Fungitoxicity of natural heterocycle glucoside vicine obtained from *Vicia faba* L. against selected microscopic filamentous fungi

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ABSTRACT

Vicine and convicine were isolated from mature seeds of *Vicia faba* L. These compounds were separated from the relevant fractions by HPLC and identified by IR, MS and NMR. Obtained vicine was used for all bioassays. Vicine in concentration of 25 µg in 1 ml of medium had significant toxic effect. The concentration of 5 µg of vicine in 1 ml of medium had significant fungistatic effect for fungi *Fusarium culmorum* and *Alternaria alternata*. The concentration of 500 ng of vicine in 1 ml of medium had significant fungistatic effect for fungi *Cladosporium herbarum* and *Botrytis cinerea*. The effective concentration of vicine in our test was significantly lower than is the concentration of vicine in seeds and pods of *V. faba*. The toxicity of vicine comparing with the toxicity of insecticide Actellic was higher by one order. Also, the fungicide Impact had a lower toxicity than vicine. Fungitoxic and fungistatic effect of vicine was comparable with some other tested fungicides and with the plant saponin digitonin. Our results confirmed that vicine is important natural fungicide.

Keywords: vicine; convicine; pirimiphos-methyl; flutriafol; propiconazole; carbendazim; fungicide bioassay; *Fusarium culmorum*; *Cladosporium herbarum*; *Alternaria alternata*; *Botrytis cinerea*

Vicine [2,6-diamino-5-(β-D-glucopyranosyloxy)-4(1*H*)-pyrimidinone, CAS RN: 152-93-2], convicine [6-amino-5-(β-D-glucopyranosyloxy)-2,4(1*H*,3*H*)-pirimidinedione, CAS RN: 19286-37-4] and their aglycones divicine [2,6-diamino-1,6-dihydro-4,5-pyrimidinedione, CAS RN: 32267-39-3] and isouramil [6-amino-5-hydroxy-2,4(1*H*,3*H*)-pyrimidinedione, CAS RN: 3914-34-9] are natural biologically active plant products (Figure 1). Vicine and/or convicine were detected in various species of family Fabaceae: *Vicia sativa* L. (Ritthausen 1876), and *Vicia faba* L. (Brown and Roberts 1972). Vicine was found also in *Pisum sativum* L. (Jamalian et al. 1977) and in *Lupinus albus* L. (Pompei and Lucisano 1976), but were not reported in other paper (Pitz et al. 1980). The highest concentration of vicine or convicine in faba beans was found in young seeds. The concentration decreased rapidly with maturity of seeds or the whole pod (Jamalian and Bassiri 1978, Pitz et al. 1981, Bjerg et al. 1985). Vicine was isolated also from species of other families, for example family Chenopodiaceae (Von Lippmann 1896), or *Cucurbitaceae* (Dutta et al. 1981).

Effects of vicine and convicine on various organisms are described and characterized in many studies. Vicine and convicine induce several human diseases, for example favism. The LD_{50} data of vicine and its aglycone (divicine) 24 hours after intraperitoneal administration were 4000 $mg.kg^{-1}$ for vicine and 149 $mg.kg^{-1}$ for divicine related to body weight of rats. The influence of vicine on insect was also studied (Deschroches et al. 1995).

There are several papers describing fungicidal properties of vicine and convicine. However, they are mostly derived from one single paper (Bjerg et al. 1984). In this paper influence of vicine, convicine and 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) in various concentrations (0.006, 0.030, 0.150%) was tested on fungi (*Botrytis cinerea* Pers. ex Fr. – pathogenic to *V. faba*, *Ascochyta fabae* Speg. – pathogenic to *V. faba* and *Pyrenophora graminae* Ito et Kurib. – apathogenic to *V. faba*) that were cultivated on agar discs. The selected concentration levels of the tested medium were related to concentrations of vicine in green plant parts of the faba beans (Bjerg et al. 1985). It has shown a growth inhibition in these tests (recorded as a related between the mycelium weight at each concentration variant against the control) for *Botrytis cinnerea* and *Ascochyta fabae*, as well as for *Pyrenophora graminea*. Vicine and convicine manifest-

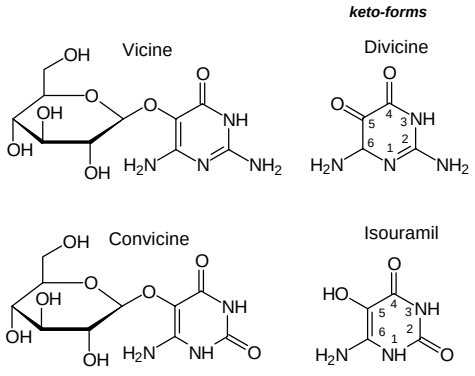

Figure 1. Structural formulas of significant natural heterocycles in *V. faba*

ed weak fungistatic effects. It is evident that the test results from *Ascochyta fabae* are not sufficiently evident. The results of the remaining two species manifested certain inhibition in the hyphae growth.

The aim of this study is to confirm or disprove that pirimidine glycosides (vicine or convicine) containing in *V. faba* might be a part of the plant resistance towards the phytopathogenic fungi.

MATERIAL AND METHODS

Isolation and identification of vicine and convicine from *Vicia faba* L.

Vicine and convicine were isolated from two different samples of *Vicia faba* L. (first one was a non-specific cultivar and the second cultivar was of genotype EEV). Mature seeds (1 kg) were milled in Wiley mill. The seed powder was successively extracted with light petroleum ether, ethyl acetate (EtOAc) and methanol (MeOH), and after that with cold and later hot (70°C) MeOH-H_2O (1:1). The combined MeOH and MeOH-H_2O extracts were evaporated and dissolved in water (500 ml). The solution was washed six times in 250 ml 1-butanol (n-BuOH) and the water portion was evaporated. The dry residue of the water portion was separated on column chromatography (CC) packed with LiChroprep RP-18 (40–63 μm). The column was eluted successively with 500 ml water, three times with 200 ml MeOH-H_2O (2:1, 1:1, 1:2) and 500 ml MeOH. Fractions (100 ml) were collected and monitored on thin layer chromatography (TLC). Crude vicine (3 g) and small quantum of convicine were obtained. Vicine crystallised from the first fraction already during the elution and evaporation of water.

Vicine was purified and convicine isolated from the relevant fractions by high performance liquid chromatography (HPLC) (performed on a Knauer instrument). Detection and analysis were carried out at 274 nm. Column (250 × 4 mm I.D.) was filled with Separon SGX C18 (particle size 7 μm). Water 92.5% with 7.5% MeOH was used as mobile phase, with flow rate 0.6 ml.min^{-1} eluted during 25 minutes. A column (250 × 8 mm I.D. or 250 × 12.7 mm I.D.) filled with Separon SGX C18 (particle size 7 μm) was used for separation and/or purification of convicine and vicine. Water was used as mobile phase with a flow rate 3 ml.min^{-1} or 4 ml.min^{-1}, respectively.

For the detection of vicine and convicine, authentic samples were used as internal standard. The identity of both compounds was confirmed by infrared spectroscopy (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR).

Bioassay of vicine

For the bioassay were used strains of the Culture Collection of Fungi of the Department of Botany, Faculty of Natural Science, Charles University, Prague, Czech Republic (Kubátová et al. 1996). Vicine toxicity was observed on *Fusarium* spp., CCF 1839 *F. culmorum* (FC), CCF 1333 *F. solanum* (FS), CCF 1699 *Cladosporium herbarum* (CH), CCF 2672 *Alternaria alternata* (AA) and CCF 2361 *Botrytis cinerea* (BC).

The cultivation methods of microscopic filamentous fungi for the bioassay on agar, or liquid malt nutrient media in test tubes, and the evaluation of the mycelium growth in the fungal cultures for each treatment of the tested substances has been formerly reported by Pavlík et al. (2000).

Toxicity of vicine was compared with pesticides Actellic 50 EC (pirimiphos-methyl), Impact 125 EC (flutriafol), Tilt 250 EC (propiconazole), Tilt CB FW (carbendazim and propiconazole), which were obtained from various manufacturers of agrochemicals (Table 1). Digitonin as a representative of plant saponins was also used for a comparison. The volumes of added solutions and the doses of the active ingredients are indicated in Table 2. The toxicity of vicine was compared with four commercial pesticides. The pesticides were used as reference material because they are used in plant protection in agriculture.

RESULTS AND DISCUSSION

HPLC analysis

Vicine and convicine was isolated from non-specific cultivars (identified by IR, MS and NMR) that were further used as reference samples for all other isolations. Vicine obtained from this preparation was used for all bioassays. As the amount of convicine was not sufficient for bioassays, we tried to isolate it from other source (the

Table 1. The characterization of pesticides and their active ingredients

Pesticides	Actellic 50 EC	Impact EC	Tilt 250 EC	Tilt CB FW*
Production	Zeneca	Zeneca	Ciba-Geigy AG	Ciba-Geigy AG
Active ingredients	pirimiphos-methyl	flutriafol	propiconazole	carbendazim and propiconazole
CAS RN	29232-93-7	76674-21-0	60207-90-1	10605-21-7
Contents	50%	125 g.l^{-1}	250 g.l^{-1}	25%, 12.5%

* data only for first active ingredient, CAS RN chemical abstract registry number

Table 2. Volume of pesticide and vicine solutions for treatments

Dilution (solution, doses)	Treatments				
Tested substances	A	B	C	D	E
Pesticides	20 µl	2 µl	2 µl	2 µl	2 µl
Pirimiphos-methyl	**	1 mg	100 µg	**	**
Flutriafol	2.5 mg	0.25 mg	25 µg	**	**
Propiconazole[1]	**	0.5 mg	50 µg	5 µg	0.5 µg
Carbendazim[2]	**	0.5 mg	50 µg	5 µg	0.5 µg
Propiconazole[2]	**	0.25 mg	25 µg	2.5 µg	0.25 µg
Vicine	**	150 µl	20 µl	4 µl	4 µl
Vicine	**	0.375 mg	50 µg	10 µg	1 µg
Digitonin	**	0.5 mg	50 µg	10 µg	1 µg
Digitonin	**	100 µl	10 µl	2 µl	2 µl

[1] Tilt 250 EC, [2] Tilt CB FW; ** test was not done

genotype EEV). From the HPLC chromatogram (Figure 2) is evident that the convicine fraction obtained from CC contained several constituents. According to the internal standards, the vicine was detected as peak 4 and convicine as peak 3. After isolating both compounds only vicine was identified (by IR and MS), but the compound corresponding to peak 3 was according to FAB (fast atom bombardment)-MS characterised as a compound with MW 336 and composition $C_{12}H_{20}O_9N_2$, not identical with convicine. The next compound corresponding to peak 2 remained also undetermined, however, it might be the same compound reported by Sixdenier et al. (1996). He also indicated a presence of an additional slower-migrating peak, corresponding with an undetermined substance (with UV absorption at 274 nm) frequent in various genotypes of faba bean. It was described in numerous papers that in this chromatographic region was detected L-DOPA. However, the analysis of MS has shown that our peak 2 was not identical with L-DOPA. The fragmentation in MS indicated the presence of glucose. In addition, other fractions obtained by CC and analysed by HPLC contained compounds that elute in the region of vicine and convicine. They are also glycosides (confirmed by MS), but different from vicine and convicine, and might be also interesting as defense compounds of faba bean plants against negative biotic factors.

Bioassay

The microscopic filamentous fungi were selected with respect to their optimal growth in experimental *in vitro* conditions. These selected phytopathogenic microorganisms, which can be well cultivated even without a host, thus belonging to facultative parasites. In contrary to Bjerg's work (Bjerg et al. 1984) we did not concentrated our attention to *Ascochyta fabae* pathogenic to *V. faba*, or other genera and species from family Fabaceae, because these fungi are adapted to the defense mechanisms of the host plants. We selected for our experiments *B. cinerea*, which we did not consider obligatory pathogenic to *V. faba*. This fungus does not cause

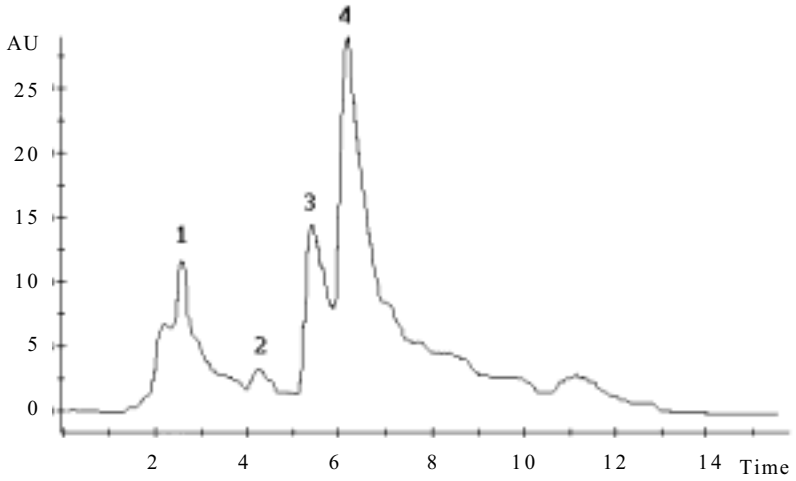

Figure 2. HPLC analyse of the convicine fraction from *V. faba* (genotype EEV) from CC; HPLC conditions: Separon SGX C18 (7.5:92.5, v/v) at 0.6 ml.min^{-1}; the peaks 1–3 undetermined substances, peak 4 vicine

Table 3. Results of the bioassay

Natural product	Treatments			
Doses vicine in 2 ml medium	0.375 mg	50 µg	10 µg	1 µg
Fungi	B	C	D	E
Fusarium culmorum	+ –	+ –	+	+ +
Alternaria alternata	+ –	+ –	+	+ +
Cladosporium herbarum	**	–	+ –	+
Botrytis cinerea	**	–	+ –	+ +

– it does not grow, + – it was slightly grown and deposited on the bottom (fungitoxic effect), + it was grown slightly (fungistatic effect), + + it was grown massively (from little effect up to no effect), ** test was not done

any serious decease of faba bean in our region (Czech or Slovak Republic), and thus has no negative impact on the yield (Zvára 1981).

In contrast with results of Bjerg et al. (1984), vicine in our bioassay showed more significant fungitoxic and/or fungistatic properties. Vicine in concentration of 25 µg in 1 ml of medium had significant toxic effect (Table 3). No mycelium growth has been observed at *C. herbarum* and *B. cinerea*. Only a few hyphae with non-continuing growth were found at *F. solani*, *F. culmorum* and *A. alternata*. They sedimented on bottom of test tube. The concentration of 5 µg of vicine in 1 ml of medium had significant fungistatic effect on fungi *F. culmorum* and *A. alternata*. The concentration of 500 ng of vicine in 1 ml of medium had significant fungistatic effect on fungi *C. herbarum* and *B. cinerea*.

Significant differences in the sensitivity are caused by differences between the used methods. Our experience (Pavlík et al. 2000) evidently indicated that it is more suitable to test pure natural compounds in a liquid medium than on agar plates. One can see the advantage when testing substances with a low solubility in water or even in organic solvents as it is in the cases of vicine, convicine or digitonin. Such poorly soluble compounds can easy precipitate in the solidifying agar medium. Therefore is the concentration of the tested compound on the agar discs or at the external paper disc method discontinued. Crystallization of the tested compound may appear at higher concentrations. The dissolved compound on the external disc can be partly diffused into the surrounding space. The major part of the space can remain without any test-germinating spores, they do not penetrate the external paper discs for a restricted time, however, after certain delay they grow in all spaces including the external discs. Therefore, the evaluation of compound is difficult, but fungal spores can grow on the whole surface of Petri dishes. Hyphae growing from this method should be done by 24 hours. On the other hand, if the test compound is applied into the fluid medium such problems do not appear. Therefore, it is possible to evaluate not only acute toxicity (by 24 hours), but also chronic toxicity after several days, as it was done in our experiments. The evaluation done after several days is very important for the study of toxicity of natural compounds in plants. This long time evaluation is free of some problems connected with the fungistatic effect. It is easily possible to isolate both, spores and mycelium from the fluid medium than from agar discs. It is also possible to evaluate both, the qualitative effect (absence of the spores germination power and destroyed hyfes of the mycelium deposited on the bottom of the test tube), and the quantity of the mycelium grown on or in the fluid medium. This test better correlates with the *in vivo* conditions. This is generally valid also for *in vivo* toxicity tests performed on pollen (Pavlík and Jandurová 1998, 1999, 2000, 2001).

The effective concentration of vicine in our test was significantly lower than is the concentration of vicine in seeds and pods of *Vicia faba*. The toxicity of vicine compared with the toxicity of insecticide Actellic was higher by one order. Also, the fungicide Impact had a lower toxicity than vicine. Fungitoxic and fungistatic effect of vicine was comparable with some other tested fungicides and with the plant saponin digitonin. The importance of comparing pesticides and fungicidal natural product with the test compound is described elsewhere (Pavlík et al. 2000). Our results compared with those of Bjerg et al. (1984) confirmed that vicine is important natural fungicide.

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ABSTRAKT

Fungitoxicita přírodního heterocyklického glukosidu vicinu z *Vicia faba* L. k vybraným mikroskopickým vláknitým houbám

Vicin s konvicinem byly izolovány ze zralých semen *Vicia faba* L. Tyto sloučeniny byly separovány z příslušných frakcí HPLC a identifikovány IR, MS a NMR. Získaný vicin byl použit pro všechny biozkoušky. Vicin v koncentraci 25 µg v 1 ml média měl významný toxický efekt. Koncentrace 5 µg v 1 ml média měla významný fungistatický efekt pro houby *Fusarium culmorum* a *Alternaria alternata*. Koncentrace 500 ng vicinu v 1 ml média měla významný fungistatický efekt na houby *Cladosporium herbarum* a *Botrytis cinerea*. Účinná koncentrace vicinu v našich testech byla významně nižší, než je koncentrace vicinu v semenech a luscích *V. faba*. Toxicita vicinu byla v porovnání s toxicitou insekticidu Actellic řádově vyšší.Také fungicid Impakt měl nižší toxicitu než vicin. Fungitoxický a fungistatický účinek vicinu byl srovnatelný s dalšími testovanými fungicidy a s rostlinným saponinem digitoninem. Výsledky potvrdily, že vicin je důležitým přírodním fungicidem.

Klíčová slova: vicin; konvicin; pirimiphos-methyl; flutriafol; propiconazol; carbendazim; fungicidní biotest; *Fusarium culmorum*; *Cladosporium herbarum*; *Alternaria alternata*; *Botrytis cinerea*

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