

Milieu-Dependent Pro- and Antioxidant Activity of Juglone May Explain Linear and Nonlinear Effects on Seedling Development

V. Chobot · F. Hadacek

Received: 3 December 2008 / Revised: 27 January 2009 / Accepted: 10 February 2009 / Published online: 5 March 2009
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Abstract Juglone, 5-hydroxy-1,4-naphthoquinone, is known for its wide range of biological activities. It has been suggested that juglone's excellent redox cycling properties contribute to this reputation. Many biological activities are nonlinear with low concentrations exerting stimulating effects, whereas only higher concentrations cause inhibition. Here, we corroborate studies on the nematode *Caenorhabditis elegans* that point out hormetic effects by showing that juglone may cause a nonlinear effect on postgerminative shoot and root growth of *Sinapis alba*. This effect was only significantly visible, however, when seedlings were stressed with methanol. Classic and modified versions of the deoxyribose assay were applied successfully to characterize antioxidative (purposeful generation of hydroxyl radicals) and prooxidative (no purposeful generation of hydroxyl radicals) activities. Variants of the assay with and without the addition of the iron chelator EDTA showed that the antioxidant activity is independent on chelation of iron ions by juglone; by contrast, the strength of the prooxidative activity depended on the chelation of iron ions by juglone. The hormetic effects of lower concentrations on germination of *Sinapis alba*, thus, may be caused by the antioxidant activities of this compound, which are especially effective when the test organism is subjected to higher oxidative challenge. The present study suggests that pronounced prooxidative activities, which are considerably accelerated by chelation of

iron ions, may contribute to the toxic effects of juglone at higher concentrations.

Keywords Naphthoquinones · *Sinapis alba* · Inhibition · Hormesis · ROS · Fenton reaction · Chelation of iron · Stress

Introduction

Although the need for greater use of modern technology and additional research on allelopathic mechanisms has been articulated (Romeo 2000), this area remains somewhat neglected relative to traditional phytotoxicity experiments. A few recent studies include those on: juglone effects on root plasma membrane H^+ ATPase activity and root water uptake (Hejl and Koster 2004); the use of polydimethylsiloxane materials to quantify levels of the photosynthesis inhibitor sorgoleone in the rhizosphere of sorghum plants (Weidenhamer 2005); the critical evaluation of the contribution of (\pm)-catechin to invasion success of spotted knapweed (Blair et al. 2005, 2006); degradation of soluble phenolics by ectomycorrhizal fungi, thus controlling species interactions in black spruce stands (Zeng and Mallik 2006); selective phytotoxicity of L-DOPA because of differential polyphenol oxidase activity in barnyard grass and lettuce, thus mediating reactive oxygen species and/or free radical species (Hachinohe and Matsumoto 2007); and differential gene expression in rice under low nitrogen due to stronger activation of genes that function in synthesis of allelochemicals (Song et al. 2008).

Often, we do not pay much attention to studies carried out many decades ago, simply because, compared to current standards, the methodologies have dramatically changed. Hans Molisch is generally regarded as the person coining

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the term allelopathy. In his experiments, he used the odor of apple peels, mainly comprised of ethylene, as a model system to explore effects of volatile emissions on the development of a range of plant species. He reported the results in his famous booklet “Der Einfluß der Pflanze auf die andere, Allelopathie” (the effect of one plant on another, allelopathy); there also exists an English translation (Molisch 2002). Molisch’s observations contain some fundamental insights, which are still under discussion today. In the summary, Molisch notes: “If seedlings are only exposed to the gas emitted by apples a relatively short time, then it becomes evident, that not an inhibition of longitudinal growth occurs but a very pronounced stimulation. Exposure to apple odor for one to five hours caused an unambiguous stimulation, whereas 24 hours caused a slight inhibition that became more pronounced with ongoing exposure”. He also provides an interpretation: “Here, the often observed rule is confirmed that poisons and irritating compounds are harmful in higher concentrations, but stimulate in diluted form”. The last sentence is especially notable, because Molisch (1937) already acknowledges a nonlinear mode of action for allelochemicals. In many instances, the response of living organisms to abiotic or biotic effectors is nonlinear, i.e., at low dosages the response is opposite to that of high ones; high doses inhibit growth and low doses stimulate growth. The latter phenomenon is also known by the term hormesis (Calabrese et al. 2007). Although it has been known for a long time, it has been largely ignored because nobody could explain it (Stebbing, 1982). Allelopathic studies that address the phenomenon of hormesis are infrequent, and then the phenomenon is addressed usually only peripherally (Romagni et al. 2000; Belz 2008). However, it has been focused on recently in ecological modeling of allelopathy (Sinkkonen 2007). In their recent survey of hormesis in plant biology, Calabrese and Blain (2009) note that allelopathy is a rapidly developing area in plant chemical biology in which hormetic responses of chemical exudates from plant roots are to be considered.

Juglone, 5-hydroxy-1,4-naphthoquinone, is classified as a strong redox cyler with high potential to react with oxygen and its reactive species. Thereby, it interferes with vital cell processes such as photosynthesis, respiration, cell division, and membrane transport (Bertin et al. 2003; Tomilov et al. 2006). Among others, one of the noted harmful effects is the alleged role in the allelopathy of walnut trees (Jose 2002). In an attempt to optimize simple tube assay procedures, thus allowing us to explore the potential redox reactions of allelochemicals, modified variants of the deoxyribose assay were used. This assay was developed as a simple screening method for hydroxyl radical scavengers (Aruoma et al. 1987; Halliwell et al. 1987). The recognized function of juglone as a redox cyler

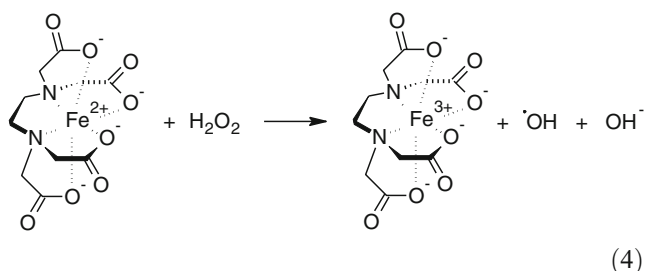
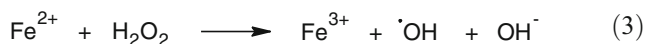
distinguishes this allelochemical as a suitable model for exploring the combined applicability of simple tube assays with biological assays to obtain further insights about the milieu-dependent reactivity of candidate molecules. In this study, the effects of juglone on seedling development of *Sinapis alba* served as the biological assay. In consideration of the proposed role of reactive oxygen species (ROS) in the mode of action of juglone, we used an agar- and filter paper-based growth assay to simulate low and high level of oxidative stress caused by variable additions of methanol (0.2 and 10%, v/v, respectively) to the medium. We expected to detect nonlinear effects (hormesis) at higher methanol levels. Hormetic abilities previously have been reported for effects of juglone on the genetic model nematode *Caenorhabditis elegans*; these effects became especially evident when *C. elegans* was pretreated with low dosages of heat, hyperbaric oxygen, or even juglone itself, and then subjected to subsequent stresses of the original or one of the other stressors (Cypser and Johnson 2002).

Materials and Methods

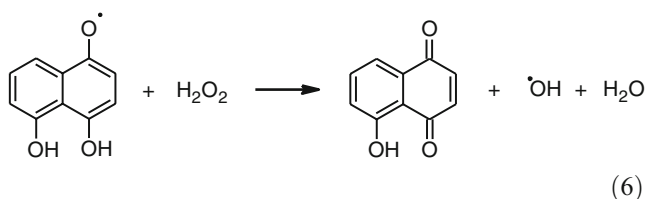
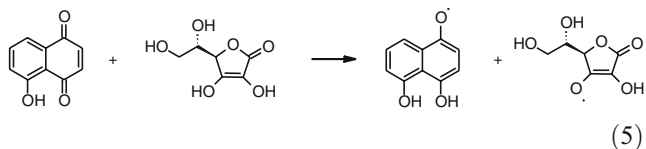
Chemicals All chemicals were obtained from Sigma Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated; water had Milli-Q quality.

Antioxidative Assays Procedures followed those described by Halliwell et al. (1987) and Aruoma (1994) for the deoxyribose assay. Juglone (Fluka, Buchs, Switzerland) was dissolved in an aqueous $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer solution (50 mM, pH 7.4) to yield final concentrations of 2–500 μM . 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 aqueous solution of FeCl_3 (50 μM) were added. In one series of the assay, those 50 μl contained 52 μM EDTA in buffer, and in the other series, only buffer was added. To start the Fenton reaction, various reactants dissolved in the above mentioned buffer systems or in water were added: 25 μl 10.0 mM aqueous solution of H_2O_2 and 25 μl 1.0 mM ascorbic acid in buffer. Standard 1.5 ml sample vials (La-Pha-Pack, Werner Reifferscheidt GmbH, Langerwehe, Germany) were used as reaction vials. The mixture was vortexed and incubated at 27°C for 60 min. Thereafter, 10 μl of 2.5% ethanolic butylated hydroxytoluene solution followed by 250 μl of 1.0% 2-thiobarbituric acid dissolved in 3% trichloroacetic acid were added to each vial to detect malonyldialdehyde, the decomposition product of 2-deoxy-D-ribose caused by the attack of hydroxyl radicals. The vials were vortexed and heated in a water bath at 85°C for 30 min. The reaction was stopped by transferring the vials into an ice water bath for 3 min. To extract the reaction product of malonyldialdehyde

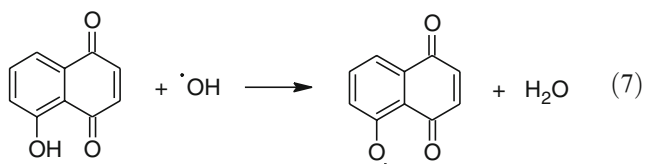
In our setup of the assay, the antioxidant activity was comparable to the variant without EDTA (Fig. 1a). This implies that chelation of iron by juglone does not affect its antioxidant activity. The reduced iron (Fe^{2+}), both in its free form (3) and as complex (4), reacts with hydrogen peroxide in the Fenton reaction and generates hydroxyl radicals, which are strong enough to decompose 2-deoxy-D-ribose to malonyldialdehyde (Aruoma et al. 1987; Aruoma 1994).



The hydroxyl radical also may arise by one electron transfers between the semiquinone of reduced juglone and hydrogen peroxide (5, 6).

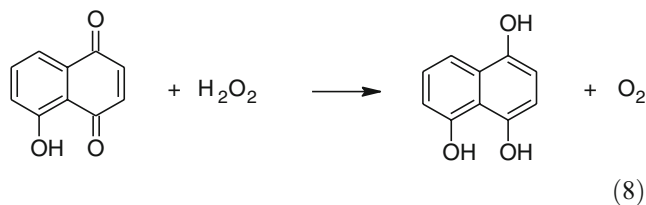


The fundamental reaction of the antioxidant activity of juglone is most likely the reduction of hydroxyl radicals to water (7).

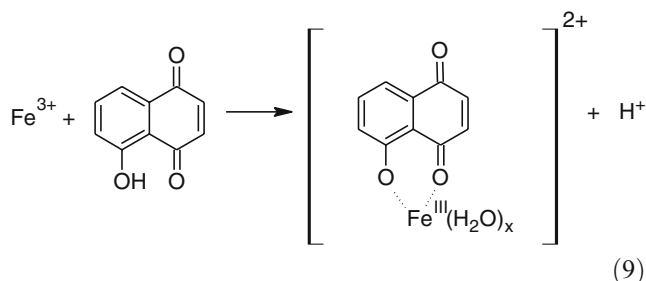


Alternatively, juglone may oxidize hydrogen peroxide to oxygen and thereby get reduced to trihydroxynaphthalene

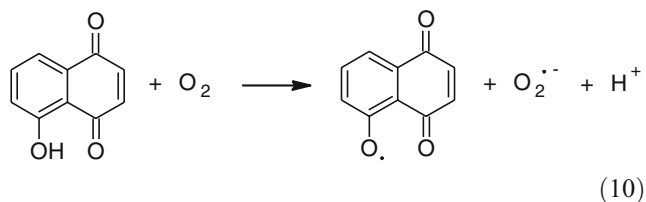
(8). The latter compound may be a more powerful reducing agent than juglone itself.



These reactions are possible at a more or less neutral pH (the pH of the aqueous buffer was adjusted to 7.4, thus resembling the milieu in the cytosol); at a lower pH, such as in the vacuole, they are less likely to work. In neutral milieu, juglone also may chelate iron ions (the chelation is both possible for iron (III) and iron (II) ions; the former are illustrated [9]). However, for antioxidant activity, chelation of iron ions by juglone did not affect the efficacy of the reaction.



In an attempt to obtain a tube assay that also facilitates the detection of prooxidative effects, the deoxyribose assay was modified. In this variant, hydrogen peroxide and ascorbic acid were not added. The duration of the assay was increased to 16 h to facilitate detection of reactions with oxygen dissolved in the aqueous buffer. Juglone showed a pronounced prooxidative effect as expected (Fig. 1b). Again, two setups were performed, one with addition of EDTA, the other without. However, the prooxidative activity of juglone was much more pronounced if EDTA was not added. This difference merits attention. Due to the absence of low concentration of free radicals, molecular oxygen is reduced to superoxide by juglone (10).



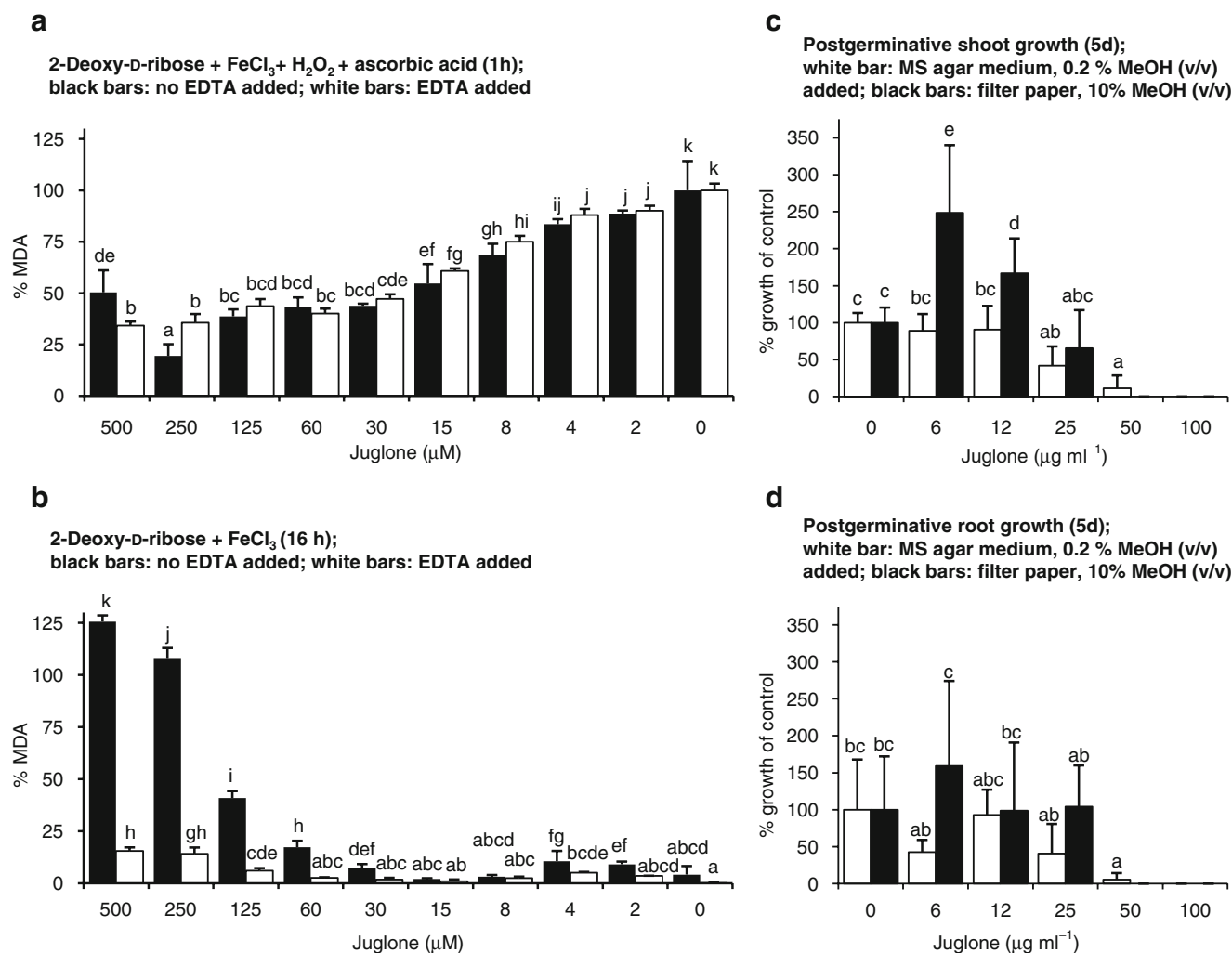
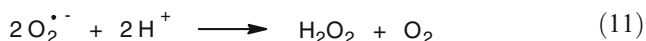


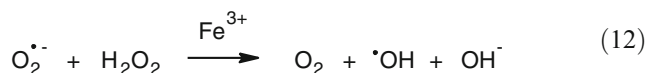
Fig. 1 **a** Antioxidative activity of juglone, quantified in % malonyldialdehyde, an oxidative decomposition product of 2-deoxy-D-ribose; *bars*, means; *error bars*, standard deviation; $N=3$, letters indicate different levels of significance (95% probability, Duncan's multiple range test). **b** Prooxidative activity of juglone; for specific information, see antioxi-

dant activity; scale of 100% MDA scale is the same as in (a). **c** Postgerminative shoot growth, quantitative biological assay; *bars*, means; *error bars*, standard deviation; $N=5$; letters indicate different levels of significance (95% probability, Duncan's multiple range test). **d** Postgerminative root growth; for specific information, see shoot growth

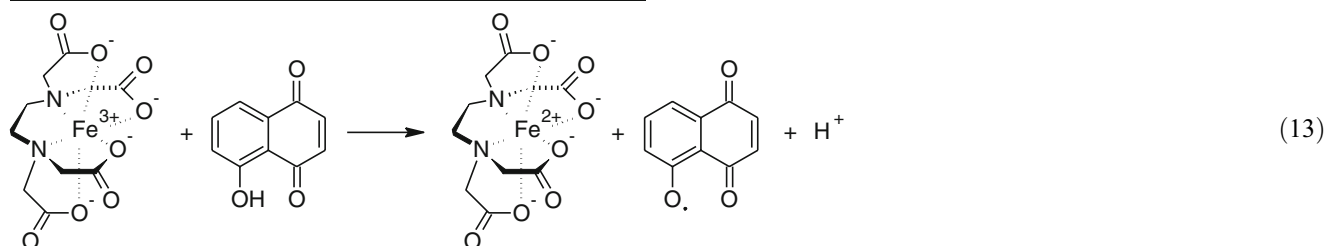
Superoxide can react with itself (dismutation of superoxide, [11]).



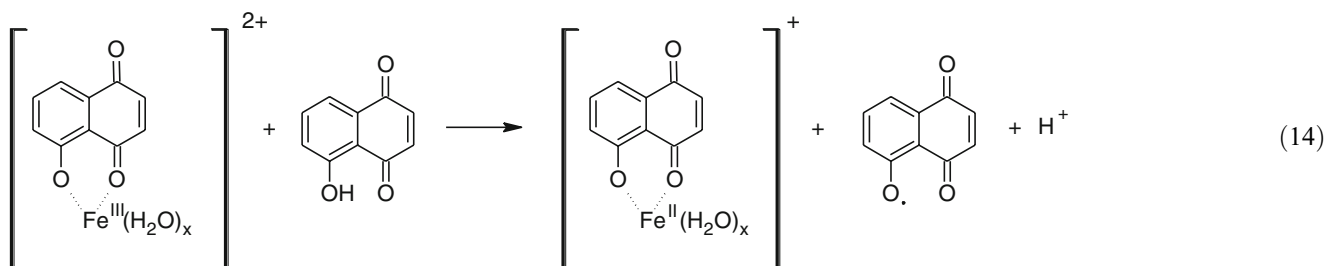
Superoxide may also directly react with the formed hydrogen peroxide generating hydroxyl radicals conditional to the presence of iron (12).



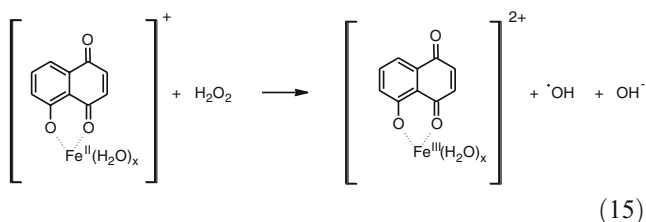
The results suggest that chelation of iron by different molecules affects the electrochemical potential required for reduction of iron (III) (Miller et al. 1990). If EDTA is added, juglone reduces the EDTA complex of iron (III) (13).



If no EDTA is added, then iron (III) is chelated by juglone and—as suggested by the pronounced activity visible at higher concentrations tested—reduced to iron (II) in the complex (14).



For the juglone–iron (III) complex, the required energy for the reduction seems to be considerably lower. As a consequence, more juglone-chelated iron (II) is available for the generation of hydroxyl radicals in a Fenton type reaction (15) than EDTA-chelated iron (II) in the other setup.



Within the tested range of concentrations, the prooxidative effect was linear. It is possible that the nonlinearity in the antioxidant setup, where no EDTA was added, may be caused by the reactions leading to the prooxidative effect as described above.

By starting from the notion that juglone is an efficient redox cyclers, we wanted to explore if juglone might cause nonlinear effects in biological assays, hormesis—a process whereby test organisms exposed to low levels of stress or toxins become relatively more resistant to subsequent challenges. In the present case, we expected low levels of juglone to ameliorate the stress caused by higher methanol concentrations. In vivo oxidation of methanol may generate superoxide, thus causing toxic effects in living organisms. The addition of methanol to aqueous solutions may not only be employed to improve dispersion of lipophilic compounds in aqueous media—as is good laboratory practice—but also to increase stress for the tested organism. Thus, to create a low- and high-stress setup of the postgermination growth assay, *S. alba* was germinated on MS agar medium (<0.2% MeOH) and on filter paper assay (10% MeOH). The results are illustrated by Fig. 1c and d. Shoot size of the seedlings exposed to higher methanol concentration was nearly half the size of seedlings exposed to the low methanol

concentration ($P=0.005$, Mann–Whitney rank test). Figure 1c and d illustrate the postgerminative growth dynamics as % in relation to the control; thus, the inhibitory effect of the higher methanol concentration is not evident, as both means of the control denote 100%.

Root development was not significantly affected by the various methanol concentrations ($P=0.689$, Mann–Whitney rank test). At lower methanol concentrations (<0.2%), increasing concentrations of juglone inhibited postgermination growth of *S. alba* seedlings clearly affecting the shoot and less evidently the root; 100 $\mu\text{g ml}^{-1}$ completely inhibited germination. The concentration of the solution of juglone in the agar was about 575 μM . At the same concentration range, the deoxyribose degradation assay suggests that juglone may cause a pronounced prooxidative effect when levels of free radicals are low (Fig. 1b). In the filter paper assay (the initial concentration of methanol was around 10%), germination was inhibited at 50 $\mu\text{g ml}^{-1}$ (287 μM). Although, at 6 $\mu\text{g ml}^{-1}$ (34 μM), a significant stimulatory effect was visible (Fig. 1c). This stimulation of lower juglone concentrations was especially visible in the shoot development, which similarly was more affected by higher methanol concentrations. The roots were less affected and, accordingly, were less stimulated by lower juglone concentrations. At the low concentrations where the stimulatory and thus hormetic effect was visible ($\pm 30 \mu\text{M}$), the deoxyribose degradation assay suggests that juglone may efficiently scavenge present hydroxyl radicals. This also reflects a mitigating effect on the stress when the seeds were exposed to high methanol concentrations.

Cypser and Johnson (2002) observed a similar hormetic effect of juglone in gerontological studies that used the nematode *C. elegans* as a test organism. In that study, pretreatment with the toxin juglone mitigated oxygen stress. Conversely, preceding oxygen stress also mitigated stress caused by the application of juglone. These authors concluded that low levels of initial oxidative stress

obviously mitigate effects of subsequent stressors that cause oxidative stress. Here, we demonstrated that juglone can scavenge hydroxyl radicals generated by Fenton type reactions during oxidative stress in a chemical assay. In our biological assay, a hormetic effect was visible only when seeds of *S. alba* were stressed with 10% methanol in the solution. The results suggest that the hormetic effect of a redox cycling compound (in this regard, juglone represents an efficient example) may be caused by scavenging ROS such as the hydroxyl radical.

Juglone is a potent bioactive compound. Can this compound, however, serve as an effective chemical defense, for example, in the case of allelopathy of the walnut tree? This question is difficult to answer, as it is difficult to develop one ideal assay to measure pro- and antioxidative activities (Halliwell and Gutteridge 1995). Hormetic activity may be determined not only by structural characteristics and concentration, but also—as our results suggest—be facilitated by the milieu in the tissues of the targeted organism. The results presented here provide ideas for explaining hormesis caused by juglone. Evolution may favor such potent redox cyclers. Gene expression studies in *Arabidopsis thaliana* and rice have shown that juglone (Mylona et al. 2007), herbicides such as paraquat and glyphosate (Ahsan et al. 2008), and even hormones such as abscisic acid (Guan et al. 2000), may by generating ROS provoke similar effects in the expression patterns of antioxidant genes. This suggests that common stress-related motifs in the promoters of these genes should be present (Mylona et al. 2007). Studies that assess the effect of allelochemicals on cell cycling (Sanchez-Moreiras et al. 2008) should increase our understanding of allelopathy.

Acknowledgements V.C. and the research carried out in this study were supported by a Lise Meitner fellowship of the Austrian Science Fund (FWF M920-B03).

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