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Seed flavonoids and anthocyanins as markers of enhanced plant defence in nodulated cowpea (*Vigna unguiculata* L. Walp.)

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ABSTRACT

Insect pests are a major constraint to cowpea production in Africa. Therefore the aim of this study was to identify cowpea material that exhibit greater pest resistance and grain yield for evaluation by farmers. About 45 cowpea genotypes were assayed for flavonoids and anthocyanins with the objective of quantifying the levels of these phenolics in seed extracts as markers for effective plant defence. The results revealed significant differences in the concentration of flavonoids and anthocyanins in seed extract. Farmer varieties such as Sanzie, Bensogla and Omondaw exhibited much higher levels of phenolics compared with improved genotypes like ITH98-46, TVu1509 and IT93K-452-1. When planted in the field in Ghana and Tanzania, the genotypes that had high concentrations of flavonoids and anthocyanins in seed extracts (e.g. Bensogla, Omondaw and IT86D-2075) showed relatively lower infestation by thrips, pod-sucking bugs, aphids and alcidodes. Providing minimum protection with insecticide spray further showed that, genotypes with low infestation by thrips and pod-sucking bugs produced more grain yield without spraying, an indication of their natural resistance to these insect pests. Furthermore, farmer-selected varieties such as Sanzie, Bensogla, and Omondaw produced more grain yield without protection than their improved counterparts. These results showed that the higher the concentration of flavonoids and anthocyanins in cowpea seed extracts, the lower the insect pest incidence on seedlings raised from those seeds. Correlation analysis further confirmed a direct relationship between high flavonoids/anthocyanins in seed extracts and enhanced insect pest.

1. Introduction

Flavonoids and anthocyanins are two major classes of biologically active secondary metabolites that are important for seedling development and plant growth (Ndakidemi and Dakora, 2003). Their synthesis and accumulation in plant tissues serve various functions ranging from acting as signals in legume symbiosis with some Rhizobiales to protectants in plant defence (Ndakidemi and Dakora, 2003), and effectors of mineral solubilisation in plant rhizospheres (Dakora and Phillips, 2002). It has been shown elsewhere that cowpea (*Vigna unguiculata* L. Walp.) and Bambara groundnut (*Vigna subterranea* L. Verdc) genotypes release significantly different concentrations of flavonoids and anthocyanins when soaked in water or aqueous methanol, and that these molecules probably serve as chemical deterrents to attack by insect pests and pathogens during germination (Ndakidemi and Dakora, 2003). Earlier reports have in fact indicated that legumes defend themselves against insect pests and diseases using isoflavonoids and anthocyanins, either as protectant phytoanticipins or directly as therapeutic phytoalexins against invading pests (Dakora and Phillips, 1996; Dakora, 2003). Cowpea, common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.), respectively, use medicarpin, phaseollin, and glyceollin as phytoalexins against pathogens and insect pests (Dakora and Phillips, 1996; Dakora, 2003). Other examples include *Lonchocarpus nicou*, *Mundelia serica*, and *Pachyrrhizus erosus*, which use the isoflavonoids rotenone, munduserone, and pachyrrizone, respectively, as phytoalexins and insecticides against pathogens and soil-borne insect larvae (Fukami and Nakajima, 1971; Dakora, 2003).

In nature, however, legumes do not use single molecules for defence against insect pests and pathogens. Rather, they use an arsenal of biological ammunition present in tissues and organs such as seeds and roots to fight insect pests and diseases. These compounds include flavonoids, anthocyanins, alkaloids, terpenoids, peptides, amino acids, steroids and/or sugar acids (Ndakidemi and Dakora, 2003). Similarly, nodulating legumes use a potpourri of signal molecules such as flavonoids, anthocyanins, alkaloids, and sugar acids present in seed and root exudates to induce expres-

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sion of nod genes (Maxwell et al., 1989; Hungria et al., 1991; Dakora et al., 1993a,b; Gagnon and Ibrahim, 1998) in order to initiate nodule formation. So far, however, few studies have examined crude seed extracts as a source of both insecticides and phytoalexins for defence against seed pathogens and/or protection of young seedlings emerging from seeds. Furthermore, few data currently exist on the relationship between the mixtures of protectant molecules present in seed extracts (or tissues) and plant defence, just as little is known about the differences between farmer varieties (i.e. genotypes selected by farmers for various useful biological traits) and improved varieties (i.e. cultivars bred for various agronomic traits).

The aim of this study was (i) to measure concentrations of flavonoids and anthocyanins present in seed extracts of different cowpea genotypes as markers for identifying materials with effective defence potential, (ii) to assess the self-defence capacity of selected genotypes against insect pests under field conditions, and (iii) to test selected genotypes with greater defence potential in the field against agronomic practices such as intercropping and high planting density.

2. Materials and methods

2.1. Source of germplasm collected

Due to lack of information on cowpea genotypes with high insect pest and disease resistance, a project funded by the McKnight Foundation was launched in June 2003 in three African countries (namely Ghana, South Africa and Tanzania) with the aim of identifying cowpea genotypes with greater pest resistance and grain yield. In order to achieve this objective, one hundred and twenty six (126) cowpea genotypes were obtained from farmers, village markets, national programmes, and gene banks, in Ghana, South Africa and Tanzania. Cowpea material was also obtained from the International Institute of Tropical Agriculture in Nigeria which has the mandate for cowpea improvement. To establish baseline data, seed extracts from 45 cowpea genotypes (randomly selected) were then assayed for flavonoids and anthocyanins as it is known that legume seed phenolic compounds can enhance plant defence against insect pests and pathogens. In this study, we report the role of seed flavonoids and anthocyanins in cowpea defence.

2.2. Experiment 1: bioassay of seed extracts from 45 cowpea genotypes for levels of flavonoids and anthocyanins

Seeds of 45 cowpea genotypes with differing seed coat colors were randomly selected from the germplasm collected from various locations and institutions, and ground to fine powder (0.85 mm). About 10 g of ground seed powder (or 0.1 g of shoot) was weighed and mixed with 50 mL (or 10 mLs in case of shoot material in centrifuge tubes) of acidified methanol prepared from a ratio of 79:20:1 MeOH H_2O HCl. The mixture was incubated for 72 h in darkness for auto-extraction, filtered through Whatman paper Number 2 and absorbance of the clear supernatant measured spectrometrically at 300, 530, and 657 nm using acidified methanol as standard. Concentrations of flavonoids were measured at 300 nm and expressed as Abs g DM^{-1} (Mirecki and Teramura, 1984), while anthocyanin concentration in seed extracts was measured as $Abs_{530} - 1/3Abs_{657}$ (Lindoo and Caldwell, 1978) and expressed as Abs g DM^{-1}.

2.3. Experiment 2: field survey for pest resistance using minimum spraying on selected cowpea genotypes at Manga, Ghana

Of the 45 cowpea genotypes tested in Experiment 1, 25 were used in a minimum spray experiment with an insecticide under rainfed conditions at Manga in Ghana, and 33 in Tanzania in order to assess genotype susceptibility or resistance to insect pests. These experiments were conducted using a randomised complete block design with four replicates. Planting was done in January 2005 in Tanzania and in August 2005 in Ghana by placing seeds in drilled holes at the required planting distance. In Tanzania, cowpea was planted on 1.05 m × 4 m plots with row-to-row spacing of 35 cm and plant-to-plant spacing of 15 cm. Cowpea plots in Ghana consisted of 4 rows each 3 m long, with row-to-row spacing of 60 cm and plant-to-plant spacing of 20 cm. The treatments included protected (sprayed) plots and unprotected (unsprayed) plots. Protected plots received two sprays of lambda cyhalothrin (Karate 2.5 EC) at flowering and again at podding, while unprotected plots were only sprayed with water as control. During plant growth, records were taken on pest incidence under both protected and unprotected conditions. At early podding, further observations were done on randomly selected cowpea plants for pest resistance. In this study, thrips (*Megalurothrips sjostedti*) infestation was assessed using ten flowers randomly harvested per plot at 50% flowering (i.e. duplicate samples each consisting of 5 flowers). The number of pod-sucking bugs was also determined at early podding and at mid pod development. At harvest, the total number of pods per plot, including those damaged by pod-sucking bugs.

2.4. Experiment 3: evaluating five cowpea genotypes for pest resistance and phenolics under intercropping and high plant density

2.4.1. Background

In this study, five cowpea genotypes (namely, Bensogla, ITH98-46, Sanzie, TVu1509 and Omondaw) were selected from Experiments 1 and 2, and further tested in the field using intercropping and high plant density as treatments to assess pest attack and tissue accumulation of flavonoids and anthocyanins. Of the five cowpea genotypes tested, three (i.e. Bensogla, Sanzie and Omondaw) consistently showed high concentrations of flavonoids in seed extracts with potential for greater pest resistance, while the other two (i.e. ITH98-46 and TVu1509) exhibited low flavonoid levels in seed extracts with potential for low pest resistance.

2.4.2. Site description of Experiment 3

The study was conducted at the Agricultural Research Council (ARC) Nietvoorbij station in Stellenbosch, South Africa, during 2005 and 2006. The experiment was set up in summer under irrigation since cowpea growth is adversely affected by frost during winter rains. The site is located at 33°54′S and 18°14′E at an elevation of 146 m above mean sea level. The experimental site had a previous history of table grape cultivation with a moderate application of P fertilizer (80 kg ha^{-1} maxfos, 20% P). The pH and plant-available nutrients measured in the soil before planting in 2005 were: pH ($CaCl_2$) = 6.2 ± 0.03, P = 18.8 ± 1.8 mg kg^{-1}, K = 137.8 ± 4.8 mg kg^{-1}, Ca = 70.5 ± 1.8 mg kg^{-1}, and Mg = 16.6 ± 0.5 mg kg^{-1}.The same parameters measured in 2006 were: pH ($CaCl_2$) = 6.2 ± 0.03, P = 18.6 ± 1.8 mg kg^{-1}, K = 136.6 ± 4.8 mg kg^{-1}, Ca = 68.7 ± 1.8 mg kg^{-1}, and Mg = 15.5 ± 0.5 mg kg^{-1}.

2.4.3. Experimental design

The experimental treatments included five cowpea genotypes (i.e. Bensogla, Sanzie, Omondaw, ITH98-46 and TVu1509), two cowpea densities (83,333 vs. 166,666 plants per hectare), and two cropping systems (monoculture vs. mixed culture). A randomised complete block design was used with a 3-factorial arrangement. Four replicates were used for each treatment with a plot size of