

(±)-Catechin: Chemical Weapon, Antioxidant, or Stress Regulator?

Vladimir Chobot · Christoph Huber ·
Guenter Trettenhahn · Franz Hadacek

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Abstract (±)-Catechin is a flavan-3-ol that occurs in the organs of many plant species, especially fruits. Health-beneficial effects have been studied extensively, and notable toxic effects have not been found. In contrast, (±)-catechin has been implicated as a ‘chemical weapon’ that is exuded by the roots of *Centaurea stoebe*, an invasive knapweed of northern America. Recently, this hypothesis has been rejected based on (±)-catechin’s low phytotoxicity, instability at pH levels higher than 5, and poor recovery from soil. In the current study, (±)-catechin did not inhibit the development of white and black mustard to an extent that was comparable to the highly phytotoxic juglone, a naphthoquinone that is allegedly responsible for the allelopathy of the walnut tree. At high stress levels, caused by sub-lethal methanol concentrations in the medium, and a 12 h photoperiod, (±)-catechin even attenuated growth retardation. A similar effect was observed when (±)-catechin was assayed for brine shrimp mortality. Higher concentrations reduced the mortality caused by toxic concentrations of methanol. Further, when (±)-catechin was tested in variants of the deoxyribose degradation assay, it was an efficient scavenger of reactive oxygen species (ROS) when they were present in higher concentrations. This antioxidant effect was enhanced when iron was chelated directly by (±)-catechin. Conversely, if iron was

chelated to EDTA, pro-oxidative effects were demonstrated at higher concentrations; in this case (±)-catechin reduced molecular oxygen and iron to reagents required by the Fenton reaction to produce hydroxyl radicals. A comparison of cyclic voltammograms of (±)-catechin with the phytotoxic naphthoquinone juglone indicated similar redox-cycling properties for both compounds although juglone required lower electrochemical potentials to enter redox reactions. In buffer solutions, (±)-catechin remained stable at pH 3.6 (vacuole) and decomposed at pH 7.4 (cytoplasm) after 24 h. The results support the recent rejection of the hypothesis that (±)-catechin may serve as a ‘chemical weapon’ for invasive plants. Instead, accumulation and exudation of (±)-catechin may help plants survive periods of stress.

Keywords Flavan-3-ol · Deoxyribose degradation · Seedling growth · Brine shrimps · Cyclic voltammetry · Stress induced morphogenic response · Stability in aqueous solutions

Introduction

Catechins are flavan-3-ols that occur widely in the plant kingdom. They are found in many fruits and vegetables, as well as beverages such as tea, and thus they are the focus of many nutritional studies (Scalbert and Williamson 2000). For instance, cocoa, which is obtained from the seeds of *Theobroma cacao*, is a major ingredient of chocolate, and is the object of many studies that explore potential health-beneficial activities of the catechins (Wollgast and Anklam 2000). Among those benefits, anti-inflammatory activity, platelet anti-aggregation, cardiovascular, cancer, and neural protection are well documented, whereas only speculation

V. Chobot · F. Hadacek (✉)
Department of Chemical Ecology and Ecosystem Research,
Faculty of Life Sciences, University of Vienna,
1090 Vienna, Austria
e-mail: franz.hadacek@univie.ac.at

C. Huber · G. Trettenhahn
Department of Physical Chemistry, Faculty of Chemistry,
University of Vienna,
1090 Vienna, Austria

exists about toxic effects (Lamuella-Raventos et al. 2005). In general, the dietary intake of flavonoids traditionally has been regarded as beneficial due to their antioxidant properties; however, supporting data predominantly has come from *in vitro* studies that applied unrealistic high concentrations. Recently, the view has changed, and flavonoids now are less assumed to act as direct antioxidants but rather as inhibitors of pro-oxidant enzymes, such as NADPH oxidases and lipoxygenases, or as chelators of transition metals that mask pro-oxidant actions of reactive nitrogen (NOS) and oxygen species (ROS) (Schewe et al. 2008).

In sharp contrast to the above, (–)-catechin, one of the four isomers of the catechin epimers with *o*-hydroxyl groups on ring B, has been implicated as a chemical weapon of spotted knapweed, *Centaurea maculosa* Lam., which is now classified as a subspecies of *C. stoebe* L., *C. stoebe* ssp. *micranthos* (Gugler) Hayek (Ochsmann 2002). This knapweed species is regarded as an invasive plant in western United States (Callaway et al. 1999; Weir et al. 2003). *Centaurea stoebe* is well known in Europe, as it is one of the more wide-spread knapweed species and is especially common in abandoned dry grasslands (Dostalek and Frantik 2008). Studies that were carried out by Bais and colleagues (Bais et al. 2003; Weir et al. 2003) claimed to provide evidence that (–)-catechin has phytotoxic properties strong enough to support potential contributions of this compound to the invasive success of *C. stoebe* in the western United States. Based on this and similar studies that involved other invasive knapweed species, the novel weapons hypothesis was developed. It states that metabolites of introduced plant species may confer competitive advantages against non-adapted native species (Callaway and Ridenour 2004). The debate concerning the efficacy and ecological relevance of the epimeric mixture of (±)-catechin that is exuded by the roots of *C. stoebe* is ongoing. Besides growth inhibitory effects against some plant species at high concentrations, (±)-catechin also may act as a growth regulator and inducer of systemic resistance at lower concentrations (Weir et al. 2003; Prithiviraj et al. 2007).

We know that diverse abiotic and biotic stresses disturb the redox equilibrium in cells of affected organisms; the thus generated ROS may trigger signal cascades that activate various genes involved in specific response reactions (Wojtaszek 1997; Foyer and Noctor 2005; Gechev et al. 2006; Noctor et al. 2007; Miller et al. 2008). However, if the cell fails to control the levels of ROS, oxidative damage of DNA is inevitable (Halliwell 2006). Iron homeostasis also is vital for the cell. This transition metal is an essential nutrient of plants, yet it often limits growth and, conversely, over-accumulation leads to oxidative stress (Walker and Connolly 2008). In cells, iron is bound to proteins, ferritins, and usually is set free by

reductive processes (Laulhere and Briat 1993). Reduced iron can react with H₂O₂ to form hydroxyl radicals (·OH), which have enormous potential to damage various organic molecules (Halliwell 2006).

Bais et al. (2003) explored the pro-oxidative effects of (–)-catechin by applying solutions that contained 100 μg ml⁻¹ to root tips of *Centaurea stoebe* and *C. maculosa*, the first a known producer of (±)-catechin, and to *Arabidopsis thaliana*, the widely-used model plant in molecular plant physiology. Only the root tips of *C. stoebe* did not show any visible symptoms of induced cell death when ROS production was monitored by imaging generation of dichlorofluorescein (DCF) in the (–)-catechin-treated root tips. Similarly, by using another fluorescent probe, indo-1, the authors showed that levels of Ca²⁺ ions increased in root tips of all tested plants except *C. maculosa* after treatment with a solution of (–)-catechin (100 μg ml⁻¹). Based on this evidence, they proposed that (–)-catechin acts a chemical weapon that contributes to the invasive success of *C. stoebe*. Initially, this study was praised as a fundamental contribution to the respectability of allelopathy (Fitter 2003). Later, however, concerns that the concentrations required for these effects were unrealistic in soil by several orders of magnitude were voiced (Blair et al. 2005, 2006). These concerns recently led to a rejection of the hypothesis that (±)-catechin acts as phytotoxin in allelopathic interactions of *C. stoebe* on the basis of experimental evidence: (1) the concentration of (±)-catechin in soil is too low; (2) (±)-catechin is unstable at pH values above 5, as found in the majority of soils; (3) (±)-catechin is decomposed by extracellular enzymes; and (4) (±)-catechin is only weakly phytotoxic but a strong antioxidant (Blair et al. 2009; Duke et al. 2009a,b). In a series of studies, the originators of the novel weapons hypothesis, concomitantly claimed further support for (±)-catechin as a phytotoxic allelochemical by showing results from experiments that indicated higher susceptibility to (±)-catechin to species in the invaded than in the native range, conditional, however, on the soil type (Inderjit et al. 2008; He et al. 2009; Thorpe et al. 2009).

In this controversy, one further problematic issue has been neglected. Caution needs to be considered when using dichlorofluorescein to detect ROS formation in tissues. The dichlorofluorescein assay is subject to a serious artifact in that it produces what it is purported to measure, i.e., ROS (Bonini et al. 2006). In the cell that possess peroxidase activity, the probe-derived radical may be oxidized, thereby producing further ROS regardless of the initial oxidant (Rota et al. 1999; Bonini et al. 2006). Consequently, the toxic properties of (–)-catechin may have been overestimated by the method applied by Bais et al. (2003). Antioxidative properties of (–)-catechin may contribute to the fact that the producing root tips of *C. stoebe* yield

negative results for ROS production in imaging of DCF-infiltrated tissue. The redox chemistry of (\pm)-catechin is complex: it has *o*-hydroxyl groups on its ring B, which may either scavenge ROS or chelate free iron ions. Recently, we have shown that pro-oxidative effects of the naphthoquinone juglone, a well-known phytotoxic compound alleged to be the allelopathic agent of the walnut tree (Jose 2002), are strongly enhanced if free iron is chelated directly by juglone (Chobot and Hadacek 2009). In this study, we applied variants of the deoxyribose degradation assay (Halliwell et al. 1987) to explore the redox chemistry of juglone in the presence of free and chelated iron ions [chelating agent ethylenediaminetetraacetic acid (EDTA)] as well as at low and high levels of ROS caused by low and sub-lethal dosages of MeOH.

By assuming that (\pm)-catechin interacts with ROS, we designed variants of seedling growth assays that resemble different stress levels. This can be achieved simply by adding the organic solvent MeOH (Chobot and Hadacek 2009). MeOH is oxidized to formaldehyde and formate, and during these reactions, ROS are formed (Dobrzynska et al. 1999). Moreover, in attempts to create more complex stress scenarios, all assays were performed simultaneously both in complete dark and in a light/dark regime. If (\pm)-catechin interacts with signal cascades in the cell, we predicted that similar effects should be visible in a plant as well as in a brine shrimp mortality assay. In a previous study, we demonstrated that nonlinear effects (hormesis) of juglone on seedling growth were due to its excellent capabilities as a redox cyler (Chobot and Hadacek 2009). To explore whether (\pm)-catechin possesses properties similar to juglone, we decided to contrast the electrochemical potentials of both compounds. This can be measured by cyclic voltammetry, at pH levels characteristic for the cytoplasm and the vacuole, 7.4 and 3.6, respectively. Cyclic voltammetry is one of the less expensive electrochemical methods that are available to study redox chemistry in combination with chemical assays (Firuzi et al. 2005; Chobot et al. 2008).

(\pm)-Catechin has been shown to be unstable in soils at pH values higher than 5 (Duke et al. 2009a). The pH-dependent instability, oxidation of (\pm)-catechin (Guyot et al. 1995), may contribute to the variable recovery rates from soils that have been reported in the literature (Blair et al. 2005, 2006; Inderjit et al. 2008; Duke et al. 2009a). To obtain information about (\pm)-catechin stability in our assay system and in the cell, we used HPLC analysis after 24, 96, and 240 h at pH 3.6 and 7.4, the vacuole and cytoplasm pH, respectively. The deoxyribose degradation assays were performed at pH 7.4.

This combination of analytical, chemical, and biological assays aimed to provide insight into possible mode of actions of (\pm)-catechin in biotic interactions, not only to

contribute to the debate about its acting as a chemical weapon for the invasive *C. stoebe* but also to understand the potential benefits of (\pm)-catechin accumulation in plant tissues and root exudation.

Materials and Methods

Chemicals Most chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated; water had Milli-Q quality.

Seedling Growth Assays Seeds of black mustard (*Brassica nigra* L.) were obtained from Flora Geissler GmbH (Fisibach, Switzerland) and of white mustard (*Sinapis alba* L.) from B and T World Seeds (Paguignan, France). Solvents were analytical grade. Vapor sterilization of seeds was performed by using commercially available bleach and concentrated HCl (Clerkx et al. 2004). Various variants of the assay were performed; one on agar medium, the other on filter paper. The assays were performed in Petri dishes (9 cm diam, Greiner Bio-One, Kremsmünster, Austria). The agar medium consisted of solidified Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962); (\pm)-catechin monohydrate (Sigma Aldrich) was dissolved in 2 ml MeOH, and was added to the agar solution after autoclaving. An identical amount of MeOH also was added to the control plates. The surface sterilized seeds (7–9) were transferred to the agar medium with a sterile forceps and actively pressed into the medium to assure maximum contact with the seed surface. Preliminary experiments indicated that effects of the test compounds caused more pronounced effects if this procedure was applied. Filter paper disks (Schleicher and Schuell 520 B 1/2, 60 mm diam) were moistened with 1 ml of a 10%-methanolic aqueous solution containing the test compound in a two-fold serial dilution from 6 to 200 $\mu\text{g ml}^{-1}$. This measure was taken to prevent crystallization of (\pm)-catechin (Perry et al. 2005b). The moistened filter papers were placed in 9 cm-Petri dishes. Surface-sterilized seeds (7–9) were transferred to the filter paper with sterilized forceps. Petri dishes were kept sealed for 48 h; then, the sealing tape was removed and MeOH was allowed to evaporate. Water was added to replenish the liquid and to keep the filter paper moistened.

For each concentration, four replicate Petri dishes were prepared. Two were incubated at $25\pm 2^\circ\text{C}$ with a 12 h photoperiod in a temperate greenhouse; the other two were kept completely in dark in the same location. Scoring was performed after 5 days. For practical reasons, of each tested concentration five representative seedlings were chosen for measurement of shoot and root development. The seedlings were photographed with an Olympus

D500 digital camera equipped with a macro lens with 10x magnification. Image analysis was carried out for shoots and roots separately by using Image J 1.36b (Wayne Rasband, NIH, USA, <http://rsb.info.nih.gov/ij/>). The mean of the pixel counts of the control seedlings was determined as 100% for the quantitative assay (Roberts and Boyce 1972).

Brine Shrimp Assay For each experiment, 0.5 g cysts of *Artemia salina* L., obtained from NovoTemia (JBL GmbH & Co. KG Neuhausen, Germany), were hatched in 25 ml saline aqueous solution (g per 100 ml: 2.3 NaCl, 0.4 Na₂SO₄, 0.07 KCl; pH 8.0±0.2, adjusted with HCl and/or NaOH). For hatching, illumination was performed with a 60 W lamp from a distance of 40 cm for 1 h. The hatched larvae (nauplii) were transferred to an incubator (±25.0°C). One hundred µl of the stock solution (300 µg ml⁻¹) were serially diluted in 96-well microplates, and 50 µl of suspension of 24-h old larvae (6–30 larvae) were added. After 24 h, the wells were scored visually for dead animals (larvae without any movement for at least 10 s) by using an Olympus BHZ 2 stereomicroscope (magnification 8–70 x). Fifty µl of 0.1 M HCl were used to kill all animals. In a second scoring survey, all animals were counted for each well to calculate the number of surviving nauplii for each concentration tested, as required for a quantal biological assay (Roberts and Boyce 1972). MnCl₂ served as a positive control. The negative control was the saline solution without test compound. One set of experiments was performed with addition of 5 % MeOH to the stock saline solution of the tested compound; for dilution, only saline solution was used.

Deoxyribose Degradation Assays (H₂O₂/Fe³⁺/Ascorbic acid) Procedures followed those described earlier for the deoxyribose assay (Halliwell et al. 1987; Aruoma 1994). Here, the ability of the test compound to interact with the reduction of iron (III) by ascorbic acid and the subsequently formed hydroxyl radicals is explored (for a description see Chobot and Hadacek 2009). Briefly, (±)-Catechin monohydrate was dissolved in an aqueous KH₂PO₄/KOH buffer solution (50 mM, pH 7.4); to 125 µl of this solution, 25 µl of a 10.4 mM 2-deoxy-D-ribose solution in the same buffer system and 50 µl of an solution of Fe³⁺ (50 µM) were added. Further, 25 µl 10.0 mM aqueous solution of H₂O₂ and finally 25 µl of 1.0 mM ascorbic acid in buffer were added to start the Fenton reaction. Malonyldialdehyde (MDA) was determined photometrically at 532 nm after reaction with thiobarbituric acid and subsequent extraction of the red pigment with 1-butanol. The blank contained the full reaction mixture without 2-deoxy-D-ribose. Assays were performed in triplicate.

Deoxyribose Degradation Assays (H₂O₂/Fe³⁺) This modification was carried out without the addition of ascorbic acid, which was replaced by the same volume of buffer. This variant allows assessing whether the tested compound can reduce iron (III) to iron (II). Scoring was performed after 1 h. The negative control was the H₂O₂/Fe³⁺/ascorbic acid system mixture that lacked the test compound. The blank contained the full reaction mixture without 2-deoxy-D-ribose.

Deoxyribose Degradation Assays (Fe³⁺/Ascorbic acid) H₂O₂ was replaced by the same volume of water. Deoxyribose degradation depended strongly on the diffusion of air oxygen into the liquid. This variant allows assessment of the interactions of the tested compound with the H₂O₂ that is formed by reduction of molecular oxygen present in the solution. Consequently, scoring was performed only after 16 h. The blank contained the full reaction mixture without deoxyribose. The negative control was the H₂O₂/Fe³⁺/ascorbic acid system mixture lacking the test compound.

Deoxyribose Degradation Assays (Fe³⁺) This modification of the deoxyribose assay was carried out without the addition of H₂O₂ and ascorbic acid, which were replaced by the same volumes of buffer or water. This allows assessment of the reducing capacity of the tested compound of molecular oxygen to H₂O₂ as well as that of iron (III) to iron (II). Scoring was performed after 16 h (diffusion of air oxygen).

Blanks were performed to assess possible reactions between thiobarbituric acid and the tested compound. The values were used to correct the readings obtained in the various modifications of the assay. The blank contained the full reaction mixture without deoxyribose. The negative control was the H₂O₂/Fe³⁺/ascorbic acid system mixture lacking the test compound (100% MDA).

To the reaction mixture of the deoxyribose degradation assay, 50 µl of Fe³⁺ solution (50 µM) were added. In one series, those 50 µl contained 52 µM EDTA dissolved in buffer, which was premixed with the aqueous FeCl₃ solution (1:1 v/v). In the other series, the EDTA solution was replaced by the same volume of the buffer. In the first series, EDTA chelated the iron ions, preventing them from being chelated by the test compound; in the second series, the iron ions were chelated by the test compound.

Cyclic Voltammetry Voltammetric curves were recorded at ambient temperature in a three-electrode µAutolab PGSTAT type III system (EcoChemie Inc., The Netherlands). The working electrode was a glassy carbon electrode (3 mm diam), Ag/AgCl (saturated KCl) was used as a reference electrode, and platinum wire as a counter electrode. The

glassy carbon electrode was cleaned with MeOH and water, and polished before every measurement. The effective scan rate of the CV was 50 mV s^{-1} . The scan potential was from -250 to $+1200 \text{ mV}$ for (\pm)-catechin at pH 3.6 and 7.4, for juglone from -400 to $+1200 \text{ mV}$ at pH 7.4 and from -400 to $+1350 \text{ mV}$ at pH 3.6. Both substances were dissolved in degassed water. The concentration was 1 mM . The solution for analyses was prepared by mixing 1 ml of the water solution with 9 ml of the degassed buffer. We used phosphate buffer pH 7.4 or acetate buffer pH 3.6. The ionic strength of the buffers was 0.22 M . The ionic strength of the acetate buffer was adjusted by K_2SO_4 . The electrolytes were degassed by argon for 10 min , and measure-

ments were carried out under argon atmosphere. Juglone was obtained from Fluka (Buchs, Switzerland).

HPLC Analysis (\pm)-Catechin monohydrate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in an aqueous $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer solution (0.1 M , pH 7.4) and $\text{CH}_3\text{COONa}/\text{CH}_3\text{COOH}$ (0.1 M , pH 3.6) to give a 1 mM solution of (\pm)-catechin. The HPLC System was a Dionex Summit equipped with a photodiode array detector (PDA) and a Famos autosampler. The column was a Phenomenex Synergi Max C12, $150 \times 2 \text{ mm}$, $5 \mu\text{m}$ particle size. The column oven was adjusted to 40°C , and the flow rate was 0.2 ml min^{-1} . Solvent A was water : MeOH : *o*-phosphoric

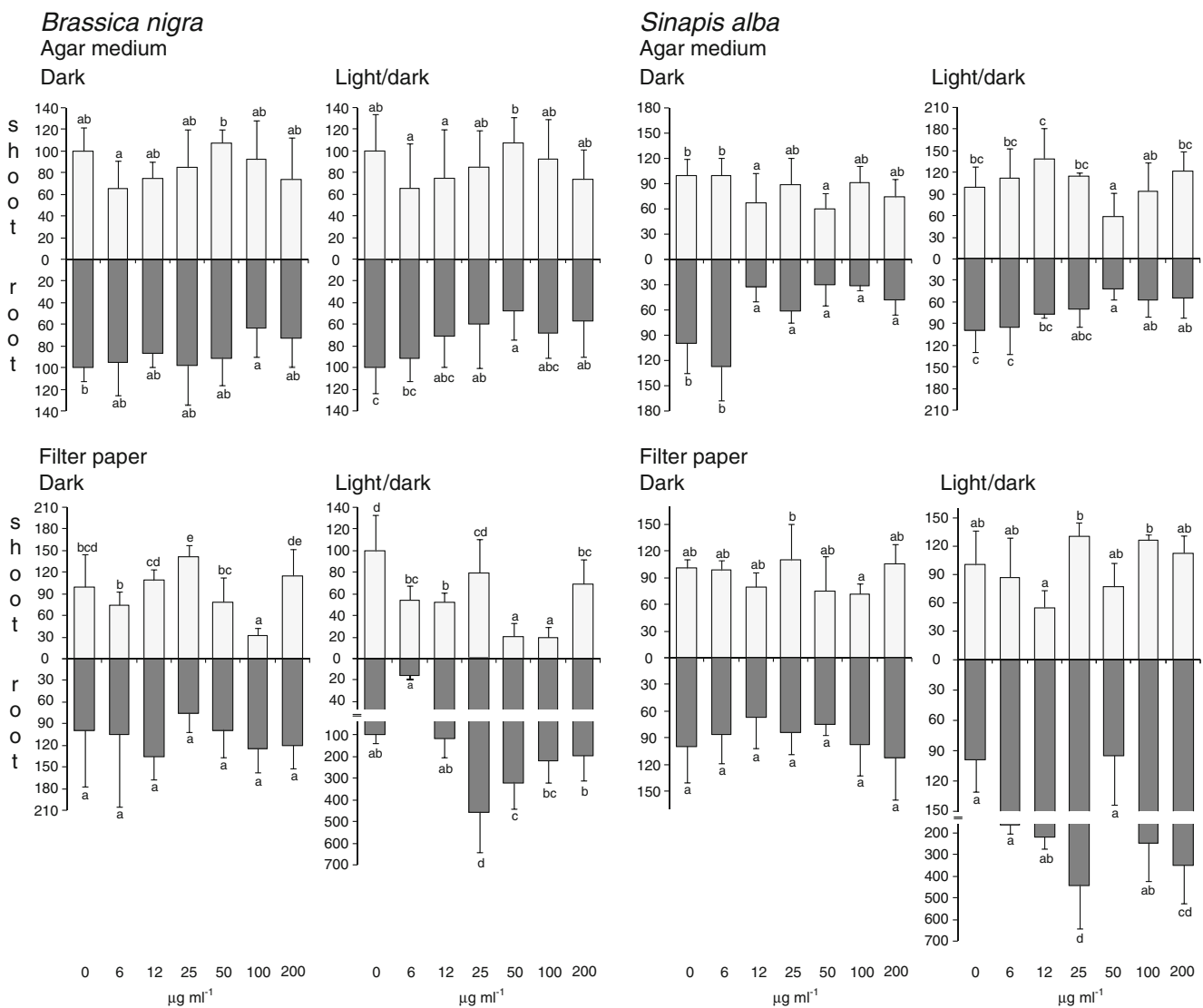


Fig. 1 Effects of (\pm)-catechin on postgerminative shoot and root growth of *Brassica nigra* and *Sinapis alba* tested on MS agar medium (0.2% MeOH, v/v) and filter paper (10% MeOH, v/v) after 5 days in temperate greenhouse at $25 \pm 2^\circ\text{C}$ in complete dark or a 12 h photoperiod. Growth is expressed as mean of the control treatment;

bars, means; error bars, standard deviation; $N=5$; letters indicate different levels of significance (95% probability, Duncan's multiple range test); 6 (19.48), 12 (38.96), 25 (81.16), 50 (162.33), 100 (324.67), 200 (649.3) $\mu\text{g ml}^{-1}$ (μM)

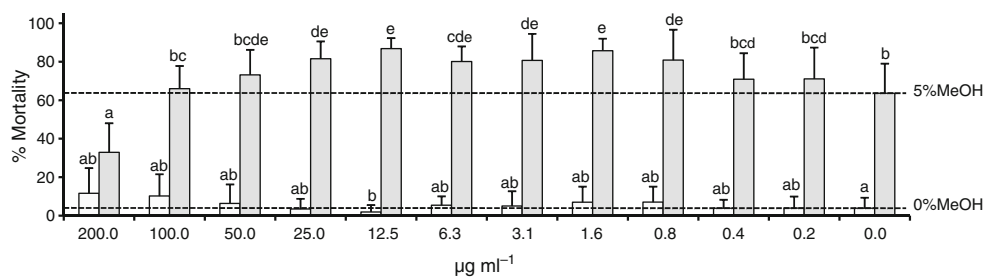


Fig. 2 Effects of (±)-catechin on mortality of 24 h-old nauplii of brine shrimp (*Artemia salina*) in saline aqueous solution after 24 h; one variant with addition of MeOH, the other with 5% (v/v); mortality rate is expressed as percentage of dead larvae; bars, means; error bars,

standard deviation; $N=8$; letters indicate different levels of significance (95% probability, Duncan's multiple range test); 6 (19.48), 12 (38.96), 25 (81.16), 50 (162.33), 100 (324.67), 200 (649.3) $\mu\text{g ml}^{-1}$ (μM)

acid (9 : 1 : 0.5, v/v/v), solvent B was pure MeOH. The gradient started with 100% of A for 2 min and then linearly changed to 100% of B within 98 min. The final concentration was held further for 10 min. Five μL of (±)-catechin solutions were injected. UV spectra were recorded from 590 to 220 nm. The experiment was repeated twice. One series only, however, is shown.

Statistics Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, MD, USA) was used to perform analyses of variance (ANOVA) with Duncan's multiple range tests at a confidence level of 95%.

Results

Seedling Growth Figure 1 summarizes the results that were obtained when (±)-catechin was applied in concentrations from 6 to 200 $\mu\text{g ml}^{-1}$ agar medium or filter paper treated with aqueous solution. In both setups, crystallization of (±)-catechin affected the testing of higher concentrations, 100 and 200 $\mu\text{g ml}^{-1}$, respectively. The two assay designs, as well as the different light regimes, specifically affected the growth of the seedlings of both brassicaeous species. Both mustards were affected similarly by specific conditions, but showed variable susceptibility. The effect that a specific concentration caused on the development of the seedlings ranged from retardation (inhibition) to induction of growth. This depended on the specific assay setup, light regime, or organ (root or shoot). The most notable inhibition was visible in white mustard seedlings, which were grown on agar medium in the dark, starting from 12 $\mu\text{g ml}^{-1}$. The presence of light slightly attenuated this effect. The black mustard seedlings were less affected. In the filter paper assay, in contrast, the presence of light caused a strong stimulation of root growth in both black mustard and white mustard, but only in the light/dark regime. In many instances, the effects were non-linear, i.e.

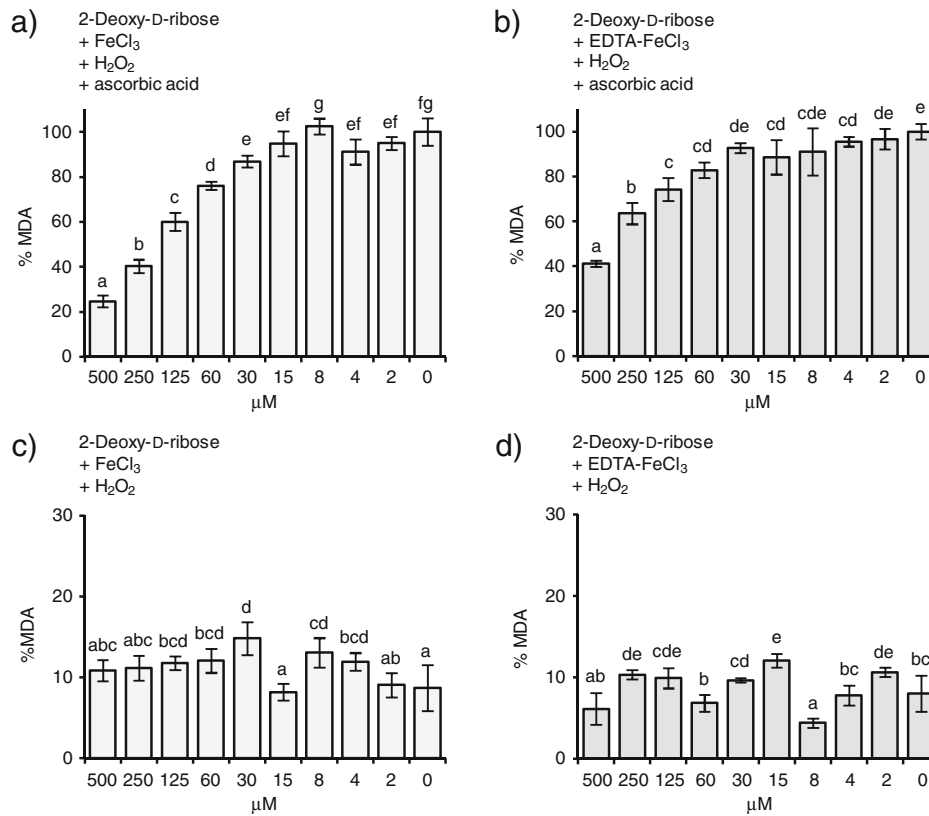
the highest concentration applied did not cause the strongest effect that was observed within the range of tested concentrations.

Brine Shrimp Assay When 5% MeOH was added to the culture medium, the mortality of the brine shrimp nauplii increased (Fig. 2). In the absence of MeOH, only one concentration (12.5 $\mu\text{g ml}^{-1}$) significantly decreased mortality rate; however, the difference of means was negligible (4 to 2%). In contrast, when 5% MeOH was added, the highest concentration tested (200 $\mu\text{g ml}^{-1}$) attenuated mortality by half (64 to 33%).

Deoxyribose Degradation Assays The results obtained in the variants of the deoxyribose degradation assay are illustrated in Fig. 3. (±)-Catechin was tested in concentrations from 2–500 μM . In the classical setup with H_2O_2 and ascorbic acid (Fig. 3a), (±)-catechin decreased the formation of MDA in a dose-dependent fashion. The effect was more pronounced, when Fe^{3+} was chelated by (±)-catechin (Fig. 3a) than by EDTA (Fig. 3b). This was evident in the difference of the means, as well as in the first concentration that differed significantly from the control. When ascorbic acid was left out, the levels of MDA decreased dramatically (Figs. 3c and d). Although some of the concentrations tested significantly differed from the control, the effects of increasing concentrations of (±)-catechin were negligible compared to the variant when ascorbic acid was present. These rather weak effects did not allow a differentiation of whether Fe^{3+} was chelated by (±)-catechin (Fig. 3c) or EDTA (Fig. 3d).

The additional variants of the assay were scored only after 16 h. Due to absence of H_2O_2 in the reaction mixtures, the observation of effects depended on the diffusion of oxygen from the air into the liquid. The presence of ascorbic acid accelerated the decomposition of deoxyribose into MDA. Again, (±)-catechin attenuated the decomposition process; if Fe^{3+} was chelated directly by (±)-catechin (Fig. 3e), the effect was more pronounced

Scoring after 1h



Scoring after 16h

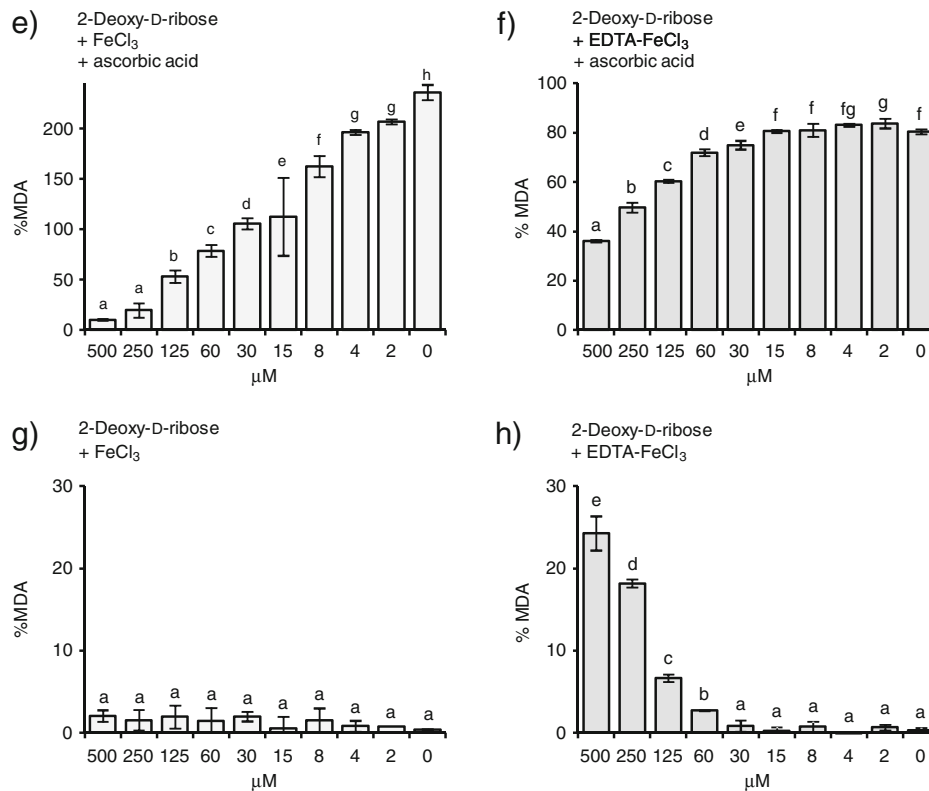


Fig. 3 Degradation of 2-D-Deoxyribose quantified by formation of malonyldialdehyde (MDA) by hydroxyl radicals generated in the Fenton reaction; in the variants (a)–(d) H₂O₂ and scoring was performed after 1 h; in variants (e)–(h) no H₂O₂ was added and formation of ROS depended on diffusion of air oxygen into the liquid of the reaction mixture, thus scoring was performed after 16 h; 100 % MDA represents the mean formation of MDA by the standard variant with addition of ascorbic acid, H₂O₂, and FeCl₃; bars, means; error bars, standard deviation; N=3; letters indicate different levels of significance (95% probability, Duncan's multiple range test); 2 (0.58), 4 (1.16), 8 (2.32), 15 (4.35), 30 (8.70), 60 (17.40), 125 (36.25), 250 (72.50), 500 (145) μM (μg ml⁻¹) (±)-catechin

than if Fe³⁺ was chelated by EDTA (Fig. 3f). If ascorbic acid was absent, the levels of MDA generally decreased; in fact, they were the lowest of all variants (Figs. 3g and h). In contrast to the variants where H₂O₂ was present (Fig. 3c and d), in this variant chelation of Fe³⁺ by EDTA (Fig. 3h) caused a significant pro-oxidative effect at the higher concentrations tested. Conversely, when Fe³⁺ was chelated by (±)-catechin, none of the tested (±)-catechin concentrations significantly differed from the control (Fig. 3g).

Cyclic Voltammetry The voltammogram of juglone (Fig. 4) shows one prominent reduction peak (reduction of quinone) and two prominent oxidation peaks (back oxidation of ring B and irreversible oxidation of ring A). The unusual high potential of the oxidation of the hydroxyl group located on ring A is caused by the strong electronegative effect of the adjacent carbonyl function on ring B, which affects the density of the electrons on ring A. The irreversibility of the oxidation of the hydroxyl group on ring A is probably caused by undetectable follow-up reactions of the semiquinone. A comparison of the voltammograms measured at pH 7.4 (cytoplasm) and 3.6 (vacuole) showed that the peak maxima shift to more positive potentials (for B from -0.18 to 0.02 V, for A from 1.01 to 1.21 V, for B' from -0.22 to 0.03 V). At lower pH, the irreversibility of the oxidation of ring A is maintained.

The cyclic voltammogram of (±)-catechin (Fig. 4) is similar to juglone. It shows a quasi-reversible oxidation of the catechol part of the molecule (ring B) and oxidation of resorcinol part of the molecule (ring A). The latter oxidation is not reversible, probably due to follow-up reactions. Similar to juglone, a comparison of the voltammograms measured at pH 7.4 (cytoplasm) and 3.6 (vacuole) showed that the peak maxima shift to more positive potentials (for B from 0.24 to 0.48 V, for A from 0.65 to 0.84 V, for B' from 0.12 to 0.28 V, respectively).

Stability of (±)-Catechin in Aqueous Solutions at Variable pH Figure 5 illustrates the obtained results. At pH 3.6, (±)-catechin remained stable in solution even after 240 h

(Fig. 5a). Peak #3 is an impurity or decomposition product that was already present in the commercial sample. At pH 7.4, the first decomposition products (e.g., #1, #4, and #7) were visible. The solution started to have a yellowish hue. After 96 h, several decomposition products were detectable. The color of the solution was now yellow (Fig. 3c). The last analysis, after 240 h, indicated that some decomposition peaks had undergone further reactions because their height decreased (e.g., #1 and #7). A comparative analysis of the UV spectra of the decomposition products revealed that peaks eluting later than 30 min were characterized by UV spectra with broad absorbance maxima above 300 nm (#5–#7) in contrast to those eluting earlier (Fig. 5b).

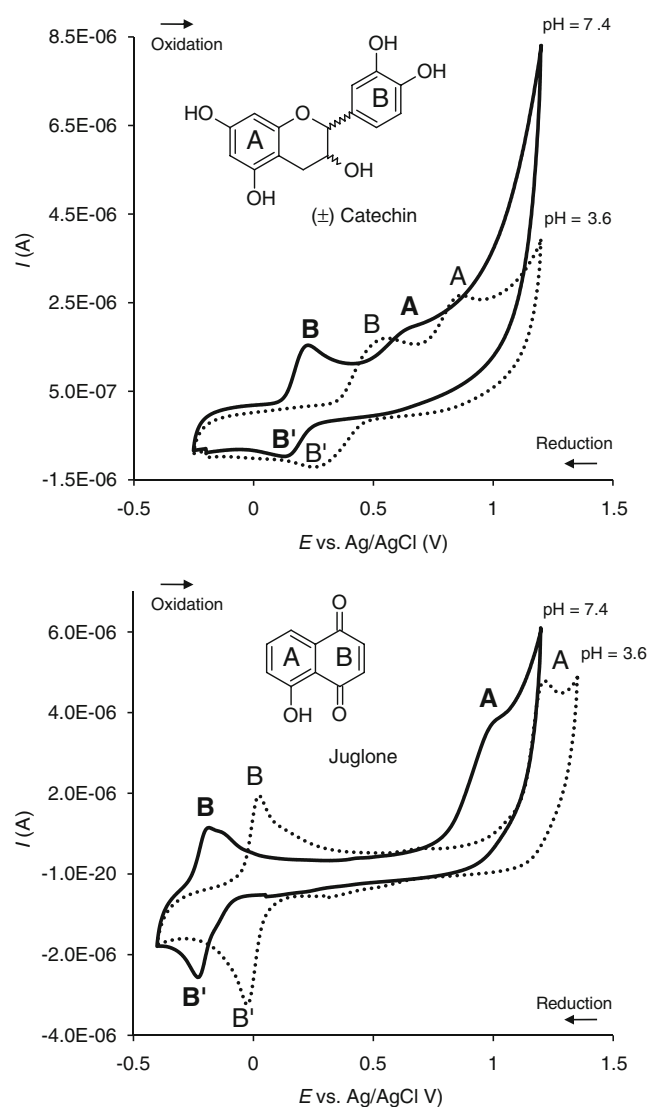
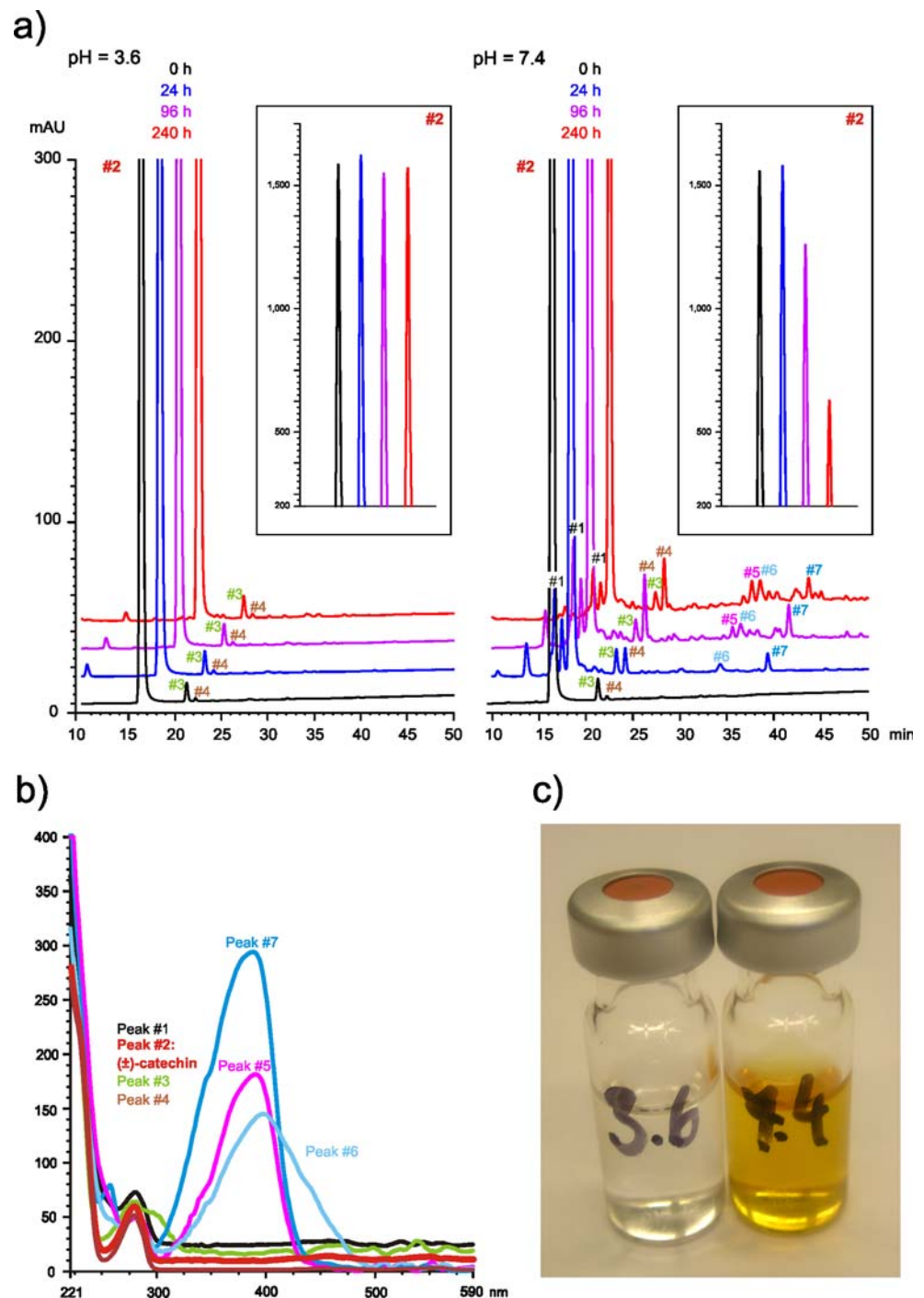


Fig. 4 Cyclic voltammograms of the flavan-3-ol (±)-catechin and the naphthoquinone juglone at pH 7.4 (cytoplasm) and 3.6 (vacuole); letters indicate peaks of oxidation and reduction and correspond to nomenclature of the phenol and quinone ring system

Fig. 5 Stability of (\pm)-catechin in aqueous solutions: (a) HPLC analyses of (\pm)-catechin at pH=3.6 and pH=7.4 at 0, 24, 96 and 240 h; (b) UV spectra of indicated peaks #1–#7, #2=(\pm)-catechin; (c) change of color in solution at pH=7.4 after 96 h compared to pH=3.6



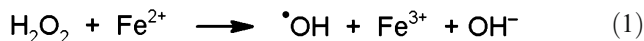
Discussion

Juglone is a well known for its phytotoxic activity (Szabo 1999; Reigosa and Pazos-Malvido 2007), and is a candidate compound for the allelopathy of the walnut tree (Jose 2002). It offers itself, thus, as positive control for the evaluation of phytotoxic effects of (\pm)-catechin. (\pm)-Catechin, however, inhibited seedling growth less efficiently by several orders of magnitude; in all variants of the seedling growth assay, juglone totally inhibited

growth at concentrations of 50 and 100 $\mu\text{g ml}^{-1}$, 0.3, and 0.6 mM, respectively (Chobot and Hadacek 2009). Significant differences to this control growth were observed starting from 6 $\mu\text{g ml}^{-1}$, 0.04 mM, depending on the setup of the assay. Although (\pm)-catechin managed to affect the growth at similar low concentrations (6 $\mu\text{g ml}^{-1}$ =0.02 mM), in some variants, it never succeeded in inhibiting seedling growth totally. Juglone is known for its pronounced phytotoxic activities at comparatively low concentrations; it even has been

suggested as a reference for an allelopathy index (Szabo 1999). (\pm)-Catechin has been shown to cause variable effects on different plant species (Weir et al. 2003; Perry et al. 2005a). In this study, we specifically explored the extent of stress level variation on seedlings. In a previous study with juglone (Chobot and Hadacek 2009), we suggested that milieu-dependent radical scavenging reactions of juglone correlate with the stress-attenuating hormetic effects of juglone under higher stress levels (when levels of free radicals are also high). In this regard, (\pm)-catechin was an even better candidate for exploration in similar assays because its lower phytotoxicity facilitated monitoring the effect across a wider range of sub-lethal concentrations. However, before the discussion of the effects observed in the biological assays may be extended to ecological implications, the effects of (\pm)-catechin in the deoxyribose degradation assay merits explanation.

The deoxyribose degradation assay was developed to detect hydroxyl radicals, which are formed in the Fenton reaction (1) (Halliwell et al. 1987; Aruoma 1994).



In this assay, ascorbic acid, which always is added as the last of all reagents, reduces iron (III) to iron (II) and starts the Fenton reaction. In case of flavonoids, chelation of iron has been implicated in contributing to the antioxidant activity (Cheng et al. 2003). By adding EDTA to the iron solution, the deoxyribose assay offers the possibility of studying the effect of the test compound when iron is either chelated by itself or by EDTA (Aruoma 1994). The iron chelated by EDTA still participates in the Fenton reaction (compare controls in Fig. 3a and b); for detailed reactions see Chobot and Hadacek (2009). Thus, depending on the setup of the assay, iron gets chelated either by (\pm)-catechin or EDTA (2).

(2)

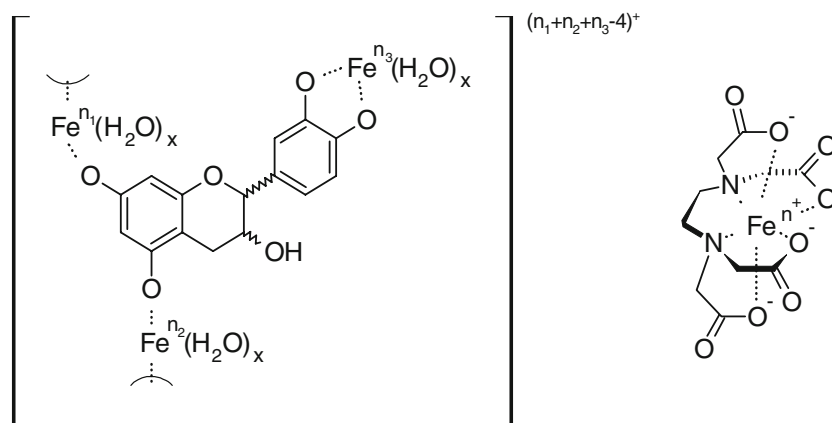
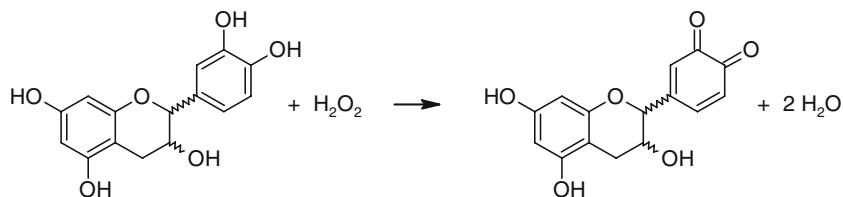


Figure 3a shows the classical variant of the deoxyribose degradation assay. The addition of ascorbic acid creates certain levels of hydroxyl radicals that trigger the formation of MDA. The mean of the control for the classical variant represents 100 % on the y axes of all bar graphs shown in Fig. 3. At lower concentrations (2–8 μM), (\pm)-catechin chelates the iron ions present in the reaction mixture (2). However, the functionality of the iron is not affected, as the levels of MDA remain unchanged. If iron is chelated by EDTA (Fig. 3b), the levels of MDA also remain unchanged. Ascorbic acid reduces iron (III) to iron

(II). With increasing concentrations of (\pm)-catechin, it starts to react with H_2O_2 . The illustrated reactions comprise only a portion of those that are possible. The decreasing levels of MDA in Fig. 3a and b may be explained by the fact that increasing amounts of (\pm)-catechin reduce H_2O_2 to water by transfer of two electrons (3). Of all ingredients in the reaction mixture, ascorbic acid has the greatest affinity for reducing iron; (\pm)-catechin does not compare. This is shown by the variants of the deoxyribose degradation assay illustrated in Fig. 3c and d. In this setup, no ascorbic acid was added.

Increasing levels of (\pm)-catechin did not reduce iron (III), neither if it was chelated by (\pm)-catechin nor by EDTA.

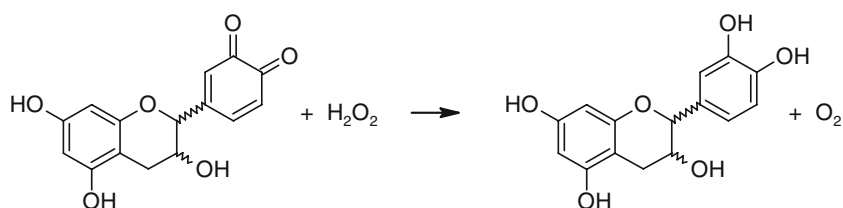
This explains the more or less constants levels of MDA when concentrations of (\pm)-catechin increased.



(3)

When (\pm)-catechin reduces H_2O_2 to water, (\pm)-catechin gets oxidized to a quinone. This quinone also may react

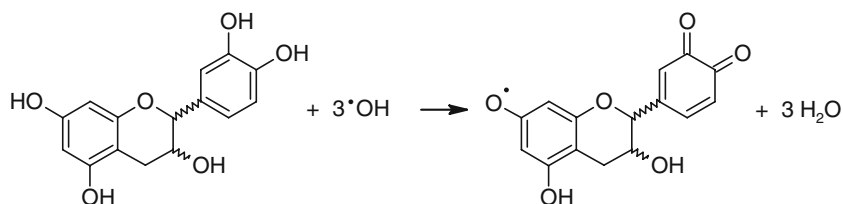
with H_2O_2 and oxidize it to molecular oxygen. During this reaction the quinone is reduced back to (\pm)-catechin (4).



(4)

Besides the previously illustrated reactions (3, 4), (\pm)-catechin also may reduce hydroxyl radicals produced by the Fenton reaction to water (5). Such reactions also

might provide the precursors for polymerization into proanthocyanidins and condensed tannins (Dixon et al. 2005).



(5)

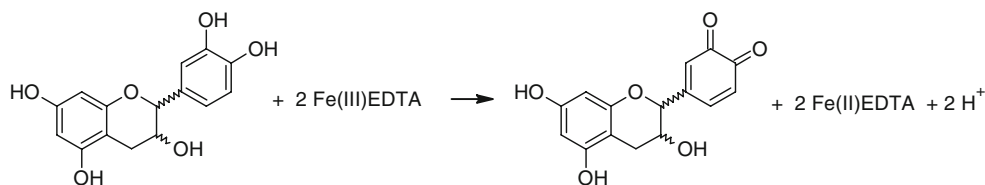
Slight differences existed between the setup with and without EDTA, which will be explained below.

The levels of MDA in Fig. 3a–d were scored after 1 h. In a second series of variants of the deoxyribose degradation assay, no H_2O_2 was added to the reaction mixture. Hydroxyl radical formation depended of the oxidation of

diffused oxygen from the air (for detailed reactions see Chobot and Hadacek 2009). Figure 3e and f illustrate that after 16 h, levels of MDA that compare or even exceed that of the standard setup with H_2O_2 could be found in the solution. Similarly, increasing concentrations of (\pm)-catechin decreased the levels of MDA. Again, the decrease

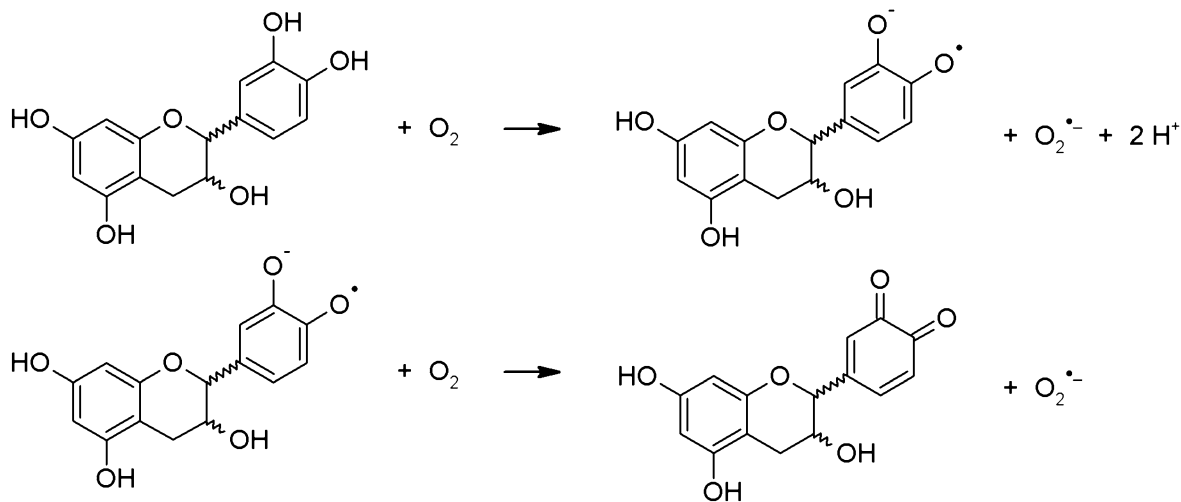
of MDA was more pronounced in the variant without the addition of the EDTA than in the variant with the addition. The variants of the deoxyribose degradation assay (shown in Fig. 3g and h) revealed that iron

chelated by EDTA may be reduced by (\pm)-catechin (6). Compared to ascorbic acid, this reaction is characterized by a much slower kinetic because the effect only became visible after 16 h.

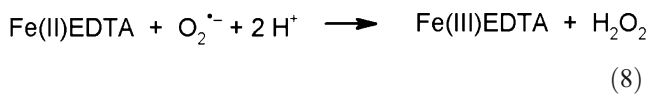


(6)

Simultaneously, (\pm)-catechin also may reduce molecular oxygen to superoxide anion radical (7), which can be reduced by iron(II) to H_2O_2 (8).



(7)



H_2O_2 may then further react with iron(II), which becomes increasingly available as rising amounts of (\pm)-catechin replenish the reduced iron(II) pool. The above outlined reactions represent possible reaction sequences involved in the pro-oxidative effect visible in Fig. 3h. However, if the iron was chelated by (\pm)-catechin, no reduction of iron in its complex with (\pm)-catechin occurred. This indicates that the reduction of the iron chelated by (\pm)-catechin is not allowed thermodynamically in contrast to the EDTA–iron complex. Consequently, the slightly higher levels of MDA in Fig. 3f compared to Fig. 3e may be

explained by underlying pro-oxidative effects. In the variants where scoring was performed after 1 h (Fig. 3a vs. Fig. 3b), a similar phenomenon is visible. However, no pro-oxidative effect is visible in Fig. 3d, the variant where ascorbic acid was not added. Although the obtained results allow no unambiguous designation of a specific reaction causing this effect, it is evident that the iron(III) in the iron–EDTA complex is more reactive than in the iron–(\pm)-catechin complex. This supports the conclusion that chelation of iron adds to the antioxidative effects of (\pm)-catechin, just as it has been postulated for other flavonoids (Yoshino and Murakami 1998; Mira et al. 2002) and (\pm)-catechin in dimethyl sulfoxide solution (Bodini et al. 2001).

What implications do these insights have for the interpretation of biological assays of (\pm)-catechin? In the

seedling development assays we carried out, we observed many non-linear effects, especially in the filter paper assay where seedlings were subjected to high MeOH concentrations in the initial phase. We varied the photoperiod and concentration of the organic solvent MeOH (low in agar medium and high on filter paper) in the test solution of (\pm)-catechin. Non-linear effects, i.e., non-dosage-dependent stimulation or inhibition of seedling growth, were visible. Such effects also were observed by Weir et al. (2003) and Prithiviraj et al. (2007) who studied the effect of (\pm)-catechin on a wide range of co-occurring species. The latter authors also attributed this to hormesis, the phenomenon whereby the compound stimulates at low concentrations and inhibits in higher concentrations; it is described by the classical statement of the famous physician Philippus Theophrastus Aureolus Bombastus von Hohenheim, better known as Paracelsus, who lived at the end of the middle ages: “All things are poison and nothing is without poison, only the dose permits something not to be poisonous” [Paracelsus (1538) Third Defensio]. Hormesis is a phenomenon that has long been known but usually has been ignored due to the fact that nobody knew how to explain it (Stebbing 1982). In a recent study, we suggested that milieu-dependent anti- and pro-oxidative activity of juglone may account for hormetic effects caused by this compound under high stress levels (Chobot and Hadacek 2009).

In the present study, we obtained results for (\pm)-catechin that showed considerable variation depending of the setup of the assay. Identical concentration levels either caused inhibition (agar medium assay) or stimulation of growth (filter paper assay), see light/dark regime in Fig. 1. The stimulation at higher concentrations was visible both in black and white mustard. Thus, our results strongly support a view that was previously voiced by Weir et al. (2003); these authors suggested that (\pm)-catechin may be a growth regulator. Plants that are exposed to sub-lethal levels of abiotic stress conditions—as in our assay and those carried out in other labs—may exhibit a broad range of morphogenic responses. Those include inhibition of cell elongation, localized stimulation of cell division, and alterations in the cell differentiation status, the stress-induced morphogenic response (SIMR). The similarity in the responses to distinct stresses is postulated as being orchestrated by increased ROS production and altered phytohormone transport (Potters et al. 2007, 2009). The results obtained from our assays resemble such stress-induced morphogenic responses. The idiosyncratic variation of the effects observed at identical concentrations could have been caused by a combination of stress factors that resulted in different levels of morphogenic responses. In contrast to clinical studies, ecological studies are usually less standardized and, as a consequence, lower inter-laboratory comparability exists. In our assays, exact reproducibility of

assay conditions—which are nearly impossible to achieve—were replaced by utilizing a low and high level stress scenario to characterize the stress-induced morphogenic response (SIMR) of our test organisms.

Artemia salina, the brine shrimps, is a crustacean. Its susceptibility to (\pm)-catechin and juglone provides information about potential ecotoxicological effects of these compounds (Nunes et al. 2006). The brine shrimp assay also provides information about intracellular generation of ROS (Matthews 1995; Chobot et al. 2006). The antioxidative effects of (\pm)-catechin visible in the chemical assays are reflected in the lower mortality rates observed at 5% MeOH concentrations in the saline aqueous solution where concentrations of (\pm)-catechin increased. Conversely, no change in mortality rates occurred when no MeOH was added to the saline solution. Within the range of tested concentrations, (\pm)-catechin did not trigger the formation of notable amounts of ROS, as is the case for juglone (IC_{50} =0.27 mM) (Matthews 1995).

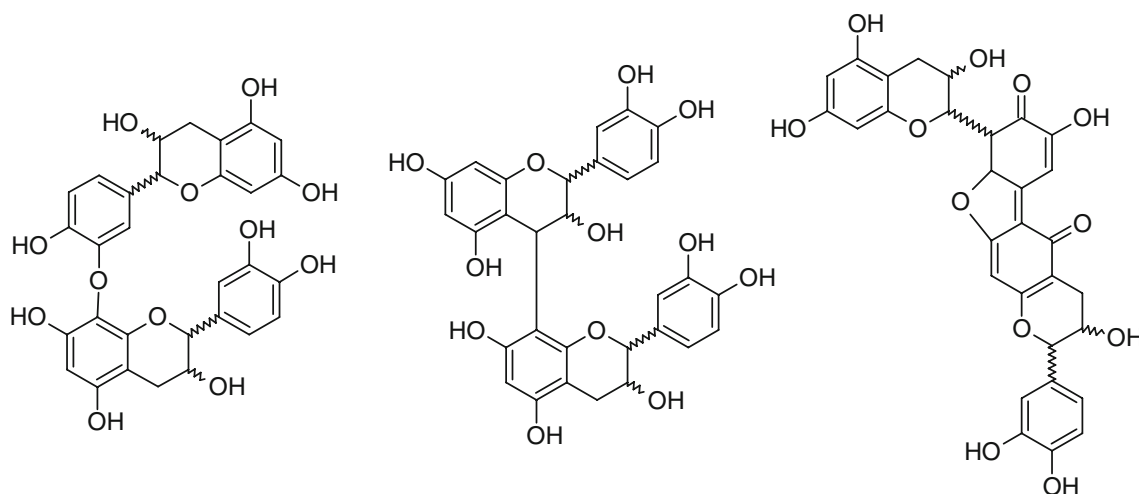
The results from the chemical and biological assays discussed thus far point more to an antioxidative role, (as pointed out by Duke et al. 2009a,b), than to pro-oxidative activity of (\pm)-catechin (as suggested by Bais et al. 2003). Antioxidative effects were more pronounced when the tested organisms were subjected to higher levels of stress; (\pm)-catechin reduced these high levels of ROS by scavenging them. Conversely, (\pm)-catechin may generate ROS, but compared to juglone, which we tested in a similar variant of the deoxyribose assay (Chobot and Hadacek 2009), the pro-oxidative effect is weak. Still, it may account for some of the inhibitory effects of (\pm)-catechin visible in the seedling growth assays.

Juglone is well known as an efficient redox cycler (Petrova et al. 1990; Bertin et al. 2003). In a recent study, we demonstrated that juglone may either scavenge or generate ROS depending on the milieu (Chobot and Hadacek 2009). The results obtained in our electrochemical experiments provide some evidence that suggest classifying (\pm)-catechin also as a redox cycler. The cyclic voltammograms of juglone and (\pm)-catechin show similarities. Both compounds displayed several oxidation peaks, one of which was reversible and accompanied by a corresponding reduction peak, as expected for a redox cycler. At pH 7.4, the peaks showed lower electrode potentials compared to pH 3.6 (Petrova et al. 1990; Martinez et al. 2005). This suggests that compounds such as juglone and (\pm)-catechin may have evolved to be most reactive in the milieu of the cytoplasm rather than the milieu of the vacuole. Juglone was characterized by reversible peaks at lower redox potentials than (\pm)-catechin. Juglone, which is a quinone, first gets reduced to trihydroxynaphthalene (Chobot and Hadacek 2009); this compound then is easily oxidized back into the quinone. (\pm)-Catechin is oxidized first to the

quinone, which then gets reduced to the phenol. In summary, these reactions require lower electrochemical potentials for juglone than for (\pm)-catechin. In the case of flavonoids, lower electrochemical potentials have been correlated with increased antioxidative properties (Kilmartin and Hsu 2003; Han et al. 2009). Consequently, the results obtained from the electrochemical studies suggest classifying (\pm)-catechin as a redox cyler, although not so efficient a one as juglone.

In the assessment of potential effects in biotic interactions, (\pm)-catechin stability is a crucial issue. This flavan-3-ol is a precursor of condensed tannins, which are widespread within plants, and arise from radical polymerization of the semiquinones of (\pm)-catechin (Dixon et al. 2005). The initial pH in our buffer solutions was 7.4, as in the cytoplasm. HPLC analyses of a 1 mM buffer solution showed that (\pm)-catechin is decomposed at pH 7.4 but not

at pH 3.6. This suggests that (\pm)-catechin is reactive in the cytoplasm but not in the vacuole. Uptake in roots is evident; brown discoloration occurs when roots are treated with aqueous (\pm)-catechin solutions (Duke et al. 2009a; unpublished results). HPLC analyses demonstrate that decomposition of (\pm)-catechin begins already in the aqueous solution. In the rhizosphere, the speed of these reactions may be increased by extracellular enzymes (Duke et al. 2009b). No phenolic acids were detected as were in studies that focus on the decomposition of (\pm)-catechin in soils (Tharayil et al. 2008). Such phenolic acids, however, may be products of reactions affected by the presence of soil microbes (Pillai and Swarup 2002). The UV-spectra of the peaks of the decomposition products detectable in the HPLC analysis indicate various dimers of (\pm)-catechin that are formed by radical polymerization of semiquinones (Guyot et al. 1996b) (9).



(9)

The last structure illustrated in this series (9) contains more conjugated double bonds than the other two dimers of (\pm)-catechin, six vs. three. This chemical characteristic is responsible for the UV spectra that shows absorbance maxima above 300 nm (#5–#7 in Fig. 5b). The HPLC analyses also indicate that only after 24 h does (\pm)-catechin start to decompose. This suggests that our chemical assays were unaffected by the instability of (\pm)-catechin, but not our biological assays. It is quite possible that such decomposition products could affect the growth of seedlings as well. For instance, decomposition products of (\pm)-catechin inhibit β -glucosidase activity (Guyot et al. 1996a) and might inhibit other enzymes as well. The tanning effect is well known.

The results from our experiments provide little support for (\pm)-catechin as a chemical weapon. Rather, they support the recent criticism of this potential function (Blair et al. 2009; Duke et al. 2009a,b): (1) the deoxyribose degradation assay indicated only weak pro-oxidative activity that does not compare favorably to pro-oxidative activities of renowned phytotoxic compounds such as juglone, and suggests that the ROS generating effect of (\pm)-catechin as shown by imaging of dichlorofluorescein might have been overestimated due to artifact formation; (2) the inhibition of seedling growth reveals more hormesis-like effects, especially under increased stress; (3) the previous hormesis-like effect may be facilitated by the pronounced antioxidative effects of (\pm)-catechin (Duke et al. 2009a); (4) the

antioxidative properties of (\pm)-catechin that are evident in the brine shrimp assay and the lack of increased mortality rates also suggest low ecotoxicological potential; and (5) (\pm)-catechin is stable only at very low pH levels. Consequently, the necessity of proving phytotoxicity in the field, an ultimate proof for allelopathy (Romeo 2000), remains still to be demonstrated for (\pm)-catechin. The many studies that claim allelopathic activity for (\pm)-catechin in the field do not provide convincing evidence that it is actually involved in this effect. The observed effects potentially could be caused by different soil chemistry in native and invasive soils that affects the availability of nutrients. Recent cross-continental studies (e.g., Inderjit et al. 2008; He et al. 2009; Thorpe et al. 2009) do not provide any attention to soil chemical characteristics, nor even to pH.

The wide occurrence of (\pm)-catechin in plants, however, suggests that it might confer benefits, either when accumulated or exuded by roots. Within tissues this benefit might be contributing to stress tolerance as many other phenolic compounds do (Close and McArthur 2002; Grace 2005; Hatier and Gould 2008). In the rhizosphere, (\pm)-catechin might protect the root tip as follows: Decomposition of soil organic matter (SOM), a process that occurs in soils containing plant litter, especially cellulose, involves transition metal-dependent generation of hydroxyl radicals by the Fenton reaction (Goodell et al. 1997, 2006; Baldrian and Valaskova 2008;). First, exuded (\pm)-catechin likely chelates iron in a fashion such that its participation in the Fenton reaction is energetically unlikely, and, second, it may scavenge free radicals potentially harmful to the root tips.

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