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Original Research

Sensitivity Differences and Accumulation of Screening Compounds in Three Conifer Plants under Enhanced UV-B Radiation

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Abstract

Responses of American arborvitae (*Thuja occidentalis*), common yew (*Taxus x media)* and juniper (*Juniperus communis*) to enhanced UV-B irradiance were studied. Control plants were grown without UV treatment, but at identical PhAR and temperature regimes. Visual symptoms of the ultraviolet effect noted on *Thuja* shoots as brown/red discolouration were correlated with the accumulation of phenolic compounds. Flavonoid glycosides identified by the retention times and UV spectra to a small degree increased their intensity. However, the biggest changes referred to the appearance of two free flavonoids with retention times of 47.7 and 48.2, and these compounds belong to effective UV absorbers. No effects on the accumulation of colourless flavonoids, at negligible changes in total phenolics and almost no symptoms during 9-week UV-irradiation in *Taxus* and *Juniperus*, indicate that in these species anatomical features and/or the constitutive phenolics might be responsible for low epidermal transmittance and for ultraviolet protection. Anthocyanins were not found in the examined conifers.

Keywords: UV-radiation, phenolic compounds, American arborvitae, common yew, juniper

Introduction

The reduction of stratospheric ozone, a thin gaseous layer, is leading to increased levels of UV-B radiation. Although its amount in solar radiation is very low, the UV-B wavelengths are highly energetic and even a small increase might modify plant growth [1-3]. Plants have evolved a repair mechanism and avoidance strategy including epidermal screening by the accumulation of phenolic compounds protecting the mesophyll tissues [4-7]. According to Tegelberg and Julkunen-Tiitto [8], flavonoids and phenolic acids were determined as high UV-B screening compounds. The other important strategy is the formation of antioxidants against the generation of oxidative stress [9, 10].

Coniferous trees, important primarily for forestry, belong to a species that is highly adapted to environmental conditions and well protected against enhanced UV radiation [11]. Even the biomass losses observed in greenhouse experiments often do not occur in their natural habitats [12, 13]. Needles of all ages, with their glaucous waxy surfaces and thick epidermal cells, are responsible for this high tolerance. Moreover, UVB-stimulated phenol accumulation in the needles of some conifers [14, 15, 16]. However, it is a more complex issue whether these are sufficient protections in young emerging needles or whether they are effective throughout the year [17].

Many ornamental conifers with varied forms and cultivars used in landscape architecture are grown under dif-

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ferent biotope conditions and at changeable solar radiation and have not been tested for their sensitivity to enhanced UV radiation. Among these, *Taxus*, *Thuja* and *Juniperus* genotypes are very commonly used and show increased shoot decay symptoms, at least in Poland. The causes of this phenomenon are not known. Plants were examined in terms of phytopathological analysis, but they turned out to be infested by pathogens only occasionally. Probably the observed symptoms may be caused by climatic and site factors. They may include, as has been shown for Norway spruce [18], increased UV-B level resulting from the reduction of the stratospheric ozone layer. In our earlier study [19] under elevated UV-B radiation *Thuja occidentalis* was the only genus with a significant (nearly 50%) reduction in monosaccharide accumulation and chlorophyll contents, but the vitality index (Rf_d) was shown to increase when compared to *Taxus* and *Juniperus*.

This study describes the effect of elevated UV-B radiation under experimental conditions on the morphology of *Thuja*, *Taxus* and *Juniperus* leaves and the accumulation of UV-protective compounds (total phenols and flavonoids), potentially responsible for the tolerance and avoidance mechanism.

Experimental Procedures

Plant Material, Growth and Irradiation Conditions

Experiments were conducted using 3- or 4-year-old seedlings of *Taxus x media 'Hicksii'* – common yew, *Thuja occidentalis* – American arborvitae and *Juniperus communis* – juniper. Plants were grown in 12-14 cm pots containing high peat and ground pine bark in a 1:1 (v:v) mixture at pH 4.5-5.5. Experiments were conducted in a growth chamber at day/night 22/18^{0}C temperature, 14/10 h photoperiod and 120 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), using fluorescence Philips 58 W/84 sun lamps. UV was supplied by TL 20W/01 RS Philips lamps with 16 kJ m^{-2} d^{-1} (750 mW m^{-2}) irradiance at the canopy level, and the photon flux density was 3.25 µmol m^{-2} s^{-1} at 315 nm (Fig. 1). The PhAR radiation (400-700 nm) was measured by FF-01 (Sonopan, Poland) quantum sensor and UV-B irradiance with a VLX 3W (Vilber Lourmat, France) radiometer.

Symptom Analysis and Sample Collection

Leaf discoloration and other symptoms were assessed weekly, until the 10th week of UV radiation. For phenolic analyses shoot sections were harvested 3, 6 and 9 weeks after the beginning of the UV-B exposure, and additionally for *Thuja* 1 and 2 weeks of irradiation. In each experiment 4 seedlings growing in individual pots were irradiated and 4 were grown at zero UV-level (control). Shoot sections were collected at successive irradiation dates. For *Taxus* and *Juniperus* the experiments were repeated twice, while for *Thuja* three times. Weighted samples were immediately frozen in liquid nitrogen and stored for several days at -20°C.

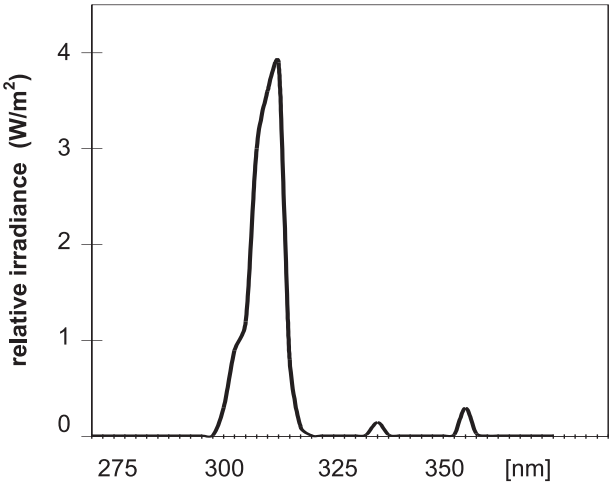

Fig. 1. Light spectrum used for ultraviolet-B irradiance.

Analyses of Phenolic Compounds

For the determination of total phenols, frozen needle samples (100 mg) were homogenized in 3 ml 80% methanol and additionally washed with 2 ml of methanol. After each step of extraction the liquids were centrifuged (30 min, 12,000 g) and the combined supernatants were evaporated in a speed vacuum concentrator (Heto Lab Equipment A/S, Denmark) to give aqueous suspensions. These suspensions were extracted with 2 x 5 ml of ethyl acetate (30 min on a Vortex shaker) and the combined ethyl acetate fractions were evaporated to dryness. Residues were dissolved in 1 ml of 80% methanol and water diluted before analysis. The phenols were determined spectrophotometrically, using Folin-Ciocalteu's reagent (Sigma Chemicals) test [20] and expressed as the coumaric acid equivalent.

Colourless flavonoids were estimated in crude methanol extracts [14] by measuring absorbance at 305 nm using a UV/VIS spectrophotometer (Perkin-Elmer Lambda 11) and expressed per g FW of needles. Moreover, absorbancy of methanol-extractable UV-B absorbing compounds (only for *Thuja* – 9 weeks after radiation) was measured from 280 to 320 nm in steps of 5 nm. Anthocyanins were measured in 0.5 N HCl extracts prepared from 200 mg samples [21].

For HPLC analysis performed according to Stobiecki et al. [22], frozen leaf samples (200 mg) were homogenized in 80% methanol at 4°C (15 ml; together with the washing volume), 3.4-dimethoxybenzoic acid (DMBA) was added as an internal standard. Extraction was performed in an ultrasonic bath for 30 min with a VirTis Model VirSonic 60 sonicator. The suspensions were filtered through a Bucher funnel and concentrated under vacuum at room tem-

perature. Pellets were redissolved in 80% methanol. The amount of injected solution used for quantitative separation was related to tissue fresh weight. Analyses were performed on a Merck Hitachi HPLC pump Model L-7000, equipped with a diode array detector Model L-7450 and a Superspher 100 RP-18 column (250 mm x 2 mm; Merck) at 0.2 µl flow per minute. UV absorbance was recorded at 310 nm. The elution protocol was carried out with two solvent mixtures: A (95% acetonitrile, 4.5% H_2O, 0.5% acetic acid, v/v/v) and B (95% H_2O, 4.5% acetonitrile, 0.5% acetic acid, v/v/v). Elution steps were as follows: 0-5 min isocratic at 10% A, 5-40 min linear gradient from 10 to 30% of A, 40-48 min linear gradient up to 100% of A, 48-60 min isocratic at 100% of A. Free flavonoids and glucosides were registered according to their UV spectra and their retention times using standards commonly used in the analyses of flavonoids and in our phytochemistry laboratory [23].

Statistical Analysis

Experimental data were subjected to a one-way analysis of variance (ANOVA) and significant differences between means were determined using the Tukey multiple range test. Moreover, standard deviations were calculated and shown in the figures.

Results

UV-B exposure caused the most distinctive symptoms in *Thuja* plants (Table 1, Photo 1). After initial chlorotic changes (2-3 weeks after irradiation) leaves assumed brown/red discolouration. In turn, *Juniperus* needles, especially the one-year olds, turned intensive green. Another symptom of UV radiation was rigidity of needles and young shoots; most intensive in *Thuja* and slight in *Taxus*, as well as roughness of *Thuja* scaly leaves.

The concentration of total phenolic compounds in *Taxus* and *Juniperus* control needles was almost twofold higher than in *Thuja* (Fig. 2). As a consequence of UV-B exposure the level of these compounds increased in all the investigated species. In *Thuja* scales due to the appearing discolouration the compounds were analyzed already starting from the first week of irradiation. In this species the increase was similar in the 9-week period of irradiation, i.e. 30-60% above the control plants. Maximum changes found in *Taxus* and *Juniperus* amounted to a 20-30% increase. Apart from total phenols the response of methanol-extractable UV-absorbing compounds measured at 305 nm (colourless flavonoids) was observed only in *Thuja* scales (Fig. 3). In *Taxus* needles the level of flavonoids did not change, whereas in *Juniperus* it decreased after 6–9-week exposure to UV radiation. Anthocyanins were not found in any of the investigated plants.

Extracts from *Thuja* scales were also estimated by absorbance measurements (Fig. 4) and subjected to qualitative chromatography analysis (Fig. 5). Concentrations of compounds ranged consistently from 280 to 320 nm, nearly twofold higher in plants growing for 9 weeks under UV-B. Based on UV absorption spectra registered during LC/UV analysis with a diode array detector (DAD), and

Table 1. Effect of UV-B radiation on the shoot morphology of three coniferous plants.

Genus	Visual symptoms (1 week after radiation)	
	3	starting from 6
Thuja – American arborvitae	chlorotic discolouration	brown/red discolouration, rigidity and roughness of needles
Taxus – common yew	no changes	rigidity of needles
Juniperus – juniper	no changes	intensively green

control

UV-B treated

Photo 1. Effect of UV-B radiation on *T. occidentalis* shoots discolouration – 9 weeks after radiation.

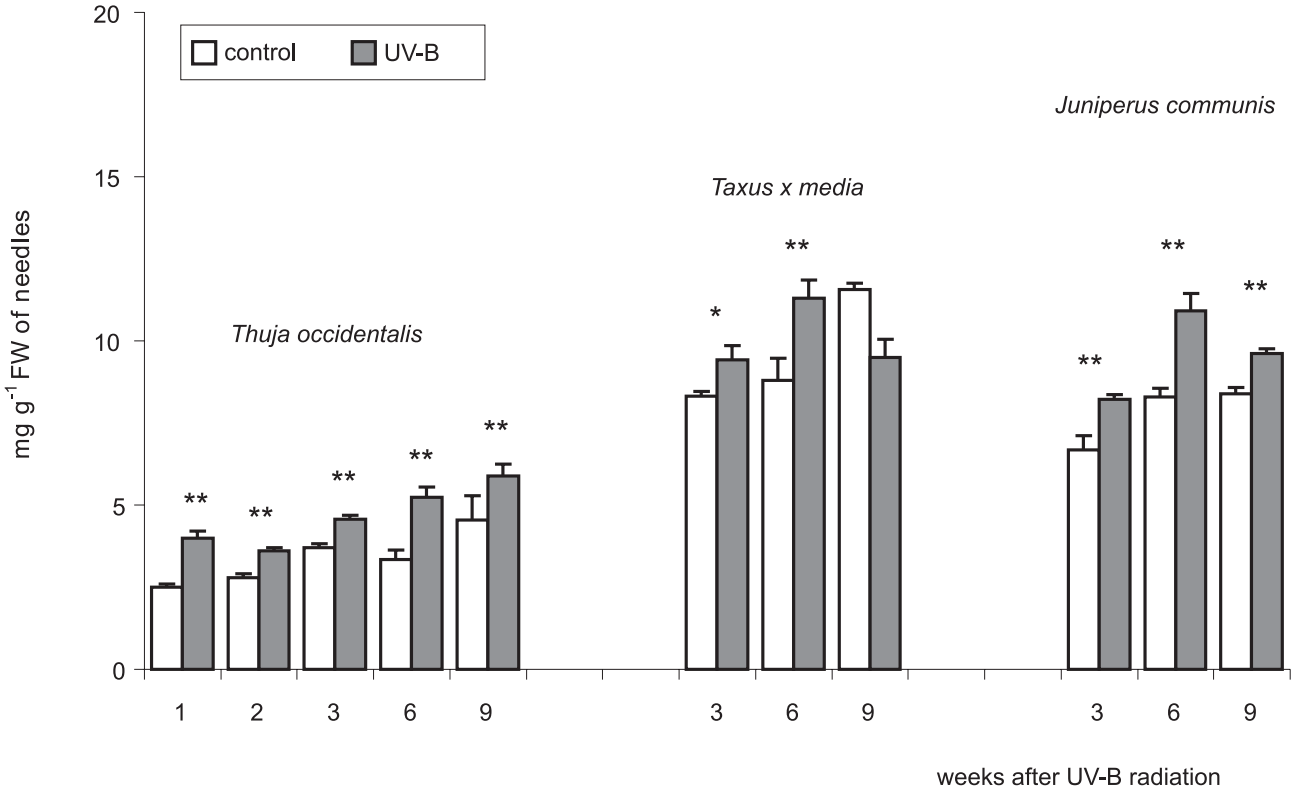

Fig. 2. Effects of UV-B radiation on accumulation of phenolic compounds in *T. occidentalis T. x media*, and *J. communis* needles. Mean ± SD. Data significantly different from control: *P< 0.05, ** P< 0.01.

retention times, flavonoid conjugates were separated as glycosides, within the range of retention time 25-45 min, and free unbounded aglycones with retention times of 47.7 and 48.2. It was possible to determine only relative quantitative changes caused by radiation stress, so UV-B induced a slight accumulation of 32.8 and 37.8 glycosides, while two free flavonoids (47.7 and 48.2 min) increased considerably. The content of a non-flavonoid substance, corresponding to retention time of 49.6 min, decreased.

Discussion

The response of *Thuja* genus to enhanced UV-B radiation, as compared to *Taxus* and *Juniperus,* involved some visual symptoms and accumulation of screening compounds: total phenols and flavonoids. These compounds and products of their oxidation probably were responsible for the colour changes noted in *Thuja* scaly leaves. Phenols were extracted using ethyl acetate, so involving the phenolic acids and condensed tannins, which – next to flavonoids – are important as UV-screening substances. In contrast, shortly after irradiation (2-3 weeks) the observed chlorotic changes resulted from a lowered chlorophyll level, which among other things led to a decreased accumulation of monosaccharides [19].

An interesting phenomenon observed in *Thuja*, and partly also in *Taxus*, was UV-B-induced stem and leaf rigidity, while in *Thuja* it was also roughness of their scaly leaves. Although one of the reasons could have been growth inhibition, such effects were not found, while the effect of UV on dry matter reduction also was not found [unpublished data].

Taking into consideration the common opinion on the relatively high tolerance of coniferous plants to UV radiation [11], in the conducted experiments a relatively high radiation dose was applied in relation to PhAR (1:40) and in relation to that found in nature. In spite of this fact, apart from discolouration and changes in rigidity and roughness of scales, such high doses did not cause injury. However all studied species are long-living and in nature their needles may last several growing seasons. Thus, also the effects of UV-B may accumulate and turn up after a longer period.

In *Taxus* and *Juniperus*, due to the structure and arrangement of needles, the limited amount of UV-B might penetrate into the mesophyll cells. Needles of these species are covered by a waxy layer, and additionally in juniper they are narrow, overlapping and arranged at a small light incidence angle. Moreover, the constitutive phenol level was higher than in *Thuja*. Thus, we consider that for this reason the changes in contents of UV-absorbing compounds were slight.

Among forest conifers the highest susceptibility was found for the *Pinus*, although in most studies enhanced UV radiation under field conditions did not reduce the productivity of those trees [24]; however, in perennials,

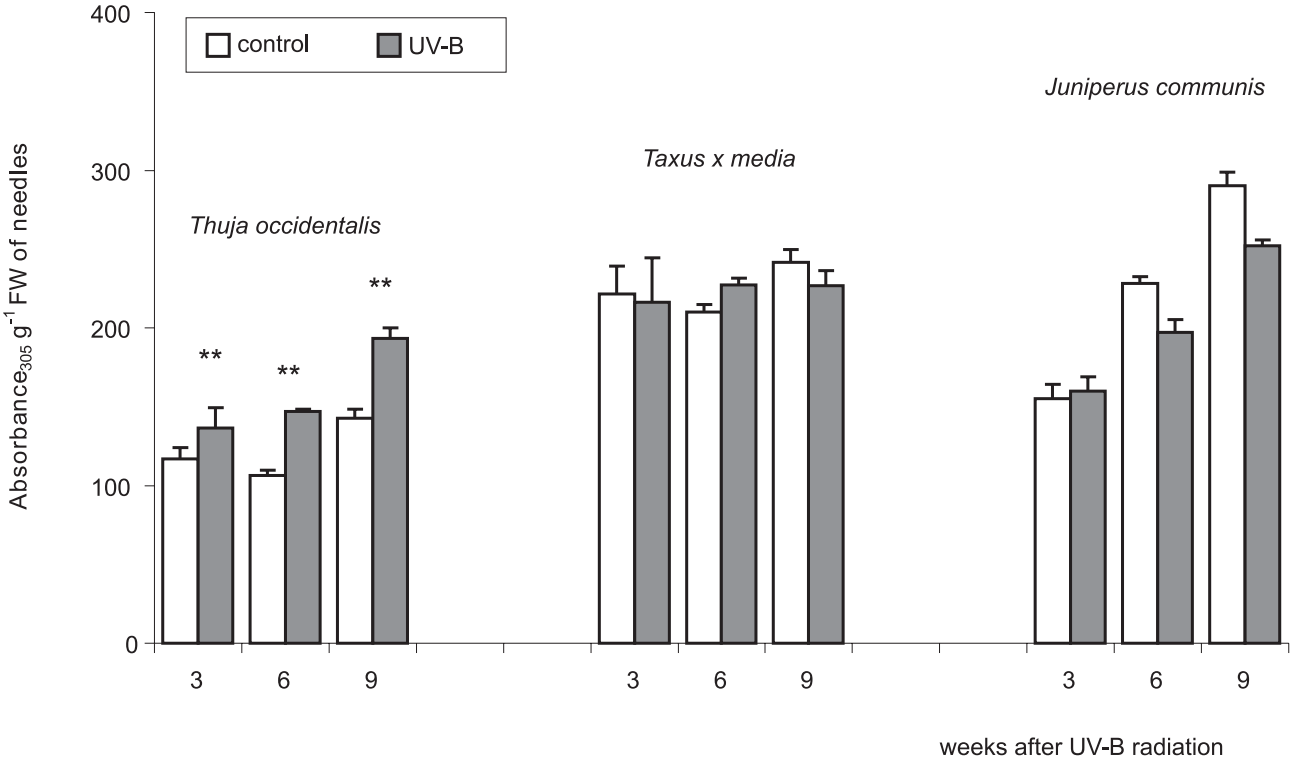

Fig. 3. Effects of UV-B radiation on accumulation of flavonoids in *T. occidentalis, T. x media*, and *J. communis* needles. Mean ± SD. Data significantly different from control: *P< 0.05, ** P< 0.01.

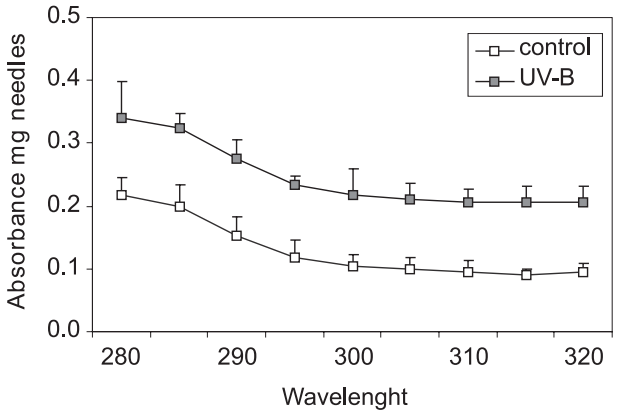

Fig. 4. Absorption spectra of UV-screening compounds of methanolic extracts of T. occidentalis scaly leaves. Mean ± SD.

especially in evergreen species the UV-B effect may be cumulative. In most investigated coniferous species the accumulation of UV-absorbing compounds was reported along with a reduction of needle area, especially in seedlings up to 3 years old [12, 15, 25].

According to a data review by Ryan and Hunt [26], the relative proportion of different flavonoids and other phenolic compounds may be more important in the protection of the plant against UV-B than their absolute concentration. Thus, apart from quantitative measurement the methanol extracts of *Thuja* needles were subjected to quality analysis. Based on the retention times detected during LC/UV analysis it was determined that two peaks corresponding to flavonoid glycosides slightly increased their intensity. However, the biggest changes referred to the appearance of free, non-glycoside flavonoids and these compounds need to be sufficiently effective UV absorbers. Probably their accumulation might be connected with the decrease of peak with retention time of 49.6, or this effect was a consequence of induction of phenylpropanoid synthesis. As an example, in a deciduous *Salix myrsinifolia* tree, the accumulation of flavonoids and phenolic acids during UV-B exposure was accompanied by a decrease in salicylate contents [8].

In most plant species almost 70% of the UV-B radiation reaching the leaf surface is attenuated before it reaches the inner tissues. The methanol-soluble UV-absorbing substances were found in the epidermis as well as in mesophyll tissues, suggesting the protective function for the underlying cells. Hoque and Remus [4] revealed by fluorescence spectroscopy and confocal laser scanning microscopy in Norway spruce needles that UV-screening mechanisms was highly complex, though connected with methanol-soluble substances of the epidermis. The UV-absorbing flavonoids and their derivatives were localized in the cell walls of the outer epidermal cell layer; moreover, a conversion of UV light into PhAR takes place in that tissue. Flavonoids exhibit free-radical scavenging activity, which might offer an additional protection to cells accumulating these compounds [27]. There is also unambiguous evidence indicating their role in the protection against excessive UV-B radiation. *Arabidopsis* mutants lacking

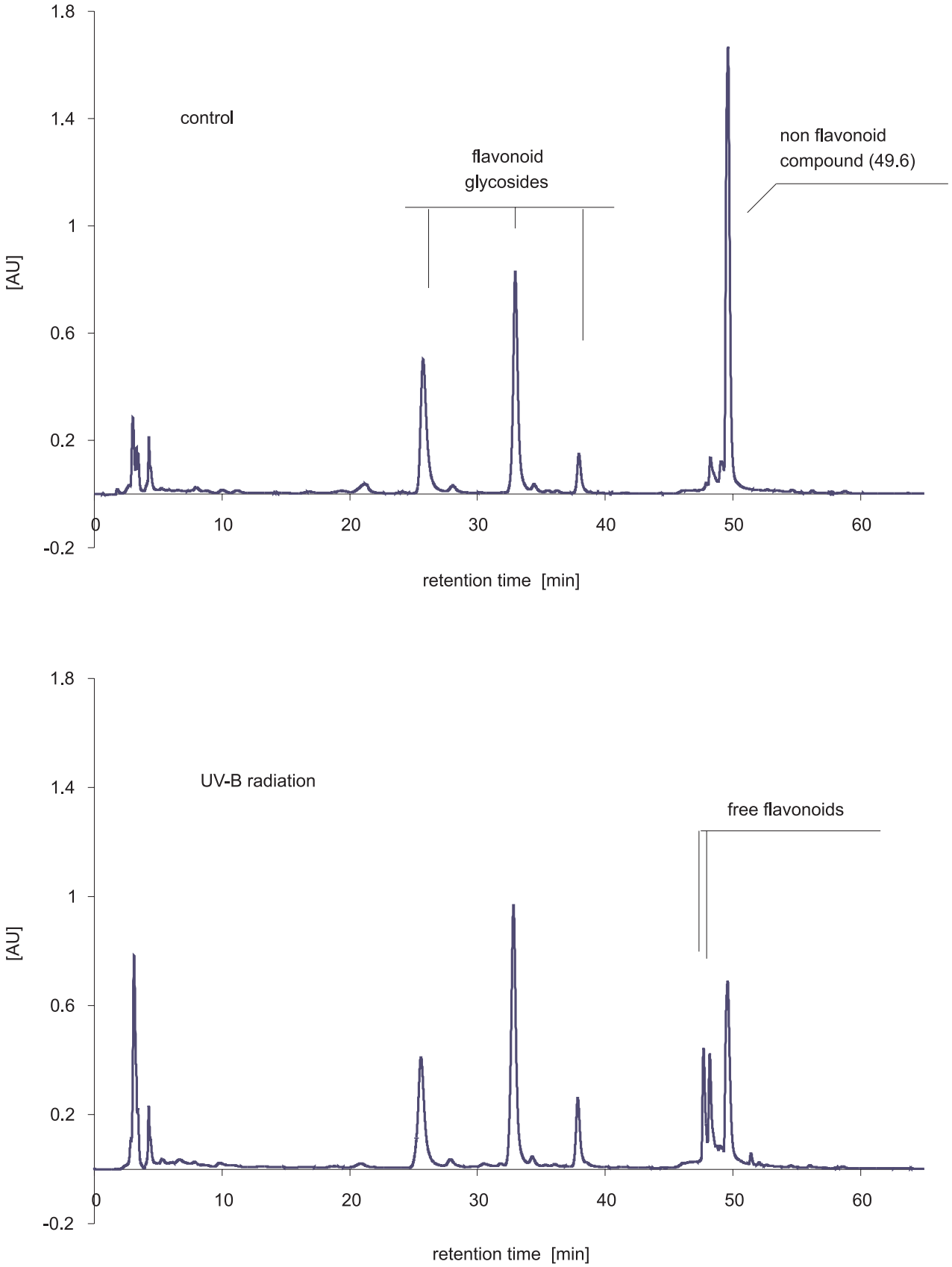

Fig. 5. HPLC chromatograms (305 nm) of methanolic extracts of *T. occidentalis* scaly leaves.

flavonoids exhibit enhanced UV injury and oxidative damages [28, 29].

It can be concluded that the studied coniferous species belong to UV-tolerant trees. Under the elevated or even high UV-B irradiation, exceeding the level on the Earth's surface (between 2 and 12 kJ m^{-2} per day) [30] no injury was recorded. Besides anatomical and morphological adaptation typical for conifers, an increase in total phenols was noted. A low constitutive level of screening compounds of *Thuja* scaly leaves was intensified by the accumulation of flavonoids – effective UV absorbers. The effect of UV radiation on brown/red discolouration of *Thuja* leaves remains uncertain, though we suggest the oxidation of phenolic compounds. It also seems rather unlikely for the dying out of conifers observed in ecosystems or in garden and park plantings to be caused by excessive ultraviolet radiation.

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