

Phenolic content as an indicator of tolerance of cowpea seedlings to *Sclerotium rolfii*

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Abstract The role of phenolics in plant tolerance to pathogen infection is well documented. The objective of the present preliminary investigation was to study phenolic metabolites involved in the tolerance or susceptibility of cowpea (*Vigna unguiculata* Walp.) cultivars to *Sclerotium rolfii* Sacc. and to use their presence as a possible screening tool. Total, free acid, ester-bound and cell wall-bound phenolics of 10 cowpea cultivars were quantified. In healthy seedlings, the tolerant cultivars displayed the higher phenol content than the susceptible cultivars. In *S. rolfii* infected seedlings, the highest increase was found from 48 h after inoculation. The net effect of inoculation was a 630% increase in total phenolics (soluble and insoluble) in the stem of tolerant cultivars while the total phenolic content increased only by 212% in the stems of susceptible cultivars. Although, no significant difference ($P = 0.05$) was detected among cultivars, in terms of free acid phenolics, the amount of ester-bound and cell wall-bound phenolics significantly

increased, therefore demonstrating a similar trend to the one observed for the total phenolic content. These preliminary results showed that the presence of phenolics before and after *S. rolfii* infection may be used as a rapid screening method for detection of tolerance to *S. rolfii* damping-off and stem rot of cowpea.

Keywords Cinnamic acids · Phenolics · Phytoalexin · Resistance · *Sclerotium* · *Vigna unguiculata*

Sclerotium rolfii Sacc. is one of the devastating soil-borne pathogens with a wide host range causing diseases in several plants (Maurya et al. 2007). In a field trial in the Ouémé valley in the Republic of Benin, variation in susceptibility among cowpea cultivars to *S. rolfii* was detected and some were more tolerant, with lower disease incidence, producing higher yields, than others (Adandonon et al. 2005a). Jain et al. (2011) indicated that phenols are induced in most of the plant tissues undergoing tolerance responses due to non-host pathogen interaction, or undergoing defensive responses due to beneficial microorganism actions. Lignin for instance and its phenolic precursors are themselves toxic to pathogens (Basha et al. 2006) and their polymerization makes cell walls thicker and stronger, which restricts penetration of cell wall by virulent pathogens. Moreover, it was discovered that dark coloured seeds tend to be more tolerant to fungal attack from storage fungi compared to white or cream coloured seeds (Kritzinger et al. 2002). Brown cowpea seeds, containing a higher concentration of soluble phenolics, mainly

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flavonoids, were found to be more tolerant to *Colletotrichum dematium* (Pers. Ex Fr) Grove (Pakela 2003) and damping-off (Aveling and Powell 2005) than white or cream cultivars. To our knowledge, the tolerance mechanism involving an increase in phenolic compounds and/or production of phytoalexins (Morrissey and Osbourn 1999) is well documented. However, little is known about the phenolic content of cowpea associated with tolerance or susceptibility to *S. rolf sii* infection. The objective of the present preliminary research was to study the phenolic compounds deployed during cowpea resistance/tolerance to *S. rolf sii* damping-off and stem rot and determine whether their presence could be used as a rapid screening method for detection of tolerance.

A virulent isolate of *S. rolf sii* (IITA 408, International Institute of Tropical Agriculture, Cotonou, Benin) and 10 cowpea cultivars with known tolerance or susceptibility to the pathogen (Adandonon et al. 2005b) were included in the study: Five tolerant, Cameroon, Kpodji, Gboto, Sèwé and Tchawé daho; two intermediate, IT83D-326-2 and Delekinwa; and three susceptible, TVU 72–74, KVX-61-1 and Tchawé kpayo. In a previous study, Adandonon et al. (2005b) classified the cultivars as tolerant or susceptible based on the cultivars response to *Sclerotium* infection using a paper-based method and disease incidence in a two-year experiment in the field. In the paper-based method, cultivars with a growth rate of more than 2 mm/day at 12 days after inoculation included all known tolerant cultivars (PEDUNE-BENIN 1995). They were also those with the lowest disease incidence (less than 9%) in the field (Adandonon et al. 2005b). Cameroon, Kpodji, Gboto, Sèwé and Tchawé daho were classified as tolerant to *Sclerotium* infection. In contrast, TVU 72–74, KVX-61-1 and Tchawé kpayo, with growth rates of less than 0.2 mm/day at 12 days and disease incidences higher than 14%, were classified as susceptible and IT83D-326-2 and Delekinwa as intermediate. As a preliminary test, Tchawé kpayo (susceptible) and Cameroon (tolerant) were used to determine the time of phenolic induction in the stems of plants infected by *S. rolf sii*. All 10 cultivars were used in all subsequent experiments.

One 5-mm diameter disc of 4-d-old *S. rolf sii* culture on PDA was placed adjacent to wounded collar of 5-day old cowpea seedlings and incubated in the greenhouse at temperatures varying between 25 to 27 °C. At 0, 6, 24, 48 and 72 h after inoculation, five seedlings ($N = 5$) were uprooted and 6-cm long stem samples were separately

cut from each seedling collar upwards. There were three replicates of five seedling stem samples harvested five times after inoculation, totalling 75 seedling stem samples. The samples were frozen in liquid nitrogen, ground into a fine powder using a mortar and pestle, placed in respective Eppendorf tubes and stored in the dark until further use. For the chemical analysis, all reagents and solvents used were of analytical grade (AR). Standards were purchased from Sigma-Aldrich (Steinheim, Germany) and the Folin-Ciocalteu reagent (FCR) was obtained from Merck (Dusseldorf, Germany). The total dry weight (DW) of each of the subsamples (0.05 g) was weighed into 1.5 ml Eppendorf tubes. The samples were separately extracted with 1 ml of methanol/acetone/water (7:7:1 v/v/v) Extracts were centrifuged at 10^4 g for 1 min in a microcentrifuge 7200 G (Denver Instrumental Company, USA) and the supernatant was retained.

The amount of total soluble phenolic compounds from the 10 cultivars was determined colorimetrically (Du Plooy et al. 2009). Quantification of phenolics was carried out using a calibration curve of gallic acid and the cell wall-bound phenolic compounds determined following methods of Regnier (1994). The identification and quantification of the extract components from the 10 cultivars was conducted using Thin Layer Chromatography (TLC) The tolerant Cameroon cultivar was used for high-performance liquid chromatography (HPLC) analysis using a Hewlett Packard 1040 series II HPLC with a normal one-wave length detector (three phases pump). A MALsil C₁₈, 5- μ m reverse-phase analytical column (250 \times 46 nm, 5 μ m particle size, Chrompack, Separations, Johannesburg) was used together with a precolumn (4.0 mm ID, 150 mm long). Samples (20 μ l) were analysed using a gradient of two solvents ranging from 7% solvent A (acetonitrile, HPLC grade), 93% solvent B (0.1% aqueous orthophosphoric acid, pH 2.6) to 70% solvent A, in 45 min. The wavelengths of the detector were 280 and 320 nm. The chromatogram was analysed with Hewlett Packard HPLC software (Star, Dos series). For statistical analysis, the general linear model (GLM) was used under SAS System and mean separations were done using the Student Newman Keuls option.

In the preliminary test used to determine the time of phenolic induction in the stems of plants infected by *S. rolf sii*, the total phenolic content did not differ significantly between inoculated and uninoculated plants of Tchawé kpayo (susceptible) until 24 h after inoculation. In contrast, the total phenolic content in the inoculated

Cameroon cultivar was significantly ($P < 0.05$) higher than that in the inoculated Tchawa kpayo cultivar at 48 h. However, for both cultivars, the highest total phenolic content reached at 48 h in inoculated plants did not differ ($P = 0.05$) from that at 72 h, indicating that the time of maximum total soluble phenolic induction in the cowpea cultivars after *S. rolfii* infection is 48 h (Fig. 1).

Total phenolic (TP) extract results (Table 1) confirm that the 10 cowpea cultivars tested have a pre-existing total phenolic content that is equal to or more than 2.4 mg gallic acid/g DW. The five tolerant cultivars, namely Cameroon, Kpodji, Gboto, Sèwé and Tchawé daho, had significantly higher ($P < 0.05$) pre-existing phenolic content than the susceptible cultivars. The results showed a significant difference ($P < 0.05$) among cultivars in terms of the total phenolic compound present in the stem cells 48 h after inoculation with *S. rolfii*. Three significantly different ($P < 0.05$) groups were observed in terms of the total phenolic content (Table 1): the first group with total phenolic content greater than 19 mg gallic acid equivalent/g DW were the five tolerant cultivars; the second group with total phenolic content between 14 and 15 mg gallic acid/g DW were IT83D-326-2 and Delekinwa; the third and last group with total phenolic content less than 10 mg gallic acid equivalent/g DW consisted of the susceptible cultivars namely TVU 72-74, K VX-61-1 and Tchawé kpayo. The tolerant cultivars had the highest ester-bound and cell wall-bound phenolic values at 48 h (Table 2). Moreover, the cell wall-bound phenolic values for all tolerant cultivars at 48 h after inoculation were more than eight-fold that when uninoculated (Table 2). Analytical HPLC chromatograms showed five peaks [1 (rt = 12.5 min); 2 (rt = 17 min), 3 (rt = 34 min), 4 (rt = 33.6 min) and 5 (rt = 37.5 min)] at 320 nm corresponding to five compounds in the inoculated Cameroon cultivar samples (Fig. 2a) (with strong peaks) that were absent in the non-inoculated samples (Fig. 2a and b). No difference was found between control and inoculated samples at 280 nm.

The role of phenolic compounds in the host/pathogen interaction has been extensively reported and summarised in several review articles (Sarma et al. 2002; Maurya et al. 2007; Jain et al. 2011; Singh et al. 2014) and constitutive phenolics are known to confer resistance indirectly through activation of post-infection responses in the host (Harborne 1988). Several studies have shown that some phenolics are inhibitors

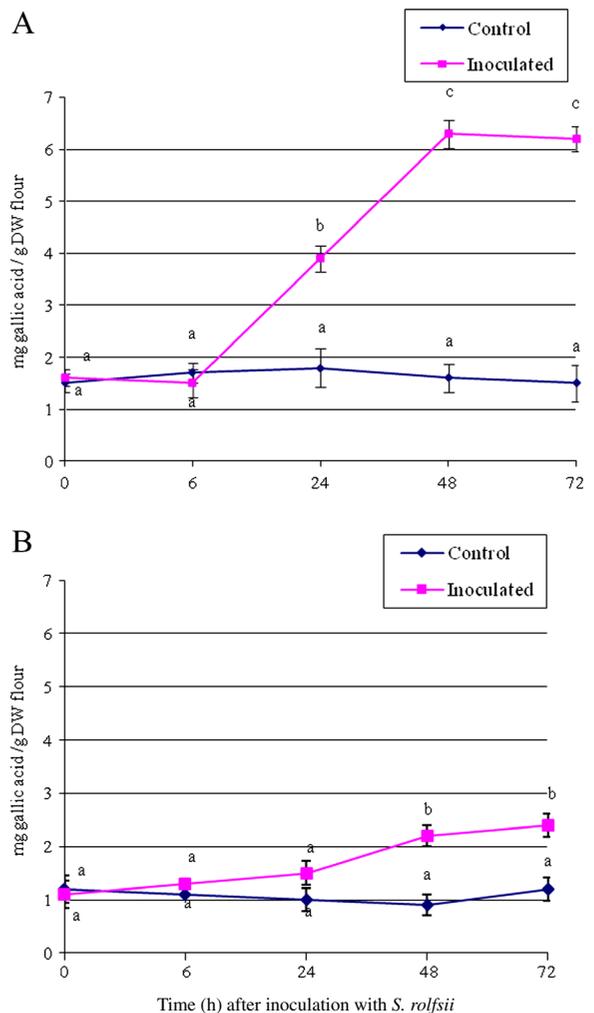


Fig. 1 Effect of time (h) after *Sclerotium rolfii* infection, on maximum total soluble phenolic induction in two cowpea cultivars: **a** Cameroon (tolerant); and **b** Tchawé kpayo (susceptible). Within a figure line, values not followed by the same letters are significantly different ($P < 0.05$) according to the Student Newman Keuls option. For Fig. 1a, LSD, P , and CV (%) values are 2.1; 0.037; and 7.5 when inoculated and 1.3; 0.34 and 5.2 when not inoculated. For Fig. 1b, the values are 0.4; 0.025 and 5.7 when inoculated and 0.84; 0.26 and 6.9 when not inoculated. Five-day old seedlings were inoculated using inoculum plug (of 4-day old culture of *S. rolfii* on PDA) placed adjacent to each wounded seedling collar. Total soluble phenolics were determined at 0, 6, 24, 48 and 72 h after inoculation. Controls were non-inoculated wounded seedlings

associated with non-host resistance whereas others are formed or increased in response to pathogen infection, and are considered to be an important component in the defence response of the host to the pathogen (Nicholson and Hammerschmidt 1992). Results in the present study showed that the total phenolic compound content in the

Table 1 Total phenolic compounds in the cells of cowpea seedlings 48 h after inoculation with *Sclerotium rolfsii*

Cultivars	Total phenolic compounds ¹ (mg gallic acid/g DW)	
	Control	Inoculated
Cameroon	3.6 ± 0.06 f	26.3 ± 0.13 d
Kpodji	3.2 ± 0.07 cd	19.8 ± 0.07 c
Gboto	3.5 ± 0.02 ef	19.4 ± 0.07 c
Sèwé	3.3 ± 0.07 de	20.4 ± 0.08 c
Tchawé daxo	3.1 ± 0.07 bcd	19.4 ± 0.08 c
IT83D-326	2.9 ± 0.05 b	14.1 ± 0.09 b
Delekinwa	3 ± 0.07 bc	14.3 ± 0.12 b
TVU72-74	2.6 ± 0.09 a	8.2 ± 0.06 a
KVX61-1	2.4 ± 0.06 a	8.4 ± 0.11 a
Tchawé kpayo	2.5 ± 0.06 a	7.8 ± 0.04 a
LSD	0.2	4.9
<i>P</i>	0.035	0.024
CV%	7.8	8.3

¹ Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ($P < 0.05$) according to the Student Newman Keuls option

cowpea seedling stems differed significantly ($P < 0.05$) among cultivars before and increased after inoculation

Table 2 Total soluble and insoluble phenolics, and their percentage compared to the total phenolic compounds in the cells of cowpea seedlings 48 h after inoculation with *Sclerotium rolfsii*

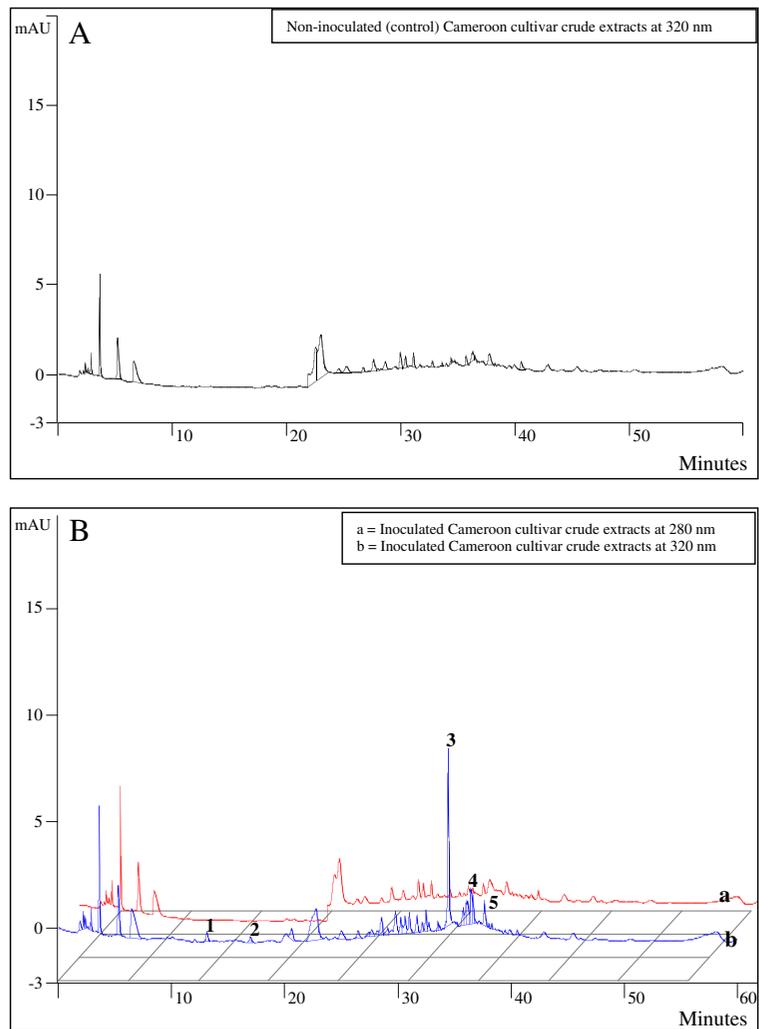
Cultivars	Total soluble phenolics ¹ (mg gallic acid/g DW)				Insoluble phenolics ¹ (mg gallic acid/g DW)	
	Free acid		Ester-bound		Cell wall	
	Control	inoculated	Control	Inoculated	Control	Inoculated
Cameroon	0.7 ± 0.07 a	1.6 ± 0.29 a (6.1%)	1.2 ± 0.31 a	4.3 ± 0.28 e (16.3%)	1.6 ± 0.13 e	20.4 ± 0.18 d (77.6%)
Kpodji	0.6 ± 0.06 a	1.5 ± 0.28 a (7.6%)	1.1 ± 0.14 a	3.7 ± 0.11 d (18.7%)	1.5 ± 0.04 de	14.6 ± 0.31 c (73.7%)
Gboto	0.4 ± 0.11 a	1.1 ± 0.09 a (5.7%)	0.9 ± 0.05 a	2.9 ± 0.07 c (14.9%)	1.6 ± 0.14 e	15.4 ± 0.24 c (79.4%)
Sèwé	0.7 ± 0.34 a	1.0 ± 0.04 a (4.9%)	0.8 ± 0.09 a	3.1 ± 0.04 c (15.2%)	1.3 ± 0.13 bc	16.3 ± 0.12 c (79.9%)
Tchawé daxo	0.8 ± 0.13 a	1.3 ± 0.25 a (6.7%)	1.1 ± 0.20 a	3.2 ± 0.19 c (16.5%)	1.4 ± 0.19 cd	14.9 ± 0.17 c (76.8%)
IT83D-326	0.7 ± 0.16 a	1.1 ± 0.05 a (7.8%)	0.9 ± 0.11 a	2.3 ± 0.10 b (16.3%)	1.3 ± 0.08 cd	10.7 ± 0.36 b (75.9%)
Delekinwa	0.6 ± 0.09 a	1.2 ± 0.22 a (8.4%)	1.1 ± 0.15 a	2.2 ± 0.23 b (15.4%)	1.2 ± 0.12 b	10.9 ± 0.01 b (76.2%)
TVU72-74	0.5 ± 0.06 a	0.7 ± 0.17 a (8.5%)	1.0 ± 0.19 a	1.7 ± 0.04 a (20.7%)	1.0 ± 0.12 a	5.8 ± 0.09 a (70.7%)
KVX61-1	0.7 ± 0.03 a	0.8 ± 0.02 a (9.5%)	1.0 ± 0.24 a	1.4 ± 0.32 a (16.7%)	1.0 ± 0.06 a	6.2 ± 0.09 a (73.8%)
Tchawé Kpayo	0.4 ± 0.09 a	0.8 ± 0.11 a (9.0%)	0.8 ± 0.07 a	1.3 ± 0.22 a (16.7%)	0.9 ± 0.20 a	5.7 ± 0.32 a (73.1%)
LSD	1.4	2.6	3.2	0.4	0.15	3.4
<i>P</i>	0.17	0.20	0.09	0.03	0.019	0.017
CV (%)	7.2	6.3	5.1	3.9	3.2	6.2

¹ Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ($P < 0.05$) according to the Student Newman Keuls option. Values in parenthesis within a row express the percentage of the phenolics compared to the total phenolic compounds in the stem cell

with *S. rolfsii*. A significant difference in total phenolic compound content was detected between the non-inoculated control and inoculated plants of the tolerant and susceptible cultivars only 24 h and 48 h after inoculation, respectively. Nandi et al. (2013) studied the hypersensitive reaction of cowpea to *S. rolfsii* finding that a greater accumulation of total phenol in cowpea plants was observed up to five days after inoculation. In addition, a strong relationship was observed between total phenol content and the tolerance/susceptibility status of the cultivars with the more tolerant the cultivar, the higher the phenolic content after inoculation.

The TLC analysis performed in the current study confirmed the presence of compounds belonging to the hydroxycinnamic family (data not shown). These results could indicate that, when *S. rolfsii* infects cowpea, several esters, especially cinnamic acids, are induced in the tolerant cultivars, which are able to withstand the infection caused by the pathogen. Sarma et al. (2002) also reported that phenolics including gallic, ferulic, chlorogenic and cinnamic acids were present in leaves, collar and roots of chickpea (*Cicer arietinum* L.) plants and the concentration increased in tolerant seedlings or was induced when plants were inoculated with *S. rolfsii* and treated with rhizobacteria. Similar results were

Fig. 2 HPLC analysis of a crude extract of a cowpea cultivar (Cameroon) 48 h after inoculation with *Sclerotium rolfsii*: Profile of the control sample at 280 nm (a), profile of the inoculated sample analysed at 280 and 320 nm (b). The graph b) showed five peaks that were absent in graph a): 1 (rt = 12.5 min); 2 (rt = 17 min), 3 (rt = 34 min), 4 (rt = 33.6 min) and 5 (rt = 37.5 min)]



found by Singh et al. (2014) who confirmed that accumulation of polyphenolic increases in both tolerant chickpea cultivars and microbial-treated susceptible cultivars infected by pathogens. Conducting research on the interaction between *S. rolfsii* and groundnut (*Arachis hypogaea* L.), Saraswathi and Reddy (2012) indicated that there was an increase in production of phenolic acids in the *S. rolfsii* diseased area of the groundnut hypocotyls compared to healthy ones. The authors further indicated that chromatographic data revealed 10 phenolic acids in healthy plants, whereas in the infected plants, six additional compounds were detected with the progression of the disease. In the case of susceptible cowpea cultivars, the production of a small amount of phytoalexins 48 h after inoculation appears to be the first attempt to stop the pathogen. However, the

concentration of these phytoalexins was not sufficient to be toxic to prevent the growth of the pathogen. It was reported that resistance occurs in plants when phytoalexins reach a concentration sufficient to restrict pathogen development (Nicholson and Hammerschmidt 1992; Kuć 1995) whereas formation of phytoalexins in a susceptible host following infection by a pathogen seems to be prevented by suppressor molecules produced by the pathogen (Bailey and Mansfield 1982). In our current study, two phytoalexins revealed by the HPLC analysis might be produced by the cowpea tolerant cultivar seedlings in response to *S. rolfsii* infection.

The content of soluble free acid found in the stem did not vary over cultivars, though it increased 48 h after inoculation. The low percentage of metabolites found in the stem is similar to previous data reported in wheat

(*Triticum turgidum* L.) (Regnier 1994). This low increase of free acid content in the stem after inoculation by the pathogen is not unusual and can be explained by the orientation of the metabolism to the esterification of these acids and further to an integration of the phenolics into the cell wall in order to protect the plant cells against the invasion (Fry 1979). After inoculation, the ester-bound and cell wall-bound phenolics were induced more than 1.5- and 8-fold, respectively, compared to the controls. These data are consistent with early reports on other crops (Regnier 1994; Chen et al. 2000; Mohammadi and Kazemi 2002) and accumulation of lignin and phenolic compounds in the host, in response to fungal attack is well known for strengthening the cell wall. The results in the present study showed phenolic compound, mainly ester-bound soluble and cell wall-bound phenolics are the major phenolics in cowpea cultivars involved in the tolerance of the plant against damping-off and stem of cowpea caused by *S. rolfisii*. These preliminary results show that phenolics present before and after *S. rolfisii* infection, could be used as screening parameters for tolerance to *S. rolfisii* damping-off and stem rot. Further research may provide more information on the characterisation and purification of the phytoalexins involved in the tolerance.

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