, we found that the TUNEL signals were

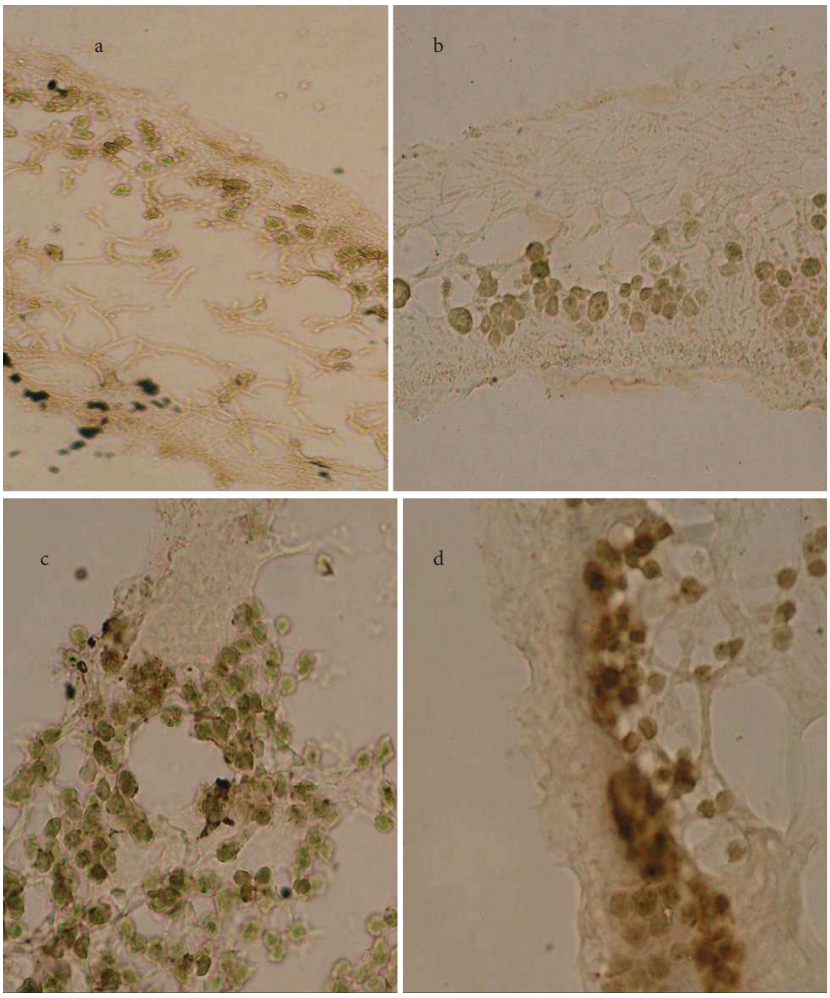

Figure 1. TUNEL staining of the sections of lichen in the control group (**a**) and in the groups exposed to UV-B at doses of 5.9 (**b**), 47.9 (**c**), and 95.9 J/cm^2 (**d**). TUNEL staining, original magnification ×40. Dark brown nuclei are TUNEL (+) cells.

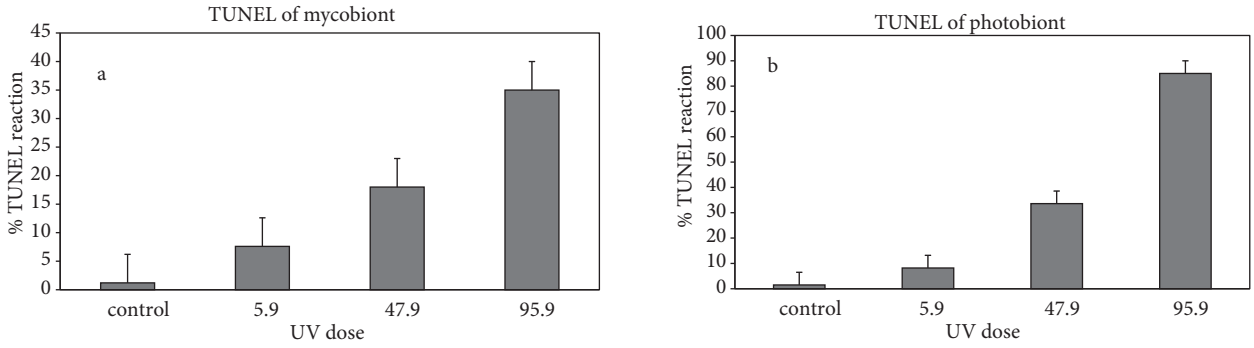

Figure 2. The spatial distribution of the TUNEL reaction in mycobiont (**a**) and photobiont (**b**) layers of *P. Semipinnata* in the control group (non-exposed) and in the groups exposed to UV-B at doses of 5.9, 47.9 and 95.9 J/cm^2.

largely complementary to the acridine orange staining signals during the different stages of cell death. It has been noticed that relatively strong acridine orange signals are detected in the non-stressed or lower stressed lichen thalli, and this observation implies that there is a low degree of DNA damage because acridine orange tends to combine with relatively intact DNA molecules and produces a very strong signal. As the DNA chain loses its structural conformation gradually in the middle and terminal stages of cell death, the acridine orange staining signal becomes increasingly weaker in comparison to the initial stage as a result of the destruction of DNA molecules into small fragments (Figure 3).

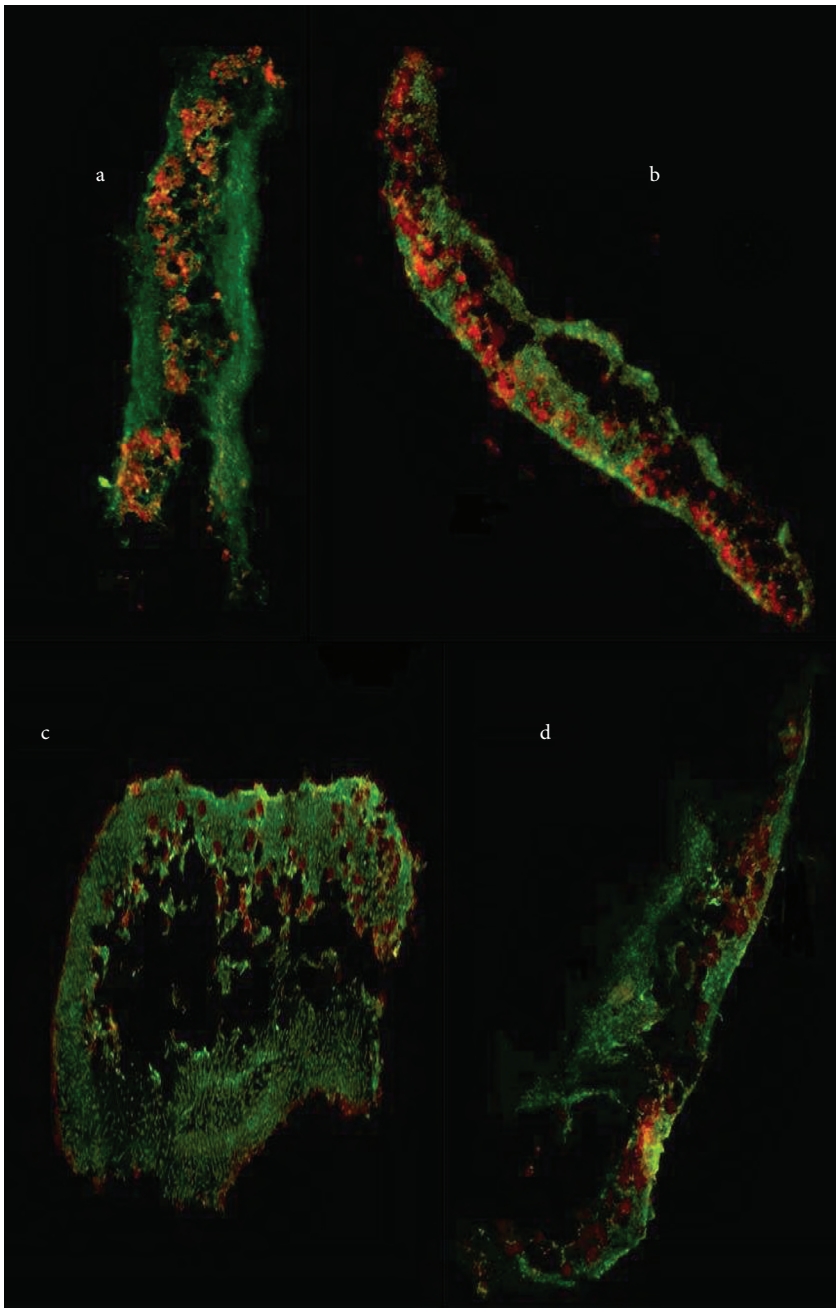

Figure 3. Acridine staining of the sections of lichen in the control group (**a**) and in the groups exposed to UV-B at doses of 5.9(**b**), 47.9 (**c**), and 95.9 J/cm^2 (**d**). Acridine staining, original magnification ×40.

In the present study, cell death was also evaluated by Evan's blue uptake. The Evans blue dye is excluded from viable cells that retain intact plasma membranes (25). We have determined that the dead cells are detected both in the photobiont and mycobiont layers as measured by Evan's blue dye. In addition, the detection of Evan's blue dye in cells precedes the detection of the TUNEL-positive cells. This observation indicates that the plasma membrane alteration is an early indicator of cells undergoing DNA fragmentation, since it occurs prior to the detection of DNA strand breaks by the TUNEL reaction. This is consistent with what has been reported previously using plant cells and Evan's blue dye (16,25). It should be noted that in plant and animal cells, Evan's blue does not discriminate between apoptosis and necrosis (12). As the number of Evan's blue-stained cells is higher than those containing TUNEL-labeled nuclei in our experiments, we cannot exclude the possibility that some of the Evan's blue-stained cells are not apoptotic, but necrotic (Figure 4).

Measurement of chlorophyll fluorescence and thermoluminescence indicated that lichens are remarkably tolerant to extreme radiation, which kills vascular plants within a day (26). The symbiotic nature of a lichen thallus raises the question of which partner is the most susceptible to excess solar radiation. Björn (27) has noted that there is no UV-B-induced morphological damage in the 2 foilose, tripartite lichens *Nephroma arcticum* and *Peltigera aphthosa*. Sass and Vass (28) found a remarkably high tolerance in the lichens *Cladonia convolute* and *Peltigera aphtosa*. The mycobiont of *Xanthoria elegans* has also been found to have a high resistance to extreme UV radiances (29). Similarly, in our study, the higher percentage of TUNEL positive nuclei and acridine orange stained cells in the photobiont of *P. semipinnata* reveals that the mycobiont layer is more resistant to UV-B stress compared to the phycobiont layer (Figure 2a, Figure 3).

Lichens contain a high proportion of secondary products, some not found in plants, algae and fungi. Many are synthesized by the mycobiont and are often deposited as crystals on the surfaces of hyphae and phycobiont cells (30). Bachereau and Asta (31) found that the screening of UV-B in the field significantly decreased concentrations of colorless acetone-soluble phenolic compounds in the medulla of *Cetraria islandica*, and assumed these components to act as UV-B screens. Whereas some cases of UV damage in parasitic and saprophytic fungi have been documented (32-34), reports on UV damage in lichenized fungi are few. Studies on *Xanthoria elegans* have shown that the mycobiont, including ascospore germination, is highly resistant to UV(29) In addition, the natural growth form of some species, such as the multi-layered thallus of the alga *Prasiola crispa* and the accumulation of dead cells on the thallus surface reported for some lichen species (35), could be a form of protection against UV-B (both in the context of DNA damage and other biochemical processes) as suggested previously for mat-forming cyanobacteria (36). In fact, both hypotheses, a mycobiont-centric UV-B protection and algal cell death as a form of protection against UV-B, have not yet been sufficiently tested for lichens.

In conclusion, this study has revealed that UV-B induces cell death in the lichen *P. semipinnata* with features similar to those of animal apoptosis. A novel finding of this study is that the TUNEL method is

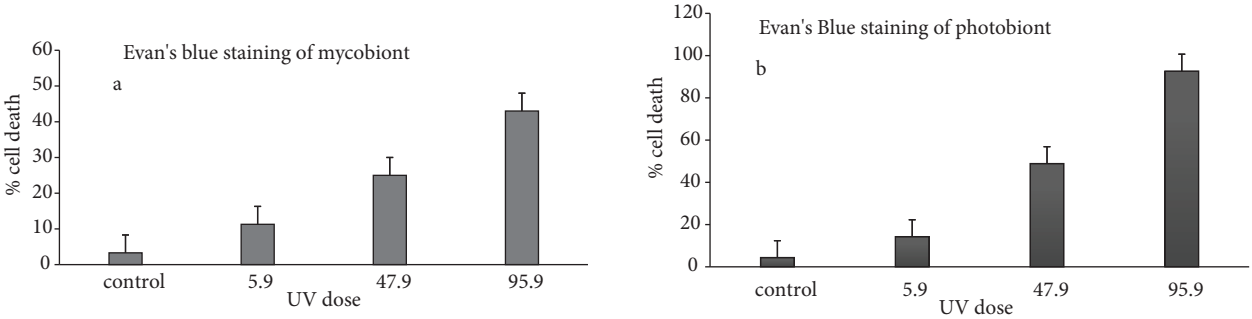

Figure 4. The percentage of cell death in the photobiont of *P. semipinnata* in the control group (non-exposed) and in the groups exposed to UV-B at doses of 5.9, 47.9 and 95.9 J/cm^2 Cell viability is assessed by staining with Evan's blue.

useful in determining the nuclear DNA fragmentation in lichen species. Further studies are required to investigate the apoptotic-like mechanisms under UV-B stress in lichen.

Acknowledgments

We wish to thank Belgin Halıcı for her excellent technical assistance.

***Corresponding author**:*

Dilek ÜNAL

Department of Molecular Biology and Genetic,

Faculty of Science and Letter,

Bilecik University,

Bilecik - TURKEY

E-mail: dilek.unal@bilecik.edu.tr

References

1. Liu SH, Fu BY, Xu HX et al. Cell death in response to osmotic and salt stresses in two rice (*Oryza sativa* L.) ecotypes. Plant Sci 172: 897-902, 2007.
2. Pennell RI, Lamb C. Programmed cell death in plants. Plant Cell 9: 1157-1168, 1997.
3. Shimamoto K, Takahashi A, Henmi K et al. Programmed cell death in plants. Plant Biotechnol J 16:49-53, 1999.
4. Pan JW, Dong AH, Zhu MY. Advances on programmed cell death in higher plants I. Hereditas(Beijing) 22: 189-192, 2000.
5. Pan JW, Zhu MY, Chen H. Aluminum-induced cell death in root-tip cells of barley. Environ Exp Bot 46: 71-79, 2001.
6. Roze LV, Linz JE. Lovastatin triggers an apoptosis-like cell death process in the fungus *Mucor racemosus*. Fungal Genet Biol 25: 119-133, 1998.
7. Ramsdale M. Programmed Cell Death and Apoptosis in Fungi. The Mycota XIII, Fungal Genomics Alistair J.P. Brown (Ed.) Springer-Verlag Berlin Heidelberg, 2006.
8. Solomon M, Belenghi B, Delledone M et al. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11: 431-443, 1999.
9. Yakimova ET, Kapchina-Toteva VM, Laarhoven LJ et al. Involvement of ethylene and lipid signaling in cadmium-induced programmed cell death in tomato suspension cells. Plant Physiol Biochem 44: 581-589, 2006.
10. Danon A, Gallois P. UV-C radiation induces apoptotic-like changes in Arabidopsis thaliana. FEBS Letters 437: 131-136, 1998.
11. Oral BH. George AJT, Haskard DO. Prevention of hydrogen peroxide and cisplatin induced apoptosis by intracellular catalase overexpression. Turk J Biol 24: 685-696, 2000.
12. Danon A, Delorme V, Mailhac N et al. Plant programmed cell death: a common way to die. Plant Physiol Biochem 38: 647-655, 2000.
13. Britt AB. DNA damage and repair in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 75-100, 1996.
14. Hall RSB, Paulson M, Duncan K et al. Water-and temperature-dependence of DNA damage and repair in the fruticose lichen *Cladonia arbuscula* ssp. Mitis exposed to UV-B radiation. Physiol Plantarum 118: 371-379, 2003.
15. Tuteja N, Singh MB, Misra MK et al. Molecular mechanisms of DNA damage and repair: progress in plants. Crit Rev Biochem Mol 36: 337-97, 2001.
16. Baker CJ, Mock NM. An improved method for monitoring cell-death in cell-suspension and leaf disc assays using Evans blue. Plant Cell Tiss Org Culture 39: 7-12, 1994.
17. Kerr JFR, Harmon BV. Definition and incidence of apoptosis: an historical perspective, in: Tomei L.D., Cope F.O. (Eds.), Apoptosis: the Molecular Basis of Cell Death, Cold Spring Harbor Laboratory Press, New York, pp. 5-29, 1991.
18. Gorczyca W, Tuziak T, Kram A et al, Detection of apoptosis DNA strands breaks in fine-needle aspiration biopsies by *in situ* end labelling of fragmented DNA. Cytometry 15: 169-175, 1994.
19. Liljeroth E, Bryngelsson T. DNA fragmentation in cereal roots indicative of programmed root cortical cell death. Physiol Plantarum 111: 365-372, 2001.
20. Wang H, Li J, Bostock RM et al. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell 8: 375-391, 1996.
21. Wolvekamp MCJ, Darby IA, Fuller PJ. Cautionary note on the use of end-labelling DNA fragments for detection of apoptosis. Pathology 30: 267-271, 1998.
22. Liljeroth E, Bryngelsson T. Earlier onset of DNA fragmentation in leaves of wheat compared to barley and rye. Hereditas 136: 108-115, 2002.
23. Moharikar S, D'Souza JS, Kulkarni AB et al. Apoptotic-like cell death pathway is induced in unicellular chlorophyte *Chlamydomonas reinhardtii* (Chlorophyceae) cells following UV irradiation: detection and functional analyses. Journal of Phycology 42: 423-433, 2006.

24. Del Carratore R, Della Croce C, Simili M et al. Cell cycle and morphological alterations as indicative of apoptosis promoted by UV irradiation in *Saccoramyces cerevisiae*. Mutat Res 513: 183-191, 2002.

25. Asai T, Stone JM, Heard JE et al. Fumonisin B1-induced cell death in Arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell 12: 1823-1835, 2000.

26. Solheim B, Zielke M, Bjerke JW et al. Effects of enhanced UV-B radiation on nitrogen fixation in arctic ecosystems. Plant Ecol. 182: 109-118, 2006.

27. Björn LO. Stratospheric ozone, ultraviolet radiation, and cryptogams. Biol Conserv 135: 326-333, 2007.

28. Sass L, Vass I. Characterization of UV-B tolerance in lichens by photosystem II electron transport measurements, in: G. Grab (Ed.), Photosynthesis; Mechanisms and Effects, Kluwer Academic Publishers, Dordrecht, pp.2381-2384, 1998.

29. De Vera P, Horneck G, Rettberg P et al. The potential of the lichen symbiosis to cope with the extreme conditions of outer space, I. Influence of UV radiation and space vacuum on the vitality of lichen symbiosis and germination capacity. Inter J Astrobiol 1: 285-293, 2003.

30. Honegger R. Ultrastructural studies in lichens. II. Mycobiont and photobiont cell wall surface layers and adhering crystalline lichen products in four Parmeliaceae. New Phytol 103: 797-808, 1986.

31. Bachereau F, Asta J. Effects of solar ultraviolet radiation at high altitude on the physiology and the biochemistry of a terricolous lichen (Cetraria islandica (L.) Ach) Symbiosis 23: 197-217, 1997.

32. Rotem J, Aust HJ. The effect of ultraviolet and solar radiation and temperature on survival of fungal propagules. J Phytopathol 133: 76-84, 1991.

33. Fargues J, Rougier M, Goujet R et al. Inactivation of conidia of *Paecilomyces fumosoroseus* by nearultraviolet (UVB and UVA) and visible radiation. J Invertebr Pathol 69: 70-78, 1997

34. Moody SA, Newsham KK, Ayres PG et al. Variation in the responses of litter and phylloplane fungi to UV-B radiation. Mycol Res 103: 1469-1477, 1999.

35. Crittenden PD. Nutrient exchange in an Antarctic macrolichen during summer snowfall snow melt events. New Phytol 139: 697-707, 1998.

36. Margulis L, Walker JCG, Rambler MB. Reassessment of the roles of oxygen and ultraviolet light in precambrian evolution. Nature 264: 620-624, 1976.