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Original Scientific Paper

Effects of juglone on pea and maize seed germination, early seedling development and detoxification enzyme activities

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ABSTRACT: Pea and maize responses to treatment with juglone were investigated by quantifying germination, radicle growth and activities of detoxification enzymes quinone reductase and glutathione transferase. Juglone, a naphthoquinone present in *Juglans* species, is one of the best-examined allelochemicals, yet mechanisms of its action have not been fully elucidated. Pea is considered to be juglone sensitive, while some publications consider maize tolerant to growing near *Juglans* species. In this study, maize and pea differed in their responses to juglone according to quinone reductase and glutathione transferase activities. A major difference was significantly higher activities of both enzymes in maize in comparison with pea. Neither soluble quinone reductase nor glutathione transferase was responsive to juglone treatment in maize, while both activities were induced by juglone in pea. Increased pea quinone reductase and glutathione transferase activities were, however, still lower than the corresponding activities in maize, indicating that constitutively high activity of detoxification enzymes could be a prerequisite for juglone tolerance.

Keywords: allelopathy, juglone, quinonereductase, glutathione-*s*-transferase

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INTRODUCTION

Naphthoquinones are electrophilic compounds known to show many biological effects, including antibacterial, antifungal (Didry *et al.* 1994; Sasaki *et al.* 2002; Clark *et al.*1990), antiviral (Tandon *et al.* 2004; Sendl *et al.* 1996), cytotoxic (Inbaraj & Chignell 2004; Castro *et al.* 2008; Babula *et al.* 2009 a, b), antimutagenic and antioxidative effects (Kumar *et al.* 2013). Juglone (5-hidroxy-1, 4-naphthoquinone), as an allelopathic substance, is present in soil near *Juglans* trees and is known to have inhibitory or toxic effects on many plant species. As other naphthoquinones, juglone expresses toxicity to sensitive plants at multiple levels – inhibition of plasma membrane H^+-ATPase (Rudnicka *et al.* 2014; Hejl&Koster 2004), impairment of photosynthesis, respiration and transpiration (Hejl *et al.* 1993; Hejl & Koster 2004; Babula *et al.* 2009a), disturbance of mitochondrial function through redox cycling, and induction of programmed cell death through disturbance of mitosis and DNA damage (Babula *et al.* 2009b). In contrast to juglone sensitive plants, a number of plant species are considered to be less susceptible or tolerant to juglone. Many of these classifications into sensitive and tolerant plants contradict each other and are often not followed by thorough scientific examinations (Scott & Sullivan 2007; Jose & Gillespie 1998b), though difference in juglone tolerance among species is evident. Pea is considered to be juglone sensitive; some horticultural and extension publications consider maize to be tolerant to juglone (Funt & Martin 1993), while other reports classify it as juglone sensitive (for review see Willis 2000). Effects of juglone at the molecular level have been more extensively investigated during the past decade (Hejl & Koster 2004; Mylona *et al.* 2007; Chi *et al.* 2011; Sytykiewicz 2011; Rudnicka *et al.* 2014), often on maize, but to our knowledge no molecular investigations have been done with pea.

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Juglone can be detoxified in plants by conjugation with glutathione, as suggested by Sytykiewicz (2011). Glutathione-*S*-transferases are multifunctional enzymes that take part in detoxification of electrophilic xenobiotics (Edwards 1996; Hatton *et al.* 1999). They are mainly cytosolic (Dixon *et al.* 2002) though microsomal forms are also present in plants (Mohsenzadeh *et al.* 2011). It has been shown that GST expression is upregulated under juglone treatment in maize (Sytykiewicz 2011) and rice (Chi *et al.* 2011). Prior to conjugation, electrophile xenobiotics should be reduced by another class of detoxification enzymes, quinone reductases (QRs). QRs are present in both membrane and soluble fractions of plant cells (Spitsberg & Coscia 1982) and catalyze reduction of electrophilic quinones such as juglone, that can subsequently undergo conjugation with glutathione or glucuronic acid (Deller *et al.* 2008). Activities of these detoxifying enzymes have not, to our knowledge, been examined in response to naphthoquinone treatment in allelopathy. Establishing physiological differences between juglone-tolerant and juglone-susceptible plants could elucidate the mechanism of tolerance and enable crop improvement by induction of natural defence mechanisms.

This study aimed to determine whether pea as juglone-susceptible, and maize, as a potentially juglone tolerant plant, respond differently to juglone treatment with regard to quinone reductase and glutathione transferase activities.

MATERIALS AND METHODS

Pea (*Pisum sativum*L., var. Kelvedon, Superior seed Co.) and maize (*Zea mays saccharata*, VPTC union F1, Superior seed Co.) were thoroughly rinsed under tap water and left to imbibe for 6 h. Seeds were then transferred to dishes with filter paper (25 seeds per dish in 4 replicates; 100 seeds for each control and treatment). Filter paper was then wetted either with distilled water or 0.5 mM juglone dissolved in warm deionized water and left to cool. Seeds were kept in the dark at 25±2°C and radicles collected after 48 h and 96 h. Radicle lengths in germinated seeds were measured while care was taken not to damage the tissue. Excised radicle tissue was then weighed, frozen in liquid N_2 and stored at -80°C until use

Tissue was ground in liquid nitrogen and extracted in a medium (10 ml per gram of tissue fresh weight) consisting of 200 mM sucrose, 1 mM DTT, 1 mM EDTA and 10 mM Hepes-KOH pH 7.8. Extracts were centrifuged at 10000 x g for 10 min and supernatants decanted. Supernatants were then centrifuged for 40 min at 100000 x g. Resulting pellets were resuspended in 250 µl of membrane resuspension medium (10 mM Hepes-KOH pH 7.5, 200 mM sucrose and 10 % w/v glycerol) and used as a microsomal fraction. Supernatants were used as the total soluble fraction.

Protein content was estimated according to Bradford (1976) modified for microtiter plate use, with BSA as standard, with aim to calculate enzyme specific activities. Quinone reductase total activity was estimated as described earlier (Wrobel *et al.* 2002), with some modifications. Both NADH and NADPH were tested as electron donors and juglone was used as an electron acceptor. The reaction mixture contained 10 mM Hepes pH 7.5, 0.2 mM NAD(P)H and 80 µM juglone. The reaction was started by adding 100 µl soluble fraction or 10 µl microsomal fraction and NAD(P)H oxidation was monitored at 340 nm, using extinction coefficient 6.23 mM^{-1}. Triton X-100 was included in the reaction mixture at 0.02% to permeabilize membrane vesicles when microsomal fractions were assayed. Non-enzymatic oxidation of NAD(P)H was monitored for 3 min in the absence of a sample and this value subtracted from all measured activities. Also, non-specific oxidation of NAD(P)H was monitored in the absence of juglone for every sample, and subtracted from juglone-specific adenylate oxidation.

Glutathione S-transferase (GST) activity was measured essentially by the method of Habig*et al.* (1974). The reaction mixture contained 100 mM potassium-phosphate buffer pH 6.5, 1 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 150 µl soluble fraction. For microsomal fractions, a 50 µl sample [How were pellets solubilised?]was assayed in the same reaction mixture containing 0.02% Triton X-100 to permeabilize microsomal membrane vesicles.

Activities were calculated with Microcal Origin (version 6.1), and significant differences were analysed using one-way ANOVA.

RESULTS

Juglone at 0.5 mM inhibited pea germination, but had no significant effect on maize germination. Pea germination was inhibited by 16% at 48 h and by 10% at 96 h after imbibition started (Table 1). No significant differences were observed in germination percentage in juglone-treated maize compared with thecontrol 48 or 96 h after imbibition started. Biomass production was lowered by juglone in both pea and maize, with a more pronounced effect on maize where average radicle fresh weight was ca. 30% lower than in the control compared with a10% decrease in pea. Radicle elongation was more affected in pea, reduced by 40% under juglone treatment, while in maize reduction in length was 15%.Growth parameter means varied among seeds obtained in different years, but the trends were consistent. Table 1 shows results from one year.

In pea soluble fractions, juglone treatment led to elevation in both NADH and NADPH dependent QR activity at 96h, while only NADPH-QR was elevated at 48 h (Fig. 1 A). On the other hand, maize soluble fractions

Table 1. Growth parameters for 96 h old pea and maize seedlings in control and juglone-treated samples. Values are means of 4 replicates (25 seeds per replicate)± SE. Asterisk denotes a statistically significant difference from the corresponding control, calculated using ANOVA test with $p<0.05$.

	Germination (%)		Radicle mass (mg)		Radicle length (cm)	
	pea	maize	pea	maize	pea	maize
Control	91.43±1.43	81.43±3.57	41.67±1.28	29.75±0.96	5.98±0.34	7.60±0.44
Juglone	80.26±1.32*	85.73±1.50	37.14±0.69*	20.46±1.17*	3.50±0.21*	6.46±0.34*

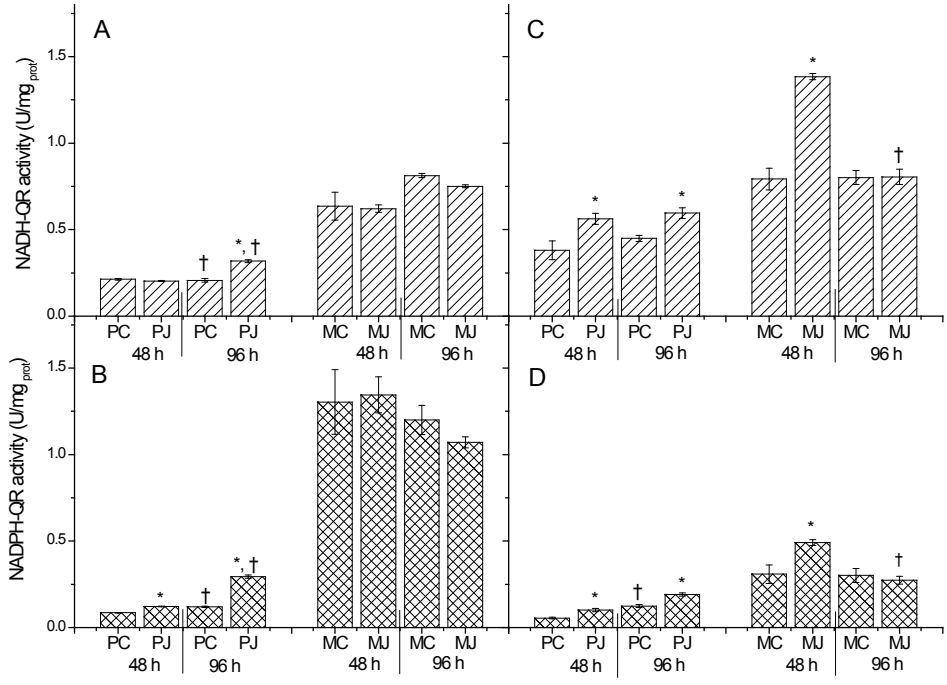

Figure 1. NADH- and NADPH-dependent quinone reductase specific activities in soluble (A, B) and microsomal (C, D) fractions isolated from 48 h old and 96 h old pea and maize seedlings expressed as units per mg protein. One unit of activity was defined as the amount ofenzyme that oxidizes 1 μmol of NADH per min. Significant ($p<0.05$) differences from the respective control are denoted by *, while significant differences between 96h and 48h seedlings are denoted by†. PC – pea control, PJ – pea juglone-treated, MC – maize control, MJ – maize juglone-treated

had significantly higher constitutive QR activity but did not react to juglone treatment. (Fig. 1 B)

Pea microsomal QR was responsive to juglone with both adenylates as electron donors, and also showed an increasing trend with age (Fig. 1 C). Maize microsomal QR activity was, as in soluble fractions, constitutively higher than in pea. Both NADH and NADPH dependent QR in maize microsomal fractions showed a sharp increase at 48 h, with subsequent decrease at 96 h (Fig. 1 D).

Pea constitutive QR activities in all fractions with both NADH and NADPH as electron donors were significantly lower than in maize, ranging from 6.6% of the corresponding maize activity for soluble NADPH dependent QR (activity in maize being 15 times higher than in pea) to 48.8% of the corresponding activity in maize for microsomal NADH-dependent activity. Both NADH- and NADPH-dependent QR activities were higher in maize than in pea, in both soluble and microsomal fractions.

Glutathione transferase activity was very low in both pea and maize microsomal fractions, an order of magnitude less than maize soluble GST activity (Fig.2, B). Juglone treatment induced no significant response of this activity. Soluble pea GST activity (Fig.2, A) was also low and decreased in 96 h compared with 48 h old seedlings. In 96 h old pea seedlings, GST activity showed a slight but significant increase upon juglone treatment. In the maize total soluble fraction, GST activity was high

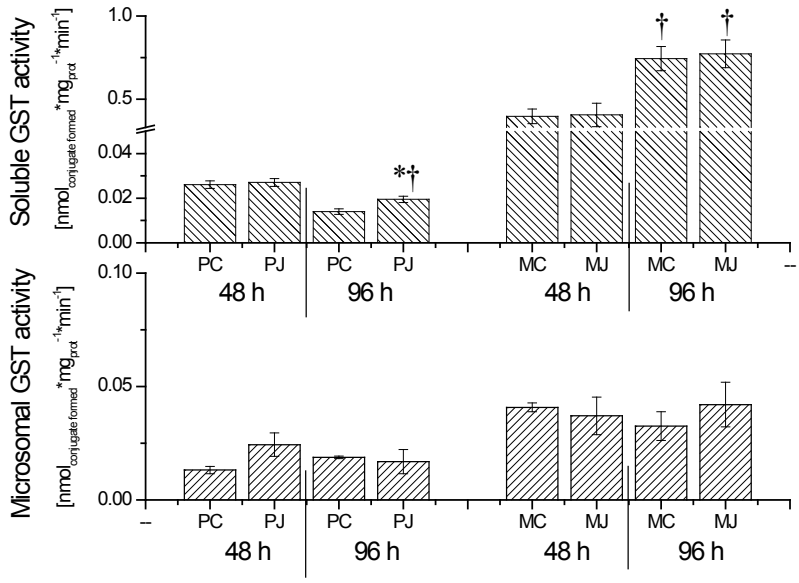

Figure 2. Glutathione transferase specific activity in soluble and microsomal fractions isolated from 48 h old and 96 h old pea and maize seedlings, expressed as units per mg protein. One unit of activity was defined as the amount of enzyme that produces 1 μmol of CDNB-glutathione conjugate per min. Significant ($p<0.05$) differences from the respective control aredenoted by *, while significant differences between 96h and 48h seedlings aredenoted by†. PC – pea control, PJ – pea juglone-treated, MC – maize control, MJ – maize juglone-treated

and increased during development, but did not react to juglone treatment.

DISCUSSION

The concentration of juglone in soil, in the vicinity of juglone-producing plants, is highly dependent on distance from the plant, presence of mycorrhizal funghi, soil type, soil drainage and amount of rainfall. Soil juglone concentrations reported in the literature vary from around 10 μM (Jose & Gillespie 1998a; Böhm *et al.* 2006) to about 1 mM (Ercisli *et al.* 2005; Rietveld 1983). Juglone concentration of 0.5 mmol per liter was used because it occurs in natural conditions and should be high enough to induce quick response even in plants relatively tolerant to this allelochemical. Juglone treatment inhibited pea germination by 10% while germination was not affected in maize in the presence of 0.5 mM juglone. Muskmelon has been shown to be juglone tolerant (up to 1 mM juglone) during germination (Kocaçalışkan & Terzi 2001), as in our experiments with maize. As suggested by Terzi (2008), this could indicate that there may be mechanism(s) for tolerance to juglone in these species' seed coats. Growth (measured as average radicle length and weight) was affected in both species.

QRs can catalyze transfer of one (QR1) or two (QR2) electrons, and both exist in plants (Greenshields *et al.* 2005; Matvienko *et al.* 2001). QR1 generates free radical species that enhance oxidative stress brought about by electrophile xenobiotic treatment, and these have been found to be upregulated in parasitic *Triphysaria* during haustoria development (Matvienko *et al.* 2001). QR2 are thought to be involved in detoxification of xenobiotics, removing reactive electrophile quinones from redox cycling thus enabling their detoxification by conjugation (Mylona *et al.* 2007, Greenshields *et al.* 2005). Also, QR2 have been suggested to take part in maintaining redox homeostasis in membranes (Beyer *et al.* 1996). QR2 in plant tissue is up-regulated upon infection (Greenshields *et al.* 2005) and exposure to allelopathic quinones (Matvienko *et al.* 2001). Our results suggest that QR induction by juglone in maize is transient; a sharp increase in QR activity appeared at 96 h when juglone was introduced at 48 h to untreated maize seedlings (our unpublished results). In pea, on the other hand, QR activity increased during development and was stimulated by juglone treatment, continually increasing its activity. Comparing soluble fractions of the two species, the control-level NADPH-dependent QR activity was 10-fold in maize compared with pea, which was consistent with low pea QR activity found earlier (Spitsberg & Coscia 1982). By measuring total activity in our experiments, we could not distinguish whether one- or two-electron transfer, or both, were responsible for the observed activities. QR activities measured in this study were specific (activity with NAD(P)H and juglone as

substrate), but that does not mean that they were catalyzed by a single enzyme. In addition to two QR types, redox systems present in plant cell membranes can *in vitro* catalyze NAD(P)H oxidation accompanied by electron transfer to quinones (Schopfer *et al.* 2008). Further analyses should elucidate which membrane fraction and which QR type was responsible for this transient increase in NAD(P)H oxidation.

GSTs are well-characterized both in maize (McGonigle *et al.* 2000) and in pea (Edwards 1996). According to Cummins *et al.* (1997), maize seedlings contain ten-fold higher GST activities toward herbicides than competing weeds of equivalent age. Pea seedlings, on the other hand, contain lower CDNB-GST activities that decrease with age (Edwards 1996). This is in accordance with our results. Various GSTs have been found to be upregulated by juglone treatment in rice (Chi *et al.* 2011), together with other defence and detoxification enzymes. Even 10 μM juglone induced significant transcriptome alterations after 1h treatment (Chi *et al.* 2011). In maize seedlings, juglone treatment led to significant, concentration-dependent increases in *Gst1* transcript level (Sytykiewicz 2011). This induction was obvious in 4d old primary roots and coleoptiles of maize, while further treatment (permanent 6 and 8 days of juglone exposure) led to decline in *Gst1* gene expression. Our results show that in maize, CDNB conjugation by GST was elevated in 96 h old seedlings compared with 48 h, but this was irrespective of juglone treatment. In pea however, although control GST activity decreased with time, at 96 h it was elevated by juglone treatment. These activities have not, to our knowledge, been investigated in pea in response to juglone treatment. In maize, other authors (Sytykiewicz 2011; Mylona *et al.* 2007) have detected *GstI* transcript level up-regulation but GST enzyme activity was not measured. It has been shown that GSTs of different subunit compositions differ in activity and substrate specificity (Edwards 1996). The discrepancy between gene expression in previous research and enzyme activity in our experiments could be the consequence of the lack of proper subunit assembly or post-translational activation to form the active enzyme. Also, this could be caused by the use of different subspecies and varieties (Terzi 2008); crop cultivars and varieties can differ in response to juglone treatment, as many of them are selected for stress and pathogen resistance, which may be effected by different detoxification and antioxidant system components.

In general, in our experiments with pea, both QR and GST activities were elevated under juglone treatment. In maize, there was a transient elevation of QR activity in the microsomal fraction upon juglone treatment. Maize GST was elevated developmentally, not in response to juglone treatment. It might be that constitutive GST activity in maize is high enough to cope with stress caused by juglone under these experimental conditions. High GST activity has been found to be responsible for herbicide selectivity, tolerant crops having up to 20-fold higher GST activities than susceptible weeds (Hatton *et al.* 1999). Thus, high constitutive GST activity in maize radicles could be responsible for juglone tolerance, at least during early development.

CONCLUSIONS

In our experiments, maize showed constitutively higher activities of both detoxification enzymes, QR and GST. NADPH-dependent QR was increased during early seedling development in pea but after two days did not increase enough to match the corresponding activity in maize. After this time, constitutive GST activity decreased in pea and increased in maize. QRs responsive to juglone treatment were mainly microsomal, while soluble GSTs were elevated in pea under this treatment. Both enzymes were responsive to juglone treatment in pea, where constitutive activities were low, while in maize with high activities, GST was not responsive to treatment. We suggest that high constitutive activity of detoxifying enzymes is, at least in part, responsible for juglone tolerance.

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Botanica SERBICA

REZIME

Efekat juglona na klijanje semena graška i kukuruza, rani razvoj klijanaca i aktivnost enzima za detoksifikaciju

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Semena graška i kukuruza su tretirana juglonom i praćeno je klijanje, rastenje radikule i aktivnosti kinon reduktaza i glutation transferaza. Juglon je naftokinon koji se javlja kod biljaka roda *Juglans.* Spada u jednu od najčešće ispitivanih alelopatskih supstanci, ali mehanizam njegovog delovanja još nije do kraja razjašnjen. Grašak je biljka osetljiva na prisustvo juglona, dok se u pojedinim radovima kukuruz smatra tolerantnim na juglon. U ovom radu su dve ispitivane vrste pokazale različite odgovore u pogledu aktivnosti kinon reduktaze i glutation transferaze na tretman juglonom. Najveća razlika je u tome što su obe aktivnosti u kontrolnoj grupi značajno veće kod kukuruza nego kod graška. Ni solubilna kinon reduktazna ni glutation transferazna aktivnost nisu se menjale kod kukuruza pri tretmanu juglonom, dok su obe aktivnosti povećane kod tretiranog graška u odnosu na kontrolu. Povećane aktivnosti kod graška su ipak znatno niže od aktivnosti kod kukuruza, pa je moguće da je visoka konstitutivna aktivnost ovih enzima jedan od preduslova za tolerantnost na juglon.

Ključne reči: alelopatija, juglon, kinon reduktaza, glutation transferaza