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A 2-ARYLBENZOFURAN FROM ROOTS OF *CICER BIJUGUM* ASSOCIATED WITH FUSARIUM WILT RESISTANCE

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Key Word Index—*Cicer*; Leguminosae; chickpea; Fusarium; wilt; isoflav-3-enes; 2-arylbenzofuran; cicerfuran.

Abstract—A new 2-arylbenzofuran, 2-(2′-methoxy-4′,5′-methylenedioxyphenyl)-6-hydroxybenzofuran (cicerfuran) has been isolated from roots of the wild chickpea *Cicer bijugum* by preparative HPLC. The structure was determined by one and two dimensional ^{1}H and ^{13}C NMR spectroscopy and Fast Atom Bombardment-Mass Spectrometry. The concentration of cicerfuran and a previously identified isoflav-3-ene, judaicin, was found to be greater in the roots of plants grown in the presence of the wilt pathogen *Fusarium oxysporum* f.sp. *ciceri* than in those grown in wilt-free soil. In addition, both cicerfuran and judaicin inhibited germination of the fungal spores when tested at natural concentrations. Cicerfuran was more potent than both maackiain and medicarpin, which are known antifungal phytoalexins in *Cicer*. The potential value of cicerfuran as a novel natural defence mechanism for the cultivated chickpea *C. arietinum* is discussed.

INTRODUCTION

The chickpea, *Cicer arietinum* L., is a major source of human and domestic animal food, particularly in the semi-arid tropics where its production is concentrated [1], and is considered to be the third most important pulse crop after dry beans, *Phaseolus vulgaris* L., and dry peas, *Pisum sativum* L. [2]. One of the major chickpea production constraints is Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceri* (Padwick) Snyd and Hans [3]. Emphasis has been placed on natural resistance to control this pathogen because other forms of management are inappropriate [4, 5]. Resistance mechanisms have been identified in some cultivated species to Fusarium wilt [6–8], but not all cultivars are resistant to all pathotypes, and reliance on the same mechanism may result in a high selection pressure in favour of tolerant pest populations, thereby compromising the durability of the resistance. In a continuation of our work to identify mechanisms of resistance to Fusarium wilt in chickpea, we have identified a new 2-arylbenzofuran, cicerfuran, from the roots of the wild species *C. bijugum*. This shows potent antifungal activity against spores of *F. oxysporum* f.sp. *ciceri* and is also induced in the presence of the pathogen.

RESULTS AND DISCUSSION

Isolation and structural determination

An unknown compound, **1**, was isolated from root extracts of *C. bijugum* by scale-up of a previously reported analytical method [9] to semi-preparative HPLC. The relatively long retention time of **1** compared to other isoflavonoids in roots of *C. bijugum* [9] suggested that it was likely to be an aglycone. Its UV spectrum was typical of either a pterocarpene or a 2-arylbenzofuran, although the prominent shoulder at 284 nm favoured the latter class of isoflavonoid [10]. The molecular structure of **1** was determined using standard ^{1}H and ^{13}C NMR experiments, including HMQC and HMBC. A summary of chemical shift assignments, together with the long range heteronuclear connectivities used to confirm the structure

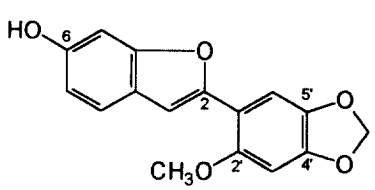

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Table 1. Summary of ^1H and ^{13}C NMR data for the 2-arylbenzofuran, **1** (δ in DMSO-d_6, 67.8 MHz, 37°)

C	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$ long-range connectivities (HMBC)
2	150.0		
3	104.4	7.10 (*s*)	111.2 (C-1′), 120.8 (C-4), 150.0 (C-2), 154.0 (C-7a)
3a	121.2		
4	120.8	7.37 (*d*, 8.6)	154.0 (C-7a), 155.4 (C-6)
5	112.1	6.71 (*dd*, 8.6, 2.3)	97.2 (C-7)
6	155.4		
7	97.2	6.92 (*d*, 2.3)	112.1 (C-5), 121.2 (C-3a), 155.4 (C-6)
7a	154.0		
1′	111.2		
2′	151.8		
3′	95.5	6.95 (*s*)	111.2 (C-1′), 141.0 (C-5′), 147.7 (C-4′), 151.8 (C-2′)
4′	147.7		
5′	141.0		
6′	104.7	7.34 (*s*)	141.0 (C-5′), 147.7 (C-4′), 150.0 (C-2), 151.8 (C-2′)
2′-OCH_3	56.4	3.91 (*s*)	151.8 (C-2′)
OCH_2O	101.4	6.05 (*s*)	

as 2-(2′-methoxy-4′,5′-methylenedioxyphenyl)-6-hydroxybenzofuran, is given in Table 1. The empirical formula of $C_{16}H_{12}O_5$ derived from NMR data was in agreement with the $[M]^+$ ion at *m/z* 284 recorded by FAB-MS. Compound **1** is thus a new 2-arylbenzofuran which we designate cicerfuran for ease of reference. The distribution of **1** is restricted to three species, *C. bijugum*, *C. judaicum* and *C. pinnatifidum* [9], and represents the first reported occurrence of 2-arylbenzofurans in *Cicer*, although several examples of this class of isoflavonoid have been found in other genera and species of Leguminosae including *Glycyrrhiza* sp. [11, 12], *Dalbergia odorifera* [13], *Sophora* sp. [14], *Onobrychis viciifolia* [15] and *Hedysarum polybotrys* [16]. It is also noteworthy that with the exception of *Dalbergia odorifera*, 2-arylbenzofurans occurred constitutively in all these sources, and were recovered from root material. This is in contrast to many earlier reports which cite the occurrence of 2-arylbenzofurans solely as phytoalexins [17].

Antifungal activity of Cicer *root compounds*

Germination of spores of *F. oxysporum* f.sp. *ciceri* was completely inhibited by treatment with **1** at a concentration of 250 μg ml^{-1}, and the effect at lower concentrations was dose dependent, as illustrated in Fig. 1. The inhibitory effect of **1** was more potent than that of both maackiain and medicarpin [8]. The co-occurrence of medicarpin and maackiain in cultivated chickpeas is considered to be the principal fungal defence mechanism [8, 18], but it is clear that **1** provides an additional resistance component in those species in which it is found. It represents, therefore, the first example of a 2-arylbenzofuran with biological activity against a pathogen specific to the plant of origin. General patterns of antimicrobial activity have, however, been reported for other 2-arylbenzofurans such as vignafuran, a phytoalexin from *Vigna unguiculata* which exhibits potent antifungal activity [17], and licocoumarone from the roots of *Glycyrrhiza uralensis* which has been shown to have antioxidant and antimicrobial activity [11].

Judaicin (7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene) and 2-methoxyjudaicin, two recently identified isoflav-3-enes. [19, 20] from the roots of *C. bijugum*, *C. judaicum* and *C. pinnatifidum* [9] were also tested for activity against spores of *F. oxysporum* f.sp. *ciceri*. The former compound was found to be less potent than both maackiain and cicerfuran in terms of inhibition of spore germination with 50% of spores germinating even when treated with 500 μg ml^{-1}. 2-Methoxyjudaicin, however, did not inhibit spore germination at any of the concentrations at which it was tested (Fig. 1).

The glucosides of maackiain and judaicin, which are the most abundant phenolic compounds in roots of these *Cicer* species [19, 20], did not inhibit germination of Fusarium spores. However, the enzymes required to deglycosylate maackiain 3-*O*-glucoside and maackiain 3-*O*-(6′-*O*-malonylglucoside) have been isolated from chickpeas [21], and production of the antifungal aglycone from the glycosides has been postulated previously [22, 23]. Storing the active aglycone as a glycoside may well provide a more accessible source of this defence component than a biosynthetic route from glycosides of formononetin and biochanin A [24].

Induced biosynthesis of Cicer *root compounds and fungal wilt resistance*

Many antifungal isoflavonoids including pterocarpans and 2-arylbenzofurans occur either as phytoalexins [17, 25], or may be induced in the presence of microbial infection if they already occur

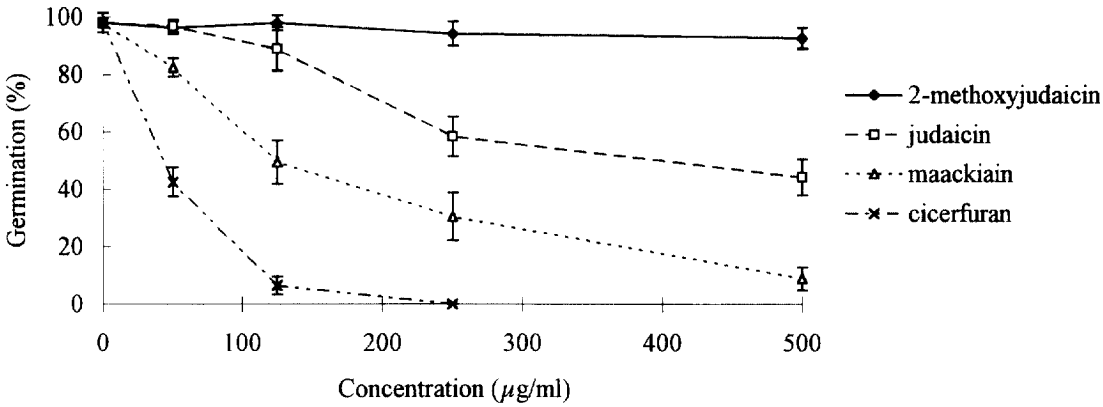

Fig. 1. Germination of *F. oxysporum* f.sp. *ciceri* after treatment with *Cicer* root compounds.

constitutively [8]. To evaluate the effects of the presence of Fusarium on the biosynthesis of judaicin, 2-methoxyjudaicin and **1**, four species, including the cultivated species *C. arietinum* and the three wild species *C. bijugum*, *C. judaicum* and *C. pinnatifidum*, were grown in either wilt-free soil or soil inoculated with *F. oxysporum* f.sp. *ciceri*. Those wild species which were reported previously to be wilt-resistant [26–28], did not show any signs of wilt after 28 days, whereas all *C. arietinum* plants growing in inoculated soil were wilting. All plants grown in wilt-free soil were growing normally.

The methanolic root extracts of wild and cultivated species from both sterile soil or inoculated soil were analysed by HPLC in order to evaluate differences between them. Authentic standards were obtained as described previously for judaicin, 2-methoxyjudaicin and maackiain [19, 20]. The concentrations of **1** and judaicin were significantly greater in the roots of all the wild species which had been grown in inoculated soil with the exception of judaicin in *C. bijugum* (Figs 2 and 3). It is likely that the increased production of these compounds represents a direct response to the fungus, as there were no differences in the soil in which these plants were grown other than the presence or absence of the pathogen. Furthermore, since **1** and judaicin occurred only in the resistant wild species and both compounds were biologically active against the pathogen at their natural induced concentration, it is reasonable to infer that they play a part in defence against the invading pathogen. Although the concentration of 2-methoxyjudaicin was also greater in the presence of the pathogen the value, if any, that this compound provides to plant defence is unknown since the compound was inactive against the pathogen at natural concentrations. Although the role of 2-arylbenzofurans as phytoalexins or induced compounds has been noted previously, for instance in the case of vignafuran, demethylvignafuran and isoperofuran [17, 29, 30], this is the first report of induction of isoflav-3-ene biosynthesis in the presence of a biological elicitor. This finding, coupled with the recent identification of the first isoflav-3-ene glycosides [19, 20], indicates a more important role for this class of isoflavonoid than has been suggested previously [31].

Conclusions

The present study has demonstrated that the 2-arylbenzofuran, cicerfuran, and the isoflav-3-ene

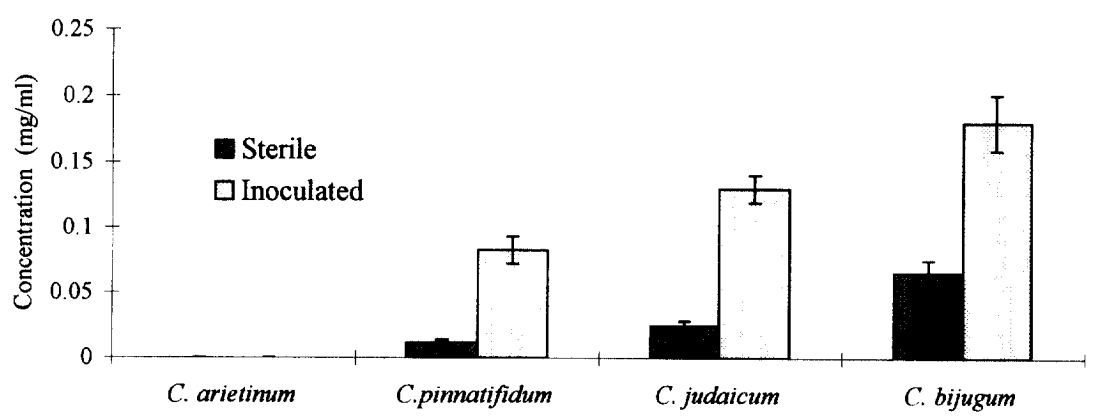

Fig. 2. Concentration (mg/g) of cicerfuran in roots of *Cicer* species grown in wilt inoculated or sterile soil.

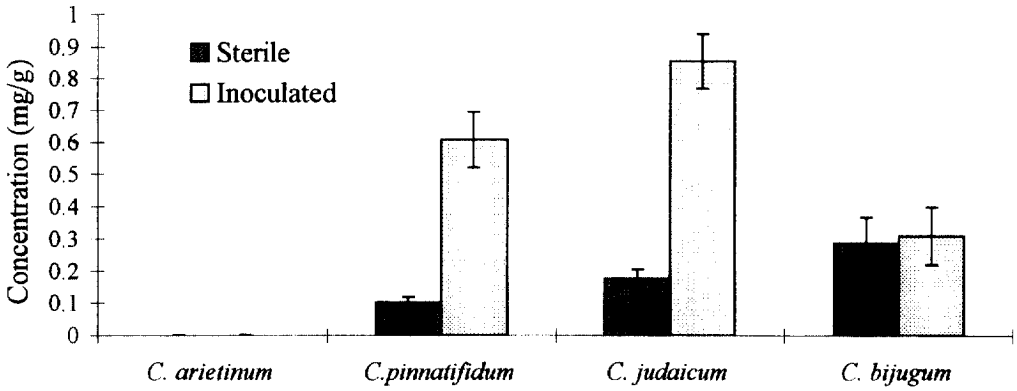

Fig. 3. Concentration (mg/g) of judaicin in roots of *Cicer* species grown in wilt inoculated or sterile soil.

judaicin, are potential defence compounds in wild relatives of chickpea. This is an important finding since it provides an additional tool for chickpea improvement and thereby the management of Fusarium wilt. While mechanisms exist in cultivated species [8], they are not effective against all races of the pathogen and additional mechanisms are required. If an additional mechanism can be exploited, the likelihood of the pathogen developing tolerance to pterocarpan defence compounds may be reduced substantially.

While wild species of crops are reservoirs of useful agricultural characters, their practical value is often compromised by sexual incompatibility between species and in particular between cultivated ones. This is the case with *Cicer* [32], although recent studies have highlighted the potential for genetic transformation (Williamson, B., personal communication) suggesting that cicerfuran may have value in chickpea improvement despite its absence from the cultivated species *C. arietinum*.

EXPERIMENTAL

Plant material

Seeds of *C. arietinum* (ICC4951), *C. bijugum* (ICCW42), *C. judaicum* (ICCW73) and *C. pinnatifidum* (ICCW88) were obtained from the Genetic Resources Unit of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) India. Plants were grown under greenhouse conditions in wilt free soil or soil inoculated with *Fusarium oxysporum* f.sp. *ciceri* race 1 (IMI361496) with 5 seedlings per pot and 5 pots per treatment. Soil was inoculated as described previously [8]. Root material was freeze-dried 28 days after sowing and milled.

Analytical and isolation procedures

Freeze-dried root material from approximately 5 g fresh wt. per species was extracted and analysed by HPLC as described elsewhere [9]. Compounds were detected by comparison of UV/VIS spectra and retention times with authentic standards [19, 20]. Analytical methods were scaled up to semi-prep. level using a Lichrocart 5 μm ODS column, 10 mm (i.d.) × 250 mm with a flow rate of 4.7 ml min^{-1}. The extract of *C. bijugum* roots (10 g) grown in wilt sick soil was injected in 150 μl aliquots directly onto the column and approximately 1.5 mg of **1** was collected manually.

General

NMR spectra were recorded at 400 or 270 and 100 or 67.8 MHz for ^{1}H and ^{13}C, respectively. Samples were dissolved in DMSO-d_6 with TMS as a primary reference. A temp. of 37° was used for all NMR experiments. FAB-MS (positive mode); 3-nitrobenzyl alcohol matrix.

Cicerfuran (2-(2′-*methoxy*-4′,5′-*methylenedioxyphenyl*)-6-*hydroxybenzofuran*) (**1**). UV λ_{max}^{MeCN} nm: 251 sh, 275 sh, 284 sh, 300 sh, 309 sh, 338, 354 sh. FAB-MS (positive) *m/z*: 284 [M]$^+$.

Fungal culture and bioassays

Spores of *F. oxysporum* f.sp. *ciceri* (IMI 361496) were transferred from potato dextrose agar to potato dextrose broth at 25° and allowed to grow. After 7 days, the spores were filtered through muslin and washed in sterilised water over a 3 μm Millipore filter to remove any auto-inhibitors to give approximately 1.0×10^6 spores ml^{-1}. The spore suspension was pipetted in 95 μl aliquots into the wells of a microtitre plate. The test compounds, which were insoluble in water, were dissolved in methanol and added to the spore suspension at 5 μl aliquots as described previously [8]. A 50 μl aliquot from each test well was transferred to a cavity slide and maintained at 100% relative humidity at 25°. Compounds were tested in the range 0–500 μg ml^{-1}. Each treatment was replicated five times. Control treatments comprised 95 μl spore suspensions with 5 μl of methanol. After 12 h, the bioassay was terminated by adding one drop of cotton blue stain. Five fields of view were chosen ran-

domly for each of the five replicates of each treatment and the mean percentage germination evaluated.

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