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Effects of triterpenoid saponins of field scabious (*Knautia arvensis* L. *Coult*.), alfalfa, red clover and common soapwort on growth of *Gaeumannomyces graminis* var. *tritici* and *Fusarium culmorum*

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ABSTRACT

Field scabious (*Knautia arvensis* L. *Coult*) was chosen for study, as it was previously identified among 500 plants as potential anti-proteolytic feed additive for ruminants and reducing the number of protozoa, due to its saponin content. Saponins from aerial parts of field scabious, alfalfa and red clover as well as roots of common soapwort were tested in agar medium to study their effects on growth of two major pathogens of cereals: *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and *Fusarium culmorum* (*Fc*). All saponins inhibited the *Ggt* growth. Most active saponins were from soapwort (IC_{50} 14.1 µg/ml) followed by alfalfa (IC_{50} 148.5 µg/ml) and red clover (IC_{50} 156.8 µg/ml), whereas field scabious were almost inactive (IC_{50} >1000 µg/ml). Saponins from alfalfa, red clover and field scabious stimulated the *Fc* growth and only the compounds from common soapwort were slightly inhibitory to *Fc* growth. In most cases, the effects of saponins on growth of both fungi was dose-dependent.

Key words: Alfalfa, common soapwort, field scabious, fungal growth inhibition, fungal growth stimulation, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici*, *Knautia arvensis,* pathogens, red clover, triterpenoid saponins.

INTRODUCTION

Five hundred plant samples and their extracts were screened in the Project of European Union 6th Programme - *Rumen*-up (Ruminal Metabolism Enhanced Naturally Using Plants) and field scabious (*Knautia arvensis* L. Coult.) - a perennial plant characteristic of temperate climate was identified as a potential anti-proteolytic feed additive for ruminants. This activity was related to its saponin content and it reduced the number of protozoa and a Patent application of *K. arvensis* in ruminant nutrition was filed (patent no. WO2005099729) (2,8,28). Therefore the secondary metabolites of field scabious were recently studied in our laboratories. Our first studies reported that methanol extract from *K. arvensis* contained a wide spectrum of secondary metabolites: phenolic

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acids, flavonoids (18) and triterpenoid saponin glycosides [mazusaponin I and mazusaponin II] (17).

Saponins are probably produced by plants to protect them against attack by pathogens as many of them show antimicrobial activity. The antifungal activity of saponins is similar to polyene antibiotics and involves the formation of complexes with sterols in fungal membranes. This results in pore formation, loss of membrane integrity and leakage of cell contents of saponin-sensitive fungi (1,19,24). The best known example of antifungal activity of saponins is avenacine A-1 – a triterpenoid saponin from oat roots (*Avena* spp.). This saponin protects the oats against infection by *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (*Ggt*), which is a problematic pathogen of wheat, barley, rye and triticale plants, which cannot synthesize saponins (24). Take-all, caused by *Ggt*, is the most significant root disease of wheat (*Triticum aestivum* L.) worldwide (6). As the use of fungicides in plant protection against *Ggt* gave unsatisfactory results, the focus has been on alternative strategies like biological controls by using the antagonistic microorganisms (3,6) and by searching for new chemicals naturally occurring in plants, including saponins *e.g.* of various *Medicago* species (13-16). Not all fungi are sensitive to saponins. Two major mechanisms of fungi resistance to saponins exist. Some fungi (*e.g.* oomycetes *Pythium* and *Phytophtora*) are resistant to the toxic effect of saponins because they have little or no sterols in their membranes, while other produce enzymes which specifically detoxify the saponins of their host plants. These phytopathogenic fungi produce specific glycosyl hydrolases that remove sugar molecules from their glycosyl chains to give products which are less toxic to fungal growth, since it decreases their amphipathic nature and thereby prevents them from disrupting membranes. Some of these enzymes are constitutively expressed, while others are induced by their saponin substrates. The degradation of avenacine A-1 by *Gaeumannomyces graminis* var. *avenae* (*Gga*), which contrary to *Ggt*, can infect oat roots, is well known example of such decomposition (19,24,25). There are also several examples that various fungi of the genus *Fusarium* [active in rhizosphere region and roots] (7), are resistant to certain saponins (1,4,5,26,27). The genus *Fusarium* includes many phytopathogenic species. One of them, *F. culmorum* (W.G. Smith) Sacc. (*Fc*) is very harmful to plants by causing as *Ggt*, foot and root rot diseases (3,7,9).

This study aimed to test *in vitro* the antifungal activity of triterpenoid saponin fraction from the aerial parts of field scabious against these two strong wheat pathogens - *Ggt* and *Fc*, in comparison with triterpenoid saponin fractions from the aerial parts of alfalfa (intensively studied in our lab), red clover and roots of common soapwort [plant well known for its detergent property and cultivated world wide] (10).

MATERIALS AND METHODS

I. Plant material

Field scabious (*Knautia arvensis* L. Coult.) plants were collected from the experimental plots using seeds from plants growing in the wild. Red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L. var. 'Radius') were collected from experimental plots using commercial seeds. Dry, ground roots of common soapwort (*Saponaria officinalis* L.) were purchased from commercial source (Herbapol in Krakow,

Poland). All voucher samples have been deposited in the Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy, Poland.

II. Fungal isolates

The isolates of *Ggt* and *Fc*, both virulent to wheat were isolated from wheat roots and used in this study.

III. Extraction and isolation of saponins fractions

Dried powdered aerial parts of *K. arvensis*, *T. pratense, M. sativa* were defatted with $CHCl_3$ and roots of *S. officinalis* with $CHCl_3$:hexane (1:1 v/v) in a Soxhlet apparatus for 48 h and extracted with 70% MeOH overnight at room temperature. The extract was filtered and the residue was extracted twice under the same conditions. The combined methanolic extract was concentrated under reduced pressure and lyophilized. The extract was suspended in H_2O and applied to a preparative column of RP-18 (100 x 60 mm, LiChroprep C18, 40-63 μm, Merck) preconditioned with water. Sugars and phenolics were removed with 40% MeOH. Saponins were then eluted from the column with 80% MeOH for field scabious, red clover and alfalfa, and 90% MeOH for common soapwort. The solvent was evaporated in vacuum and the residue was lyophilized, obtaining crude mixture of saponins.

IV. Antifungal activity

Samples (500 mg) of saponin fractions of each plant were dissolved in 100 ml MiliQ water and then sterilized by microfiltration using sterile Whatman 25 mm GD/X PVDF syringe filters with pore size 0.2 μm. The different concentrations of sterile saponin solutions were mixed with sterile water to the final volume of 10 ml and then these solutions were added to 40 ml of warm (50°C) agar nutrient medium. This medium contained 48.75 g of Potato Dextrose Agar (Difco) and 1.25 g of Yeast Extract (Difco) in 1000 ml (PDA+YE). After thorough mixing the agar medium with the saponin solutions, 15 ml aliquots of these mixtures (~40°C) were poured into Petri dishes (9 cm dia). After solidification, the agar plates were kept overnight in dark to dry surface of medium and then 6 mm discs of *Ggt* and *Fc* grown on the same agar medium were put after turning upside down in the centre of the medium surface. Final concentrations of saponins in the agar medium were: 0, 50, 100, 200, 500 and 1000 μg/ml (0, 0.005, 0.01, 0.02, 0.05 and 0.1%). Additionally, only in the case of *S. officinalis* and *Ggt*, the experiment was repeated with a set of lower concentrations of saponins: 0, 10, 20, 30, 40, 50 μg/ml (0, 0.001, 0.002, 0.003, 0.004 and 0.005%). Radial mycelial growth was estimated by two perpendicular measurements of colony diameter on three Petri plates minus 6 mm of initial diameter of fungal discs, after 2-8 days of *Ggt* incubation and after 2-6 days of *Fc* incubation, both at 25°C. Plates with the soapwort saponins in the agar medium were incubated with *Fc* for additional two days due to slower growth of the fungus. Fungal growth inhibition or stimulation by saponin addition to medium were also calculated. In *Ggt*, concentrations of saponin fractions at which 50% (in comparison to the control growth value = 100%) fungal growth inhibition occurred (IC_{50}) were calculated from dose-response curves using the regression analysis (Excel). In *Fc*, the concentration of saponins at which 25% fungal growth inhibition occurred (IC_{25}) was calculated only for common soapwort.

Statistical analysis: Results of the radial mycelial growth after 8 (*Ggt*) and 6 or 8 (*Fc*) days of incubation were subjected to ANOVA and means were compared using Tukey's multiple range test (Statgraphics). Statistical significance was declared at $P = 0.05$. All determinations were done in six replicates.

RESULTS AND DISCUSSION

***Gaeumannomyces graminis* var. *tritici* (*Ggt*)**

***K. arvensis* :** *Ggt* was found sensitive to the triterpenoid saponins and the fungal growth rate was dose-dependent for all plants (Figs. 1 and 2). The weakest negative effect on the *Ggt* growth was observed for saponins from aerial parts of *K. arvensis*. The two lowest concentrations (50 µg/ml and 100 µg/ml) of saponins did not inhibit the fungal growth. Growth was inhibited by 10-30% at 200-1000 µg/ml (Figs. 1A and 2A).

Alfafa : The saponins from alfalfa aerial parts at 500 µg/ml and 1000 µg/ml completely inhibited the *Ggt* growth (Fig. 1B). At 200 µg/ml, the *Ggt* isolate started to grow after 8 days (9 mm colony dia). The lowest concentration of saponins (50 µg/ml) had little effect (26% of fungal growth inhibition after 2 days of incubation and 8% after 8 days). At 100 µg/ml, *Ggt* growth was delayed for two days and the growth inhibition gradually decreased to 21% after 8 days (Fig. 2B). It suggested that the saponins from alfalfa aerial parts induced the ability of the fungus to deglucosylate these substrates. Osborne (24) also reported that many isolates of *Ggt* have very weak ability to deglucosylate avenacin A-l, which was detectable only after prolonged incubation with the substrate. In these fungi, DNA sequences that cross-hybridize to the *AVN1* gene encoding avenacinase in *Gga* were found.

Red clover: Saponins from aerial parts of red clover had strange effect on *Ggt* isolate growth. In two lowest concentrations (50 and 100 µg/ml), the effect was non-significant after 8 days, although there was 1-day delay in growth and gradual fungal growth inhibition was noticed. However, the higher concentrations (200-1000 µg/ml) completely inhibited the growth of fungus until the end of 8-day incubation period (Figs. 1C and 2C).

***S. officinalis* :** The strongest negative effect was observed for root saponins of common soapwort (Figs. 1D, 2D and 3D), when the fungus did not show any symptoms of its growth on the medium containing 100-1000 µg/ml of the saponin fraction (results not presented). These saponins at 10-50 µg/ml also adversely affected the fungal growth (Fig. 1D). Inhibition of the fungal growth by soapwort saponins was 75% at 20 µg/ml, 90% at 30 µg/ml and 95% at 40 µg/ml during the experiment. However, inhibition of fungal growth exposed to 10 µg/ml of saponins gradually decreased from 35% after 3 days to 9% after 8 days (Fig. 2D).

The following regression equations describe the relationships between saponin concentrations and inhibitions of *Ggt* growth:

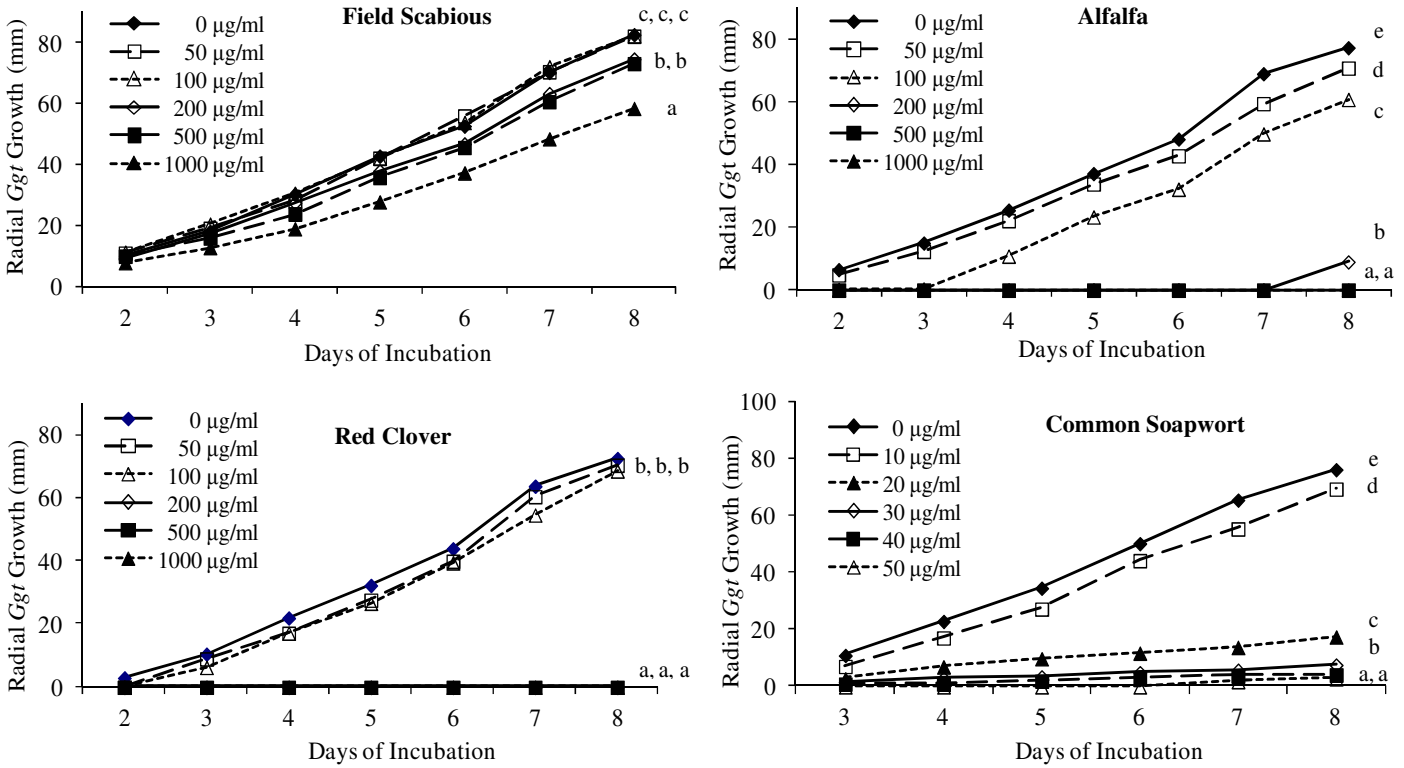

Figure 1. Effects of saponin content in PDA+YE agar medium on colony radial growth of the *Ggt*. The fungal colonies diameter after 8 days, marked with the same letter are not significantly different at $P = 0.05$.

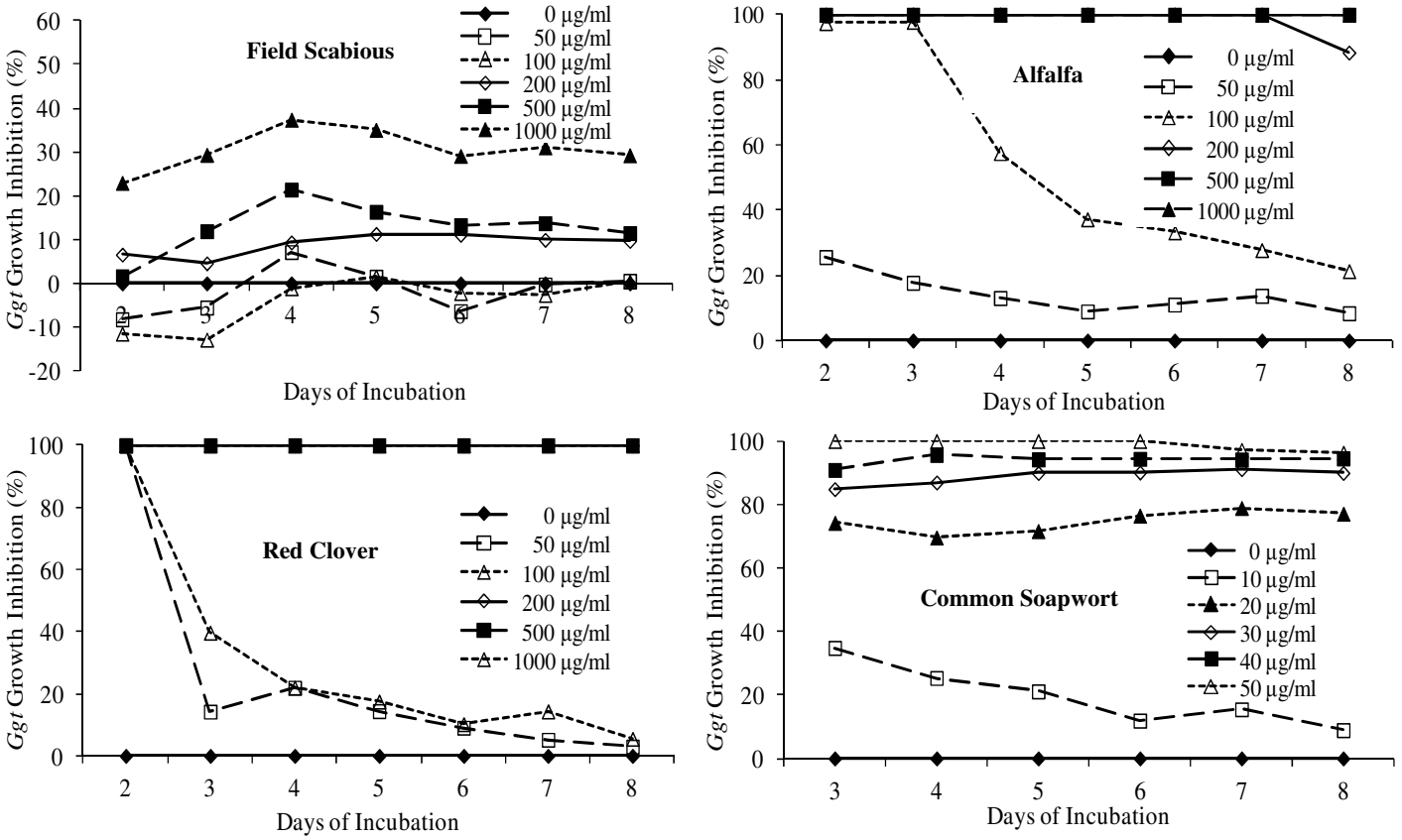

Figure 2**.** Inhibitory effects of saponins in PDA+YE agar medium on *Ggt* growth (in %).

Linear for field scabious ($y = 0.0289x - 0.3325$, $R^2 = 0.95$) and common soapwort ($y = 3.3799x - 6.7031$, $R^2 = 0.90$)

Quadratic for alfalfa ($y = 0.0022x^2 + 0.005x + 0.7524$, $R^2 = 1.00$) and red clover ($y = 0.004x^2 + 0.3239x + 2.4791$, $R^2 = 0.99$).

Where x : Concentration of saponins, y : Growth inhibition (%)

The IC_{50} concentrations of triterpenoic saponins calculated from these equations were: 14.1 µg/ml for roots of common soapwort, 148.5 µg/ml for aerial part of alfalfa, 156.8 µg/ml for aerial parts of red clover and >1000 µg/ml for aerial parts of field scabious.

***Fusarium culmorum* (*Fc*)**

***S. officinalis* :** The sensitivity of the *Fc* isolate was opposite to *Ggt*. At the end of incubation period, only the saponins from roots of common soapwort had weak inhibitory effects on *Fc* growth (Fig. 3D). This effect was dose-dependent, although it was statistically significant only for two highest saponin concentrations (500 and 1000 µg/ml). After 8 days of incubation, the rates of *Fc* growth inhibition on the agar nutrient medium containing 50, 100, 200, 500 and 1000 µg/ml were 4, 6, 11, 23 and 30%, respectively (Fig 4D). The saponin concentration at which 25% fungal growth inhibition occurred (IC_{25}) was 539 mg/l, obtained from the regression equation ($y = -0.00003x^2 + 0.0622x + 0.2043$, $R^2 = 1.00$). Fungus responded differently to saponins from roots of common soapwort at the beginning and end of incubation period. Saponins stimulated the growth of *Fc* isolate after 2 days and fungal growth-stimulation was gradually replaced by fungal growth-inhibition (Fig. 4D). The products of the saponin degradation by the fungus were probably more toxic to the microorganism than the initial substrate. Although fungal glycosidases are intended to detoxify plant saponins, but they can make the saponins more toxic *e.g.* by removing sugar molecules from the glycosyl chains. Such phenomenon can be observed in oats. Avenacoside is stored in the plant as the bisdesmoside, which has a relatively low disrupting capacity of both plant and fungal membranes. After wounding the plant tissues, endogenous hydrolyzing enzymes can remove one of the sugar chains to produce a monodesmosidic compound, which is toxic to fungi and probably also to plant cells (24).

Alfalfa: In general, *Fc* reacted to alfalfa saponin fraction by a moderate increase in colony diameter up to 26-27% by the highest dose of saponins after 2 and 3 days of incubation. The stimulation of fungal growth was dose-dependent after 4 days, but at the end of incubation period, the colony diameters of *Fc* isolate growing on two highest doses of saponins were lower than those of 100 and 200 µg/ml saponin concentrations (Figs. 3B and 4B). After 6 days, the diameters of fungus growing on 100-1000 µg/ml of saponin doses were significantly higher than control and 50 µg/ml series (Fig.3B).

Red clover, *K. arvensis*: Addition of red clover and the field scabious *(K. arvensis)* saponins into the agar medium also stimulated the growth of *Fc*. This stimulation was dose-dependent, reaching 50% at the highest saponin concentration after 2-6 days for red

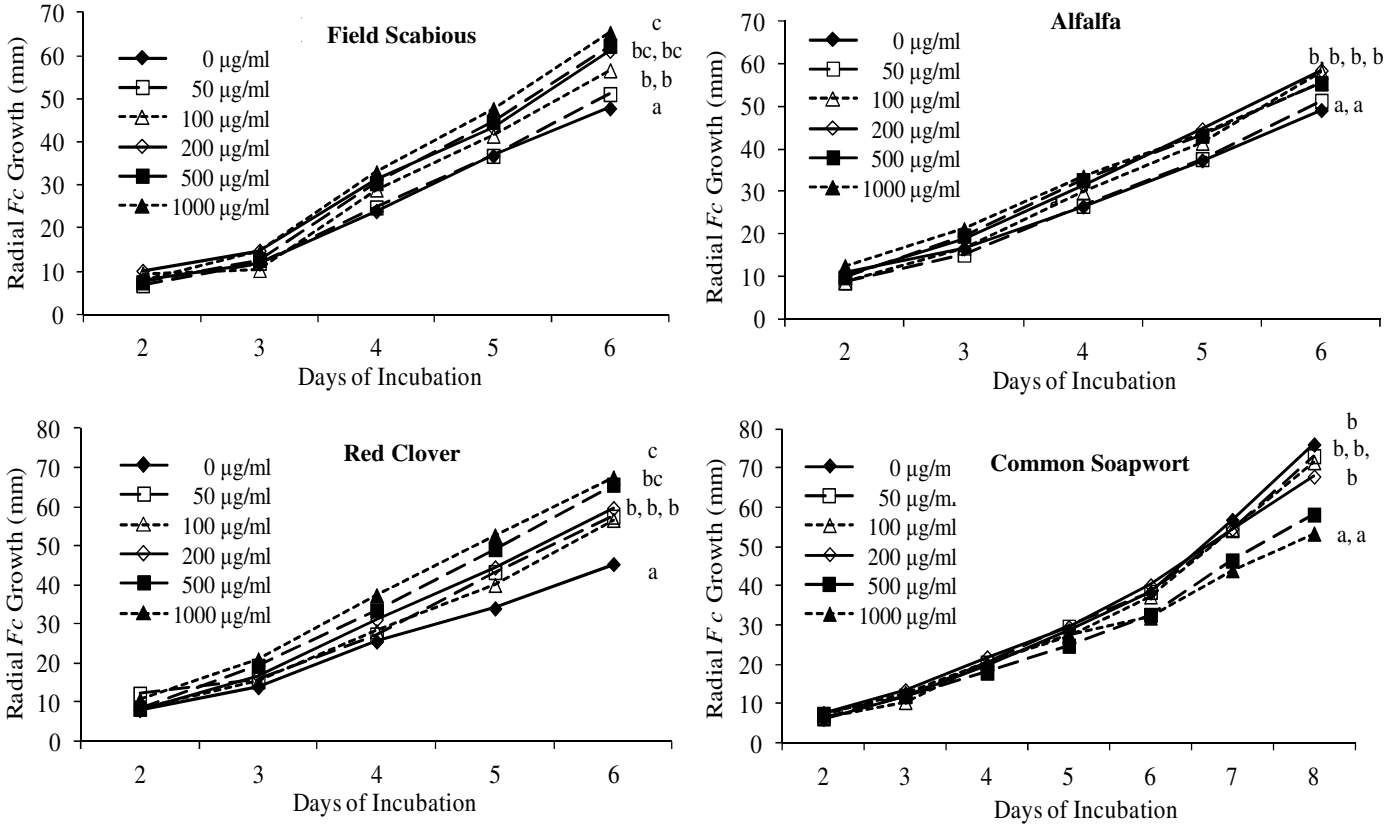

Figure 3. Effects of saponin content in PDA+YE agar medium on radial colony growth of *Fc* isolate**.** The diameters of fungal colonies after 6 or 8 days, marked with the same letter are not significantly different at $P = 0.05$.

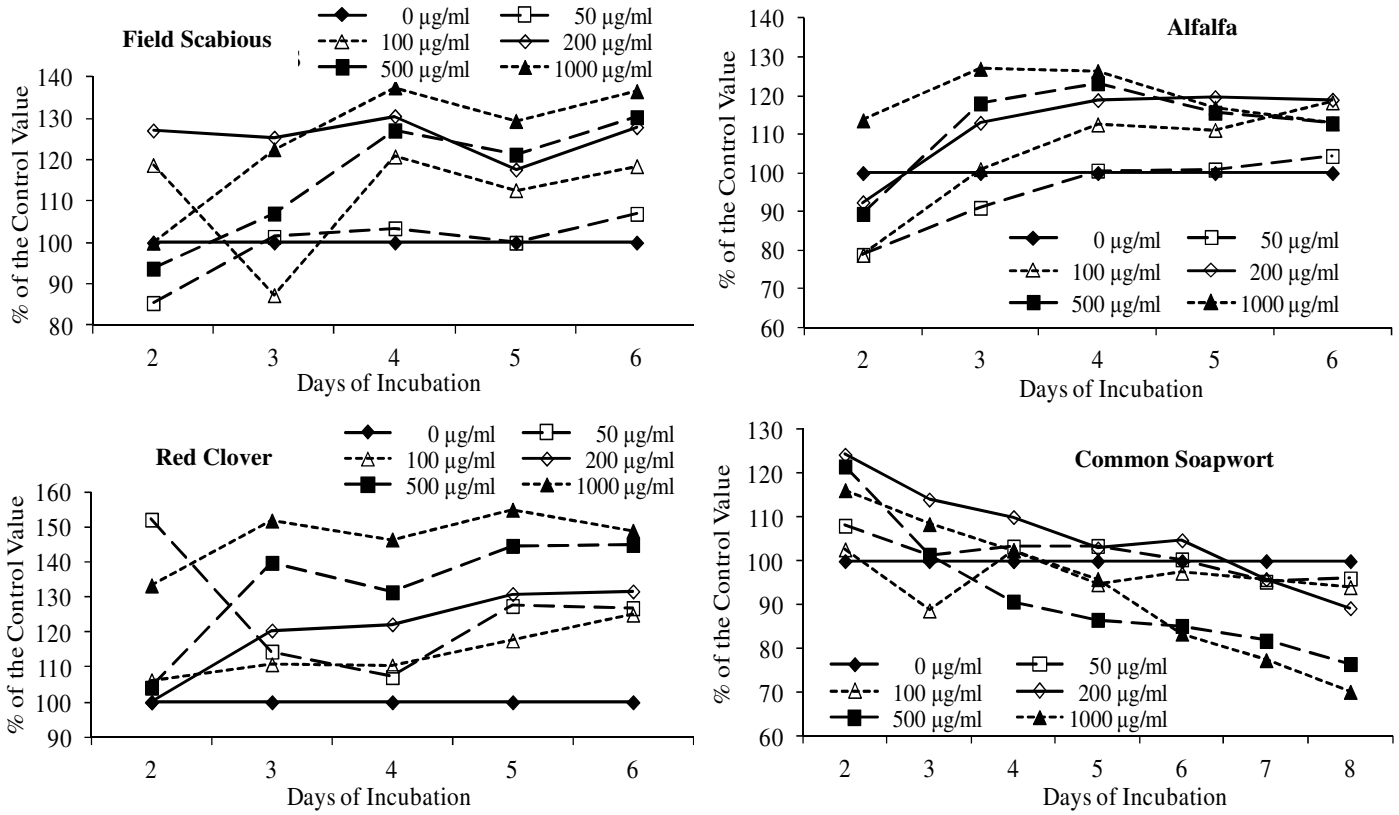

Figure 4. Inhibitory effects of saponins on *Fc* growth (in % in comparison to the control) on PDA+YE agar medium.

clover and 30-40% for field scabious after 4-6 days. On the 6th day, colony diameters of both fungi were significantly higher than control for all saponin experimental series (Figs. 3A, 3C, 4A and 4C).

Comparison of the activity of saponins from these four plant species showed that the effect was dependent on the saponin source. Most active against *Ggt* were saponins from soapwort, followed by those from alfalfa and red clover, whereas saponins from field scabious were almost inactive. Soapwort saponins were the only ones to inhibit *Fc* growth, whereas the saponins from other plants (especially from red clover) stimulated the growth of this fungus. This is consistent with previous findings that triterpene saponins are not highly active against most of *Fusarium* species (22).

Considering these facts it was expected that soapwort saponins having quilaic acid, gipsogenin and gipsogenic acid as an aglycone parts (10,11,12) had antifungal activity. It also concerned alfalfa top saponin having medicagenic acid and zanhic acid as an aglycone part (20), but red clover saponins based on soyasapogenols were biologically inactive (23). Mazusaponins were the dominant aglycones of field scabious (17).

These differences in antifungal activities of the studied saponin fractions of field scabious, alfalfa, red clover and soapwort can be due to the structural differences among the saponins. Sapogenins were responsible for antimicrobial activity. Sugar substituents may modulate this activity and usually monodesmosides are more active than bisdesmosides. Also, functional groups *e.g.* COOH present in the prosapogenin molecule increase this activity, but there is no general rule (21,22). To explain these differences, further research with the use of individual glycosides is needed.

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