Accumulation of Phenolic Compounds and Phytoalexins in Sliced and Elicitor-Treated Cotyledons of *Cicer arietinum* L.

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Cicer arietinum, *Ascochyta rabiei*, Elicitor, Isoflavones, Phytoalexins

Upon slicing cotyledons of chickpea, *Cicer arietinum* L., accumulate the pterocarpan phytoalexins medicarpin and maackiain. Treatment of this tissue with an elicitor from the phytopathogenic deuteromycete *Ascochyta rabiei* (Pass.) Lab. greatly enhances accumulation of the pterocarpans and of other isoflavones and flavonoids. Isolation, chromatographic purification and structural elucidation by spectroscopic techniques of 16 phenolic compounds is described. Cotyledons induced for phytoalexin biosynthesis readily accumulate the isoflavones daidzein, formononetin, calycosin and pseudobaptigenin which are thought to be intermediates in pterocarpan formation.

Introduction

Chickpea (*Cicer arietinum* L.) is an important crop plant of dryland agriculture in Asian and African countries [1, 2]. The main constitutive phenolic compounds of this plant are biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) and formononetin (7-hydroxy-4′-methoxyisoflavone). These isoflavones (Fig. 1) have been reported to occur as aglycones, the 7-0-glucosides and, as most prominent constituents, the isoflavone 7-0-glucoside-6″-0-malonate esters [3, 4].

Upon infection with *Helminthosporium carbonum* [5], *Nectria haematococca* [6] or *Ascochyta rabiei* [7] chickpea plants accumulate the pterocarpan phytoalexins medicarpin and maackian (Fig. 1). Similarly, chickpea cell suspension cultures when treated with yeast extract [8] or fungal elicitors [9] rapidly synthesize these two rather widespread occurring pterocarpans [10, 11].

In addition to fungal infection or elicitor application [12, 13] phytoalexin accumulation in plant tissues can also be induced by the application of heavy metal ions [14–17], UV-light [18], yeast extract [8, 19] or various xenobiochemicals [20, 21]. In several cases formation of phytoalexins is accompanied by the additional accumulation of various other polyphenolic compounds including intermediates of phytoalexin biosynthesis [21–23]. Medicarpin and maackian biosynthesis has been investigated by feeding experiments with Cu^{2+}-treated seedlings of *Trifolium pratense* [24–27] and *Medicago sativa* [26, 27]. Furthermore, these investigations suggested that in case of medicarpin biosynthesis formononetin is first hydroxylated in position 2′ and then reduced to the corresponding isoflavanone vestitone. A subsequent reductive step will lead to the pterocarpan medicarpin. In maackiain biosynthesis an additional hydroxylation reaction in the 3′-position is required for the formation of the methylenedioxy moiety [24].

Recently we have presented enzymatic evidence for both the reductive step yielding an isoflavanone [28] and the hydroxylation reactions [29] involved in pterocarpan biosynthesis.

We have now continued our earlier studies [9] on the elicitor-induced accumulation of polyphenolic compounds in chickpea tissue. In sliced cotyledons treated with an elicitor preparation from *A. rabiei* the two phytoalexins accumulated together with the isoflavones formononetin and biochanin A and numerous other phenolic compounds. This paper reports the isolation, purification and structural elucidation of most of these compounds. Some of them are believed to be intermediates in the biosynthesis of medicarpin and maackiain.

Abbreviations: HPLC, high performance liquid chromatography; TMSi, trimethylsilyl; FG, formononetin 7-0-glucoside; BG, biochanin A 7-0-glucoside; FGM, formononetin 7-0-glucoside-6″-0-malonate; BGM, biochanin A 7-0-glucoside-6″-0-malonate; RT, retention time; GC-MS, gaschromatography-mass spectroscopy; E, elicitor; TLC, thin-layer chromatography.

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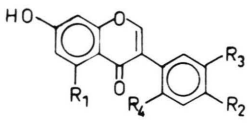


$R_1 = R_3 = R_4 = H$, $R_2 = OH$ Daidzein

$R_1 = R_3 = R_4 = H$, $R_2 = OCH_3$ Formononetin

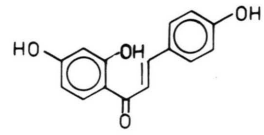
$R_1 = R_3 = H$, $R_4 = OH$, $R_2 = OCH_3$ 2'-OH-Formononetin

$R_1 = R_4 = H$, $R_3 = OH$, $R_2 = OCH_3$ Calycosin (3'-OH-Formononetin)

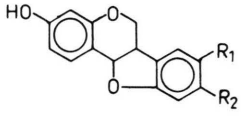
$R_1 = OH$, $R_3 = R_4 = H$, $R_2 = OCH_3$ Biochanin A

$R_1 = R_3 = OH$, $R_4 = H$, $R_2 = OCH_3$ Pratensein

$R_1 = R_4 = H$, $R_2 = R_3 =$ -O-CH_2-O- Pseudobaptigenin

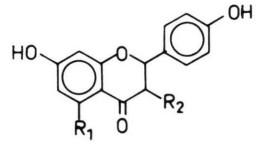


Isoliquiritigenin



$R_1 = H$, $R_2 = OCH_3$ Medicarpin

$R_1 = R_2 =$ -O-CH_2-O- Maackiain



$R_1 = OH$, $R_2 = H$ Naringenin

$R_1 = H$, $R_2 = OH$ Garbanzol

Fig. 1. Structures of phenolic compounds isolated from elicitor treated sliced cotyledons of *Cicer arietinum* L.

Materials and Methods

Plant material and treatment of cotyledons

Chickpea seeds of the Kabuli type were obtained from a commercial source. The seeds (800 g) were surfaced-sterilized first with 70% ethanol, then washed with water and finally soaked in 5% sodium-hypochlorite (w/v) for 15 min. After careful removal of the sterilizing agent with sterile destilled water the seeds were kept on wet paper in 200 ml Erlenmeyer flasks in the dark at 25 °C.

After 24 h the seeds were sliced [9] and incubated with an autoclaved solution (50 mg/5 ml water per 10 g seeds) of an elicitor of *Ascochyta rabiei*. The isolation of the elicitor was described in an earlier report [9].

The sliced cotyledons were incubated with the elicitor solution in large sterile petri dishes under moist conditions for 48 h. Control assays were incubated without the elicitor under the same conditions in sliced or unsliced form.

Extraction of the phenolic compounds

After incubation for 48 h the cotyledons were extracted with cold acetone and subsequently with methanol in a Waring Blendor as described [3]. Identical aliquots of all extracts were chromatographed by HPLC-methods as described by Köster *et al.* [3].

Isolation and purification of phenolic compounds

The extracts from the sliced and the elicitor treated cotyledons were first chromatographed on polyamide (MN-polyamide SC 6, Machery and Nagel, Düren, FRG). The column was eluted with a water/methanol gradient using solutions of the following composition: 100% water, 20% methanol, 40% methanol, 60% methanol, 80% methanol, 100% methanol, 100% methanol/1% ammonia. Compounds from all fractions were further purified by TLC, silica gel GF 254 (Merck, Darmstadt) with the following solvent systems.

S1: toluene : methanol : ethylacetate : petrolether (6:3:2:1);

S2: chloroform : isopropanol (10:1);
S3: dichloromethane : methanol (15:1);
S4: pentene : diethylether : acetic acid (75:35:3);
S5: formic acid : ethylacetate : methylethyl-ketone : water (10:50:30:10).

The phenolic substances were detected under UV light (+NH_3), or by spraying with Fast Blue Salt [30] and diazotized *p*-nitroaniline reagents.

Chemicals

All reference compounds were from the collection of the institute.

Identification of compounds

Biochanin A and formononetin together with their 7-0-glucosides and the 7-0-glucoside-6″-0-malonates were identified according to our previous reports [3, 4].

All other compounds were characterized by their UV-spectroscopic data using diagnostic reagents and a comparison with literature values [31]. Whenever possible TLC-cochromatography with reference compounds in 5 different solvents was performed. GC-MS analyses of the trimethylsilyl derivatives of phenolic compounds was performed as described [32]. Glycosides were hydrolyzed (2*N* H_2 SO_4) and aglycones isolated as usual for further spectroscopic analyses.

Spectroscopic data of those compounds which had hitherto not been isolated from chickpea cotyledons are given as follows.

Daidzin. UV: λ_{max} 256, 312 (sh) nm; λ_{max} ($NaOCH_3$) 256, 272 (sh) 320 (sh) nm; λ_{max} ($AlCl_3$) 258, 304 (sh) nm; λ_{max} ($AlCl_3$/HCl) 257, 303, 362 (sh) nm; λ_{max} (NaOAc) 256, 322 (sh) nm; λ_{max} (NaOAc/H_3BO_3) 254, 318 (sh) nm.

Naringin. UV: λ_{max} 283, 325 nm; λ_{max} ($NaOCH_3$) 245, 284, 360 nm; λ_{max} ($AlCl_3$) 308, 375 nm; λ_{max} ($AlCl_3$/HCl) 306, 375 nm; λ_{max} (NaOAc) 283, 329; λ_{max} (NaOAc/H_3BO_3) 283, 329 nm.

Garbanzol. UV: λ_{max} 274, 310 nm; λ_{max} ($NaOCH_3$) 250, 297 (sh) 334 nm; λ_{max} ($AlCl_3$) 265 (sh), 308, 343 (sh); λ_{max} ($AlCl_3$/HCl) 275, 310 nm; λ_{max} (NaOAc) 255 (sh), 282, 334 nm; λ_{max} (NaOAc/H_3BO_3) 275, 312 nm.

MS (TMSi) *m/e* (rel. int.): 488 (35), 473 (30), 400 (4), 280 (100), 208 (8).

Daidzein. UV: λ_{max} 248, 252, 260, 300 (sh) nm; λ_{max} ($NaOCH_3$) 258, 288 (sh), 328 (sh) nm; λ_{max} ($AlCl_3$) 248, 258, 260 (sh), 302 (sh) nm; λ_{max} ($AlCl_3$/HCl) 248, 258, 260 (sh), 302 (sh) nm; λ_{max} (NaOAc) 255, 310, 330 (sh) nm; λ_{max} (NaOAc/H_3BO_3) 262 (sh) 303 nm.

Calycosin. UV: λ_{max} 249, 260 (sh), 296 (sh) nm; λ_{max} ($NaOCH_3$) 255, 330 (sh) nm; λ_{max} ($AlCl_3$) 249 (sh), 260 (sh), 291 nm; λ_{max} ($AlCl_3$/HCl) 240, 248 (sh), 291 nm; λ_{max} (NaOAc) 255, 284 (sh), 329 (sh) nm; λ_{max} (NaOAc/H_3BO_3) 250, 286 (sh) nm.

MS (TMSi) *m/e* (rel. int.): 428 (100), 413 (24), 398 (95), 191/192 (30) [23].

Naringenin. UV: λ_{max} 287, 325 (sh) nm; λ_{max} ($NaOCH_3$) 245, 275, 325 nm; λ_{max} ($AlCl_3$) 274 (sh), 312, 374 nm; λ_{max} ($AlCl_3$/HCl) 272 (sh), 310, 370 nm; λ_{max} (NaOAc) 284 (sh), 324 nm; λ_{max} (NaOAc/H_3BO_3) 289, 322 (sh) nm.

Pratensein. UV: λ_{max} 262, 290 (sh) nm; λ_{max} ($NaOCH_3$) 270, 321 nm; λ_{max} ($AlCl_3$) 272, 312 (sh), 371 nm; λ_{max} ($AlCl_3$/HCl) 272, 312 (sh), 371 nm; λ_{max} (NaOAc) 269, 325 (sh) nm; λ_{max} (NaOAc/H_3BO_3) 263, 290 (sh) nm.

Pseudobaptigenin. UV: λ_{max} 241 (sh), 250, 262 (sh), 295, 345 (sh) nm; λ_{max} ($NaOCH_3$) 258, 297 (sh), 333 nm; λ_{max} ($AlCl_3$) 241 (sh), 248, 262 (sh), 296 nm; λ_{max} ($AlCl_3$/HCl) 241 (sh), 248, 262 (sh), 296 nm; λ_{max} (NaOAc) 259, 295 (sh), 334 nm; λ_{max} (NaOAc/H_3BO_3) 251, 262 (sh), 296 nm.

MS (TMSi) *m/e* (rel. int.): 354 (100), 339 (32), 209 (4), 146/147 (40) [38].

Unidentified compound. UV: λ_{max} 238 (sh), 278, 314 nm; λ_{max} ($NaOCH_3$) 248 (sh), 278, 346 nm; λ_{max} ($AlCl_3$) 288 (sh), 303, 360 nm; λ_{max} ($AlCl_3$/HCl) 285, 303, 360 nm; λ_{max} (NaOAc) 278, 314 nm; λ_{max} (NaOAc/H_3BO_3) 278, 314 nm.

MS (TMSi) *m/e* (rel. int.): 446 (6), 356 (18), 341 (22), 223/224 (22), 222 (100), 209 (4).

Isoliquiritigenin. UV: λ_{max} 255 (sh), 295 (sh), 358 nm; λ_{max} ($NaOCH_3$) 262 (sh), 280 (sh), 319 (sh), 425 nm; λ_{max} ($AlCl_3$) 258 (sh), 320, 381 (sh), 423 nm: λ_{max} ($AlCl_3$/HCl) 320 (sh), 376 (sh), 422 nm; λ_{max} (NaOAc) 278 (sh), 332, 393 nm; λ_{max} (NaOAc/H_3BO_3) 286, 380, 443, 476 nm.

Results and Discussion

Shortly after sowing cotyledons of chickpea start to accumulate the isoflavones biochanin A and formononetin together with their 7-0-glucosides and their 7-0-glucoside-6″-0-malonate esters [9]. The accumulation of pterocarpan phytoalexins can be induced in these tissues under strictly aseptic conditions by wounding of the cotyledons. Subsequent treatment of these sliced cotyledons with a crude elicitor obtained from the mycelium of the chickpea pathogenic fungus *Ascochyta rabiei* (Pass.) Lab. (syn. *Mycosphaerella rabiei* Kovachevski) leads to a dramatic increase in the level of the phytoalexins but also of numerous other aromatic compounds [9]. Sliced and elicitor treated cotyledons of chickpea were now used to isolate several phenolic compounds, especially various biosynthetic precursors of the pterocarpan phytoalexins medicarpin and maackiain.

The stimulatory effect of wounding and of subsequent elicitor treatment on polyphenol accumulation in chickpea cotyledons can readily be demonstrated by HPLC. Acetone/methanol extracts of germinated but uninjured cotyledons lead to a relatively simple HPLC-diagram (Fig. 2A) which shows the presence of the isoflavones formononetin and biochanin A together with their glucosyl conjugates. The conjugates FG, FGM and BGM were again shown to be the major phenolic compounds in the chickpea cotyledons [9] (Table I). Fig. 2B demonstrates the effect of wounding of the cotyledons on the accumulation of the isoflavones and various other polyphenols including the phytoalexins.

This treatment of cotyledons not only led to the appearance of several new compounds but also to an enhanced accumulation of formononetin, biochanin

Table I. Compounds isolated from elicitor treated chickpea cotyledons with their HPLC retention factors and their concentration depending areas of the integrated UV-signals measured at 261 nm. Control: values obtained from uninjured cotyledons. Data obtained with extracts from sliced cotyledons and sliced elicitor treated cotyledons indicate stimulation of polyphenol accumulation according to treatment.

R_t	compound	control	sliced cotyledons	sliced + E cotyledons
10.05	Daidzin	n.d.	3866	3513
11.15	Naringin	n.d.	3020	4644
15.38	Garbanzol	1960	8451	10860
18.56	FG	32114	48670	60840
20.09	Daidzein	2490	9141	10597
22.76	Calycosin	3617	32146	24414
23.43	FGM	16741	98626	95790
25.31	BG	8390	15856	13315
27.70	Naringenin	689	1804	4567
29.09	BGM	37194	81177	57086
31.36	Pratensein	n.d.	2595	3651
33.46	Pseudobaptigenin	525	3471	5501
34.46	Formononetin	1132	4356	11359
36.73	Maackiain	n.d.	582	1954
38.00	Medicarpin	n.d.	473	3261
39.76	not identified	n.d.	1700	4409
40.08	Biochanin	5265	6732	13822

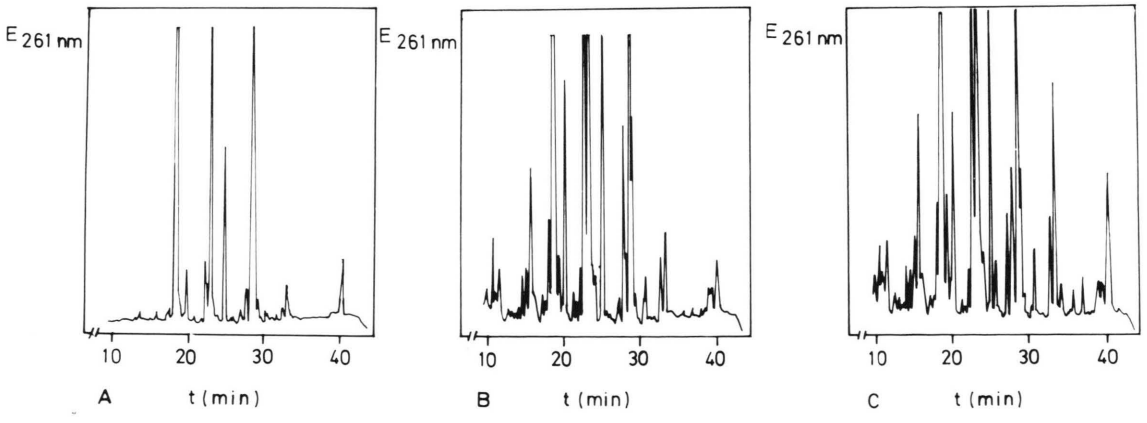


Fig. 2. HPLC diagrams obtained with extracts of chickpea cotyledons. A) Control assay with unsliced cotyledons; B) extract from sliced, and C) extract from sliced and elicitor treated cotyledons. All aliquots were measured under identical conditions at 261 nm.

A and their glucosyl conjugates (Table I). The HPLC-diagram of an extract obtained from sliced and elicitor treated cotyledons is shown in Fig. 2C. The figure documents that the elicitor has led to further stimulation of the accumulation of the phytoalexins and other aromatic compounds (Table I).

The accumulation of phytoalexins induced by wounding of the cotyledons may be interpreted by the action of endogenous elicitor material liberated from the plant cells [33, 34]. This elicitor material is possibly generated from pectic structures of the cell wall by hydrolytic enzymes [35]. Induction and stimulation of phytoalexin formation by wounding of plant tissues has also been observed in several other cases [34, 36] though it should not be considered as a common rule for all plants [37].

In order to study the accumulation of putative biosynthetic precursors of medicarpin and maackiain we have isolated and purified the phenolic compounds from sliced elicitor treated cotyledons. Extracts were prepared with acetone/methanol as described by Köster *et al.* [3, 4]. The phenolic compounds from nearly 800 g elicitor treated cotyledons were fractionated by polyamide column chromatography as described under "Materials and Methods". The fractions eluted with 20% and 40% methanol contained the isoflavone-glucosides of formononetin, biochanin A and daidzein (*i.e.* daidzin; Fig. 1) which were identified by UV-spectroscopy and co-chromatographie with authentic samples. Naringenin-glucoside (naringin) was also found in these methanol fractions.

Most of the phenolic aglycones were found in the fraction obtained after elution with 60% and 80% methanol. Identification of these constituents was again performed by co-chromatography and UV-spectroscopy using authentic samples and diagnostic reagents which resulted in the identification of the following products: biochanin A, formononetin, daidzein, pratensein, naringenin, isoliquiritigenin and the two phytoalexins medicarpin and maackiain (Fig. 1).

Two other isoflavones, calycosin and pseudobaptigenin (Fig. 1) were identified by UV and GC-MS-spectroscopy based on a comparison with literature values. The spectrum of the TMSi derivate of calycosin (3′-hydroxyformononetin) is characterized by a mass of 428 and the M^+ signal as the base peak. Therefore, this spectrum can be distinguished from the spectrum of 2′-hydroxyformononetin, an intermediate of medicarpin biosynthesis, because this latter compound is characterized by a weak M^+ and a predominant M^+-15 ion [22, 23]. The spectrum of the TMSi derivative of pseudobaptigenin showed a M^+-ion at 354 and a characteristic fragmentation pattern. The data from this spectrum are in line with the mass spectrum reported for the TMSi derivate of pseudobaptigenin [38]. Calycosin and pseudobaptigenin are considered to be intermediates in the biosynthesis of maackiain [24].

The dihydroflavonol garbanzol was identified by UV- and GC-MS-spectroscopy. This compound is a known constituent of chickpea though it normally occurs in very low amounts only [39]. Despite of good UV- and GC-MS-spectroscopic data the chemical structure of another aglycone (Table I, R_T 39.76 min, "not identified") has not yet been elucidated because sufficient amounts for NMR investigations could not be obtained.

Flavonols such as kaempferol, quercetin and isorhamnetin which were previously isolated from aerial parts of chickpea plants [40] could not be detected in the cotyledons.

The eluate obtained from the polyamide column with 100% methanol with a trace of ammonia contained the isoflavone-glucoside-6″-0-malonates FGM and BGM.

The aforementioned aglycones and conjugates can all be separated (Fig. 2A–C) and quantitatively determined by HPLC analyses. The areas of the integrated UV-signals of the HPLC diagrams recorded at 261 nm allow a comparison of the amounts of compounds isolated from the uninjured, the sliced and the elicitor treated cotyledons. Table I where the compounds are listed according to their R_T values presents such a comparison to demonstrate the elicitor caused stimulation of polyphenol accumulation in the chickpea cotyledons. In the untreated controls several compounds could not be detected. Mechanical wounding of the chickpea cotyledons led to an induction of the phytoalexin biosynthesis and to a pronounced increase of the total phenolic content. Subsequent treatment with an elicitor from *A. rabiei* causes phytoalexins and other phenolic aglycones to accumulate to an even higher level, whereas the accumulation of biochanin A and formononetin conjugates was not further effected.

Wounding and elicitor treatment not only influenced the biosynthesis of the aglycones and the

conjugates of 5-desoxyisoflavones (daidzein, formononetin, calycosin, pseudobaptigenin, medicarpin, maackiain) but also significantly stimulated production of the aglycones and derivatives of 5-hydroxyisoflavones (biochanin A, pratensein) and other flavonoids (garbanzol, naringenin). Examples for a parallel stimulation of the biosynthesis of 5-hydroxy and 5-desoxyisoflavones and of relevant biosynthetic enzymes has also been found in other plants [41–43]. Similarly, elicitor treatment of chickpea cell suspension cultures results in a pronounced stimulation of the chalcone synthase required for the formation of naringenin, biochanin A or pratensein though these compounds are not involved in the concomitantly induced biosynthesis of medicarpin and maackiain [44].

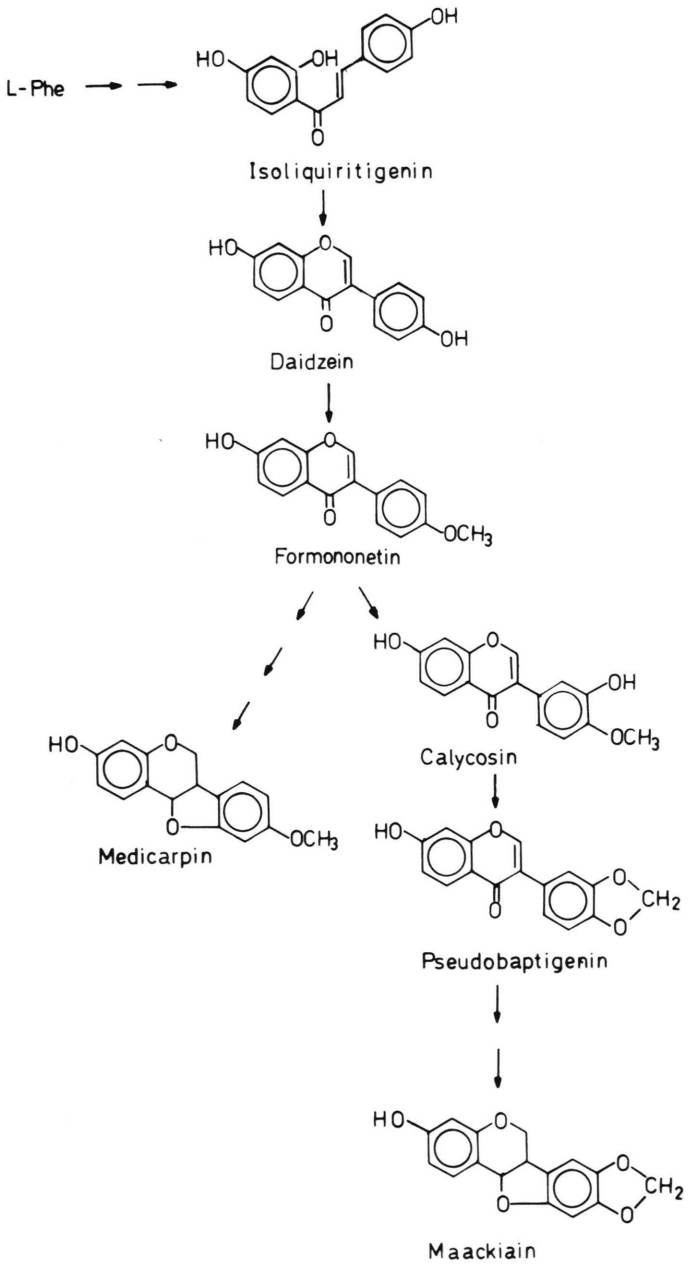


Fig. 3. Postulated biosynthetic sequence to pterocarpan phytoalexins *via* trihydroxychalcone and 5-deoxyisoflavones. All compounds were isolated from elicitor treated chickpea cotyledons.

The present knowledge of medicarpin and maackiain biosynthesis [45] and the recent characterization of enzymes involved in late stages of pterocarpan phytoalexin formation [28, 29] allow further interpretation of the observed accumulation of several compounds as listed in Table I. Isoliquiritigenin, daidzein, formononetin, calycosin and pseudobaptigenin are readily arranged in a biosynthetic sequence leading to maackiain (Fig. 3). Conversion of formononetin to medicarpin would require formation of 2′-hydroxyformononetin and 2′-hydroxydihydroformononetin (*i.e.* vestitone). Our HPLC analyses of extracts obtained from elicitor treated chickpea cotyledons have, however, shown that these two compounds accumulated, if at all, in only trace amounts insufficient for substantial purification. Such differences in the accumulation behaviour of 2′- and 3′-hydroxyformononetin remain to be elucidated.

In general, our results have again demonstrated that elicitor treatment of plant tissues may result in a pronounced activation of polyphenol biosynthesis and that so far unknown intermediates of phytoalexin formation tend to accumulate.

Acknowledgements

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