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MEDICAGENIC ACID AND SAPONINS IN CALLUS AND SUSPENSION CULTURES OF ALFALFA

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Key Word Index—*Medicago sativa*; *M. truncatula*; Fabacea; callus culture; saponins; medicagenic acid.

Abstract—Saponins have been extracted from callus and suspension cultures from *Medicago sativa* and *M. truncatula*. Acid hydrolysis of the saponins has provided soyasapogenol B and medicagenic acid as the main genins; they were identified by TLC and HPLC.

INTRODUCTION

Tissue culture preparation of saponins has aroused interest in at least two domains, which are the production of bioactive compounds [1] and the study of biosynthesis [2]. As part of investigations on alfalfa antifeedant saponins, we have examined the saponin content of all parts of the plant (root, stem and leaves, seeds) and interestingly we found that the chemical nature of the saponins differed according to their location [3, 4]. In the hope of better understanding the formation and evolution of the saponins, their nature and occurrence in callus and suspension cultures from alfalfa were investigated.

RESULTS AND DISCUSSION

The present work was performed on calli from two clones of *Medicago sativa* (125–1 and E1) and on suspension cultures from the Jemmalong cultivar of *M. truncatula*. Plants were grown from seeds in the greenhouse with a 16-hr photoperiod. Twenty-one strains were investigated: 18 calli initiated from petioles of *Medicago sativa* and cultured on two different media (UM and B II) and three suspension cultures initiated from leaves and seeds of *Medicago truncatula*.

Calli were extracted with boiling water and the saponins were subsequently transferred into butanol according to the usual saponin extraction procedure. The extracts showed on TLC, the typical pinkish colours of alfalfa saponins upon sulphuric acid spraying but low yields (0.002 to 0.35%) and complexity of the mixtures precluded any characterization of these compounds in the pure state. The extracts were thus hydrolysed under acidic conditions to liberate the triterpenes (0.01 to 0.16%); identification of the genins was performed by TLC and analytical HPLC. TLC revealed the presence in several cultures of medicagenic acid [5] and soyasapogenol B [6]; no hederagenin was detected. HPLC analysis of three samples showed peaks corresponding to medicagenic acid.

In conclusion, it is worth noting that even isolated cells and calli are able to make saponins with highly oxidized genins such as medicagenic acid. As far as antifeedant activity is concerned, it would be interesting to be able to block the metabolism at a low oxidation level since most saponins with soyasapogenins are devoid of the deleterious activity.

EXPERIMENTAL

Explants (leaves for *M. truncatula*, petioles for *M. sativa*) were taken from the last unfolded leaves, surface sterilized with 7% calcium hypochlorite for 10 min and rinsed twice with sterile water. Petioles and leaves were cut into small pieces of *ca* 2 mm length. They were then cultured in large Petri dishes (Φ 90 mm) containing either UM medium with 2 mg 2,4-D and 0.25 mg kinetin per litre or B II medium with 2 mg 2,4-D and 2 mg kinetin per litre as previously described [7]. Cultures were maintained at 28° for *M. sativa* and at 26° for *M. truncatula* with 16 hr photoperiod under 14 W/m^2 illumination. Calli were subcultured each month.

Suspension cultures of *M. truncatula* were initiated with ca 2 g of fresh callus per 40 ml of UM liquid medium in 250 ml Erlenmeyer flasks and maintained at 26° on a giratory shaker at 80 rmp. Subcultures were done every three weeks by adding 10 ml of the suspension to 35 ml of fresh medium.

Extraction. After 2,3,4 or 6 months of culture, calli (fr. wt = 160–450 g) were refluxed in boiling water for 3 hr. After filtration, the aq. soln was extracted × 3 with *n*-BuOH. The butanol phases were evapd to dryness and the residue dissolved in MeOH. The soln was filtered and evapd to give the crude saponins. Further purification was obtained by suspension in MeOH and pptn by addition of five volumes of Et_2O. The yield of saponins varied between 0.06 and 0.35%. When the weight was sufficient, dialysis against H_2O was performed. For example dialysis of 719 mg *n*-BuOH extract left 9 mg of pure saponin (0.002% of the fr. wt).

After 3 months of culture, suspension cultures (fr. wt = 72–490 g) was extracted with *n*-BuOH; the *n*-BuOH phases were evapd to dryness. The extraction method was the same as

the one used for calli. The yields of crude saponin residue were between 0.05 and 0.31% (0.002% after dialysis).

Partial hydrolysis of saponins. The saponin mixture was dissolved in 2% aq. $HClO_4$ and heated at 140°C in a thick wall sealed tube for 2 hr. After cooling, the sapogenin ppt was filtered, rinsed with H_2O and dried *in vacuo* over P_2O_5. The average yield of sapogenins were comprised between 0.01 and 0.16% (9 runs).

Sapogenin identification by TLC. The different residues corresponding to calli and suspensions cultures, and reference samples were applied to a silica gel plate. After elution with a mixture of $CHCl_3$–MeOH (9:1) and development by spraying with aq. H_2SO_4 and heating, medicagenic acid and soyasapogenol B were detected.

Analytical HPLC. A reference soln was prepared with medicagenic acid and hederagenin at a concentration of 0.025% for each genin. The sample soln was prepared by dissolving 10 mg of residue in 5 ml MeOH. The HPLC of genins was performed on an instrument equipped with a Hypersil 5 ODS reversed phase column (C_{18}) (15 cm × 4.6 mm), mobile phase: MeOH–H_2O–HCOOH (1500:500:1), flow rate 2 ml/min, injected vol. 20 μl; UV detection at 210 nm. Under these conditions, the R_t of medicagenic acid is 5.2 min, R_t of hederagenin is 10.6 min.

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