Seed coat phytochemistry of both resistant and susceptible seeds affords some protection against the granivorous beetle *Callosobruchus maculatus*.

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Abstract-The seed coat lies at the interface between the internal structures of the seed and the external environment and thus represents a key arena in the study of seed-herbivore interactions. *Callosobruchus maculatus* is a cosmopolitan pest of legume seeds, and under post-harvest conditions, females interact directly with the seed testa prior to laying their eggs. Here we investigate the effect of chemical extracts of the seed coat of the resistant *Phaseolus vulgaris* and the susceptible *Vigna unguiculata* beans on egg laying preferences and larval development of *C. maculatus*. Seed coat extracts contained phenolic, glycoside and alkaloid compounds. Upon re-incorporation of extracts into artificial host beans it was found that that several seed coat extracts from both the resistant and susceptible varieties reduced female oviposition and disrupted larval growth and development. However, none of the extracts assayed resulted in complete ovipositional or developmental failure suggesting that complete resistance in *P. vulgaris* is derived from other physical or chemical properties of the seed

and/or seed coat that function either alone or synergistically. Further work is required to elucidate the importance of synergistic interactions between different physiological defence mechanisms on overall plant (seed) resistance.

Key Words: Kidney beans; Phytochemicals; Volatiles; Bioactivity; Bruchidae.

1. Introduction

High concentrations of nutrients in the endosperm make seeds an ideal source of food for a range of animals (Fenner & Thompson 2005). This in turn results in counter-selection on plants to evolve adaptations that reduce the likelihood of seeds being detected (Porter 2013) or consumed or digested (Rodgerson 1998). These defences are frequently found in the seed coat as this represents the interface between the internal structures of the seed and the external environment (Souza & Marcos-Filho 2001; Zeng et al. 2004).

The seeds of many legumes, for example the cowpea (*Vigna unguiculata*), form an essential element in the diet of human populations (Fatokun 2002). However, post-harvest losses to granivorous insects, especially bruchid beetles can be as high at 90% (Umeozor 2005). One of the most important bruchid pests is *Callosobruchus maculatus* (Coleoptera: Bruchidae). Under laboratory conditions, female *C. maculatus* readily lay their eggs on several species of host seed. However, the first instar larvae can only penetrate the testa of relatively few species (Janzen 1977), indicating the seed coat of many legume species affords protection against bruchid beetles.

A key methodological approach in understanding the mechanisms by which seeds protect themselves against herbivory has been to compare the physical and chemical profiles of resistant and susceptible seeds. For example, Silva et al. (2004) found little difference in the thickness or levels of cyanide in the seed coat of the resistant red kidney bean (*P. vulgaris*) and the susceptible cowpea. By contrast, they did find high levels of vicilin in the seed coat of *P. vulgaris*, suggesting that this protein affords protection against herbivory by

C. maculatus. However, the phytochemistry of the seed coat of *P. vulgaris* and *V. unguicuata*

differ in several other respects (Abdel-Sabour et al. 2010), rendering it difficult to pinpoint

the exact mechanism of phytochemical defense without bioassays of different seed coat

fractions.

The incorporation of allelochemicals into artificial seeds (Janzen et al. 1977; Shade et

al. 1986; Macedo et al. 1993; Soares et al. 2007; Hudaib et al. 2013) allows those chemical

groups that confer resistance to bruchid infestation to be identified. This method was first

used by Janzen et al. (1977) to study which phytochemical groups confer seed resistance to

C. maculatus. They found alkaloids and non-protein amino acids to be highly toxic to larval

development. Vicillins-like 7S storage proteins have been found in the testa of various

legumes and linked to seed resistance to herbivory (Oliveira et al. 1999; Moraes et al. 2000;

Souza et al. 2012), as have lectins, lectin-like α-amylase inhibitors and arcelin (Ishimoto &

Kitamura 1989).

However, seeds defend themselves with an arsenal of secondary compounds (Janzen

1977; Odeyemi et al. 2008) that sometimes work synergistically to reduce granivory (Bekele

& Hassanali 2001). Thus, assays of the toxicity of other phytochemical groups on the growth

and development of *C. maculatus* are necessary to understand more fully the mechanisms by

which seeds avoid herbivory and to identify potential targets for the development of resistant

cowpea cultivars (Srinivasan & Durairaj 2007). Here we report the results of phytochemical

screens of seed coat extracts derived from the resistant *P. vulgaris* and the susceptible *V.

unguiculata*, in conjunction with assays of their bioactivity against *C. maculatus*, using

artificial seeds.

2. Materials and methods

2.1 Insect cultures. The beetles, *Callosobruchus maculatus* (Coleoptera: Bruchidae), used in

this study were derived from Niamey, Niger (Eady et al. 2000) and have been under

laboratory culture on black-eyed beans (*Vigna unguiculata*) for approximately 14 years at 27°C and ~35%rh. Females used in the experimental protocols reported here were all originally virgin and approximately 48h post eclosion. Virgins of known age were collected by isolating egg-laden seeds in the individual wells of 25 cell repli-dishes. All females were mated to virgin males prior to being used in the experiments.

2.2 Seed coat extraction. Two kilograms of black-eyed beans (*V. unguiculata*) and 2kg of red kidney-beans (*P. vulgaris*) were soaked in distilled water for 30 and 45 min respectively to remove the seed coat. The seed coats were left to dry at room temperature before being milled into fine flour. Solvent extraction methods based on solvent polarity (Doughari, 2012) were used to extract different phytochemical groups from the seed coat flours. Where yield was expected to be low, Soxhlet extraction was applied (Jayaprakasha et al., 2001). Four solvents were used: 70% ethanol, 100% methanol, 100% acetone and 100% chloroform. For each solvent 100g of milled seed coat was added to 500ml of solvent and the extraction process continued until the solvent filtrate appeared colourless, indicating most soluble constituents have been extracted (Audu et al., 2007). Extracts were suction-filtered and evaporated to dryness under vacuum in a rotary evaporator (Rotavapor® R-210/R-215) at the boiling point of the solvent. The percentage yield for each extract was calculated as the weight of the dry extract divided by the initial dry weight of the ground seed testa. Dry extracts were kept at 2°C prior to use in bioactivity assays.

2.3 Volatile and phytochemical analysis of extracts. Volatiles from the different extracts were analysed using headspace injection; volatiles of different bean extracts were analysed by GC/MS (Shimadzu GC/MS-QP 2010 S) equipped with a SUPELCOW AX[TM10] capillary column (30m x 0.25mm; 0.25µm film thickness) with helium as the carrier gas at a flow-rate of 1ml/min. The oven temperature programmed from 50°C, hold 5min, 50 -240°C ramp at 5°C/min and 240°C hold on for 20min. The injector and detector maintained at 250°C.

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Headspace injection volume was 0.5ml and 4:1 split mode. The mass spectra were taken over the M/Z (35-500) range with an ionizing voltage of 70 eV. Identification of volatiles was performed by NIST / EPA / NIH 0.5 Mass Spectral Library search based on their Kováts retention indices. The Kováts retention indices for the temperature-programmed chromatography are calculated according to Kovats (1958).

2.4 Phytochemical screening tests and bioassays of seed coat extracts. Extracts from both legumes seed coat were subjected to standard phytochemical screening methods (Harborne, 1973; Odebiyi and Sofowora, 1978; Edeoga et al., 2005). Artificial seeds were prepared as described by Hudaib et al. (2013). Briefly, decorticated *V. unguiculata* seeds were ground into fine flour, which was then mixed with water (20ml per 100g) to produce a dough that was subsequently shaped into artificial seeds. Seed coat extracts were added to the *V. unguiculata* flour at 2.5g extract /100g flour prior to making the dough. Artificial seeds containing no extract were the control.

Two females were allowed to oviposit for 3h on either 10 artificial control seeds or 10 artificial seeds loaded with one of the respective seed coat extracts (*i.e.* females not offered a choice of seeds). Each treatment was replicated 20 times. Following oviposition, the number of eggs laid on the seeds was counted. The egg-laden beans were incubated at 27ºC, 35% rh and the number and timing of emerged offspring determined. Upon emergence, virgin male offspring were isolated in Petri dishes and their longevity (at 27 °C and 35% rh) determined. To control for potential micro-environment effects, Petri dishes containing the virgin males were rotated daily. Male (offspring) elytra length, a proxy for body size (Wilson & Hill 1989) was recorded.

2.5 Statistical analysis. An ANOVA was used to determine the effect of treatment (extract) on female fecundity and offspring size (elytra length) whilst larval development time was analysed via a Kruskal-Wallis test using IBM SPSS version 21.0 (IBM Corp., Armonk, NY.).

Binomial logistic regression and Cox proportional hazards model (performed in R version 2.15.2) were used to analyse egg-to-adult survival and offspring longevity, respectively (Crawley 2002). Male elytra length was included as a covariate in the latter model.

3. Results

3.1 Phytochemistry of seed coat extracts. Phytochemical screening of the extracts (Doughari, 2012) revealed the seed coats of both *P. vulgaris* and *V. unguiculata* to be rich in a variety of phytochemicals (Table 1). No qualitative differences were detected, although extracts of *P. vulgaris* tended to yield more positive readings, indicating higher concentrations in comparison to *V. unguiculata.* Kovats retention indices (RI) revealed the presence of *D*-limonene in the methanolic extract of *P. vulgaris* (RI = 1197) whilst Dimethyl-disulphide (RI = 1073) and Hexanal (RI 1197) were detected in the methanolic extract of *V. unguiculata* (Fig.1). No volatiles were detected in the chloroform, ethanol or acetone extracts using this method.

3.2 Bioassays of seed coat extracts. The addition of seed coat extracts to artificial seeds significantly affected the number of eggs laid (*ANOVA*: $F_{(6,125)} = 12.74$, $P < 0.001$); the addition of methanol and chloroform extracts of both *V. unguiculata* and *P. vulgaris* reduced female oviposition (Fig. 2a). Larval development time was also affected by the addition of seed coat extracts to artificial seeds (*Kruskal-Wallis; Chi-Square* = 18.08, *df* = 6, *P* = 0.006; Fig. 2b); the addition of *V. unguiculata* chloroform extract and *P. vulgaris* ethanol extract resulted in the greatest reduction in development time (Fig. 2b). Logistic regression with a logit link function and binomial error (Crawley 2002) revealed a significant effect of treatment on egg-to-adult survival; Δ deviance = 34.91, *df* = 1, *P* < 0.05. The lowest egg-to-adult survival rates were associated with the polar (methanol and ethanol) extracts of *P. vulgaris* (Fig. 2c). Offspring size (male elytra length) was also affected by the addition of seed coat extracts: *ANOVA* $F_{(6, 124)} = 21.54$, $P < 0.001$ (Fig. 2d). Chloroform extracts derived

from the testa of both seeds resulted in small offspring as did the methanol extract of *P. vulgaris*. A *Cox proportional hazard model* revealed a significant effect of treatment (*Likelihood ratio* =13.9, *df* = 6, *P* = 0.03) but not male size on male longevity (Likelihood ratio = 7.49, *df* = 7, *P* = 0.38). Addition of the *P. vulgaris* chloroform extract resulted in elevated male longevity (Fig. 2e). Male lifespan (eggs to adult death) was affected by treatment; *Kruskal Wallis Test*; *Chi-Square* = 23.18, *df* = 6, *P* = 0.001. Beetles that completed their development on artificial seeds loaded with the methanol and ethanol extracts of *P. Vulgaris* tended to have a reduced lifespan, whilst those grown on artificial seeds containing the chloroform fraction of *P. vulgaris* had the longest lifespan, despite the adults being relatively small (Fig. 2f).

4. Discussion

The results suggest the phytochemical profile of the seed coat of both resistant and susceptible varieties of bean afford some protection against granivorous beetles. Extracts of both the resistant *P. vulgaris* and the susceptible *V. unguiculata* diminished the propensity of female *C. maculatus* beetles to lay eggs and impacted larval growth and development. The chloroform extracts of both *P. vulgaris* and *V. unguiculata* reduced female oviposition. These extracts contained phytosteroids, a class of triterpeniods that can interfere with the metabolic pathways in insects (Després et al., 2007) and that are known to deter oviposition in insect pests. For example, application of cucurbitacin to host leaves deterred oviposition in the European cornborer (*Ostrinia nubilalis*) and the beet armyworm (*Spodoptera exigua*) (Tallamy et al 1997). Ingestion of phytoecdysteroids has also been shown to reduce larval weight in the Indian meal moth (*Plodia interpunctella*) (Rharrabe et al., 2010), possibly via phytoecdysteroids acting as Na/K-ATPase inhibitors (Després et al., 2007). Despite the methanolic extracts of *P. vulgaris* and *V. unguiculata* having quite different phytochemical profiles, both exhibited oviposition deterrent properties. Saponins were detected in the seed

coat of *V. unguiculata* but not *P. vulgaris*. Saponins have known insecticidal activity, affecting the passage of food through the gut, the gut microflora, the uptake of sterols and membrane permeability (De Geyter et al 2007). However, the saponin containing extracts of *V. unguiculata* had little effect on larval growth and development (Fig. 2). Thus the type, concentration and profile of saponins (Ha et al. 2013) is likely to affect their bioactivity.

By contrast the methanolic extract of *P. vulgaris* contained phenolic compounds and terpenoids. Terpenoids are known to interact with the cholinergic system, the GABA system and the octopaminergic system, all of which could account for their insecticidal properties (Ratten 2010), whilst phenolic compounds have been shown to inhibit mitochondrial activity (Ratten 2010). That insect oviposition is deterred by the presence of terpenoids has been shown in the diamondback moth (*Plutella xylostella*) by Qui et al (1998). Phenolic compounds have also been shown to deter oviposition in the bruchid beetle *Callosobruchus chinensis* (Upasani et al 2003; Salunke et al 2005). The GCMS analysis of the methanolic extracts of both bean types revealed the presence of both D-limonene and dimethyl disulphide. D-limonene has known oviposition deterrent properties (Hudaib et al 2010) whilst dimethyl disulphide (a neurotoxin; Dugrarot et al 2003) has been shown to deter oviposition in the cabbage root fly (*Delia radicum*) (Ferry et al 2009). Thus the anti-oviposition properties of the methanolic extract could result from several phytochemical compounds within this extract.

Larval survival was lowest on the artificial seeds that incorporated the *P. vulgaris* methanolic extract with the most likely candidate antibiosis phytochemicals being the terpeniods and phenolics (see above). This extract also resulted in the smallest offspring (as determined by elytra length) providing further evidence that this extract was toxic to *C. maculatus* larvae. Offspring size was also affected by the addition of the chloroform extract to the artificial seeds. This extract contained steroids, some of which have anti-feedant

properties (Rharrabe et al., 2010; Jing et al. 2012). The 70% ethanolic extract of *P. vulgaris* also resulted relatively low egg-to-adult survival. This could be due to the high levels of tannins and saponins (see above) found in this fraction. Onuh and Onyenekwe (2008) found high levels of tannins and saponins in the seed coats of resistant *V. unguiculata* cultivars. The addition of the chloroform extract of *P. vulgaris* to artificial seeds resulted in an increase in adult longevity despite causing a reduction in adult size at eclosion. This is surprising because size and longevity tend to be positively associated in *C. maculatus* (Eady et al. 2007). The enhanced longevity of these males could be a result of plant sterols being a limited essential resource for insects (Janson et al 2009) or through the reported anti-aging properties of some plant sterols (Tada et al. 2009). Alternatively, low doses of toxins could potentially modulate stress response pathways such that the body is primed to combat other forms of environmental stress (hormesis), resulting in increased longevity (Gems & Partridge 2008).

Previously, resistance to *C. maculatus* infestation has been shown to be due to the presence of vicilin-like 7S storage globulins in the seed coat (Silva et al. 2004) and cotyledon of *P. vulgaris* (Macedo et al. 1993). Here, we show that several secondary metabolites, present in the seed coats of both resistant and susceptible varieties of beans, can have a negative effect on the fitness of the seed parasite *C. maculatus*. Identification of the mechanisms by which plants defend themselves against pests is a key step in process of breeding resistant varieties of food legumes (Keneni et al. 2011).

Acknowledgments

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TABLE 1

Phytochemicals detected in the extracts of black-eyed bean seed-coat (BEB) and kidney-bean seed-coat (KB); + is positive, ++ is moderately positive, +++ is highly positive expressing the intensity of each phytochemical group in the extracts.

Phytochemical test	70% Ethanol		100% Methanol		100% Chloroform		100% Acetone	
	BEB	KB	BEB	KB	BEB	KB	BEB	KB
Saponin	++	+++	++					+
Tannins(Phloba)	+	++						
Phenolics	++	+++		++				
Terpenoids	+	+++		+++				
Steroids					+	++		
Glycosides(Cardiac)	+	++						
Flavonoids	++	+++		++				
Alkaloids 1	++	+++		++				
Alkaloids 2				++				

Figure captions

Fig. 1 Total Ion chromatograms (TIC) of (a) 100% methanol extract of black-eyed beans and (b) 100% Methanol extract of kidney beans.

Fig. 2 Mean + SE a) fecundity, b) development time (days), c) egg-to-adult survival d) male offspring elytra length (mm), e) male offspring longevity (days) and f) male offspring

lifespan (days) of C. maculatus in relation to experimental treatment. BEBC, BEBM and BEBE represent the black-eyed bean chloroform, methanol and ethanol extracts whilst KBC, KBM and KBC represent the kidney bean chloroform, methanol and ethanol extracts.