Elicitation of Pterocarpan Phytoalexins in Cell Suspension Cultures of Different Chickpea (*Cicer arietinum* L.) Cultivars by an Elicitor from the Fungus *Ascochyta rabiei*

Helmut Keßmann, Susanne Daniel, and Wolfgang Barz

Lehrstuhl für Biochemie der Pflanzen, Westfälische Wilhelms-Universität, Hindenburgplatz 55, D-4400 Münster, Bundesrepublik Deutschland

Cell suspension cultures of two chickpea (*Cicer arietinum* L.) cultivars, resistant and susceptible towards the chickpea pathogen *Ascochyta rabiei*, were compared with regard to elicitor-induced changes in phytoalexin and isoflavone accumulation. The elicitor was isolated from fermenter-grown mycelium of *A. rabiei* and it mainly consisted of glucose, mannose and N-acetylgalactosamin.

Time course and dose response studies on elicitor action demonstrated that the cell culture of the resistant cultivar ILC 3279 accumulated large amounts of the pterocarpan phytoalexins medicarpin and maackiain within 8 h. The cell culture of the susceptible cultivar ILC 1929 accumulated only small amounts of the phytoalexins some 12 h after elicitor treatment. Growth of the cell cultures and the accumulation of isoflavones and isoflavone conjugates were not altered by elicitor treatment except for a higher accumulation of formononetin 7-O-glucoside-6″-O-malonate in cell culture ILC 3279 subsequent to the maximum of phytoalexin accumulation.

Introduction

Biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) and formononetin (7-hydroxy-4′-methoxyisoflavone) are the main phenolic constituents of chickpea where they occur as aglycones, 7-O-glucosides and, predominantly, as the 7-O-glucoside-6″-O-malonates [1, 2]. The pterocarpans medicarpin and maackiain have been identified as the phytoalexins of chickpea [3, 4] (Fig. 1). The 5-deoxyisoflavone formononetin is a central intermediate in the biosynthesis of both phytoalexins [5−8] though constitutively accumulating formononetin is not considered to be a precursor for phytoalexin accumulation [9, 10].

The most important disease of chickpea ("Ascochyta blight") is caused by the deuteromycete *Ascochyta rabiei* (Pass.) Lab. [11]. After infection of plants with spores medicarpin and maackiain accumulated in the resistant cultivar ILC 3279 to much higher levels than in the susceptible cultivar ILC 1929
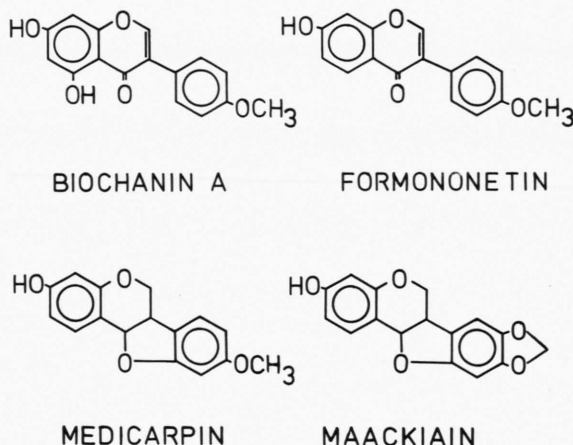

Fig. 1. Structures of isoflavones and pterocarpans mentioned in the text.

[12]. In contrast to the phytoalexins, biochanin A and formononetin together with their glucosyl conjugates accumulated in both chickpea cultivars in the same qualitative and quantitative pattern. Thus isoflavone accumulation was not affected by the infection process [12].

In cell suspension cultures established from these two chickpea cultivars biochanin A and formononetin together with their conjugates also accumulated

in the same pattern and in similar quantities as observed in the plants [10]. Transfer of such cells into a medium containing yeast extract led to a much higher accumulation of the phytoalexins in cell culture ILC 3279 than in cell culture ILC 1929 [10].

The present study was undertaken to characterize the biochemical responses of the cell cultures of chickpea cultivars ILC 3279 and ILC 1929 towards an elicitor from *A. rabiei* mycelium. Time course and dose response studies of elicitor action on phytoalexin and isoflavone accumulation as well as on several growth parameters will be presented.

Materials and Methods

Cell suspension cultures

Cell suspension cultures of the chickpea cultivars ILC 3279 and ILC 1929 were propagated in the dark on modified PRL-4c medium as previously described [10]. Elicitation experiments have clearly been described [7, 8, 10].

Cultivation of fungus and preparation of elicitor

Ascochyta rabiei (CBS number 534.65) was grown on chickpea seed meal-glucose medium according to previous reports [13]. The fungus was cultivated in 200 ml or 1000 ml Erlenmeyer flasks at 22 °C ± 2 °C under daylight on a rotary shaker (100−150 rpm) for 4−6 days. To obtain large quantities of mycelium, *A. rabiei* was grown in a fermenter (BIOSTAT, Braun, Melsungen, F.R.G.; working volume 10 l). The fermenter was filled with 7.5 l medium, autoclaved (121 °C, 60 min) and then 1.5 l preculture was added. The fermentation conditions were as follows: stirring velocity 70−75 rpm, ventilation rate 6 l air/ min, temperature 22 °C ± 0.5 °C under daylight. The fermentation was stopped after 60−65 h. Prior to inoculation 1 ml NIAX antifoam (Union Carbide) had been added. Average yield of mycelium was 250−400 g fr. wt./fermenter batch.

A crude polysaccharide fraction was prepared from the fermenter grown mycelium of *A. rabiei* as described by Ayers *et al.* [14] with the exception that the proteins were precipitated with trichloroacetic acid (10% w/v). After excessive dialysis against water, the high molecular weight fraction was lyophilized and used as elicitor. From 100 g mycelium (fresh weight) 0.5−1.0 g elicitor was obtained. This preparation was devoid of proteins as detectable by the Bradford method [15]. The elicitor was ap-

plied to the cell cultures in aqueous solution (1 ml/ 40 ml culture); controls were given 1 ml water.

Determination of isoflavones and phytoalexins

Phytoalexins, isoflavone aglycones and isoflavone conjugates were isolated from the cells and the growth media and quantitatively determined by HPLC as previously described [1, 2, 10].

Results

Elicitation of phytoalexins

Application of a polysaccharide elicitor preparation isolated from the chickpea pathogenic fungus *A. rabiei* to cell suspension cultures of cultivars ILC 3279 and ILC 1929 [10] led to the accumulation of medicarpin and maackiain both in the cells and in the growth medium (Fig. 2). According to previous investigations [16; Daniel and Barz, unpublished], the elicitor was applied to the cell cultures on day 3 of the growth cycle. Medicarpin was again found to be the major product.

The response of the two cell culture lines towards elicitor treatment was quantitatively different. After addition of 1.5 mg elicitor/40 ml culture the maximum of phytoalexin accumulation in cells and nutrient media of the cell culture ILC 3279 was reached 8 h after elicitor application (Fig. 2) whereas in the culture of the susceptible cultivar ILC 1929 this maximum was observed some 4 h later (Fig. 2). In case of the cell culture ILC 3279 the total amount of phytoalexins at the time of maximum accumulation was 2−4 times higher than in the culture ILC 1929 (Fig. 2). In the cells of cell culture ILC 3279 a second optimum of phytoalexin accumulation was observed some 72 h after elicitor treatment.

The data presented in Fig. 2 were obtained after addition of 1.5 mg elicitor/40 ml culture because this amount represents the optimum for the cell culture ILC 1929 according to dose response studies shown in Fig. 3. Higher amounts of elicitor led to a continuously increased accumulation of phytoalexins in the cell culture ILC 3279 but not in the cell culture ILC 1929. Thus, application of 5 mg elicitor/40 ml culture to cell culture ILC 3279 resulted in the accumulation of some 220 nmol medicarpin/g fr.wt. in the cells and 77 nmol medicarpin/g fr.wt. in the medium. In contrast, in the cell culture ILC 1929 only 30 nmol medicarpin/g fr.wt. could be detected in the cell extract and approximately 4−5 nmol/g fr.wt. in the
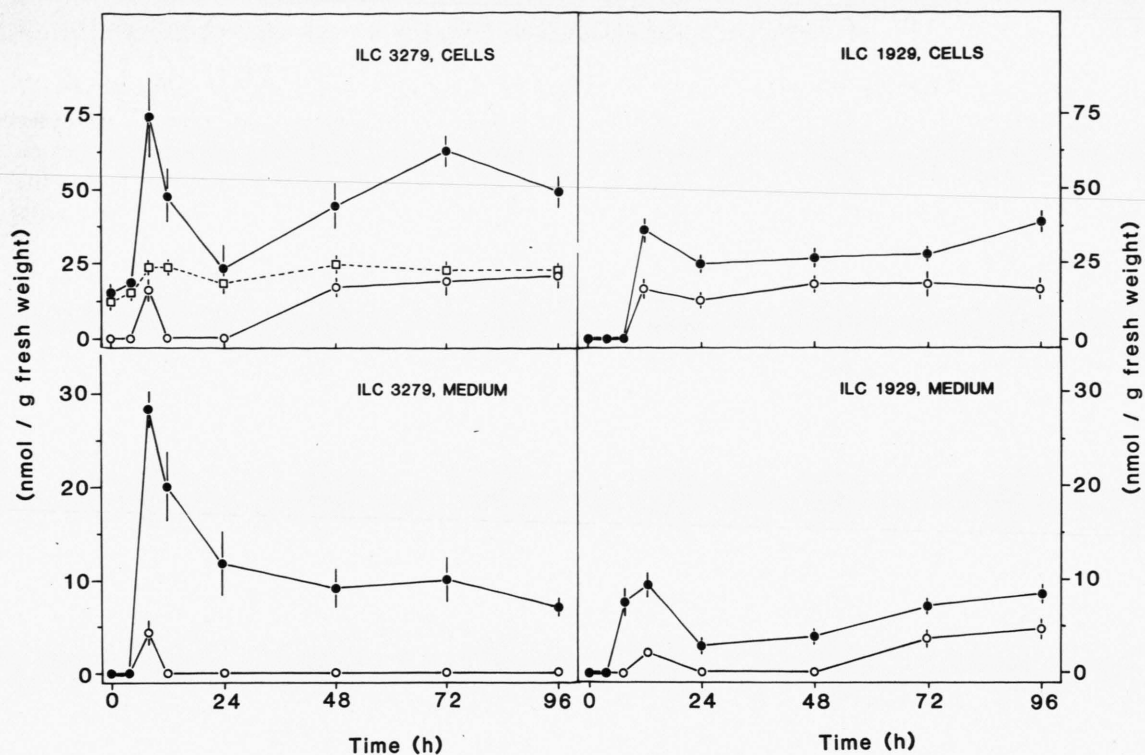
Fig. 2. Accumulation of medicarpin (●) and maackiain (○) in cells and growth medium of chickpea cell cultures ILC 3279 and ILC 1929 after application of *A. rabiei* elicitor (1.5 mg/40 ml culture) on day 3 of the growth cycle. ILC 3279 cell cultures accumulate some medicarpin constitutively (□———□) ($n = 3$).
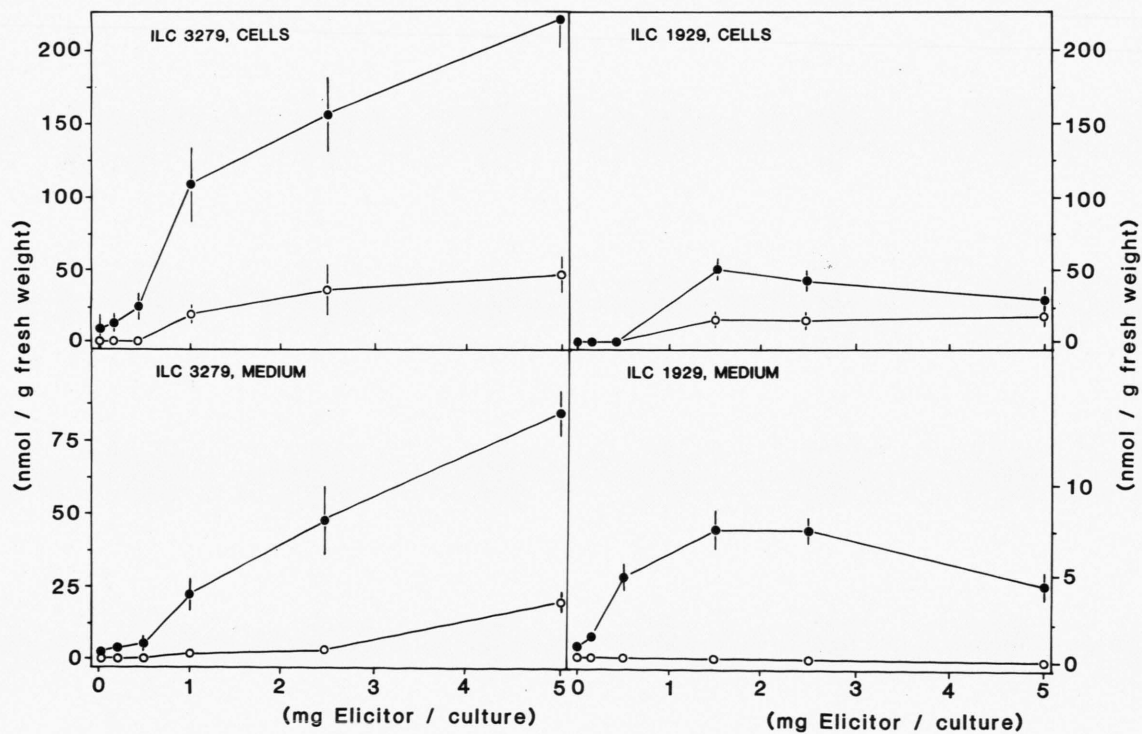

Fig. 3. Accumulation of medicarpin (●) and maackiain (○) in chickpea cell cultures ILC 3279 and ILC 1929 depending on the amount of *A. rabiei* elicitor added on day 3 of the growth cycle. Cultures ILC 3279 were analyzed 8 h and cultures ILC 1929 12 h after elicitor application ($n = 3$).

medium under otherwise identical conditions. In case of the minor phytoalexin maackiain the difference between the two cell culture lines was less prominent. Interestingly, increasing amounts of the elicitor did not alter the time course of phytoalexin accumulation as shown in Fig. 2 [17; Daniel and Barz, unpublished].

The ether extract of the growth medium from elicitor-treated cell cultures contained substantial amounts of the phytoalexins but only small amounts of formononetin and biochanin A (always less than 3 nmol/g fr.wt.) (Fig. 4). This observation indicates
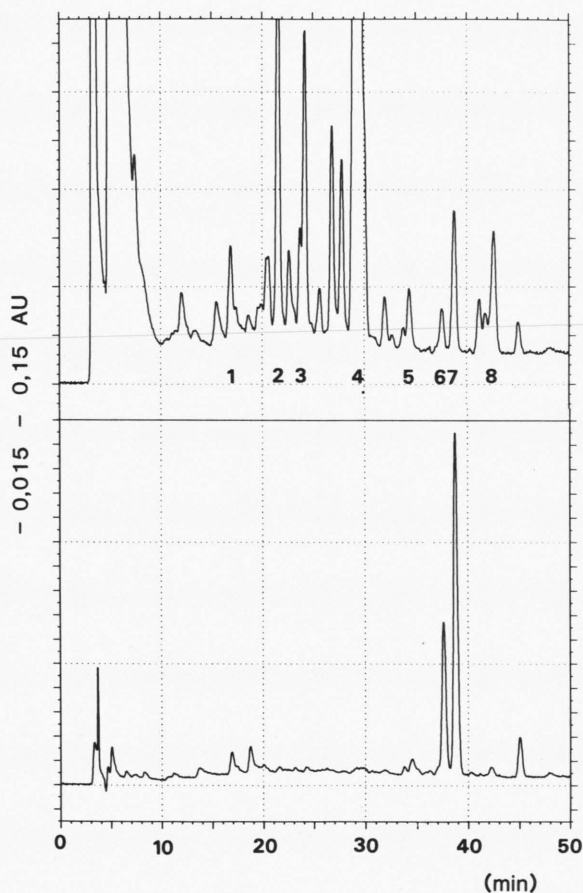

Fig. 4. HPLC chromatograms of cell extract (upper chart) and growth medium (lower chart) of chickpea cell culture ILC 3279 after application of *A. rabiei* elicitor. Elicitation was performed on day 3 of the growth cycle for 8 h. Prior to HPLC the cell extract was diluted 1:7.5. The numbered peaks represent the following compounds: 1, formononetin-7-O-glucoside; 2, FGM; 3, biochanin A-7-O-glucoside; 4, BGM; 5, formononetin; 6, maackiain; 7, medicarpin; 8, biochanin A.

that phytoalexins are excreted from the cells and are not transferred into the growth medium by cell lysis. The amount of phytoalexins measured in the cell extract cannot be explained as material absorbed to the outside of the cell wall because washing of the cells with petrolether never removed more than 10% of the phytoalexins normally measured in the cell extracts. Control experiments had shown that no significant destruction of the cells occurred by this treatment and that phytoalexins are readily soluble in petrolether.

The elicitation studies of chickpea cell suspension cultures further revealed that treatment of the cell cultures with *A. rabiei* elicitor had no negative effect on several growth characteristics, *e.g.* increase of fresh weight, soluble cellular protein as well as conductivity, phosphate concentration and pH-value of the growth medium (data not shown). Browning of the cells has never been observed even after application of such large amounts of elicitor as shown in Fig. 3.

Accumulation of isoflavones

In addition to the quantitative measurements of the phytoalexins (Fig. 2, 3), the concentrations of the isoflavones biochanin A and formononetin together with their 7-O-glucosides and 7-O-glucoside-6″-O-malonates were also determined in the extracts of the elicitor-treated chickpea cell culture lines (Fig. 5). In untreated cell cultures of both chickpea genotypes the isoflavones and the isoflavone conjugates accumulate in the same qualitative and quantitative pattern [10] as observed in the intact plant [12].

In the cell culture ILC 3279 the accumulation of formononetin, biochanin A and BGM was not significantly altered by elicitor treatment (Fig. 5) whereas formononetin 7-O-glucoside-6″-O-malonate accumulated to a substantially higher level subsequent to the maximum of phytoalexin accumulation (Fig. 2, 3). The 7-O-glucosides of biochanin A and formononetin occurred in both cell culture lines to relatively low levels only and no significant changes in the accumulation of these two compounds were measured due to elicitor treatment (data not shown).

In the cell culture ILC 1929 the accumulation of isoflavones and isoflavone conjugates was not affected by elicitor treatment. This was demonstrated both by time course and dose response studies (data not shown).
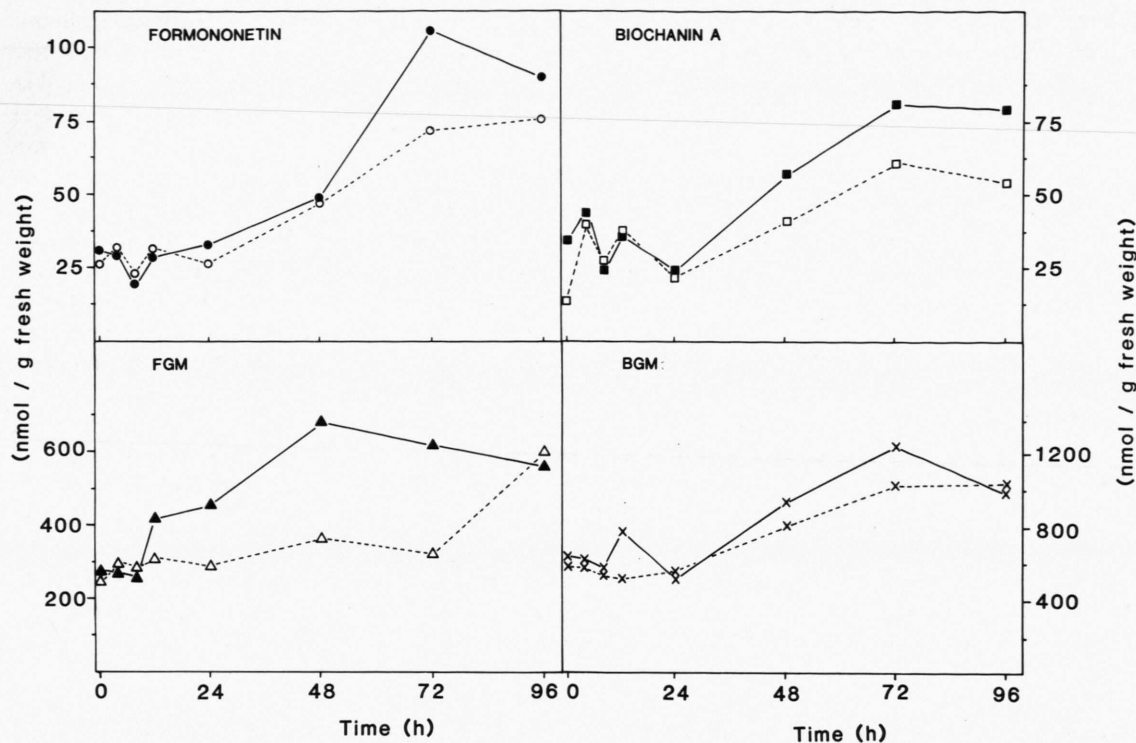
Fig. 5. Accumulation of biochanin A and formononetin together with their 6″-O-malonyl-7-O-glucosides (BGM, FGM) in the cell culture of chickpea cultivar ILC 3279 after addition of *A. rabiei* elicitor (———) (1.5 mg/40 ml culture) on day 3 of the growth cycle. Controls (– – –) were given water ($n = 3$, max. deviation $\pm24\%$).

Treatment of the cell cultures with more than 5 mg elicitor/flask led to a dramatic and irreproducible decrease in isoflavone content in both cell culture lines, whereas phytoalexins still accumulated to a similar level as found after application of 5 mg elicitor/40 ml culture. Toxic effects of the elicitor and/or the accumulated phytoalexins [18] may be responsible for these observations.

The differences between the two cell culture lines as described in Fig. 2–5 were found to be stable properties as shown by repetition of the experiments some 6, 12 and 18 months later [17, 19; Tiemann, Meier and Barz, unpublished].

Discussion

Cell suspension cultures of chickpea cultivars ILC 1929 and ILC 3279 represent of suitable experimental system to study the elicitation of pterocarpan phytoalexins and the metabolic regulation of isoflavone and isoflavone conjugates accumulation [7, 8,

17–19]. Furthermore, the cell suspension cultures seem to express genotypic differences which had previously been measured as characteristic properties of the intact plants [10, 12].

The elicitor used was a crude polysaccharide fraction. Proteins had to be removed since no elicitor activity could be measured if proteins were still present. This observation may point to the presence in *A. rabiei* mycelium of proteins which can suppress elicitor activity [20]. Gaschromatographic studies revealed the elicitor preparation mainly consists of glucose, mannose, N-acetylgalactosamine or galactosamine and small amounts of galactose (Keßmann and Barz, unpublished). Future studies have to show if a branched glucan is responsible for the elicitor activity as shown for the elicitor from *Phytophthora megasperma* f.sp. *glycinea* [21, 22].

Treatment of the cell culture ILC 3279 with this elicitor preparation resulted in a pronounced accumulation of both phytoalexins with a maximum at appr. 8 h after elicitor application (Fig. 2). Time

course and dose response studies have shown that the cell culture ILC 1929 accumulated substantially lower amounts of phytoalexins with a maximum even 4 h later than observed with the cell culture ILC 3279. Repetition of these experiments over a period of at least 18 months have shown that these differences are stable properties of the two cell culture lines. Such quantitative differences between the two chickpea cultivars with regard to the induced accumulation of phytoalexins have also been observed at the level of the natural host-pathogen interaction [12]. The molecular basis of these differences is presently under investigation. In such studies the chickpea cell cultures will serve as an ideal experimental system due to the stability of the genotypic difference at the level of the undifferentiated cell.

Phytoalexin accumulation in chickpea cell cultures is comparably well induced by either the *A. rabiei* elicitor or yeast extract [10]. Therefore it seems to be unlikely that a "host specific elicitor" [21, 23] is responsible for the differential phytoalexin accumulation.

Rapid accumulation of phytoalexins in the cells and in the medium is followed by a pronounced decrease (Fig. 2). The phytoalexins excreted into the growth medium are slowly metabolized by peroxidases [16] which tend to accumulate in plant cell culture media [24]. Peroxidative destruction of phenolic compounds such as medicarpin and maackiain is well documented [25]. However, the quantitative differences between the two chickpea cell culture lines in the elicitor-induced phytoalexin accumulation (Fig. 2, 3) cannot be explained by different rates of peroxidative destruction because this reaction proceeds at identical rate in both cell cultures [16; Daniel, Jaques and Barz, unpublished].

Comparison of phytoalexin accumulation in chickpea cell cultures and whole plants as induced by *A. rabiei* elicitor (Fig. 2, 3) or caused by spore infection [12] shows that the cell culture ILC 3279 produces 5−10 times more phytoalexins per g fr.wt. This is most likely due to the fact that in cell cultures practically all cells are exposed to the inducing agent [23]. This difference in quantity represents one major advantage in using cell cultures for biochemical investigations of host-parasite interactions though the results obtained with cell cultures must be verified at the level of the natural host-pathogen interaction [26, 23].

Furthermore, the chickpea cell cultures facilitate comparative studies on elicitor effects on both the inducible phytoalexin and the constitutive isoflavone metabolism. Isoflavones and isoflavone conjugates accumulate in both cell culture lines in the same qualitative and quantitative pattern as observed in the whole plant [10, 12]. Among these constitutively formed compounds only FGM in cell culture ILC 3279 accumulated to significantly higher levels under elicitor treatment (Fig. 4). Formononetin is a central intermediate in the biosynthesis of medicarpin and maackiain [5−7]. The enhanced accumulation of FGM may indicate that excess formononetin formed during elicitor-induced phytoalexin biosynthesis is funneled into conjugate formation. This assumption is corroborated by the observation that in cell culture ILC 3279 the enzyme activity for the specific isoflavone 7-O-glucosyltransferase [27] is dramatically stimulated by elicitor treatment [16] (Daniel, Hinderer and Barz, unpublished). Quantitative comparison of Fig. 3 and 5 shows that the amount of phytoalexins produced were most likely not formed from constitutively accumulated formononetin though highly specific enzymes for isoflavone conjugate degradation have been described in chickpea [19, 28].

Such data and previous results [10] lead to the assumption that elicitor application induces the complete biosynthetic pathway required for pterocarpan phytoalexin formation. This has already been demonstrated for several enzymes of the general phenylpropanoid pathway [16, 19] and three enzymes specifically involved in late steps of pterocarpan phytoalexin formation [7, 8, 19]. Elicitor induction of complete biosynthetic routes of phytoalexins has already been demonstrated in other studies employing plant cell cultures [23, 29]. Present studies are especially directed towards elucidating the metabolic regulation and relation of the inducible phytoalexin and the constitutive isoflavone biosynthetic routes.

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