

Composition and Quantitation of Saponins in Alfalfa (*Medicago sativa* L.) Seedlings

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The concentrations of individual saponins in germinating alfalfa seeds and seedlings between 1 and 16 days of growth were determined by HPLC. It was shown that monodesmosidic medicagenic acid glycoside was synthesized after 4 days of germination and subsequently followed by bidesmosidic saponin production. The total saponin concentration increased from 2.12 $\mu\text{mol/g}$ of dry matter at the beginning of germination to around 6 $\mu\text{mol/g}$ after 8–16 days of seedling growth. It was concluded that previous reports on saponin concentration in alfalfa seedlings (8–10% in dry matter) as measured by biological tests were highly overestimated; the real concentration is several times lower.

Keywords: *Medicago sativa*; seedlings; saponins; germination; secondary metabolites

INTRODUCTION

Alfalfa (lucerne) is a valuable source of a high-quality protein in temperate climates. The aerial parts of the plant may be used as a forage crop in the form of green feed, hay, or pellets. The human consumption of lucerne is generally low, but in some countries there has been an increasing interest in using alfalfa as sprouts for green salads or in the form of tablets or juices (Oakenfull, 1980; Malinow et al., 1982) for their effect on serum cholesterol. The influence on serum cholesterol is believed to be due to the triterpene saponins occurring in seeds, roots, and aerial parts. In general these saponins are regarded as antinutrients, being especially harmful for monogastric animals due to their effect on the palatability of the feed (Cheeke, 1983) or due to their influence on the digestion and absorption processes (Lu and Jorgensen, 1987; Oleszek et al., 1994). Mucosal irritation and effects on liver and kidney are common properties of saponins (Kawaguchi et al., 1994), and thus estimation of the safety is essential for the use of any saponin or saponin containing products as a food or food additives.

During the past few years there has been great progress made in the chemistry of saponins in roots and aerial parts of alfalfa (Oleszek, 1996). A number of individual soyasapogenol B, hederagenin, medicagenic acid, and zanhic acid glycosides have been isolated and their structure-dependent hemolytic (Oleszek, 1990), antifungal (Polacheck et al., 1986; Oleszek et al., 1990b, 1992), and intestine membrane depolarizing activities (Oleszek et al., 1994) have been established. Moreover, on the basis of available saponin standards, a new analytical HPLC procedure for their determination has been developed (Nowacka and Oleszek, 1992, 1994). This analytical procedure clearly showed that some previous literature data on saponin concentrations in plant material, as measured by biological tests, were overestimated due to the structure-dependent activities of individual compounds and their differential distribution in plant parts. It has been reported that a small

variation in highly active, monodesmosidic saponins may strongly influence the results (Nowacka and Oleszek, 1994).

There has been very limited information available on the concentration and composition of saponins in alfalfa sprouts. It was documented that alfalfa seeds are free of biologically active saponins and contain only soyasapogenol B glycosides showing limited activity (Jurzysta, 1973). However, during germination and juvenile seedling growth, rapid synthesis of biologically active saponins was reported (Pedersen, 1975; Gorski et al., 1991). As measured by bioassays, the concentration of saponins in seedlings reached a very high level: up to 8–10% of dry matter (Fenwick and Oakenfull, 1983; Price et al., 1987; Gorski et al., 1991). Thus the aim of the present work was to determine the occurrence of individual saponins in alfalfa seedlings and verify early biological data on their quantitation with new analytical techniques.

MATERIALS AND METHODS

Plant Material. Seeds of alfalfa (*Medicago sativa* var. Boja) were germinated on a filter paper in glass Petri dishes (1 g per dish) at room temperature (21–22 °C, 80% relative humidity). The papers were kept moist by subirrigated paper wicks. Forty eight plates were prepared, and three plates were terminated each day for 16 days. The germinated seeds or seedlings were freeze-dried and powdered.

Chemicals. Acetonitrile and methanol were of HPLC grade (Baker, Lodz, Poland); bromophenacyl bromide and 18-crown-6 were from Sigma (St. Louis, MO).

HPLC Analysis. *Extraction.* For extraction, 200 mg of powdered material was refluxed for 1.5 h with 10 mL of aqueous 30% MeOH. The extract was centrifuged, and supernatant was made up to 10 mL with 30% MeOH.

Purification. A 5 mL amount of each extract was passed through a C₁₈ SepPak cartridge (Waters Associates) preconditioned with 5 mL of 30% MeOH. The cartridge was washed with 5 mL of 30% MeOH, and saponins were removed with 5 mL of HPLC grade MeOH.

Derivatization for HPLC. After evaporation of the solvent, saponins were derivatized with 4-bromophenacyl bromide (Oleszek et al., 1990a). For the determination of zanhic acid

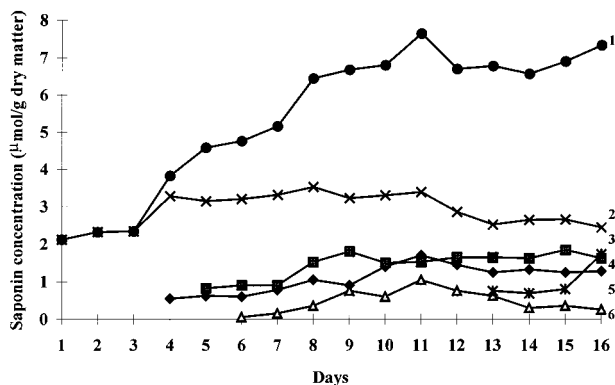


Figure 1. Saponin concentration in germinating alfalfa seeds: 1, total saponin concentration; 2, soyasaponin I; 3, zanhic acid tridesmoside; 4, 3Glc,28Glc medicagenic acid; 5, 3Glc medicagenic acid; 6, 3GlcA,28AraRhaXyl medicagenic acid.

tridesmoside a previously described procedure was adopted (Nowacka and Oleszek, 1994).

Identification and Quantitation. The saponins derivatized with 4-bromophenacyl bromide were analyzed by a HPLC system (Waters) consisting of a 616 pump, a 600S controller, and a 996 photodiode array detector operating at 260 nm. The Millennium Chromatography Manager was used to monitor chromatographic parameters and to process the data. Separations were performed on a 5 μm (250 \times 4.6 mm i.d.) Eurospher 80 C₁₈ column (Säulenteknik, Germany). Chromatographic runs were carried out using a mobile phase (ACN/H₂O) gradient as previously described (Nowacka and Oleszek, 1992). Three independent chromatographic runs were performed for each extract, and saponins were identified by comparing their retention times with those of authentic standards (Oleszek et al., 1990a,b, 1992). Quantitation was based on external standardization by employing calibration curves in the range of 0.25–2 mg/mL of reference compounds.

Statistical Analysis. Each sample was submitted to three replicate analyses, and results were subjected to the ANOVA test. The relative standard deviation for saponins was 7–8% ($n = 5$).

Trichoderma viride Bioassay. Bioassay experiments to measure the growth in the diameter of colonies of *T. viride* in the presence of 500 mg of powdered seedlings per 100 mL of growing medium were performed according to a previously described procedure (Oleszek et al., 1990b).

RESULTS AND DISCUSSION

Ungerminated seeds of alfalfa var. Boja contained only one saponin detectable by HPLC. From the retention time, this was identified as soyasaponin I, occurring at a concentration of 2.12 $\mu\text{mol/g}$ of dry matter. This concentration was quite stable during the germination period (curve 2, Figure 1) with some fluctuations in the range of 2.5–3.5 $\mu\text{mol/g}$ observed in the seedlings at an age of 4–13 days. A similar concentration of soyasaponin I (2.8 $\mu\text{mol/g}$) could be found in aerial parts of mature alfalfa plants (Nowacka and Oleszek, 1994); roots contained only 0.5 $\mu\text{mol/g}$ of dry matter. In this study, intentionally, no distinction was made between soyasaponin I and chromosaponin I, which according to recent reports is a genuine compound occurring in the germinating seeds of some legumes (Massiot et al., 1992; Kudou et al., 1993; Tsurumi et al., 1992). Soyasaponin I is thought to be an artifact from its DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) conjugate, chromosaponin I, which may degrade easily during the sample preparation (Massiot et al., 1992). Under the conditions of hot extraction and derivatiza-

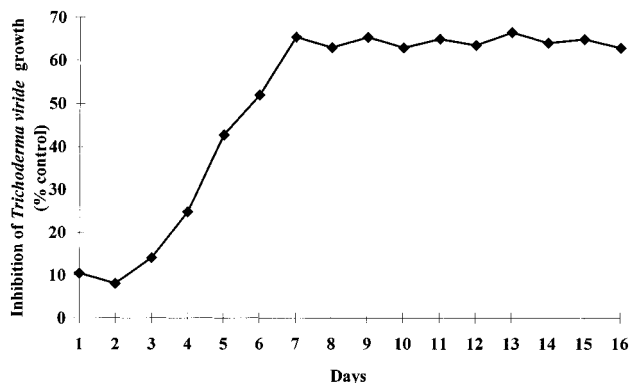


Figure 2. Inhibition of *Trichoderma viride* growth by the plant material from germinated alfalfa seeds (500 mg/100 mL of growing medium).

tion applied in this research, chromosaponin I, if present, degraded totally to soyasaponin I.

On the fourth day of germination the synthesis of medicagenic acid glycoside was observed. The first compound of this group was medicagenic acid 3-*O*-glucoside (3Glc Ma) (curve 5, Figure 1). Its concentration was increasing gradually from 0.54 to 1.7 $\mu\text{mol/g}$ between the fourth and eleventh days of germination. Then the concentration decreased to the stable level of 1.2–1.3 $\mu\text{mol/g}$. On the fifth day 3-*O*-glucuronopyranosyl–medicagenic acid–28-(arabinopyranosyl-rhamnopyranosyl-xylopyranosyl) ester (3GlcA,28AraRhaXyl Ma) and on the sixth day 3-*O*-glucopyranosyl–medicagenic acid–28-glucoopyranosyl ester (3Glc,28Glc Ma) were detected (curves 4 and 6, respectively). Their concentrations ranged between 0.82 and 1.8 and between 0.06 and 1.04 $\mu\text{mol/g}$, respectively, and after 10 days showed quite stable levels. Zanhic acid tridesmoside (curve 3, Figure 1) appeared for the first time after 12 days of seedling growth at a concentration which was comparable to medicagenic acid glycosides.

The sequence of the synthesis of medicagenic acid glycosides shows clearly that at first the monodesmosidic glycosides are being produced and then bidesmosidic forms appear. The glycoside of zanhic acid, which is a 16-OH derivative of medicagenic acid, is formed much later, after 12 days of germination. It is not yet known whether medicagenic acid is a precursor of zanhic acid synthesis, but the present sequence of their appearance in germinating seeds and seedlings may suggest such a possibility. In general after a small increase at the beginning of the synthesis of a particular compound, all of them approached the stable molar concentration per 1 g of dry matter after a few days of seedling growth.

The same applies to the total saponin content in the seedlings (curve 1, Figure 1). During the first 8 days there was a gradual increase in the total saponin concentration from 2.12 to 6 $\mu\text{mol/g}$, and afterward this remained at the same level of around 6 $\mu\text{mol/g}$. This corresponds to a total saponin concentration of 0.6% in dry matter and differs from previous reports indicating the concentration of saponins in alfalfa seedlings at 8–10% of dry weight (Fenwick and Oakenfull, 1983; Price et al., 1987; Gorski et al., 1991). Previous reports were based on biological evaluations, and to explain these differences, the *T. viride* tests were performed (Figure 2). They clearly showed that only plant material originating from the seedlings being between the third and sixth days of germination inhibited fungus

in a linear manner; older seedlings gave smaller diameters of the colonies. At the seventh day and onward on seedlings, totally inhibited fungus growth and evaluation of saponin concentration were not possible. This fungus reaction correlated with the synthesis of 3Glc Ma. As shown previously, 3Glc Ma has extremely high activity against *T. viride*, and total inhibition occurs at a concentration of 0.25–0.30 $\mu\text{mol}/100\text{ mL}$ of growing medium, which corresponds to an amount of 0.5–0.6 $\mu\text{mol}/\text{g}$ of dry matter (Oleszek et al., 1990b). This is the concentration that can be found in the seedlings between fourth and seventh days of the growth. After this period, the concentration of 3Glc Ma exceeded the critical value at which the growth of the fungus was totally restricted.

Thus, biological tests used for determination of saponins in alfalfa seedlings produce results which are drastically overestimated. Similar overestimations occurred when biological tests were used for alfalfa root saponin determination (Nowacka and Oleszek, 1994). Analytical procedures applied in this research showed clearly that concentrations of saponins in alfalfa seedlings are not much different from levels in mature alfalfa plants of varieties recognized as intermediate in saponin level. This might be important for the nutritionists as well as for the consumers who include alfalfa sprouts in the diet. The saponin concentration in sprouts is not as high as previously reported and is unlikely to create a hazard for the consumers health.

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