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The origin of copper-induced medicarpin accumulation and its secretion from roots of young fenugreek seedlings are regulated by copper concentration

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ABSTRACT

Isoflavonoid pterocarpans, like medicarpin (M), are produced by leguminous plants in response to biotic or abiotic elicitation from either their glycosidic conjugate pools or by de novo synthesis. In an attempt to clarify M origin in response to copper elicitation, intact *Trigonella foenum-graecum* (fenugreek) seedlings and cell suspension cultures were treated with $CuCl_2$, and the accumulated isoflavonoid aglycones and their glycosides were determined by High Performance Liquid Chromatography (HPLC). Our results show that copper induces the de novo synthesis of M in a concentration dependent manner. In roots, where copper accumulated in high amounts, only part of M was formed de novo, while another part was formed at the expense of its malonyl glucoside (MGM). In contrast, when tissue copper concentration was low, like in shoots, or in roots treated with low $CuCl_2$, M was formed only by de novo synthesis. The increase of phenylalanine ammonia lyase activity (PAL) as well as the accumulation of chalcone synthase (*CHS*) and vestitone reductase (*VR*) specific transcripts are consistent with the de novo synthesis of M induced by copper. The non-linear negative correlation of the studied copper concentrations to the amount of M excreted in the seedling growth medium suggests the existence of an M secretion process which is regulated by copper concentration. The possible involvement of an ATP-dependent transporter in the copper-induced M excretion is discussed.

1. Introduction

Isoflavonoids are particularly prevalent in the Papilonoideae subfamily of the Leguminosae. Isoflavonoid phytoalexins are important plant resistance factors that are induced by biotic and abiotic elicitors. Different legume species produce different classes of isoflavonoid phytoalexins, like the pterocarpan medicarpin (M) in *Medicago sativa* [1] and *M. truncatula* [2,3], and maackian and formononetin (F) in *Trifolium repens* [4].

Isoflavonoids accumulate constitutively usually in the forms of glucosides and malonyl glucosides, primarily in plant roots but also in shoots [1,3,5,6]. In contrast, accumulation of their free forms in most cases is induced upon microbial or insect attack or upon abiotic elicitation, like UV light and heavy metals [1]. The metabolic relationship between pre-existing conjugates and free isoflavonoids produced in response to abiotic elicitors, like $CuCl_2$, has been studied in leguminous plants. In roots from elicited seedlings, their glycosidic conjugates served as metabolic pools, which released by hydrolysis the corresponding free aglycones [4,7]. However, whole alfalfa seedlings responded to copper (1 or 3 mM $CuCl_2$) elicitation by synthesizing M de novo [8]. Pterocarpan biosynthesis has been studied extensively, the enzymes leading to M biosynthesis are well characterized, and most of the corresponding genes have been cloned [1]. Phenylalanine ammonia lyase (PAL) is the enzyme which introduces phenylalanine to the phenylpropanoid pathway, chalcone synthase (CHS) is involved in the first step committed to flavonoid biosynthesis, while vestitone reductase (VR) catalyzes the penultimate step of M biosynthesis (Fig. 1). The expression of *PAL*, *CHS* and *VR* has been verified in healthy roots or cell suspension cultures of *Medicago* spp. [9–11], but *VR* is absent from shoots or leaves. Upon biotic and abiotic elicitation their activities increased in parallel to M synthesis [9,11–16].

The difference in the origin of copper-induced accumulation of M between tissues of the same or related species prompted the systematic investigation on M accumulation in response to copper

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Abbreviations: CHS, chalcone synthase; cDNA, complementary DNA; RP-HPLC, reverse phase high performance liquid chromatography; M, medicarpin; MGM, medicarpin-3-*O*-glucoside-6″-malonate; PAL, phenylalanine ammonia lyase; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; VR, vestitone reductase.

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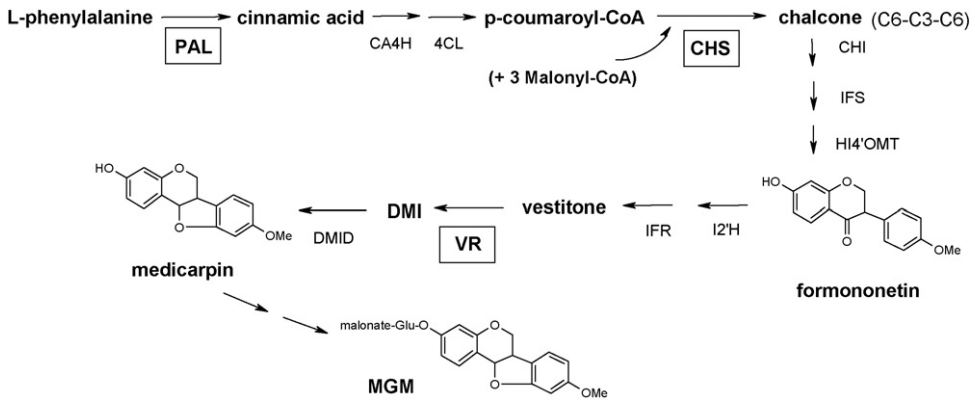

Fig. 1. Outline of the biosynthetic pathway leading to medicarpin. Enzyme and compound abbreviations used: PAL, phenylalanine ammonia lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: coenzymeA ligase; CHS, chalcone synthase; IFS, isoflavone synthase; HI4′OMT, 2-hydroxyisoflavone 4′-*O*-methyltransferase; I2′H, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; VR, vestitone reductase; DMID, 7,2′-dihydroxy-4′-methoxy-isoflavanol dehydratase. DMI, 7,2′-dihydroxyisoflavanol; MGM, medicarpin-3′-*O*-glucoside-6-*O*-malonate. Multiple arrows indicate two or more reactions.

elicitation. In view of the fact that *Trigonella foenum-graecum* L. (fenugreek) whole seedlings produced M in response to abiotic elicitation [17], we conducted a systematic investigation on M metabolism in roots, shoots and the growth medium of fenugreek seedlings and in fenugreek cell suspension cultures in response to copper elicitation. M accumulation was correlated with PAL activity and the expression of *CHS* and *VR* genes.

2. Materials and methods

2.1. Plant material and cell cultures

Trigonella foenum-graecum L. (fenugreek) seeds were purchased from a local market. Surface sterilized seeds were sown on moistened filter paper under sterile conditions and maintained at 25 ± 1 °C with a 16 h period of light ($5.3\ W\ m^{-2}$) in a growth chamber. All experiments with seedlings and cell cultures were performed at the same temperature and light conditions.

Cell suspension cultures were initiated from callus cultures that were developed from roots of 4-day-old fenugreek seedlings [18]. For their initiation about 2 g of calli were transferred into 250 ml flasks containing 50 ml of culture medium containing 1.5 mg L^{-1} benzylaminopurine (BAP), 1.0 mg L^{-1} napthalene acetic acid (NAA) and 3% sucrose in Murashige and Skoog (MS) medium [19] pH 5.8. Cultures were maintained on an orbital shaker (120 rpm) and were subcultured initially 4 weeks after initiation of the liquid culture. The following subcultures were performed at 2 weeks intervals transferring 25 ml of the culture into 25 ml of fresh medium. Cultures were used for experiments between 4 and 7 subcultures after initiation from callus.

2.2. Elicitation and inhibition experiments

Four days after subculture, suspension cultures were elicited with copper by adding sterile $CuCl_2$ at a final concentration of 0.5 mM. An equal amount of water was added to control cultures. Cells were harvested by vacuum filtration, frozen in liquid N_2 and stored at −80 °C until analysis.

Six-day-old seedlings were treated hydroponically with $CuCl_2$ of different concentrations (0, 0.01, 0.05, 0.1, 0.5 and 1 mM). Seedlings (12 per vial) were placed into sterile vials, containing 120 ml of sterile water or $CuCl_2$, so that only their roots were immersed in the growth solution. At least 4 vials per treatment were used. After the stated period, growth media and seedlings were collected, their roots were washed with distilled water and blow dried, and the excised roots and shoots (200–300 mg/sample) were weighted and stored at −80 °C until analysis. For the inhibition assays, 5-day-old seedlings were transferred into vials containing 120 ml sterile de-ionized water (control), filter-sterilized potassium cyanide (100, 250 or 500 μM) (KCN, Sigma–Aldrich) or sodium orthovanadate (100, 250 or 500 μM) (OV, Sigma). After 24 h of incubation, $CuCl_2$ was added to the media to a final concentration of 0.01 mM and seedlings were incubated for another 24 h. All experiments were performed at least in triplicates.

2.3. Copper analysis

The copper content of shoots and roots of fenugreek seedlings treated with 0.1 mM $CuCl_2$ was determined by atomic absorption spectroscopy (Spectra AA 20 plus, VARIAN) after microwave wet digestion of the dried material in supra-pure concentrated nitric acid (70–71%). Before wet digestion roots were washed thoroughly with 10% HNO_3 to exchange surface-bound copper.

2.4. Isoflavonoid extraction, analysis and identification

Plant material was extracted with 80% methanol (1:10 w/v) with pestle and mortar. After centrifugation (4000 rpm, 10 min) the collected supernatants were filtered through nylon membrane filters (0.45 μm Titan2, SUN-SRi) and subjected to RP-HPLC analysis. Cells from cell cultures were extracted with 80% methanol (1:10 w/v) by ultrasonication for 30 min. The extracts were centrifuged (4000 rpm, 15 min) and the filtered supernatants were used for HPLC analysis. Cell suspension culture or seedling growth media were adjusted to pH 2.5 and were extracted twice with an equal volume of ethyl acetate. The organic fractions were pooled, evaporated to dryness under reduced pressure at 40 °C using rotoevaporator system (Buechi Rotavapor R-114), and the residues were redissolved in 1 ml methanol, filtered and subjected to analysis. All extracts were analyzed by HPLC using a quarternary gradient pump (PU-2089, Jasco, Japan) connected to a multi-wavelength detector (MD-2015, Jasco, Japan) under the following chromatographic conditions: 20 μl of sample were injected onto a Lichrosorb RP 18, 5.0 μm, 250 × 4 mm column; elution profile: 0–25 min, 55% A in B, 25–30 min 100% A, 30–35 min 100% A, 35–40 min step return to 100% B, 40–45 min re-equalibration with 100% B. [solvents: (A) acetonitrile and (B) water with 0.1% acetic acid]; flow rate: 1.5 ml min^{-1}; detection: at 283 nm. Isoflavonoid quantification was based on a calibration curve, plotting peak area as monitored at 283 nm against known concentrations of medicarpin.

Identification of formononetin (F) was based on UV absorbance spectrum, retention time and on co-chromatography of extracts with an F standard solution (Extrasynthése, France). In order to check the presence of F conjugates (F-glucoside and/or F-malonylglucoside), malonate-free extracts of roots and shoots were prepared. Two ml of each filtered methanolic extract were heated in a sealed vial at 80 °C for 16 h. This procedure converts the malonyl-glucosides of flavonoids into the corresponding glucosides [20]. After incubation samples were allowed to cool at room temperature for 1 h, then re-filtered and subjected to HPLC analysis and co-chromatographed with formononetin-7′-*O*-glucoside (FG, Extrasynthése, France).

M and MGM were isolated using semi-preparative HPLC (Supelcosil™ column SPLC-18, 25 cm × 10 mm, 5 μm, from Supelco-USA, flow rate 2.5 ml min^{-1}, same elution profile) and identified by modern spectroscopic means (1D- and 2D-NMR, MS). A Bruker AC200, a Bruker AC400 and a Bruker AC600 spectrometers were used in obtaining the NMR spectra. Chemical shifts are given in δ values with TMS as internal standard. The 2D experiments (COSY, COSY LR, HMBC and HMQC) were performed using standard Bruker micro programs. APCI and ESI-MS were recorded in the positive and negative mode on a MSQ Thermo Finnigan spectrometer.

Medicarpin, 3-hydroxy-9-methoxypterocarpan (M): Amorphous powder; $C_{16}H_{14}O_4$; ESI and APCI-MS(+) *m/z*: 271 $[M+H]^+$; ^{1}H NMR (MeOD, 400 MHz) δ 3.55 (1H, *ddd*, $J_{6ax,\ 6a}$ = 10.9 Hz, $J_{11a,\ 6a}$ = 6.2 Hz, $J_{6eq,\ 6a}$ = 5.0 Hz, H-6a), 3.75 (1H, *dd*, $J_{6eq,\ 6ax}$ = 11.0 Hz, $J_{6a,\ 6ax}$ = 10.9 Hz, H_{ax}-6), 3.70 (1H, *s*, –OCH_3), 4.23 (1H, *dd*, $J_{6ax,\ 6eq}$ = 11.0 Hz, $J_{6a,\ 6eq}$ 5.0 Hz, H_{eq}-6), 5.49 (1H, *d*, *J* = 6.2 Hz, H-11a), 6.42 (1H, *d*, *J* = 2.5 Hz, H-4), 6.45 (1H, *dd*, *J* = 8.8, 2.5 Hz, H-8), 6.46 (1H, *d*, *J* = 2.5 Hz, H-10), 6.55 (1H, *dd*, *J* = 8.4, 2.5 Hz, H-2), 7.18 (1H, *d*, *J* = 8.8 Hz, H-7), 7.36 (1H, *d*, *J* = 8.4 Hz, H-1); ^{13}C NMR (MeOD, 50 MHz): δ 132.2 (C-1), 109.8 (C-2), 159.1 (C-3), 103.6 (C-4), 156.4 (C-4a), 66.5 (C-6), 39.5 (C-6a), 119.1 (C-6b), 126.1 (C-7), 107.4 (C-8), 161.6 (C-9), 97.5 (C-10), 160.1 (C-10a), 79.5 (C-11a), 113.4 (C-11b), 55.5 (–OCH_3).

Medicarpin-3-*O*-glucoside-6″-*O*-malonate (MGM): Colorless amorphous powder; $C_{25}H_{26}O_{12}$; ESIMS(−) *m/z*: 540 $[M+Na]^-$, APCIMS(−) *m/z*: 431 $[M\text{-malonate}]^-$ and 269 $[M\text{-malonate-Glu}]^-$; ^{1}H NMR (DMSO, 600 MHz): δ 2.86 (2H, *s*, H-2″), 3.10–3.60 (5H, *m*, Glc-2,3,4,5,6-H), 3.64 (1H, *ddd*, $J_{6ax,\ 6a}$ = 10.9 Hz, $J_{11a,\ 6a}$ = 6.2 Hz, $J_{6eq,\ 6a}$ = 5.8 Hz, H-6a), 3.65 (1H, *dd*, $J_{6eq,\ 6ax}$ = 11.7 Hz, $J_{6a,\ 6ax}$ = 10.9 Hz, H_{ax}-6), 3.68 (1H, *s*, –OCH_3), 4.28 (1H, *dd*, $J_{6ax,\ 6eq}$ = 11.7 Hz, $J_{6a,\ 6eq}$ 5.8 Hz, H_{eq}-6), 4.82 (1H, *d*, *J* = 7.4 Hz, H-1″), 5.60 (1H, *d*, *J* = 6.2 Hz, H-11a), 6.43 (1H, *dd*, *J* = 8.2, 2.4 Hz, H-8), 6.52 (1H, *d*, *J* = 2.7 Hz, H-4), 6.53 (1H, *d*, *J* = 2.4 Hz, H-10), 6.71 (1H, *dd*, *J* = 8.6, 2.7 Hz, H-2), 7.24 (1H, *d*, *J* = 8.2 Hz, H-7), 7.38 (1H, *d*, *J* = 8.6 Hz, H-1); ^{13}C NMR (DMSO, 150 MHz): δ 133.9 (C-1), 110.4 (C-2), 159.8 (C-3), 103.8 (C-4), 157.1 (C-4a), 65.9 (C-6), 39.9 (C-6a), 122.2 (C-6b), 125.1 (C-7), 106.1 (C-8), 160.7 (C-9), 104.1 (C-10), 148.9 (C-10a), 78.0 (C-11a), 114.3 (C-11b), 55.3 (–OCH_3), 100.3 (C-1′), 73.3 (C-2′), 78.3 (C-3′), 71.0 (C-4′), 78.8 (C-5′), 62.1 (C-6′), 167.8 (*CO*CH_2–COOH), 39.8 (CO*CH_2*–COOH), 169.8 ($COCH_2$–*CO*OH).

2.5. PAL extraction and assay

Samples (300 mg fresh weight) were ground to a paste in 3 ml 60 mM sodium borate buffer (pH 8.8) containing 5 mM β-mercaptoethanol and 3% (w/v) polyvinylpyrrolidone (PVP). The paste was centrifuged at 4 °C for 15 min at 12,000 rpm and the supernatant was used as a source of crude enzyme. The total protein concentration in the enzyme extracts was determined using the Bradford assay [21].

PAL activity was determined according to Beaudoin-Eagan and Thorpe [22]. The reaction mixture contained 0.2 ml of the enzyme preparation and 5.5 μmol of L-phenylalanine in 0.8 ml 60 mM borate buffer (pH 8.8). The reaction started by the addition of the enzyme extract and after incubation at 40 °C for 120 min the reaction was terminated by the addition of 50 μL 5N HCl. Absorbance at 290 nm was recorded against control incubation mixtures lacking phenylalanine. The amount of *trans*-cinnamic formed was estimated using a standard curve of this compound. Enzyme activity was expressed in μmol cinnamic acid mg $protein^{-1}$ h^{-1}. Triplicate assays were performed for each extract.

2.6. Semi-quantitative RT-PCR and DNA sequence analysis

Total RNA from fenugreek (*Trigonella foenum-graecum* L.) tissues or cell suspension cultures was extracted with the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) and treated with 10U DNase I (Roche Applied Science) for 10 min at 37 °C. First strand cDNA was synthesized from 1 μg total RNA with Expand Reverse Transcriptase (50 U/μL, Roche) according to the manufacturer's protocol. In order to amplify *CHS* and *VR* specific fragments, degenerated primers were designed using sequence information from known *CHS* and *VR* genes. Initial PCR was performed in a mixture of 50 μL that contained 1 μL of first-strand cDNA, 0.4 mM dNTP's, 1X PCR buffer, 2.5 U Expand High Fidelity DNA Polymerase (Roche) and 10 pmol each of the gene-specific degenerated primer. The following thermocycling program was used: initial denaturation at 94 °C for 2 min, followed by 25 and 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 5 min. Degenerated primers were as follows: for *CHS*, 5′-AA(G/A)GGTGCTCGTGTGCT(G/T)GTTG-3′ and 5′-AAGTCCAGGTCCAAA(G/C)CCAAA(C/T)-3′, and for *VR*, 5′-CAGG(T/A)TT(T/C)CTTGGTTCATGG-3′ and 5′-ATCACTCCAA(T/A)CA(C/G)TCTCATCC-3′. The amplified PCR products were subsequently cloned and sequenced. BLAST analysis (National Center for Biotechnology Information) revealed high homology to known *CHS* and *VR* sequences deposited in the databases. Based on the sequence information, specific fenugreek *CHS* and *VR* primers were consequently designed for the RT-PCR experiments. Reaction conditions were as above, except of the annealing temperature which was 54 °C. The *18S* rRNA gene was used as an internal control for RNA calibration. To verify the exponential phase of RT-PCR amplification, 15, 20, 25 and 30 cycles were tested for each gene, and data was collected at 16 cycles for *18S* rRNA and at 25 cycles for *CHS* and *VR*. All experiments were performed in triplicate. Primers were as follows: for *CHS* 5′-AGTCCTTCTCCTGTTGTCTT-3′ and 5′-TGGAGATGGAGTTGCTGCAC-3′, for *VR* 5′-AACAGCAGAACCACTTGAAG-3′ and 5′-GTTCATGGATCATCAAGAGTC-3′ and for *18S* rRNA 5′-TTGTGTTGGCTTCGGGAT CGGAGTAAT-3′ and 5′-GCACCACCACCCATAGAATCAAGAA-3′.

DNA was sequenced by the dideoxy chain termination method with the use of an automated sequencer model 377 (Applied Biosystems).

3. Results

3.1. Identification of isoflavonoids and their accumulation in fenugreek cell cultures and during the course of seedling growth

The main constituents of the methanolic extracts from roots and shoots of young fenugreek seedlings were isolated with semi-preparative RP-HPLC and identified by modern spectroscopic means as M and MGM.

M showed a protonated molecular ion peak at *m/z* 271 in the APCI(+) and ESIMS(+), revealing the molecular weight of 270 Da. Its ^{1}H NMR spectrum showed a set of protons signals [δ 3.53 (1H, *ddd*, *J* = 10.9, 6.2 and 5.0 Hz, H-6a), 3.61 (1H, *dd*, *J* = 11.0, 10.9 Hz, H_{ax}-6), 4.23 (1H, *dd*, *J* = 11.0, 5.0 Hz, H_{eq}-6) and 5.49 (1H, *d*, *J* = 6.2 Hz, H-11a)] characteristic of a pterocarpan skeleton [23]. The spectrum

also exhibited the presence of two sets of ABX-type aromatic resonances at δ 6.42 (1H, *d*, *J* = 2.5 Hz, H-4), 6.55 (1H, *dd*, *J* = 8.4, 2.5 Hz, H-2) and 7.36 (1H, *d*, *J* = 8.4 Hz, H-1) allocated to the A ring protons and at δ 6.45 (1H, *dd*, *J* = 8.8, 2.5 Hz, H-8), 6.46 (1H, *d*, *J* = 2.5 Hz, H-10) and 7.18 (1H, *d*, *J* = 8.8 Hz, H-7) allocated to the B ring protons. The ^{1}H NMR spectrum also revealed a methoxyl proton signal at δ 3.70 which was assigned at C-9 based on correlations observed in the COSY LR and HMBC spectra. The COSY and HMQC experiments were also employed for the structural elucidation of M. Spectral data are in full agreement with literature [24].

MGM showed a $[M+Na]^-$ ion peak at *m/z* 540 by ESIMS(−) revealing its molecular weight to 518 Da. A *pseudo*-molecular ion peak at *m/z* 269 [APCI(−)] was also evident in the mass spectrum corresponding to the aglycon, M. Moreover, an additional peak at *m/z* 431 was detected signifying the loss of the malonate unit. The ^{1}H NMR spectrum of MGM was similar to that of medicarpin, except for the signals exhibited by the sugar and malonate protons. The ^{1}H NMR spectrum of MGM showed the proton signals of pterocarpan basic skeleton of medicarpin and the two sets of ABX-type aromatic protons corresponding to the A and B ring protons as well as the methoxyl proton singlet resonance. In addition, an anomeric proton resonance at δ 4.82 (1H, *d*, *J* = 7.4 Hz) was observed in the ^{1}H NMR spectrum indicating the presence of a β-glucosyl moiety. The H-2/H-3/H-4/H-5 and H-6 protons of glucose were detected in the high-field region 3.10–3.60 ppm. Finally, the signal of the characteristic methylene of malonate moiety was observed at the ^{1}H NMR spectrum as a singlet at 2.86 ppm. At the HMBC spectrum the correlations between the protons of the methylene group and the two carboxylic carbons (167.8 and 169.8 ppm) of the malonate moiety were observed. The positions of the methoxy, β-glucosyl and malonate units in MGM were determined from the COSY LR and HMBC spectra. The COSY and HMQC experiments were also employed for the conformation of the structure. Spectra data were in full agreement with the literature [25].

RP-HPLC analysis of 6 day old fenugreek seedlings showed the presence of MGM in shoots and roots, while trace amounts of M and F were found only in roots. Co-chromatography of the methanolic and the malonate-free extracts with FG showed that no FG or FGM were present.

In order to determine the accumulation of these compounds during seedling growth, fenugreek seedlings were separated into roots and shoots 4, 6, and 11 days after sowing and the methanolic extracts from each part were analyzed by RP-HPLC. Four days from sowing MGM concentration in roots was 10.2 nmol g^{-1} FW and reached 111.4 nmol g^{-1} FW at day 11. Free M was undetectable in roots of 4-day-old seedlings and accumulated at very low levels in 11-day-old seedlings. In shoots from 4-day-old seedlings MGM was detected in trace amounts and increased during seedling growth. However, its concentration 11 days from sowing was considerably lower (22.3 nmol g^{-1} FW) compared to that of the roots, while no free M was detected (Fig. 2). HPLC analysis of fenugreek cell suspension cultures showed the presence of M, MGM, F, part of which were detected in the culture medium (Table 1).

3.2. Effect of copper elicitation on the accumulation of isoflavonoids in fenugreek seedlings and cell suspension cultures

Treatment of 6-day-old fenugreek seedlings with 0.5 mM $CuCl_2$ resulted in a progressive increase in the levels of M in the roots, while there was a concomitant decrease of MGM and a slight increase in F, part of which were detected in the growth medium (Fig. 3). When treatment lasted for 6 h, MGM concentration in roots was reduced to approx. 1/3, while M concentration increased

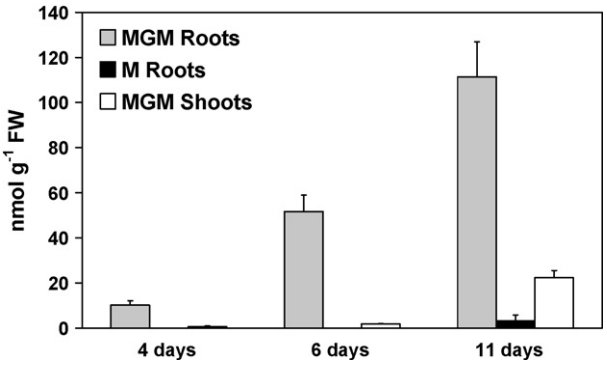

Fig. 2. MGM and M concentrations in the roots and shoots during the course of fenugreek seedling development. Values are the mean of at least three experiments ± S.E.

Table 1
MGM, M and F accumulation in cells and the medium of cell suspension cultures from fenugreek roots, as affected by a 24 h treatment with 0.5 mM $CuCl_2$.

	MGM	M	F
Control cells	15.3 ± 2.3	8.5 ± 1.3	3.8 ± 0.3
Cu cells	10.1 ± 1.7	49.4 ± 10.6	2.4 ± 1.9
Control medium	1.8 ± 0.6	7.4 ± 2.9	1.3 ± 0.3
Cu medium	3.0 ± 0.8	134.2 ± 7.1	n.d.

Values are the mean ± S.E. of at least three experiments. n.d.: not detected.

(from 2.4 to 9 nmol g^{-1} FW), and traces of F were detected. In shoots copper induced M formation (3.2 nmol g^{-1} FW), while in the seedling growth solution M concentration increased from 7.1 to 20.5 nmol g^{-1} FW (Fig. 3A). Longer treatment with $CuCl_2$ resulted in a further reduction of MGM concentration in roots while the amount of M in roots, shoots, and the seedling growth solution progressively increased (Fig. 3B and C). A 24-h-treatment resulted in a significant increase of M in both roots and shoots (from 4.1 to 69.6 and from 0 to 155.9 nmol g^{-1} FW, respectively). M accumulation in roots was accompanied by a 93% reduction of MGM (from 51.7 to 3.4 nmol g^{-1} FW). Interestingly, in shoots MGM increased (from 1 to 6.9 nmol g^{-1} FW) in parallel to M accumulation (Fig. 3C).

In a more detailed study, a range of concentrations of $CuCl_2$ (0.01, 0.05, 0.1, 0.5 and 1.0 mM) were fed hydroponically to fenugreek seedlings for 24 h, and the concentrations of MGM, M, and F were determined (Fig. 4). Although copper induced an increase in M and F accumulation in roots, F content increased at a much lower extent compared to that of M. When the elicitor concentration was low (0.01 mM), M concentration increased in roots approx. by 22 times (Fig. 4A), while MGM and F concentrations did not change significantly. Increasing elicitor concentration from 0.01 to 1.0 mM resulted in a progressive reduction of MGM (from 41.5 to 3.0 nmol g^{-1} FW) as well as of free M (from 92.1 to 30.4 nmol g^{-1} FW) (Fig. 4A). At the same time, F increased from 1.4 to 6.6 nmol g^{-1} FW. Interestingly however, the accumulation of M in shoots increased with increasing copper concentration (in 1 mM $CuCl_2$-treated seedlings M concentration reached 181 nmol g^{-1} FW), along with a slight increase of MGM concentration (Fig. 4B). Atomic absorption spectrophotometry revealed that a 2 or 4-h-exposure of fenugreek seedlings to 0.1 mM $CuCl_2$ did not result in a significant increase of copper concentration in shoots (from 11 to 14 or 19 μg g^{-1} DW, respectively) while in roots copper increased from 11 to 379 or 840 μg g^{-1} DW, respectively.

In order to investigate whether fenugreek cell suspension cultures accumulate M in response to copper elicitation we treated cell cultures with 0.5 mM for 24 h and analyzed their isoflavonoid

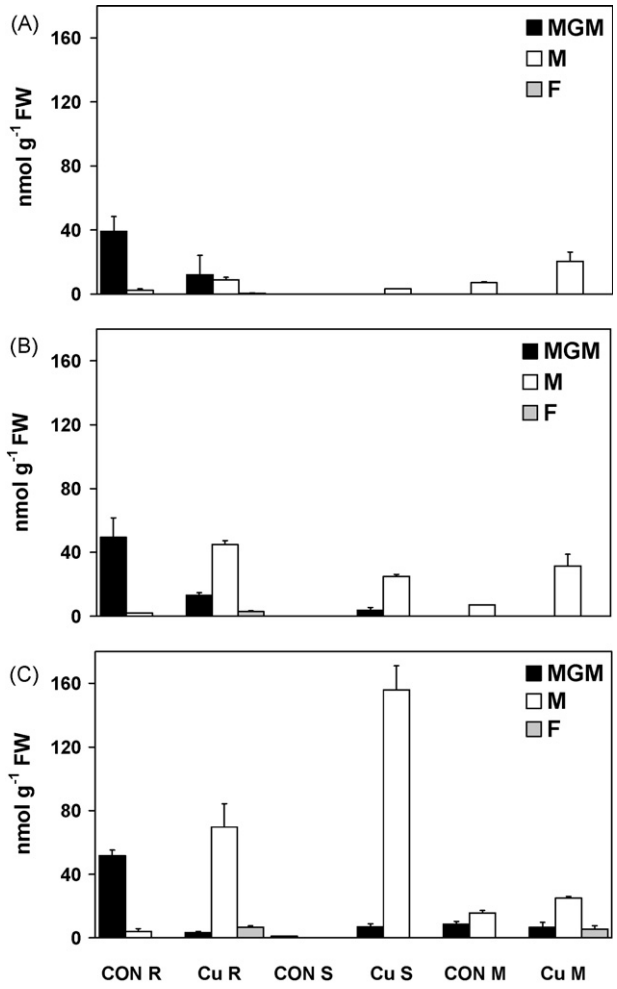

Fig. 3. MGM, M and F concentrations in the roots, shoots and the seedling growth medium as affected by elicitation of 6-day-old fenugreek seedlings with 0.5 mM $CuCl_2$ for 6 (A), 12 (B), and 24 h (C). At 0 h roots contained MGM (54.7 ± 7.2 nmol g^{-1} FW) and trace amounts of M and F, shoots contained only MGM (1.9 ± 0.1 nmol g^{-1} FW), while the seedling growth medium contained only M (6.3 ± 1.3 nmol g^{-1} FW). Values are the mean of at least three experiments ± S.E.

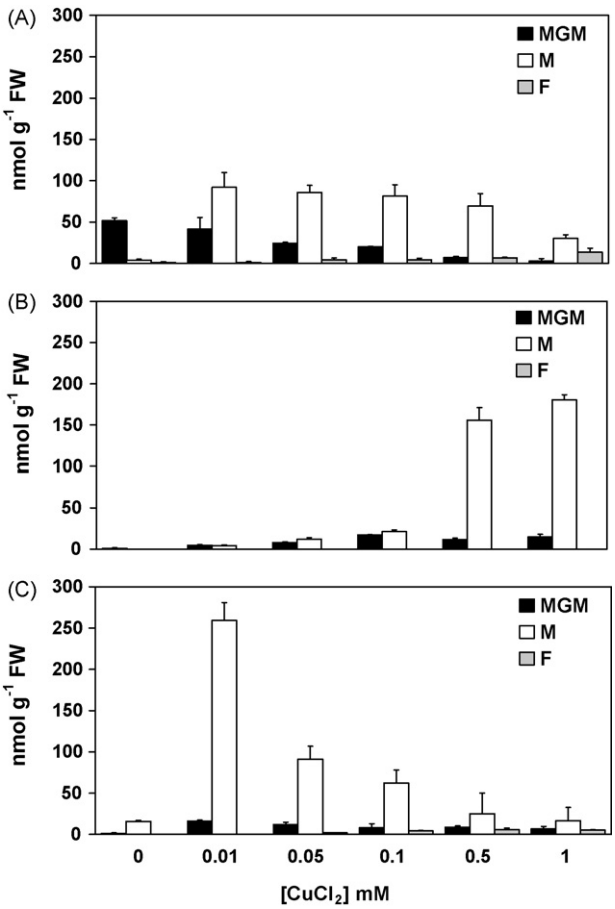

Fig. 4. MGM, M and F concentrations in roots (A), shoots (B), and the seedling growth medium (C) as affected by a 24-h-elicitation of 6-day-old fenugreek seedlings with several concentrations of $CuCl_2$. Values are the mean of at least three experiments ± S.E.

profile. MGM was detected mainly in cells (15.3 nmol g^{-1} FW), while small amounts were secreted in the culture medium (1.8 nmol g^{-1} FW). M and F were present in smaller amounts in non-elicited cells (8.5 and 3.8 nmol g^{-1} FW, respectively) and in the medium (7.4 and 1.3 nmol g^{-1} FW, respectively). Copper treatment resulted in approx. 10-fold increase of M, 67% of which was secreted in the culture medium, while MGM concentration decreased slightly (from 15.3 to 10.1 nmol g^{-1} FW) and F remained almost unchanged (Table 1).

3.3. Copper-induced excretion of isoflavonoids

Part of M, MGM, and F which were formed in roots were excreted in the seedling growth medium. When seedlings were treated with 0.01 or 0.1 mM $CuCl_2$, the amount of M excreted in the seedling growth medium was approx. 73% or 43%, respectively of total M (M in roots plus that in the growth medium). However, M excretion decreased with increasing copper concentration (from 259.4 to 16.3 nmol g^{-1} in 0.01 and 1 mM $CuCl_2$-treated seedlings, respectively) (Fig. 4C). Apart from M, MGM and F were also excreted in the growth solution. The amount of MGM in the growth solution decreased with increasing copper concentration, while that of F increased slightly.

If M exudation is mediated by ATP-binding cassette (ABC) transporters, as shown for the isoflavonoid genistein [26] ATP and ATPase must play a central role. In order to investigate the possible involvement of ATP and ATPase in the copper-induced M excretion, we studied the modification of M excretion in the presence of an ATPase and an ATP-synthesis inhibitor (orthovanadete and KCN, respectively). The results showed a significant decrease in M excretion in the presence of orthovanadate or KCN. At concentrations from 100 to 500 μM of orthovanadate, M excretion was reduced by approx. 44%. The application of the same concentrations of KCN resulted in a progressive inhibition of M excretion, which reached 98% at 500 μM KCN (Fig. 5). Both inhibitors did not affect significantly seedling growth at the concentrations used.

3.4. PAL activity and transcript levels of CHS and VR

PAL, which directs Phe to the phenylpropanoid pathway, was present in both roots and shoots of fenugreek seedling. PAL activity of roots or shoots increased in parallel to the increases in M accumulation, and there was a significant correlation between PAL activity and net M formation. The highest activities were observed in roots of 0.01 mM $CuCl_2$-treated seedlings and in shoots of 0.5 mM $CuCl_2$-treated ones, coinciding to the highest net M accumulation (Table 2).

In order to correlate the HPLC data with the expression of specific genes in the M biosynthetic pathway, we used a semi-quantitative reverse transcription (RT) PCR approach. The copper-induced accumulation of *CHS* and *VR* (an early and a late gene of the

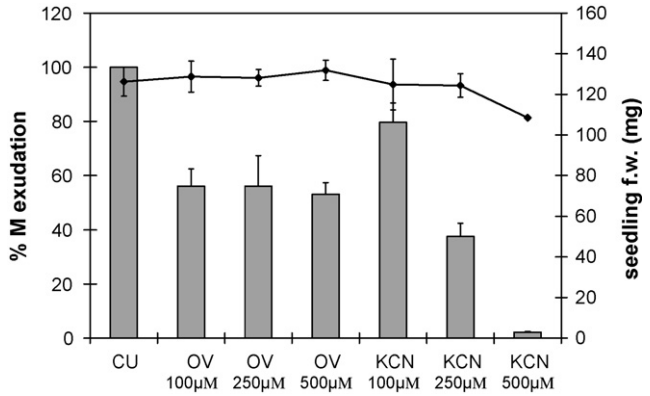

Fig. 5. Effect of Na_3VO_4 or KCN on root and shoot fresh weights and on the % of copper-induced M exudation of fenugreek seedlings (bars). Five-day-old seedlings were incubated for 24 h in the presence or absence of Na_3VO_4 (100, 250 or 500 μM) or KCN (100, 250 or 500 μM) and then treated with 0.01 mM $CuCl_2$ for further 24 h.

Table 2
PAL activity and net M formation in fenugreek roots and shoots as affected by copper elicitation.

	PAL activity (μmol CA mg^{-1} protein $^{-1}$ h^{-1})	Net M (nmol g^{-1} FW)
Roots		
Control	5.3 ± 3.0^a	4.1 ± 0.8^a
0.5 mM $CuCl_2$	5.1 ± 1.4^a	37.4 ± 4.9^b
0.1 mM $CuCl_2$	8.5 ± 1.0^a	71.2 ± 15.5^c
0.01 mM $CuCl_2$	17.0 ± 0.4^b	329.5 ± 25.5^d
Shoots		
Control	0.6 ± 0^a	1.0 ± 0.3^a
0.5 mM $CuCl_2$	7.2 ± 4.0^b	194.2 ± 14.9^b
0.1 mM $CuCl_2$	2.1 ± 0.3^b	38.3 ± 2.6^c
0.01 mM $CuCl_2$	0 ± 0^c	7.5 ± 0.5^d

Fenugreek seedlings were treated hydroponically with the stated concentrations of $CuCl_2$ for 24 h. Net M values represent the difference between amounts of M + MGM in control and copper treated seedlings. (The amount of M and MGM in roots includes that excreted in the growth medium.) Values are the mean ± S.E. of three experiments. Different letters signify significance at (at least) $p < 0.05$ (Student's *t*-test).

M biosynthetic pathway, respectively) transcripts were monitored on reverse transcribed RNA, extracted from 6-day-old fenugreek seedlings and cell suspension cultures, after a-6-h elicitation. Degenerated primers from known *CHS* and *VR* genes were initially used in order to amplify and clone the corresponding fragments from fenugreek. After verifying the sequences, fenugreek specific primers were used in order to carry out the semi-quantitative RT-PCR. As shown in Fig. 6, transcript levels of *CHS* and *VR* were increased in root tissues treated with 0.01 mM $CuCl_2$. However, their expression decreased progressively at higher copper concentrations (from 0.1 to 1 mM). On the contrary, shoot tissues responded weekly to low copper concentrations (0.01 and 0.1 mM). *CHS* and *VR* transcripts started to accumulate at 0.5 and 1 mM $CuCl_2$.

4. Discussion

The isoflavonoid constituents of fenugreek seedlings have been studied to date only in roots of 4-week-old *Rhizobium*-infected seedlings [27]. In the present study we show that MGM is a constitutive constituent in fenugreek suspension cultures as well as in roots and shoots of young seedlings, where it accumulates in a developmental manner. In contrast, free M is present only in small amounts in roots and cell cultures while it is absent from shoots. The trace amounts of MGM and the absence of M in the shoots of 7-day-old seedlings are in accordance to the very low levels of *VR* gene transcripts. In shoots, copper induces the upregulation of *CHS* and *VR* and the increase of PAL activity along with the accumulation of both M and MGM, in a concentration-dependent manner. Thus M formed in shoots of fenugreek seedlings in response to copper may originate from de novo synthesis. However, the possibility that a small amount is transported to the shoots from roots cannot be excluded.

The origin of copper-induced M in roots varies with copper concentration. At a concentration of 0.01 mM, copper induces an increase in free M without any change in MGM or F and a concomitant increase of PAL activity and *CHS* and *VR* transcript levels. Since no FGM or FG were present in roots, it is reasonable to speculate that this accumulation of M is derived exclusively from de novo synthesis and not from its conjugate pool. Higher copper concentrations induce an increase of F and a progressive reduction of both MGM and M compared to 0.01 mM $CuCl_2$, which is in accordance to the observed reduction of PAL activity and *CHS* and *VR* transcript levels. Taken together, the above data suggest that M origin in roots is differentially regulated by copper. While at low copper concentration M originates from de novo synthesis, as elicitor concentration increases de novo synthesis is gradually replaced by MGM hydrolysis.

The accumulation of isoflavonoid free aglycones and the decrease of their conjugates is a usual defense mechanism in response to elicitation [28]. Increases in isoflavonoid aglycones in response to elicitation have been attributed to either their de novo synthesis and/or to the hydrolysis of their conjugates. In chickpea cell suspension cultures, elicitation with moderate amounts of yeast extract induced the de novo synthesis of medicarpin and maakian, part of which accumulated in the form of the corresponding conjugates. Elicitation of higher yeast extract concentrations resulted in the accumulation of aglycones only, which derived from the hydrolysis of their conjugates [29]. In contrast, the copper-induced accumulation of maakian and formononetin [4] or medicarpin [7] in roots of red clover or alfalfa seedlings, respectively, was attributed solely to the hydrolysis of the corresponding isoflavonoid conjugates and not

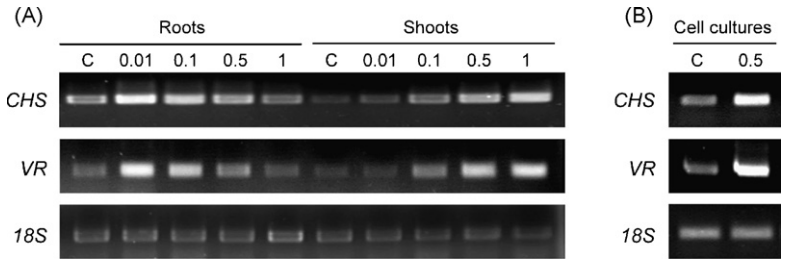

Fig. 6. RT-PCR analysis of *CHS* and *VR* gene expression. Semi-quantitative RT-PCR of steady state *CHS* and *VR* mRNA in roots and shoots of 6-day-old fenugreek seedlings and cell cultures. (A) Six-day-old seedlings treated with 0.01, 0.1, 0.5, or 1.0 mM $CuCl_2$. (B) Cell cultures treated with 0.5 mM $CuCl_2$. Total RNA was prepared from roots, shoots or cell cultures 6 h after elicitation. Expression of the *18S* rRNA gene was monitored as a control. C, control.

to de novo synthesis. Our results suggest that in fenugreek seedlings, depending on tissue and copper concentration, part or all of copper-induced M derives from de novo synthesis. The copper-induced increases in PAL activity and the up-regulation of the M pathway genes, *CHS* and *VR*, are in accordance with the above hypothesis.

The different response to copper elicitation between shoots and roots of fenugreek seedlings may reflect the different Cu^{2+} levels between these tissues. Copper accumulates mainly in roots [30–32]. Long exposure of *Lupinous albus* roots to 20 or 62 μm $CuSO_4$ showed that copper concentration in roots was approx. 2 or 6 times higher, respectively, than in the aerial parts [33]. In the primary leaves of *Phaseolus vulgaris* seedlings, 48 h after 15 or 50 μM Cu supply to the roots, copper concentration was 15 or 30 μg g^{-1} DW, respectively [30], while in roots elevated amounts of copper were already observed from the very first hour of exposure, reaching 400 or 1.400 μg g^{-1} DW (from 15 μg g^{-1} DW), respectively after 48 h of treatment [32]. Similarly, the measurements of copper concentrations in fenugreek shoots and roots of $CuCl_2$-treated fenugreek seedlings show that shoots suffered from considerably milder copper stress than the corresponding roots. Thus, it can be argued that low tissue copper concentration results in M de novo synthesis (even when tissues possess a considerable pool of its conjugate), while high concentration favours M production through MGM hydrolysis.

In cell suspension cultures and seedlings, a considerable amount of the produced M is exuded in the feeding medium (Table 1 and Fig. 4C). One of copper effects is the production of free radical hydroxyls, which in turn react to cause membrane lipid peroxidation resulting in membrane damage [34,35]. Thus it could be postulated that M exudation upon copper elicitation would be the result of a non-specific leakage out of the root cells. If this were the case, M exudation would increase with increasing copper concentration. However, M concentration in the fenugreek seedling growth medium decreased significantly as copper concentration increased in a dose-dependent manner (Fig. 4C), suggesting that M exudation was regulated by copper concentration through an M-specific transport system. Root exudation is an active process, controlled by transporters [36], by which plants interact with the rhizosphere. The isoflavonoid genistein can bind Cu^{2+} ions and its exudation in the presence of copper, may play a detoxifying role [33] leading to the assumption that M exudation may have a similar role. Sugiyama et al. [26] have shown that *Rhizobium*-induced secretion of genistein is ATP-dependent and involves ATP-binding cassette-type transporters. Similarly, the observed reduction of the copper-induced M excretion by fenugreek roots in the presence of KCN or orthovanadate may imply the involvement of such transporters (Fig. 5).

In conclusion, the origin of copper-induced M accumulation in roots and shoots of fenugreek seedlings is dependent on tissue copper concentration. Low internal copper concentrations triggered M biosynthesis in both shoots and roots and M export from root cells. At higher concentrations copper induced MGM hydrolysis in roots to contribute to M accumulation, while M biosynthesis and exudation were restricted. The similar response to copper elicitation of fenugreek seedlings and suspension cultures makes the latter an appropriate experimental tool to study the copper-induced M exudation in the environment.

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