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Highly Variable Insect Control Efficacy of *Tephrosia vogelii* Chemotypes

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ABSTRACT: *Tephrosia vogelii* has been used for generations as a pest control material in Africa. Recently, two chemotypes have been reported based on the occurrence (*chemotype 1*) or absence (*chemotype 2*) of rotenoids. This could have an impact on the efficacy and reliability of this material for pest control. We report that *chemotype 2* has no pesticidal activity against *Callosobruchus maculatus* Fabricius (family Chrysomelidae) and that this is associated with the absence of rotenoids. We present a first report of the comparative biological activity of deguelin, tephrosin, α-toxicarol, and sarcolobine and show that not all rotenoids are equally effective. Tephrosin was less toxic than deguelin which was less active than rotenone, while obovatin 5-methyl ether, the major flavonoid in *chemotype 2* was inactive. We also report that in *chemotype 1* the occurrence of rotenoids shows substantial seasonal variation.

KEYWORDS: *Tephrosia candida, Callosobruchus maculatus, flavanones, rotenoids, deguelin, pesticidal plants, bruchids, cowpea weevil*

INTRODUCTION

Bruchid beetles are well documented, highly destructive pests of stored beans and cowpea seeds.[1] In large stores, they are effectively controlled using commercial insecticides and fumigation. These control measures are, however, not always appropriate, available or affordable to small-holder farmers who rely on a variety of pesticidal plants to control storage pests.[2] *Tephrosia vogelii* Hook f. (family Leguminosae) is a particularly important example since it is used widely as a pesticide[3,4] but also enriches soil quality through nitrogen fixation and as a green mulch,[5,6] so is particularly well suited for poorly resourced small-scale farmers.

It is widely assumed, though surprisingly not reported, that the insecticidal activity of *T. vogelii* is due to the foliar rotenoids of which tephrosin and deguelin are the most abundant according to earlier studies.[7,8] Although rotenone has been extensively studied, there is surprisingly little data directly evaluating the biological activity of the other major *T. vogelii* rotenoids tephrosin and deguelin against insects. This information is important since the biological activity of rotenoids can vary significantly.[9] There is additional confusion in understanding the mechanisms of insect toxicity in *T. vogelii* since a recent study[10] reported that the insecticidal activity of this species against bruchids was specifically not associated with rotenoids. Chemical variation within plant species can affect their use, for example, as medicines or as pesticides[11] and fully understanding which compounds are effective, where they occur in the plant, and at what times of the year is important for cultivation, harvesting, and application. Variation in the rotenoid content of *T. vogelii* is known,[12] but recent work has identified two distinct chemotypes within African populations of *T. vogelii* cultivated on farms in Africa. The first contains rotenoids, whereas the second contains no rotenoids at all but instead produces prenylated flavanones, flavones and flavanols.[8] The biological activity of these flavonoids is not known. Thus, the application of *T. vogelii* may be unreliable since up to 25% of plants cultivated in parts of Africa studied so far are the chemotype containing no rotenoids and therefore may be ineffective despite widespread use.[8]

The specific aim of this study was to determine if the insecticidal activity of *T. vogelii* chemotypes 1 and 2 differed, which compounds accounted for any insecticidal activity of *T. vogelii*, whether temporal or spatial chemical variation affected activity and sustainable use, and opportunities to optimize the application of *T. vogelii*.

MATERIALS AND METHODS

Plant Material. Leaf material of *chemotype 1* and *chemotype 2* of *T. vogelii*, as defined earlier, [8] used in all bioassays was collected from the World Agroforestry Centre (ICRAF) Chitedze, Malawi where it is cultivated as an experimental fallow crop for soil fertility[6] as well as for use as a pesticide.[3] Herbarium vouchers for this material *Stevenson 10071* K (ICRAF Acc No. 02976 - Kasungu, Malawi) = *chemotype 1* and *Stevenson 10072* K (ICRAF Acc No. 02972 - Madagascar) = *chemotype 2* have been deposited at the herbarium, Royal Botanic Gardens, Kew (K).

Branches of *T. vogelii* were cut and shade dried for two weeks, whereupon the leaves were stripped from branches and stored dry. The leaf material of the two chemotypes differed in its chemistry as reported earlier.[8] *Chemotype 1* was characterized by the presence of

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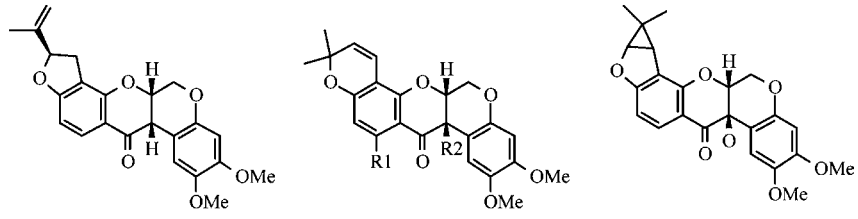
T. vogelii - chemotype 1 compounds

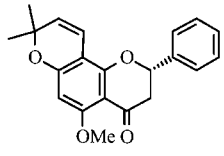


1 Rotenone

2 R_1 = H, R_2 = OH; Tephrosin
3 R_1 = R_2 = H; Deguelin
4 R_1 = OH, R_2 = H; α-Toxicarol

5 Sarcolobine

T. vogelii - chemotype 2 compounds



6 Obovatin-5-*O*-methylether

Figure 1. Chemical structures of the most abundant compounds identified in *T. vogelii chemotypes 1* and *2*.

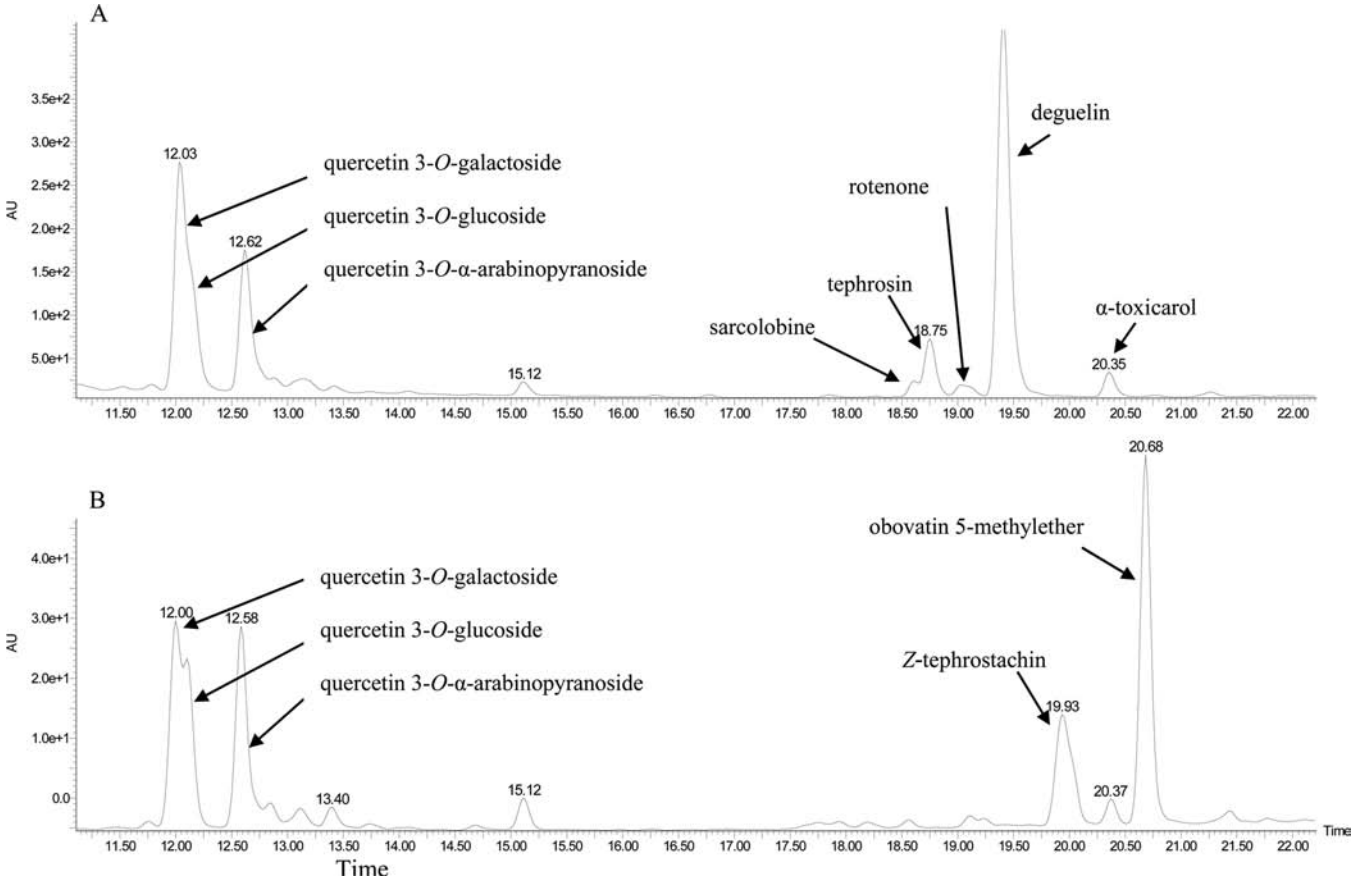


Figure 2. HPLC UV-max plot chromatograms from analyses of *T. vogelii chemotypes 1* and *2* showing differences in the chemical profile of flavonoid aglycones. All labeled aglycones are restricted to that chemotype. (A) *T. vogelii chemotype 1*, (B) *T. vogelii chemotype 2*.

rotenoids including rotenone (**1**), tephrosin (**2**), deguelin (**3**), α-toxicarol (**4**), and sarcolobine (**5**), whereas *chemotype 2* was characterized by the absence of rotenoids and the occurrence of prenylated flavanones including, obovatin 5-methylether (**6**) along with 6 other recently reported flavanones and the flavone *Z*-tephrostachin (Figures 1 and 2). Compounds **2**–**6** were isolated in sufficient quantities for biological evaluation against bruchid adults while rotenone was purchased (Sigma-Aldrich Ltd., Dorset, U.K.).

To determine potential geographic and seasonal variations in the occurrence of rotenoids in *T. vogelii chemotype 1*, plant material was collected at three times related to seasonal rainfall patterns (January,

March, June 2010) from 13 farm locations in Malawi (Table 1) for chemical analysis.

Table 1. Location of Samples of *T. vogelii* Collected in Malawi for Determining Chemical Constituents and Potential Spatiotemporal Variation in Presence/Abundance of Constituents

		location		
village	flower color	latitude south	longitude east	elevation
Chawaye	white	10°46′5.76″	34°1′35.76″	1212
Choma	white	11°17′48.66″	34°0′33.54″	1322
Kakoko	white	11°28′6.24″	33°58′4.86″	1306
Lumbwezi	purple	10°43′49.8″	34°2′12.54″	1304
Lunyangwa 1	white	11°25′39″	34°2′45″	1356
Lunyangwa 2	purple	11°25′39″	34°2′45″	1356
Luweni	white	10°44′5.1″	34°2′27.84″	1279
Malepula 1	white	11°27′14.28″	33°57′9.66″	1302
Malepula 2	white	11°27′16.14″	33°57′20.4″	1282
Maloto 1	white	10°45′39.24″	34°2′18.78″	1229
Maloto 2	purple	10°45′42.84″	34°2′19.56″	1221
Mkombezi	white	10°56′27.84″	33°58′42.06″	1031
Phwezi	white	10°54′7.02″	34°2′14.16″	1023

Chemical Analysis and Compound Isolation. All leaf material of *T. vogelii* (20 g) for analysis, compound isolation and to determine seasonal variation in chemistries was extracted by soaking in MeOH for 24 h at room temperature (21 °C) and the extract filtered through Whatman grade 1 paper. To determine the effects of detergents on the efficiency of extraction of biologically active compounds from leaves, 10 mg of *chemotype 1* was extracted at room temperature in 1 mL of either (i) water, (ii) 1% Tween 20, (iii) 5% Tween 20, or (iv) MeOH.

All extracts applied to HPLC columns were first passed through 0.45 μm nylon acrodisc filters. Chemical analysis of the filtered samples was carried out using a LC-MS detector (Waters ZQ Alliance System) with a 150 mm × 4.0 mm i.d. Luna C18(2) column with 5 μm particle size (Phenomenex, Macclesfield, U.K.) operating under gradient conditions, with A = MeOH, B = H_2O, C = 1% HCO_2H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30 °C and flow rate of 0.5 mL/min. The MS detector was fitted with an atmospheric pressure chemical ionization (APCI) source operated in positive mode under standard conditions and the source voltages tuned for optimal transmission of protonated rutin. Compounds were identified by comparison of their *in situ* UV-PDA and mass spectra during LC-MS analysis with authenticated standards as identified and reported in detail earlier[8] using the following spectral characters: rotenone (**1**) UV (LC-PDA) λmax nm, 301; (MS) m/z, 395.4 $[M + H]^+$; tephrosin (**2**) UV (LC-PDA) λmax nm, 272, 300, 314 sh; (MS) m/z, 433.4 $[M + Na]^+$; deguelin (**3**) UV (LC-PDA) λmax nm, 270, 301, 319; (MS) m/z, 395.4 $[M + H]^+$; α-toxicarol (**4**) UV (LC-PDA) λmax nm, 274, 297, 310; (MS) m/z, 411.2 $[M + H]^+$; sarcolobine (**5**) UV (LC-PDA) λmax nm, 301; (MS) m/z, 433.2 $[M + Na]^+$; obovatin 5-*O*-methylether (**6**) UV (LC-PDA) λmax nm, 270, 295, 348; (MS) m/z, 337.4 $[M + H]^+$.

For compound isolation, the MeOH extracts of each chemotype were taken to dryness using rotary evaporation, redissolved in 10 mL of MeOH (≡2 g plant material/mL), and passed through 0.45 μm nylon acrodisc filters prior to HPLC (Waters System 600E pump and 996 PDA detector). Isolation of compounds was carried out on a 250 mm × 10 mm i.d., 10 μm particle size Luna C-18(2) column (Phenomenex, Macclesfield, U.K.) operating under gradient conditions, with A = MeOH and B = H_2O; A = 25% at t = 0 min, A = 100% at t = 20 min, and A = 100% at t = 40 min; column temperature 30 °C and flow rate 4.7 mL/min. *Chemotype 1* yielded **2** (3.5 mg; t_R = 19.7 min), **3** (9.0 mg; t_R = 20.6 min), **4** (0.8 mg; t_R = 21.6 min), and **5** (0.8 mg; t_R = 18.3 min), while *chemotype 2* yielded **6** (15.0 mg; t_R = 23.0 min). All compounds were isolated initially for NMR as reported earlier[8] and identified prior to bioassay. Other compounds in *chemotype 2* could not be isolated in sufficient quantities for bioassays.

Insects. Whole organic cowpea seeds (*Vigna unguiculata* L.) were frozen at −20 °C for one week to kill any existing infestation and then stored at 4 °C to prevent further infestation. Three weeks prior to use, commodities were equilibrated to experimental conditions in a controlled temperature and humidity room (25 ± 1 °C, 60% rh, 4:10 light/dark cycle). The stock culture of *Callosobruchus maculatus* was kept at the Natural Resources Institute in Kilner jars containing 500 g of cowpea seed. The culture was maintained in controlled-environment facilities at 25 ± 1 °C and 60% rh, under a 14:10 light/dark cycle. Development time from reculturing to first emergence of adults was approximately 25 days. Reculturing took place as soon as possible after first emergence of adults from each generation, with 100−150 newly emerged adults being transferred into the new culture jar each time. Adults were sexed according to the shape of the abdomen and markings on the elytra.[13] Newly emerged insects of known age were obtained by sieving infested cowpea seeds to remove all adults, with subsequent emergences collected daily into separate containers marked with the date of emergence. Insects used for experiments were collected from the culture using an aspirator to minimize damage to insects.

Adult Bruchid Mortality in Cowpea Containing Dry Plant Material. Leaf material of both chemotypes was freshly ground as required using a commercial grinder (KIKA mill) (Janke & Kunkel GmbH & Co. KG, Germany) that produced a powder for direct use by admixing with cowpeas at different weight-to-weight ratios. To determine the potential effects of exposure time and concentration of crude leaf material of the two chemotypes of *T. vogelii* upon bruchid survival, small glass vials (10 mL) containing 5 g of cowpea seed (approximately 25 seeds) were used for each replicate, with five replicates per treatment containing dry powdered leaf (5.0, 2.0, 1.0, 0.2, 0.1 and 0.02% w/w). Ten unsexed adult insects, 2−3 days old, were added to each vial. Mortality of the adults was assessed at 24, 48, and 72 h.

Contact Toxicity of Crude Extracts and Pure Compounds. A similar bioassay was employed to investigate the contact toxicity of the different compounds **1**−**6** present in the two different chemotypes of *T. vogelii* alongside the corresponding crude plant extracts. A 10% crude extract was made using 10 g of powdered leaf extracted in 100 mL of acetone (Sigma-Aldrich Ltd., Dorset, U.K.) for 24 h, filtered (Whatman #1), and serially diluted to 1% and 0.1%. Compounds **1**−**6** were dissolved in acetone and diluted to 500, 100, and 10 ppm. To contend with the limited quantity of some compounds available from chemical isolation, each compound and crude extract was pipetted as a 75 μL aliquot into a glass vial (10 mL) and sealed. The vial was then turned manually to coat the whole internal surface of the vial with the test material. The vials were then opened and the acetone was allowed to evaporate (1 h) before introducing insects and closing vial with screw cap. Treatments were replicated 10 times unless otherwise indicated, and each replication contained five unsexed adults, 2−3 days old. Sarcolobine, α-toxicarol, and obovatin 5-methyl ether were replicated 5 times due to the limited amounts of compound. Preliminary experiments indicated that bruchids were quickly knocked down by the rotenoids, appearing to be dead, but still responsive if gently touched with a fine camel hair brush. If removed from the treatment, a small proportion of these insects would recover from the treatment. Therefore, insect responses were recorded after 72 h exposure as alive, dead or inactive. Knocked down beetles were regarded as inactive if a gentle touch with a fine camel hair brush stimulated movement of some legs or antennae but without them able to walk or oviposit.[14] Live adults were counted as those that moved actively or those that moved actively when induced to move with a camel hair brush. Insects were recorded as dead if they did not show any response after a gentle touch with a fine camel hair brush.

Effects of Rotenoids on Oviposition and F1 Emergence. The rotenoids previously reported to be most abundant (deguelin, tephrosin) in *chemotype 1* were evaluated for their effects on bruchid oviposition and F1 emergence using a similar methodology as used

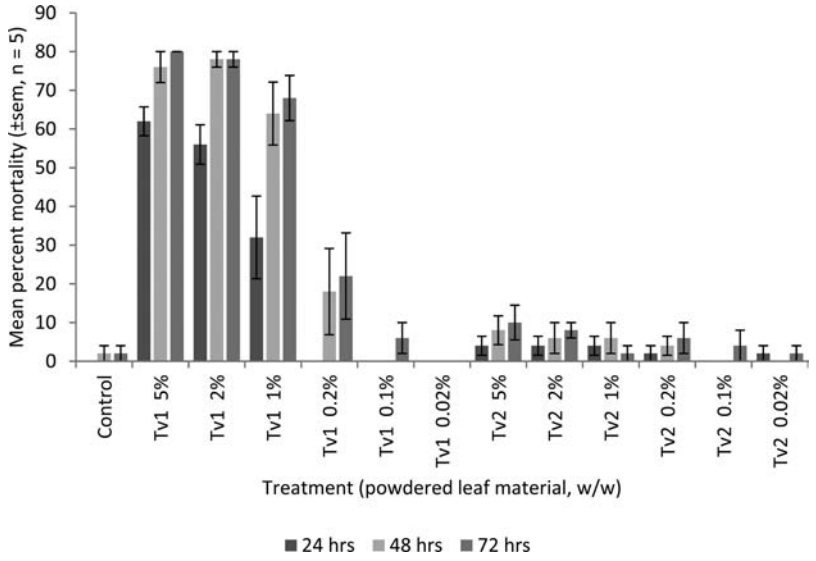


Figure 3. Effect of *T. vogelii* leaf powder admixed with cowpea seed (% w/w) on survival of *C. maculatus* adults over 24 to 72 h using two different chemotypes of the plant, Tv1 where rotenoids are present and Tv2 where rotenoids are absent.

with admixed crude leaf powders of *T. vogelii*. Their activity was compared with rotenone, reported previously to be a minor component from the plant. Compounds were applied to cowpea seed as an acetone extract in different concentrations (500, 100, and 10 ppm) by placing the cowpea seed in a round bottomed flask in a rotary evaporator to ensure even coating of the extract on to the seeds (5 mL extract per 25 g cowpea) and then air-dried on foil for 1 h. Treated cowpeas (5 g) were placed in a glass vial (10 mL) and 5 mated females 2−3 days old were introduced and each bioassay replicated 5 times per treatment. The insects were removed from each vial after 72 h and the number of eggs laid on the cowpea seeds was recorded. Starting 25 days later, the number of insects emerging in the F1 from the seeds was recorded over a two week period.

Data Analysis. Mean numbers of insect survival, mortality, oviposition and emergence were separated by ANOVA with SNK or LSD and dose responses were generated with the GLM regression features in XLSTAT version 2011.3.02. Spatiotemporal analysis by altitude and geographic reference was carried out by categorizing data in to three altitude ranges (<1200 m, 1200−1300 m, >1300 m) and three spatial clusters (North, Central, South) before carrying out an ANOVA to determine whether the abundance of any of the rotenoids were affected by their physical location or collection time.

RESULTS AND DISCUSSION

Adult Bruchid Mortality in Cowpea Containing Dry Plant Material. The exposure of adult bruchids to *chemotype 1* admixed with cowpeas led rapidly to high levels of mortality and concurs with earlier work testing this plant material against bruchids[10] (Figure 3, Table 2). Mortality was dose dependent. Increasing concentration and exposure period increased mortality of bruchids when exposed to *chemotype 1*. The highest concentration of the crude leaf powder, 5% (w/w), killed approximately 80% of the insects within 72 h, and regression analysis calculated a LC_{50} of 0.55% (w/w) for this exposure time. Exposure of the insects to *chemotype 2*, which contained prenylated flavanones and no rotenoids, did not significantly increase mortality compared to the control treatments, even at the highest concentration of 5% (w/w) plant material. The variation in bruchid mortality when exposed to crude powders of the different chemotypes of *T. vogelii* were likely due to the presence or absence of specific compounds in each chemotype, notably the absence of rotenoids accounting for the lack of insecticidal efficacy in *chemotype 2*. This contradicts the earlier assertion[10] that rotenoids were not responsible for the biological activity of *T. vogelii* against bruchids.

Table 2. Comparison of the Number of Surviving Adult *C. maculatus* When Exposed to Different Amounts of Powdered Leaf of *T. vogelii* of Two Different Chemotypes Admixed with Cowpea Seed

	mean survival (±SE)[a]		
treatment	24 h	48 h	72 h
Untreated control	10.0 ± 0.00 a	9.8 ± 0.20 a	9.8 ± 0.20 a
chemotype 1 0.02%	10.0 ± 0.00 a	10.0 ± 0.00 a	10.0 ± 0.00 a
chemotype 1 0.1%	10.0 ± 0.00 a	10.0 ± 0.00 a	9.4 ± 0.40 a
chemotype 1 0.2%	10.0 ± 0.00 a	8.2 ± 1.11 ab	7.8 ± 1.11 ab
chemotype 1 1%	6.8 ± 1.06 b	3.6 ± 0.81 cde	3.2 ± 0.58 cde
chemotype 1 2%	4.4 ± 0.51 c	2.2 ± 0.20 de	2.0 ± 0.20 e
chemotype 1 5%	3.8 ± 0.37 cd	2.4 ± 0.40 de	2.0 ± 0.00 e
chemotype 2 0.02%	10.0 ± 0.00 a	9.8 ± 0.20 a	9.8 ± 0.20 a
chemotype 2 0.1%	10.0 ± 0.00 a	10.0 ± 0.00 a	9.6 ± 0.40 a
chemotype 2 0.2%	9.8 ± 0.20 a	9.6 ± 0.25 a	9.4 ± 0.40 a
chemotype 2 1%	9.8 ± 0.20 a	9.6 ± 0.25 a	9.4 ± 0.40 a
chemotype 2 2%	9.6 ± 0.25 a	9.4 ± 0.40 a	9.2 ± 0.20 a
chemotype 2 5%	9.6 ± 0.25 a	9.2 ± 0.37 a	9.0 ± 0.45 a

[a]ANOVA with repeated measures for duration and Newman-Keuls (SNK) with 95% confidence where mean values followed by the same letter are not significantly different from each other.

Recommended applications for dry plant materials in stored products are typically around 5% (w/w),[15] but for many plant species particularly in larger stores, and especially those collected from wild sources, this quantity of material is impractical. The LC_{50} calculated for *chemotype 1* indicates that lower quantities of *T. vogelii* could be used effectively without compromising efficacy and while reducing exposure of users and consumers to plant material.

Contact Toxicity of Crude Extracts and Pure Compounds. Crude extracts of the two chemotypes showed similar toxicity trends (Figure 4, Table 3) as recorded for the corresponding powdered plant material bioassay above. However, the comparative levels of mortality were overall

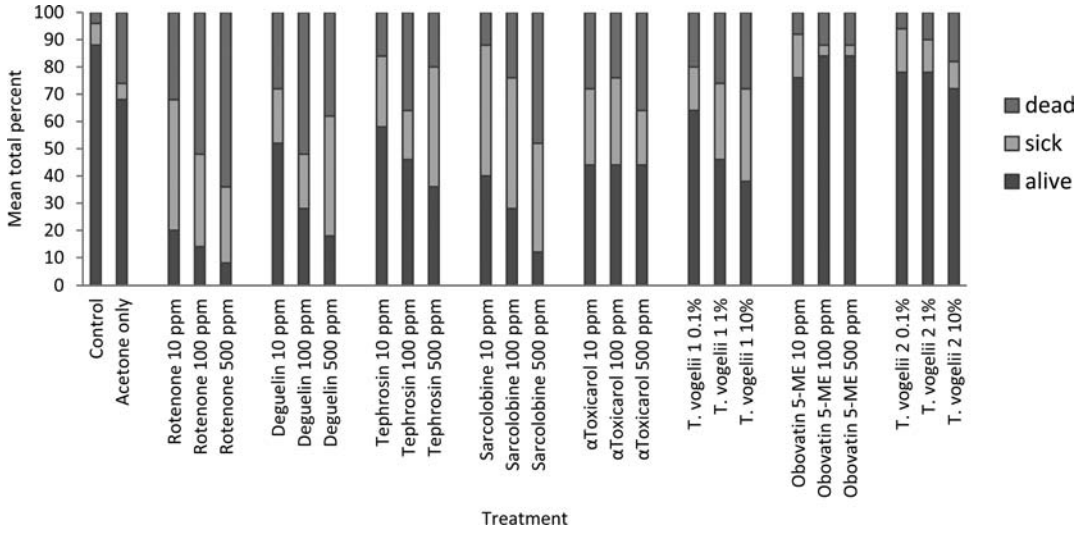


Figure 4. Proportion of *C. maculatus* found dead, inactive or alive after 72 h exposure to different concentrations of crude acetone extracts of *T. vogelii* (*chemotypes 1* and *2*) and pure compounds. Rotenone, deguelin, tephrosin, sarcolobine and *α* toxicarol are rotenoid compounds found in *T. vogelii chemotype 1*, whereas obovatin 5-methyl ether is the main flavanone compound found in *T. vogelii chemotype 2*.

Table 3. Comparison of the Number of Surviving Adult *C. maculatus* When Exposed to Different Rotenoid Compounds Present in *T. vogelii chemotype 1* and the Main Flavanone Compound in *T. vogelii chemotype 2* with Controls and Crude Extracts of Each *T. vogelii* Chemotype

treatment	mean survival (±SE)[a]	*N*
Control	4.4 ± 0.31 a	10
Obovatin 5-methyl ether 500 ppm	4.2 ± 0.20 ab	5
Obovatin 5-methyl ether 100 ppm	4.2 ± 0.20 ab	5
*T. vogelii*2 0.1%	3.9 ± 0.23 ab	10
*T. vogelii*2 1%	3.9 ± 0.23 ab	10
Obovatin 5-methyl ether 10 ppm	3.8 ± 0.37 ab	5
*T. vogelii*2 10%	3.6 ± 0.31 ab	10
Acetone only	3.4 ± 0.60 abc	10
*T. vogelii*1 0.1%	3.2 ± 0.33 abc	10
Tephrosin 10 ppm	2.9 ± 0.28 abc	10
Deguelin 10 ppm	2.6 ± 0.43 bcd	10
*T. vogelii*1 1%	2.3 ± 0.42 bcd	10
Tephrosin 100 ppm	2.3 ± 0.37 bcd	10
α-Toxicarol 10 ppm	2.2 ± 0.37 bcde	5
α-Toxicarol 100 ppm	2.2 ± 0.20 bcde	5
α-Toxicarol 500 ppm	2.2 ± 0.37 bcde	5
Sarcolobine 10 ppm	2.0 ± 0.32 bcde	5
*T. vogelii*1 10%	1.9 ± 0.32 cde	10
Tephrosin 500 ppm	1.8 ± 0.53 cde	10
Deguelin 100 ppm	1.4 ± 0.43 cde	10
Sarcolobine 100 ppm	1.4 ± 0.24 cde	5
Rotenone 10 ppm	1.0 ± 0.47 de	10
Deguelin 500 ppm	0.9 ± 0.28 de	10
Rotenone 100 ppm	0.7 ± 0.30 de	10
Sarcolobine 500 ppm	0.6 ± 0.40 de	5
Rotenone 500 ppm	0.4 ± 0.22 e	10

[a]Newman-Keuls (SNK) with 95% confidence where mean values followed by the same letter are not significantly different from each other.

lower for extracts compared to powdered plant. For example, the combined percentage of dead and inactive bruchids was 60% for the highest extract concentration of 10% (w/v) for *chemotype 1* (Figure 4). Extracts of *chemotype 2* demonstrated mortality that did not differ significantly from the control. Treatments evaluating contact toxicity of the pure compounds showed that rotenone was the most toxic compound of those tested (Figure 4, Table 3) but, since it occurred only as a relatively minor component in the leaves of *chemotype 1* (Figure 2) as reported earlier,[8] is unlikely to fully explain the biological activity of *chemotype 1*. Exposure of bruchids to deguelin, the most abundant compound in the crude extract, while less toxic than rotenone, resulted in significant mortality, while tephrosin was significantly less toxic than deguelin. Sarcolobine and to a lesser extent, toxicarol, were also toxic to bruchids, but their particularly low relative abundance in the plant would suggest they play a relatively minor role in the overall efficacy of *chemotype 1*. The LC_{50} for tephrosin was calculated at approximately 200 ppm, whereas the LC_{50} values for rotenone, deguelin, sarcolobine and toxicarol were below 10 ppm. The effect of obovatin 5-methyl ether against bruchids did not differ significantly from the control or the extract of *chemotype 2*.

Effects of Rotenoids on Oviposition and F1 Emergence. The effects of rotenone, deguelin and tephrosin on the oviposition and F1 emergence were both inhibitory and toxic (Figure 5). Few eggs were laid on cowpea seed treated with rotenone, and there was no adult emergence in the F1 even at the lowest concentration of rotenone tested (10 ppm). Oviposition was significantly higher on cowpea seed treated with deguelin than those treated with rotenone and higher still on cowpea seed treated with tephrosin. Owing to the low numbers of eggs laid and emergence, no significant differences were recorded between the different concentrations tested; however, in all cases the number of eggs and emerged adults was significantly lower than on the untreated control (ANOVA with SNK, $F = 6.6$, df = 10, $P < 0.0001$).

Despite extensive research on rotenoids against many biological systems including anticancer agents[16] and against other multicellular organisms of importance to man,[17,18] this is the first report of the biological activity of deguelin, toxicarol, sarcolobine and tephrosin compared with rotenone on an insect and importantly shows that not all rotenoids are equally effective. Thus, their relative abundance in different provenances or in material harvested at different times of the year is

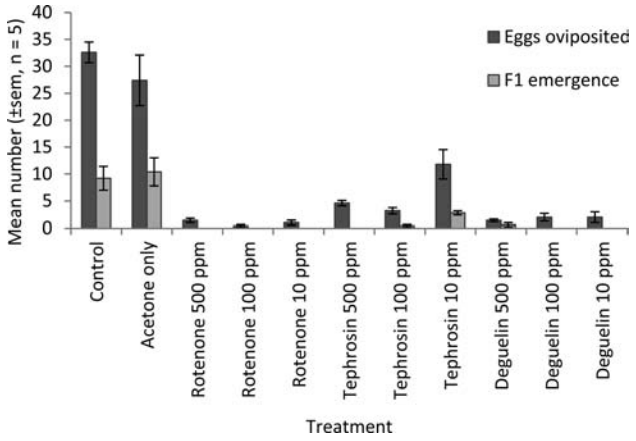


Figure 5. Observed changes in oviposition and emergence of *C. maculatus* when exposed to cowpea seed treated with three different rotenoids. Each replicate (n = 5) contained 5 females and 25 g of cowpeas. The total number of eggs was counted after 3 days and the total F1 emergence was counted over 2 weeks from the start of emergence (25 days from oviposition).

important in selecting elite material for promoting its use. We also demonstrate that the activity in *T. vogelii* is attributable to rotenoids and contradict earlier work suggesting they were not responsible for the activity. This is an important fact to establish categorically since the problems associated with the abundance of an inactive chemotype can now be overcome by a simple analysis prior to promotion.

The toxicity and deterrent effects of crude extracts reported above indicate that *T. vogelii* could be used as a liquid application in a similar manner to some commercial materials such as Actellic Super. This provides opportunities to optimize use, such as applying to storage sacks rather than directly to the grain, particularly where the grain is stored for food and not for replanting in the following season. This would separate potentially toxic plant chemicals from the stored product which is intended for food. Currently little effort is invested in training for safe use of pesticidal plants. The material available to most small holder farmers in Africa for the purpose of making extracts for application is water. Comparative analysis of the extraction efficiency of water carried out as part of the present study indicated that rotenoids are only sparingly soluble in water. Methanol (100%) extracted 10.0 times as much deguelin as water. The extraction of the *T. vogelii* plant material with Tween 20, however, increased the extraction efficiency of water for rotenoids. With 1% Tween 20, the extraction of deguelin in water was 2.8 times more efficient, and with 5% Tween 20, it was 4.9 times more efficient. This means that 5% Tween 20 extracted only half as much deguelin as 100% methanol but 5 times as much as water alone. Thus, if farmers use *T. vogelii* as an extract, they could improve insecticidal efficacy and reduce the required amount of plant material simply by incorporating a detergent such as liquid soap which is widely available in Africa. The incorporation of a liquid soap will also act as both surfactant and spreading agent for the plant extract and so will optimize application via the sprayer and further improve the evenness and efficiency of the application.

Spatiotemporal Variation of Rotenoids within *Chemotype 1*. Analysis of samples collected at three different times of year (Jan, Mar, Jun 2010) from 13 different farms in Malawi (Table 1) showed significant differences in seasonal occurrence of rotenoids and in the relative abundance of tephrosin, deguelin and rotenone (Figure 6, Table 4, ANOVA with LSD $P < 0.05$). There were no significant effects regarding the location (distance and altitude) of the samples, nor any significant interactions between season and location (ANOVA $F = 0.32$, df = 2, $P > 0.05$). On average, deguelin was more abundant than rotenone, and the least abundant of these three was tephrosin, contradicting earlier reports.[10] Deguelin and tephrosin production was seasonal, with deguelin highest in January and tephrosin highest in June. This inverse relationship in production was statistically significant as verified by linear regression ($r^2 = 0.14$, $F = 6.13$, df = 1, P = 0.018). Rotenone production was relatively constant across the three sampling times (Table 4). On the basis of these results and our bioassay data, we can argue that farmers should be harvesting *T. vogelii* leaves in January, when deguelin is highest in content. While

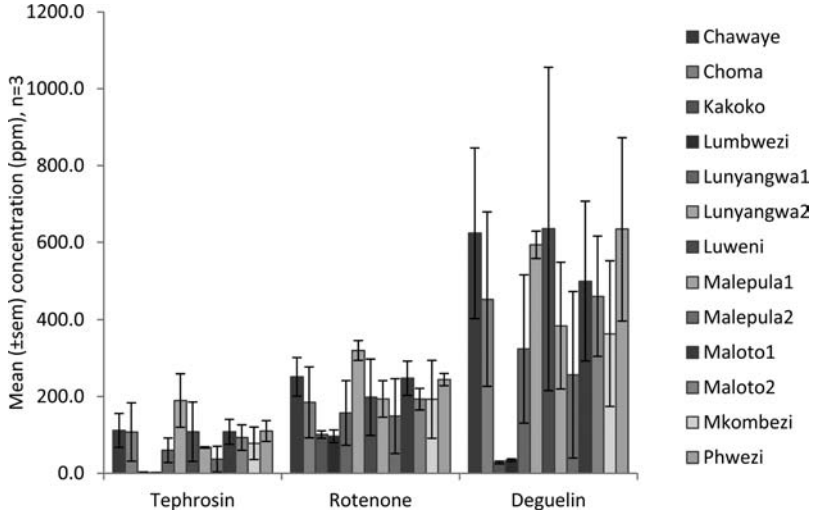


Figure 6. Mean production of rotenoids within leaves of *T. vogelii chemotype 1* collected from 13 farmsteads across northern Malawi at three different times of year (January, March and June 2010). ANOVA with Fisher's LSD showed mean rotenoid abundance within the leaves of *T. vogelii* was highest for deguelin followed by rotenone and tephrosin ($P < 0.05$). Relatively higher standard error among samples of deguelin is due to the strong seasonality of production (see Table 4).

Table 4. Effect of Season on the Production of Rotenoids in Leaves of *T. vogelii*

	mean (±SEM) concentration (ppm) from 13 locations[a]		
collection time	tephrosin	rotenone	deguelin
January 2010	48.3 ± 8.16 a	235.5 ± 26.36 a	665.4 ± 115.91 a
March 2010	75.0 ± 21.74 a,b	158.6 ± 36.37 a	267.2 ± 75.64 b
June 2010	124.2 ± 28.03 b	189.2 ± 26.53 a	288.1 ± 58.99 b

[a]ANOVA with Fisher's LSD and 95% confidence. Mean values in each column followed by the same letter are not significantly different from each other.

the occurrence of tephrosin is lowest at this time of year, it is less bioactive than deguelin or rotenone; hence, its contribution to the overall efficacy of *T. vogelii* is diminished by its relatively low abundance and efficacy vs. the much higher abundance and efficacy of deguelin.

The altitude of the growing locations sampled for *T. vogelii* varied by approximately 300 m (lowest to highest), and the distance between sample sites was maximally 100 km. Neither altitude nor distance was able to explain any of the chemical variability among sites or seasons (ANOVA $F = 0.32$, df = 2, $P > 0.05$). Although it was not significant, there was a noticeable trend in the data for all rotenoids measured to decline in abundance with every 100 m increase in altitude. Further sampling over a more diverse altitude range could help confirm the potential effects of altitude variation on rotenoid production in *T. vogelii* leaves. Similarly, there was some noticeable physical clustering of sample sites by their geo-reference; however, amalgamating samples into three clusters by distance did not help elucidate the observed variance. Other factors such as soil type and rainfall were not measured but may help explain some of the variation. Our data can also not exclude the prospect of inherent variation in the production of rotenoids within *chemotype 1* or, indeed, the presence of other distinct chemotypes. Notwithstanding the clear seasonality of production demonstrated, further research would be required to understand how other biotic and abiotic factors affect rotenoid production and the relative importance of elite material selection in propagating superior rotenoid producing *T. vogelii* stock material.

Rotenone was earlier reported to have the highest biological activity of 29 rotenoids as an inhibitor of NADH:ubiquinone oxidoreductase, while deguelin was significantly less active and tephrosin less active still.[9] This supports our findings regarding the relative activity of these compounds against bruchids. However, the conclusions drawn by Koona and Dorn [10] indicating the biological activity of *T. vogelii* was the result of compounds other than rotenoids are inconsistent with our findings and are surprising. Our results establish, to our knowledge for the first time, that deguelin and to a lesser extent tephrosin and rotenone are the toxic principles in *T. vogelii* to bruchids, and that the absence of rotenoids from *chemotype 2* renders it inactive and should not be promoted for pest control. The biological activity of *T. vogelii* is due to the presence of rotenoids, and the selection of elite material should be based on the relative occurrence of these compounds, particularly selecting provenances higher in deguelin rather than tephrosin. Although obovatin 5-methyl ether has reported antiplasmodial activity,[18] it has not been reported previously to have insecticidal properties and was shown here also to have no activity against bruchids. Thus, the dramatic differences in efficacy between the two *T. vogelii* chemotypes with rotenoids or prenylated flavanones are concurrent with existing scientific literature on the pesticidal efficacy of flavonoids.

Despite earlier reports on the variation in the relative abundance of rotenoids in *T. vogelii*,[19,20] there are no previous reports describing the importance of chemical variability in *T. vogelii* with respect to efficacy. Delfel et al.[12] reported some variation in the occurrence of rotenoids in *T. vogelii* but these were relatively minor compared to those reported for *Chemotypes 1* and *2*.[8] Cabizza et al.[21] evaluated cubé resin (chemically similar to *T. vogelii*) formulations on olives and asserted that the rotenoid composition of plant based pesticides derived from *Lonchocarpus* spp. was variable depending on the source material and the extracts used to prepare them and that this may affect their biological efficacy. While our data concur with this finding, we also warn that some material of *T. vogelii* can be completely ineffective. The use of *T. vogelii* in pest management needs to be provided by well-informed extension services who can advise on the most effective material to cultivate and how best to use it and this needs the support of good quality analytical facilities.

The use of rotenone as a botanical insecticide in Europe and North America has now largely ceased with the exception of a very few specific uses. The European Commission [EC] Directives on pesticide registration and use, that has required reregistration of all agricultural chemical products, resulted in withdrawal of rotenone albeit for commercial reasons rather than its toxicity. WHO classifies rotenone as moderately toxic with equivalent acute mammalian toxicity to many currently accepted products including pyrethroids. Rotenone is thought to have an oral lethal dose of between 300 and 500 mg/kg in humans.[24] Thus, for plant material with a total rotenoid content in dry leaf of around 1% by weight and assuming all rotenoids are equally toxic to rotenone (although most are considered less toxic according to Fang and Casida[9]), a 70 kg male would need to consume more than 2 kg of dry plant material in one sitting. Thus, exposure to plant material at concentrations typically used is in reality unlikely to present acute dangers to users in sub-Saharan Africa where its use is widespread still.[25] However, more recently the association between rotenone and Parkinson's disease has raised serious concerns about chronic effects associated with its use. This was initially proved through intravenous administration of the pure compound to rats under laboratory conditions.[26] However, over many decades of use, commercial rotenone products have presented little hazard to humans, and while not considered a cause of Parkinson's disease, a more recent study suggests chronic exposure to agricultural levels of rotenone in the USA was associated with Parkinson's disease.[27] Tanner et al.[27] argue that commercial synthetics such as permethrin with the same mode of action as rotenone, that is, mitochondrial complex I inhibition, may present similar risks for development of Parkinson's disease. In any event, all progress on the use of Tephrosia for African farmers must focus in large part on reducing exposure to the potential hazards while making its use more efficient. Use should be promoted as with other pesticides (appropriate

equipment) and use could be improved based on adapted applications such as treating bags not grain.

The determination of the specific components that are biologically active in the plant is important since it is now possible to select elite materials. Where farmers wish to use *T. vogelii* for pest control they should use *chemotype 1*. However, *chemotype 2* may be useful where farmers wish to use *T. vogelii* for its soil enriching qualities on fallow land adjacent to rivers or lakes since this will reduce the potential impacts of rotenoids on aquatic organisms.

Since *chemotype 1* and *2* were harvested in adjacent fields, the chemical differences between them are most likely inherent rather than environmental. The origins of *chemotype 2* are not clear, although it was assigned incorrectly to *Tephrosia candida* at the source institute, World Agroforestry Centre (ICRAF).[8] The ICRAF accession number suggests the original material was collected in Madagascar as *T. candida* and interestingly the flavonoid aglycone chemistry is similar to that in verified *T. candida* suggestive of earlier hybridization. However, based on current knowledge, it is not possible to substantiate this since the sequence data reported for both chemotypes differ equally from *T. candida*. [8]

As a consequence of this misnaming *T. vogelii* as *T. candida*, numerous papers published on the soil enriching properties of *T. candida* in Africa over the past decade have almost certainly, in fact, been evaluating *chemotype 2* of *T. vogelii* (e.g., Jama et al.[22]). Elsewhere, Lapointe et al.[23] reported *T. candida* to be more deterrent to the Diaprepes root weevil, *Diaprepes abbreviatus,* than *T. vogelii*. The promotion of this chemotype, along with the assumptions about its insecticidal activity, means that as much as 25% of the available *T. vogelii* in Malawi is ineffective for pest control uses. It is therefore critical where this species is promoted that extension services ensure the correct chemotype is used.

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