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Antifungal activity of saponins originated from *Medicago hybrida* against some ornamental plant pathogens

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Summary

Antifungal activity of total saponins originated from roots of *Medicago hybrida* (Pourret) Trautv. were evaluated *in vitro* against six pathogenic fungi and eight individual major saponin glycosides were tested against one of the most susceptible fungi. The total saponins showed fungitoxic effect at all investigated concentrations (0.01%, 0.05% and 0.1%) but their potency was different for individual fungi. The highest saponin concentration (0.1%) was the most effective and the inhibition of *Fusarium oxysporum* f. sp. *callistephi*, *Botrytis cinerea*, *Botrytis tulipae*, *Phoma narcissi*, *Fusarium oxysporum* f. sp. *narcissi* was 84.4%, 69.9%, 68.6%, 57.2%, 55.0%, respectively. While *Fusarium oxysporum* Schlecht., a pathogen of *Muscari armeniacum*, was inhibited by 9.5% only. Eight major saponin glycosides isolated from the total saponins of *M. hybrida* roots were tested against the mycelium growth of *Botrytis tulipae*. The mycelium growth of the pathogen was greatly inhibited by hederagenin 3-*O*-β-D-glucopyranoside and medicagenic acid 3-*O*-β-D-glucopyranoside. Medicagenic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside and oleanolic acid 3-*O*-[β-D-glucuronopyranosyl(1→ 2)-α-L-galactopyranosyl]-28-*O*-β-D-glucopyranoside showed low fungitoxic activity. Medicagenic acid 3-*O*-a-D-glucopyranosyl-28-*O*-β-D-glucopyranoside, hederagenin 3-*O*-[α-L-rhamnopyranosyl(1→ 2)-β-D-glucopyranosyl(1→ 2)-β-D-glucopyranosyl]- 28-*O*-β-D-glucopyranoside and hederagenin 3-*O*-α-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside did not limit or only slightly inhibited growth of the tested pathogen. While 2β, 3β-dihydroxyolean-12 ene-23-al-28-oic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside slightly stimulated mycelium growth of *B. tulipae*.

Key words: *Medicago hybrida*, root saponins, fungicidal activity, plant pathogens

INTRODUCTION

It is commonly known that use of some fungicides can create dangers to health and to our natural environment. Therefore, during the last decade, more and more studies have been carried out on the possibility of using natural plant-based substances, which would be less toxic than those synthetic chemicals. A considerable number of world publications have drawn attention to the possibility of using saponins, as natural fungicides (Hostettmann and Marston, 1955; Oleszek, 1996). Searching for new plant originated substances, a couple of years ago, we screened twenty nine *Medicago* species and found that a few of them were very rich in fungicidal saponins (Jurzysta and Waler, 1996; Jurzysta and Biały, 1999). Consistently, chemical structure and fungicidal activity of saponins of species such as *M. sativa* (Levy et al. 1989, Biały et al. 1999; Martyniuk et al. 1996; Saniewska et al. 2001; 2003), *M. arabica* (Martyniuk et al. 2004; Biały et al. 2004; Martyniuk et al. 2004; Saniewska et al. 2005), *M. arborea* (Tava et al. 2005) and *M. hybrida* (Biały et al. 2006) have been studied.

Recently, we isolated from roots of *Medicago hybrida* fourteen triterpene saponins and established their structures (Biały et al. 2006), but did not study their biological activities. In the present study, antifungal activity of total saponins from roots of this species was evaluated *in vitro* against six fungi and eight individual major saponin glycosides were tested against *Botrytis tulipae*, one of the most susceptible fungus.

MATERIALS AND METHODS

Total saponins and saponin glycosides

Total saponins and their individual glycosides were isolated from roots of *M. hybrida* (Pourret.) Trautv. according to the procedure described by Biały et al. (2006). Shortly, the ground plant material was defatted with chloroform, and then extracted with methanol under reflux. After removal of alcohol, the residue was dissolved in a small volume of water and the solution was placed on a LiChroprep RP 18 column. The column was washed with water and with diluted methanol until colourless solution was obtained. Total saponins were eluted with methanol and dried. Then the obtained total saponins were fractionated on a silica gel column by eluting with *n*-butanol saturated with water, and individual saponin glycosides were separated from the fractions by means of reversed-phase chromatography on LiChroprep RP-18 columns eluting with aqueous methanol solutions. Saponin structures were established on the basis of hydrolysis and spectral evidence, including IR, optical rotations, NMR and FAB-MS analyses (Biały et al. 2006).

The total saponins and the following saponin glycosides (Fig. 1) were studied for their antifungal activity:

1. Hederagenin 3-*O*-β-D-glucopyranoside

2. Medicagenic acid 3-*O*-β-D-glucopyranoside

3. Medicagenic acid 3-*O*-β-D-glucopyranosyl-28-*O*-β-D-glucopyranoside

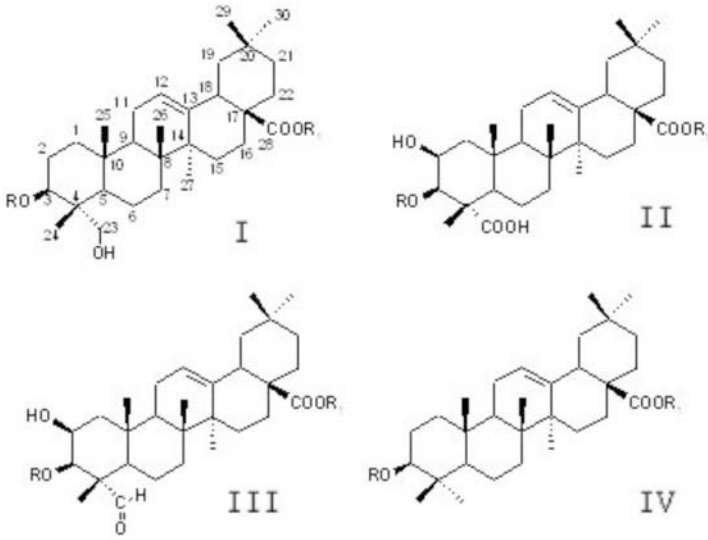
4. Hederagenin 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside

5. Hederagenin 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside

6. 2β,3β-dihydroxyolean-12ene-23-al-28-oic acid 3-*O*-β-D-glucuronopyranosyl- 28-*O*-β-D-glucopyranoside

7. Medicagenic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D- glucopyranoside

8. Oleanolic acid 3-*O*-[β-D-glucuronopyranosyl(1→2)-β-D-galactopyranosyl]-28-*O*-β-D-glucopyranoside



Saponins	Aglicone	R	R_1
1	**I**	β-D-Glc	H
2	**II**	β-D-Glc	H
3	**II**	β-D-Glc	β-D-Glc
4	**I**	β-L-Rha(1→2)-β-D-Glc(1→2)-β-D-Glc	β-D-Glc
5	**I**	β-D-GlcA	β-D-Glc
6	**III**	β-D-GlcA	β-D-Glc
7	**II**	β-D-GlcA	β-D-Glc
8	**IV**	β-D-Gal(1→2)-β-D-GlcA	β-D-Glc

Fig. 1. Structure of saponins **1 8** (I, hederagenin; II, medicagenic acid; III, 2β,3β dihydroxyole an 12ene 23 al 28 oic acid; IV, oleanolic acid).

Table 1.

Inhibitory effect of total saponins originated from roots of *Medicago hybrida* L. on the mycelial growth of some pathogenic fungi.

Pathogenic fungi	Days of incubation	Inhibition of mycelium growth (%) at a saponin concentration of		
		0.01 %	0.05 %	0.1 %
Botrytis cinerea	4	76.9 ab	80.0 a	69.9 b
Botrytis tulipae	5	77.1 a	80.8 a	68.6 a
Fusarium oxysporum f. sp. *callistephi*	7	36.6 c	59.6 b	84.4 a
Fusarium oxysporum f. sp. *narcissi*	7	39.4 c	48.7 b	55.0 a
Fusarium oxysporum , *a* pathogen of *Muscari armeniacum*	7	5.5 b	11.8 a	9.5 ab
Phoma narcissi	5	33.0 c	52.9 b	57.2 a

Means in row followed by the same letters are not significantly different at P=0.05 according to Duncan's test.

Table 2.

The effect of individual saponins, indicated in Materials and Methods as numbers **3** **8**, on the mycelium growth of *Botrytis tulipae* after six days of incubation (% of control).

onin **3**	Saponin **4**	Saponin **5**	Saponin **6**	Saponin **7**	Saponin **8**
.0	+41.5	0.0	+9.0	-5.7	-8.7
.0	-30.6	-2.4	+28.0	-11.3	-15.0
.0	+35.7	-22.0	-44.4	-0.8	-10.9
.0	+22.3	-26.8	-49.0	-22.8	-10.7

"+" stimulatory effect, " " inhibitory effect

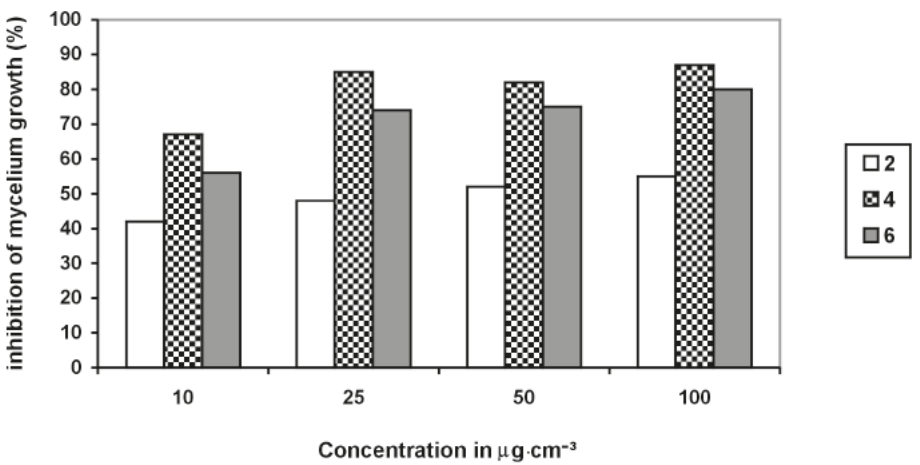


Fig. 2. Inhibitory effect of hederagenin 3 *O* β D glucopyranoside **(1)** in different concentrations on *in vitro* mycelium growth of *Botrytis tulipae* after 2, 4 and 6 days of incubation.

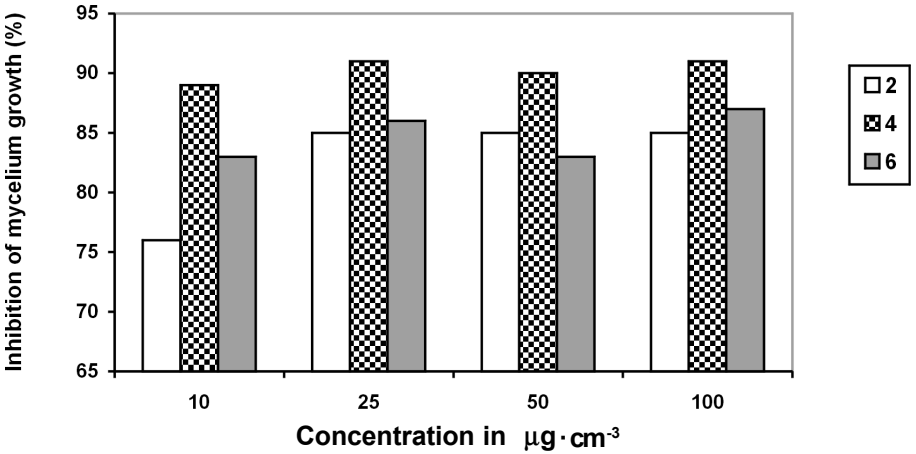


Fig. 3. Inhibitory effect of medicagenic acid 3 *O* β D glucopyranoside **(2)** in different concentration on *in vitro* mycelium growth of *Botrytis tulipae* after 2, 4 and 6 days of incubation.

***In vitro* growth of some pathogenic fungi in the presence of saponins**

Botrytis cinerea Pers. [*Botryotinia fuckeliana* (de Bary) Whetzel], *Botrytis tulipae* (Lib.) Lind, *Phoma narcissi* Aderh. [syn. *Stagonospora curtissi* (Berk.) Sacc.], *Fusarium oxysporum* Schlecht. f. sp. *callistephi* (Beach.) Snyd. et Hans., *Fusarium oxysporum* Schlecht. f. sp. *narcissi* Snyd. et Hans., and *Fusarium oxysporum* Schlecht., a pathogen of *Muscari armeniacum*m, were used for investigation. The total amount of saponins originated from roots of *Medicago hybrida* at final concentrations of 0.01%; 0.05% and 0.1% were previously dissolved in 5 cm^3 distilled and sterilized water and added to potato-dextrose-agar (PDA-Merck) after sterilization at a temperature of about 50°C. The single saponins isolated from roots of *M. hybrida* at final concentrations of 10, 25, 50 and 100 $\mu g \cdot cm^{-3}$ were dissolved in 3 cm^3 of 75% methanol and added to 100 cm^3 of PDA after sterilization. Five mm diameter plugs taken from 7-day-old culture of tested fungi, were placed in the middle of 90 mm Petri dishes containing PDA medium supplemented with the tested compounds and control without saponins. The plates were incubated at 25°C in darkness. The diameter of colonies was measured within 4, 5 or 7 days-incubation depending on the fungus being tested. Five dishes were used for each treatment and the experiment was repeated twice. To analyze the differences between mean values, Duncan's test was used, with a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

The total saponins at concentrations of 0.01%, 0.05% and 0.1% showed strong fungitoxic effect against all of the investigated fungi (Table 1). However, there were variable effects on the growth of those fungi. Saponins greatly inhibited the growth of *Fusarium oxysporum* f. sp. *callistephi*, *Botrytis cinerea*, *Botrytis tulipae*, *Phoma narcissi*, *Fusarium oxysporum* f. sp. *narcissi* and linear growth of the mycelium of these

fungi, treated with 0.1% solution of saponins, was inhibited by 84.4%, 69.9%, 68.6%, 57.2%, 55.0%, respectively, in relation to the control culture. While, linear growth of the mycelium of *Fusarium oxysporum* Schlecht., a pathogen of *Muscari armeniacum* was inhibited only by 9.5%.

It should be mentioned that different concentrations of saponins similarly inhibited mycelium growth of *Botrytis cinerea* and *Botrytis tulipae*, or higher concentration slightly less inhibited the mycelium growth of *B. cinerea* (Table 1). It is suggested that lower concentrations of saponins were sufficient to block all active places (receptors?) in mycelium hyphae. The mechanism of inhibitory effect of saponins on mycelium growth is unknown. It is also possible that higher concentrations of saponins may interact with other endogenous compound(s) of mycelium and finally the inhibitory effect on mycelium growth is slightly decreased.

According to our knowledge, saponins of *M. hybrida* have never been studied for fungicidal properties, although we have recently shown that saponins of this species possess an insecticidal activity as high as *M. arabica* or *M. murex* saponins (Szczepanik et al. 2004). On the other hand it is known that both of these species are also rich in highly fungicidal saponins (Martyniuk et al. 2002; Saniewska et al. 2005).

The individual saponin glycosides (Fig. 1) exerted different effects on the mycelium growth of *Botrytis tulipae*. Two of them: hederagenin 3-*O*-β-D-glucopyranoside **(1)** and medicagenic acid 3-*O*-β-D-glucopyranoside **(2)** had the strongest inhibitory influence against the mycelium growth of *B. tulipae* on PDA medium (Fig. 2 and 3). Hederagenin 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside **(5)**, medicagenic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside (7) and oleanolic acid 3-*O*-[β-D-glucuronopyranosyl(1→ 2)-β-D-galactopyranosyl]-28-*O*-β-D-glucopyranoside **(8)** showed low fungitoxic activity against the mycelium growth of the pathogen (Table 2). Medicagenic acid 3-*O*-β-D-glucopyranosyl-28-*O*-α-D-glucopyranoside **(3)** and hederagenin 3-*O*-[α-L-rhamnopyranosyl(1→ 2)-β-D-glucopyranosyl(1→ 2)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside (4) did not limit or stimulate the growth of the tested pathogen (Table 2). 2β,3β-dihydroxyolean-12ene-23-al-28-oic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside **(6)** slightly stimulated mycelium growth of *B. tulipae* at a low concentrations and inhibited growth at higher concentrations (Table 2). There are many examples in the literature showing that monodesmosidic saponins possessing a single sugar chain at C-3 exhibit very high biologically activities in comparison with bidesmosidic saponins which have two sugar chains. For example medicagenic acid 3-*O*-β-D-glucopyranoside shows large fungitoxic activity against many plant pathogens (Levy et al. 1986; Martyniuk et al. 1996, 2005; Saniewska et al. 2003). While, bidesmosidic glycosides of medicagenic acid exhibit very low activity.

It can be seen that *M. hybrida* saponins consist of both monodesmosidic and bidesmosidic glycosides (Fig. 1). Looking at the structure of these bidesmosidic glycosides, from the chemical point of view, it is easy to notice that alkaline hydrolysis of most of them could eliminate sugar chains from the ester linkage at C-28 and induce transformation of low biologically active saponins being bidesmosides into high active monodesmoside. Thus, this procedure allows the creation of highly biologically active medicagenic acid 3-*O*-β-D-glucopyranoside from the saponin 3. Similarly,

bidesmosidic saponins **5, 6** and **7** could be converted into monodesmosides: hederagenin 3-*O*-β-D-glucuronopyranoside, 2β, 3β-dihydroxyolean-12ene-23-al-28-oic acid 3-*O*-β-D-glucuronopyranoside and medicagenic acid 3-*O*-β-D-glucuronopyranoside, respectively. Antifungal activity of these hypothetical saponins should be studied but on the basis of data in the literature these saponins are expected to be highly biologically active.

In conclusion, saponins of *Medicago hybrida* have been shown to possess significant antifungal activity and roots of this plant could be a rich source of natural fungicides.

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Aktywność antygrzybowa saponin korzeni *Medicago hybrida* w stosunku do kilku patogenów roślin ozdobnych

S t r e s z c z e n i e

Oszacowano *in vitro* aktywność sumy saponin pozyskanych z korzeni *Medicago hybrida* na wzrost grzybni sześciu patogenów i przetestowano osiem dominujących glikozydów saponinowych względem *Botrytis cinerea*. Suma saponin wykazała fungistatyczny wpływ przy wszystkich badanych stężeniach (0,01%, 0,05% i 0,1%) ale ich aktywność była różna dla poszczególnych grzybów. Najwyższe stężenie (0.1%) saponin było najbardziej efektywne i wyrażało się silnym hamowaniem *Fusarium oxysporum* f. sp. *callistephi* (84,4%), *Botrytis cinerea* (69,9%), *Botrytis tulipae* (68,6%), *Phoma narcissi* (57,2%), *Fusarium oxysporum* f. sp. *narcissi* (55,0%). Podczas, gdy *Fusarium oxysporum* Schlecht, patogen pochodzący z *Muscari armeniacum* był zahamowany zaledwie w 9,5%. Osiem dominujących glikozydów saponinowych wyodrębnionych z sumy saponin testowano w stosunku do *Botrytis tulipae*. Wzrost liniowy grzybni tego patogena był silnie zahamowany przez dwa glikozydy: 3-*O*-*β*-D-glukopyranozyd hederageniny i 3-*O*-*β*-D-glukopyranozyd kwasu medikagenowego. 3-*O*-*β*-D-glukuronopyranozylo-28-*O*-*β*-D-glukopyranozyd kwasu medikagenowego i 3-*O*-[*β*-D-glukuronopyranozylo(1→2)-*α*-L-galaktopyranozylo]-28-*O*-*β*-D-glukopyranozyd kwasu oleanolowego wykazywały niską fungitoksyczność. 3-*O*-*β*-D-glukopyranozylo-28-*O*-*β*-D-glukopyranozyd kwasu medikagenowego, 3-*O*-[α-L-rhamnopyranozylo(1→ 2)-*β*-D-glukopyranozylo(1→ 2)-*β*-D-glukopyranozylo]-28-*O*-*β*-D-glukopyranozyd hederageniny i 3-*O*-*β*-D-glukuronopyranozylo- 28-*O*-*β*-D-glukopyranozyd hederageniny nie wpływają lub tylko śladowo wpływają na wzrost badanego patogena. Podczas, gdy 2*β*,3*β*-dihydroxyolean-12ene-23-al-28-karboksy 3-*O*-*β*-D-glukuronopyranozylo- 28-*O*-*β*-D-glukopyranozyd lekko stymuluje wzrost *B. tulipae*.