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UV-B induced changes of phenol composition and antioxidant activity in black currant fruit (*Ribes nigrum* L.)*

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Summary

Information on UV-B elicitor mediated changes on phenolic composition and antioxidant activity of black currant (*Ribes nigrum* L.) are scanty. In the present study physiological ripe black currant fruits were harvested and exposed to UV-B radiation with different exposure and adaptation times. The influence of UV-B on phenolic profile and quantitative composition as well as on the corresponding antioxidant activity was investigated. Antioxidant activity was screened with electron spin resonance spectrometry (ESR), while phenolic compound composition was conducted by HPLC analysis.

Total phenol content and phenolic composition (flavonols, anthocyanins, hydroxycinnamic and hydroxybenzoic acids) increased to a large extent during UV-B treatment, irrespective of the adaptation time. Anthocyanins are concluded to absorb UV radiation within a short time, meanwhile flavonols and phenolic acids are assumed to have an impact on antioxidant protection of UV-B mediated tissue damage. Moreover, antioxidant activity significantly correlated with different phenolic compounds and increased to a similar extent by UV-B exposure.

Introduction

Polyphenols belong to the secondary plant metabolites and are present in a wide range of fruits and vegetables. Based on the chemical structure phenolic compounds are marked by a broad spectrum of health-promoting functions such as antioxidant, antimutagenic and anticarcinogenic properties (HALLIWELL, 1996). There is clear evidence for an association between the free-radical binding character of phenols and the prevention of degenerative diseases, e.g. cancer and cardiovascular diseases (WORLD CANCER RESEARCH FUND, 1997; BAZZANO et al., 2002). Berry fruits such as black currant (*Ribes nigrum* L.) are a rich source of phenolic compounds (KÄHKÖNEN et al., 2001; BENVENUTI et al., 2004). The antioxidant activity of black currant fruits is based on the high content of flavonols (myricetin and quercetin derivatives) (ANTTONEN and KARJALAINEN, 2006) and anthocyanins (delphinidin and cyanidin derivatives) (SLIMESTAD and SOLHEIM, 2002). Moreover, hydroxycinnamic acids (caffeic acid and coumaric acid) and hydroxybenzoic acid (gallic acid and hydrobenzoic acid) also contribute to the antioxidant activity, but to a smaller extent (HÄKKINEN et al., 1999; KÄHKÖNEN et al., 2001; MÄTTÄÄ et al., 2003).

Up to now, research on antioxidant phenolic plant compounds in black currant was mainly focused on the effect of cultivars, seasonal timing and cultivation practices (KÄHKÖNEN et al., 2001; MIKKONEN et al., 2001; MOYER et al., 2002; ANTONNEN and KARJALAINEN, 2006; TABART et al., 2006). However, information on changes in phenolic composition and antioxidant activity in black currant as affected by ultraviolet-B (UV-B) radiation is scanty.

In recent years, increased UV-B radiation (280-315 nm) by air pollution-induced ozone depletion has raised awareness of the effects of UV-B on the ecosystem. Though only a small portion of the total

solar spectrum, UV-B has a large photobiological effect (KERR et al., 2003). There are many reports on potential consequences of an increase in UV-B radiation on plants, however, a rather limited understanding of the effect on secondary plant metabolites is present. The increasing exposure of plants to UV-B causes protective mechanisms in plant response to this anthropogenic source of stress. Additionally to morphological changes the protective stress response triggers distinct changes in the plant's secondary metabolism resulting in an accumulation of various phytochemicals (e.g. phenolic compounds) and their antioxidative activity (SCHREINER and HUYSKENS-KEIL, 2003; EICHHOLZ et al., 2005; HUYSKENS-KEIL et al., 2006; SCHREINER and HUYSKENS-KEIL, 2006). Anthocyanins and flavonoids have various physiological functions (i.e. antioxidative and antitumor activity) and reveal UV protective effects (HIGASHIO et al., 2001; INDORF, 2001; KOLB et al., 2001; HIGASHIO, 2005). In general, the synthesis of phenolic compounds can also be stimulated by UV exposure in postharvest as it was reported for example for quercetin in onion and strawberry, flavonoids in brokkoli and black currant (HIGASHIO et al., 2005; COSTA et al., 2006; HUYSKENS-KEIL et al., 2006). However, UV stress mediated changes in phenol profile and antioxidant activity are dependent on the duration of stress, adaptation time of plants to UV stress and physiological status of the plant at the time of UV exposure.

This study contributes to an assessment of a short-term UV-B stress induced impact on the dynamics of secondary plant metabolites in postharvest, exemplarily demonstrated on phenolic composition and antioxidant activity of black currant. Furthermore, the effect of targeted UV application in postharvest for increasing health-promoting phytochemical compounds and thus, nutritional value of fruits and vegetable products is focused.

Material and methods

Plant material and Experimental setup

Three years old plants of *Ribes nigrum* L. cv 'Titania' (n=120) were cultivated in 10 L containers with Compo Sana® rose substrate, (COMPO GmbH & Co. KG, Münster) and Manna Lin M fertilizer (Wilhelm Haug GmbH & Co. KG, Ammerbuch, Germany). Fruits were harvested at a fully developed ripening stage (according to the maturity index as described below) in July 2006. Special attention was paid on picking berries of the appropriate maturity stage according to fruit firmness and maturity index (sugar-acid-ratio) considering sample collection from all sections of the bush.

After picking, one part of fruit samples (250 g PE-trays) was subjected to UV-B radiation for 60, 90 or 120 min using an UV-B fluorescence light source (FL 20SE, 305-310 nm) with an average fluency rate of 8.2 Ws m⁻² at a distance of 30 cm to fruits. After an adaptation time of 2 or 22 h fruits were shock-frozen in liquid nitrogen and kept at -25 °C before freeze drying (Christ Alpha 1-4, Christ; Osterode, Germany). Dried samples were grinded for subsequent extraction and analysis of total phenolic content and phenolic composition, i.e. flavonoid subclasses (flavonols, anthocyanins, hydroxycinnamic and hydroxybenzoic acids). The experiment was conducted with three

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replicates per UV-B treatment and adaptation time. Non-treated fruits (250 g with three repetitions) were used as control.

Maturity index

Soluble solids of 150 g fruit per treatment with three replications were determined using a hand refractometer (Leo Kübler GmbH, Germany). For the determination of titratable acidity fruits were homogenized (Ultra-Turrax T25, Ika-Labortechnik, Staufen, Germany). Thereafter, 2.5 grams of the homogenate were dissolved with 2.5 mL of distilled water. Titratable acidity was determined by titration with 0.1 N NaOH until pH 8.1 and expressed as percent total organic acid on basis of citric acid being the main acid component in black currant fruits.

Phenolic compounds

Analysis of phenolic compounds

For the analysis of phenolic compounds, extraction was conducted following the method described by KÄHKÖNEN et al. (2001) and CONNOR et al. (2002) using acidified methanol (0.1% hydrochloric acid). An aliquot of 0.5 g grinded sample was mixed vigorously with 3 mL of acidified methanol (0.1% hydrochloric acid) and centrifuged for 15 minutes at 3000 rpm. The supernatant was collected in a 10 mL volumetric flask. The residue was treated again twice with 3 mL acidified methanol and 15 minutes centrifugation. Supernatants were collected and standardised to a final volume of 10 mL. The extracts obtained were used for the determination of the phenol content, composition, and antioxidant activity.

Determination of the total phenol content

Total phenol content in the fruit extracts were determined using the Folin-Ciocalteu method with results expressed as milligrams gallic acid equivalents (GAE) per gram of dry matter. Absorbance was measured at 765 nm (LKB-Novaspek II, Pharmacia, Freiburg, Germany). Appropriate dilutions of extracts were prepared by duplicate with distilled water (1+29; v,v). The mixture develops a dark blue color and was left to react for one hour. Duplicate lecture of samples were measured spectrophotometrically at 765 nm.

Analysis of the phenolic compounds using HPLC-DAD

In the present study, the concentrations of two major flavonoid subclasses, flavonols and anthocyanins, and two phenolic acid groups, hydroxycinnamic and hydroxybenzoic acids, were determined by HPLC-DAD. An analytical Hewlett Packard 1100 series HPLC instrument equipped with an autosampler, quaternary HPLC pump and diode array detector was used. Analytical separation of the phenolic compounds was carried out on a 250 mm x 4.6 mm, 5 µm, Fluofix 120E column (Wako Pure Chemical Industries, Osaka, Japan) with a two solvent mobile phase (eluent A = water/acetic acid/acetonitrile (94.5 + 0.5 + 5; v,v,v); eluent B = acetic acid/acetonitrile (5 + 95; v,v). The eluent gradient used for all extracts is described in Tab. 1.

The identification of phenolic compounds on the basis of the compound group's characteristic absorption wavelength was conducted according to KÄHKÖNEN et al. (2001). Two milliliter aliquots of the extract (50 mg DM mL⁻¹) were concentrated by drying with nitrogen and then re-dissolved to a volume of 500 µL with HPLC grade methanol. The injection volume was 20 µL, and the flow rate 1 mL min⁻¹. Contents of anthocyanin (detection wavelength 520 nm), flavonol (365 nm), and hydroxycinnamic acid (320 nm) were quantified as cyanidin-3-glucoside, rutin, and chlorogenic acid equivalents, respectively. Total concentration of each representative subclass was calculated from the

Tab. 1: Analysis of phenolic compounds using HPLC-DAD gradient

Time (min)	Eluent B (%)
0-4	0-4
4-5	2-4
5-10	2-4
10-25	4-8
25-40	8-22
40-45	22-28
45-50	28
50-55	28-45
55-60	45
60-61	45

concentration obtained from a 1 mM standard solution of the compounds mentioned above. Results were expressed as milligrams per gram dry matter (DM).

Determination of the antioxidant activity by Electron Spin Resonance Spectrometry (ESR)

The degradation of a stable synthetic radical, Fremy's salt (potassium nitrosodisulfonate), in presence of antioxidants in the black currant extracts was monitored with ESR as applied by RÖSCH et al. (2003). Appropriate extract dilutions (1+29; v,v) were prepared and 100 µL aliquots were allowed to react for 20 min with an equal volume of a solution of Fremy's salt (1 mM in phosphate buffer, pH 7.4). ESR spectra of Fremy's radical were obtained with a Miniscope MS100 spectrometer (Magnettech GmbH, Berlin, Germany). The antioxidant activity expressed as mM Fremy's salt reduced by 1 g dried sample (mM per g DM), was calculated by comparison with a control reaction with 100 µL Fremy's salt 1 mM and 100 µL phosphate buffer.

Statistical analysis

All data were statistically analysed (ANOVA) with SPSS 13.0 (SPSS Inc., USA). Significant differences were determined by Tuckey's test ($p < 0.05$). The mean variability was indicated by the standard deviation.

Results and discussion

Total phenol content in the ripe black currants studied were found to be slightly lower in comparison to the contents reported in the literature, revealing 14-18 mg/g DW and 22-27 mg/g DW, respectively (KÄHKÖNEN et al., 2001). This might be due to genetic differences of the plant material, differences in the physiological ripening stage of the fruit at the time of harvest and/or environmental conditions during plant growth.

The biosynthesis of phenolic compounds was affected by UV-B radiation in postharvest (Fig. 1). UV-B treatments resulted in a continuous increase of phenols showing a peak at 90 minutes. However, adaptation times did not significantly affect total phenol content. The rapid physiological response of phenols to UV radiation in postharvest is also reported for other horticultural products, e.g. *Brassica* spp., *Vaccinium* spp. (HUYSKENS-KEIL et al., 2006). It is assumed that an UV stress mediated increase of phenylalanin ammonia-lyase activity occurs leading to an acceleration of the biosynthesis of phenolic compounds (PLUSKOTA et al., 2005).

Anthocyanin synthesis in black currant was promoted by increasing exposure time of UV-B (Fig. 2). The highest increase of anthocyanin (60%) was found at 120 min UV treatment, irrespectively of the adap-

tation time. Similar results were reported for strawberries and peaches, where UV mediated increase of anthocyanins was associated with an increase in phenylalanin ammonia-lyase activity (KATAOKA and BEPPU, 2004; HIGASHIO et al., 2005).

In addition to phenylalanin ammonia-lyase induction, UV radiation increases also the activity of other enzymes involved in flavonoid synthesis: chalcon synthase, chalcon isomerase, and dihydroflavonol-4-reductase (TOMAS-BARBERAN and ESPIN, 2001). In the present study also flavonols were affected by the UV-B treatment (Fig. 3). With increasing UV-B exposure periods flavonol synthesis was activated

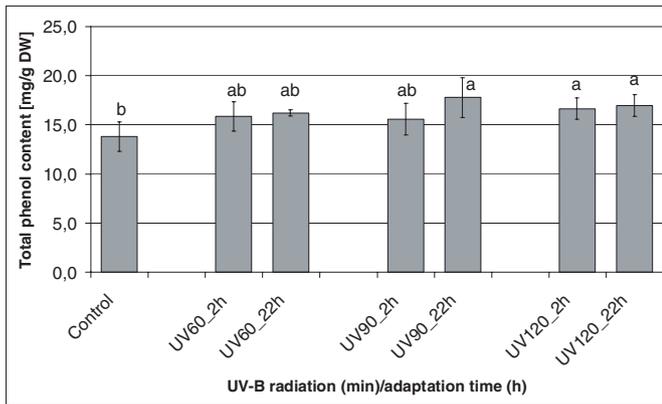


Fig. 1: UV-B induced changes of total phenol content in black currant fruits. Vertical bars indicate significant differences $P < 0.05$ (Tukey Test).

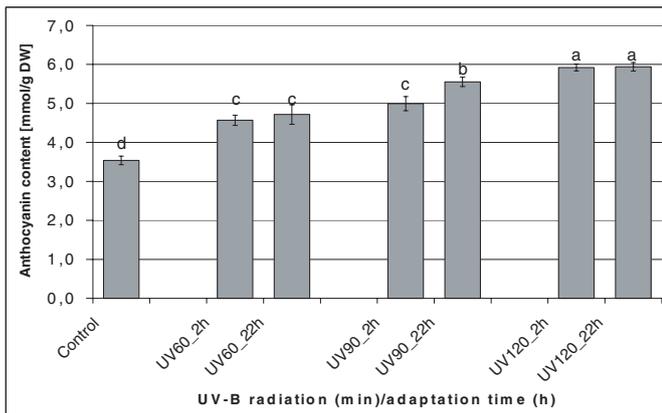


Fig. 2: UV-B induced changes of anthocyanin content in black currant fruits. Vertical bars indicate significant differences $P < 0.05$ (Tukey Test).

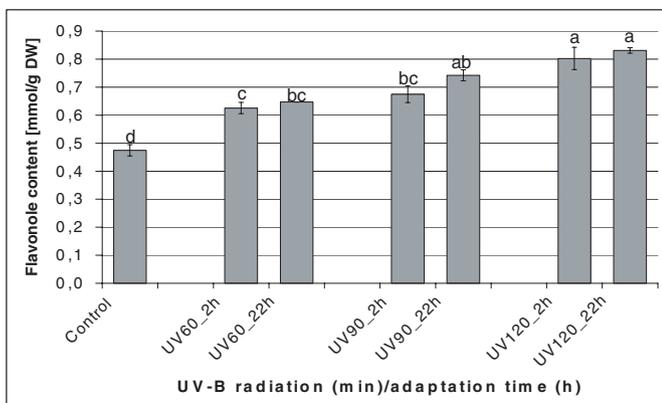


Fig. 3: UV-B induced changes of flavonol content in black currant fruits. Vertical bars indicate significant differences $P < 0.05$ (Tukey Test).

revealing a drastic increase of 76% after 120 minutes. The results demonstrate that flavonols showed a similar response to UV in comparison to anthocyanins.

Hydroxycinnamic and hydroxybenzoic acids are biochemical precursors of flavonols and anthocyanins in the shikimate pathway. Therefore, both precursors showed an early reaction to UV exposure: hydroxycinnamic acid increased by 98% after 60 min UV-B exposure, whereas the dominating hydroxybenzoic acid only increased by 35% (Fig. 4). Correlation between total phenol content and phenolic compounds revealed a strong correlation with hydroxybenzoic acids ($r=0.71, p<0.01$), anthocyanins ($r=0.75, p<0.01$) and flavonols ($r=0.66, p<0.01$). No correlation was found between total phenol content and concentration of hydroxycinnamic acid.

Antioxidant activity in harvested black currant fruits was significantly induced (21%) by UV-B radiation, however only after 120 min UV exposure with an adaptation time of 22 h (Fig. 5). Tendentially, antioxidant activity accelerated with increasing adaptation times revealing a longer biochemical reaction time to UV mediated stress.

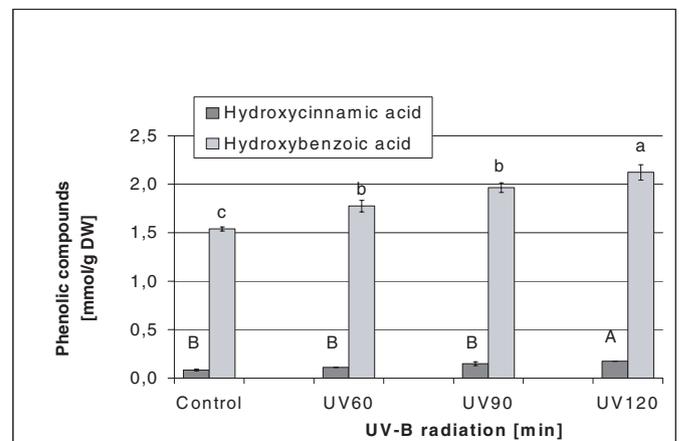


Fig. 4: UV-B induced changes of hydroxycinnamic and hydroxybenzoic acids in black currant fruit. Vertical bars indicate significant differences $P < 0.05$ (Tukey Test).

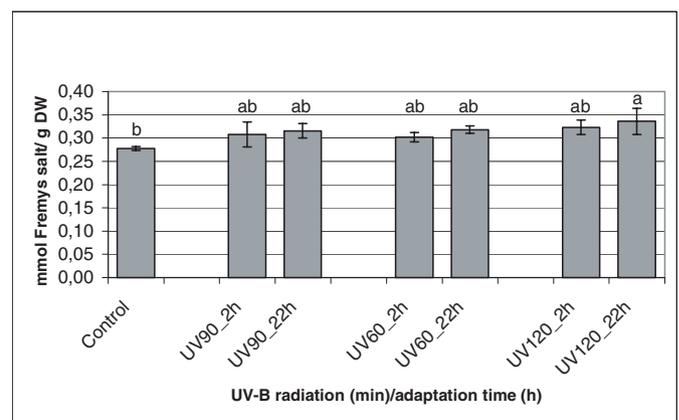


Fig. 5: UV-B induced changes of antioxidant activity in black currant fruit. Vertical bars indicate significant differences $P < 0.05$ (Tukey Test).

A strong correlation between antioxidant activity (ESR) and the different phenolic compounds were found: total phenols ($r=0.63, p<0.01$), anthocyanins ($r=0.76, p<0.01$); hydroxybenzoic acids ($r=0.71, p<0.01$) and flavonols ($r=0.70, p<0.01$). No correlation was found between ESR and hydroxycinnamic acid, presumably due to their low contribution and thus low antioxidant activity. RICE-EVANS et al. (1995) also

reported a low antioxidant activity of caffeic acid in comparison to the high antioxidant activity of anthocyanins (delphinidin and cyanidin), flavonols (quercetin and myricetin) and hydroxybenzoic acids (gallic acid) in black currant.

In conclusion, the results demonstrate that the synthesis of phenolic compounds and associated antioxidant activity of black currant fruits is stimulated by a short term UV-B radiation in postharvest. Anthocyanins are concluded to be able to absorb UV radiation within a short time and thus have a UV-B protective effect in black currant as it is also known for other fruits (JAAKOLA et al., 2004; YANG et al., 2005; HAGEN et al., 2007). Flavonoids have various physiological functions such as antioxidant or antitumor activity as well as UV protective effects (HIGASHIO et al., 2001; KOLB et al., 2001; HIGASHIO, 2005). In the present study, flavonols and phenolic acids are also assumed to have an impact on the antioxidant protection of UV-B mediated tissue damage in black currant. Further studies on the dynamic of UV mediated changes in secondary plant compounds and antioxidant activity of black currant will be conducted.

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