Stimulation of the Growth and the Triterpenoid Saponin Accumulation of *Saponaria officinalis* Cell and *Gypsophila paniculata* Root Suspension Cultures by Improvement of the Mineral Composition of the Media

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Highly glycosylated pentacyclic triterpenoid saponins were produced by in vitro liquid culture of two Caryophyllaceae species: *Saponaria officinalis* L. and *Gypsophila paniculata* L. *S. officinalis* cells were subcultured in a modified Murashige and Skoog medium and *G. paniculata* roots in a modified Gamborg medium. The improvement of the mineral composition of new liquid media was achieved through a method based upon techniques used in soilless cultures. This improvement concerned the macronutrient and especially the micronutrient composition of the media. This new mineral formulation was calculated using macronutrient and micronutrient requirements of whole plants. These media, called MH media (media from hydroponic conditions: MH2 for *S. officinalis* cells and MH3 for *G. paniculata* roots), increased fresh weight biomass and accumulation of triterpenoid saponins in in vitro cultures, respectively, by 350−700 g L^{-1} of liquid medium and 2.5−4.4 mg (g of fresh weight)$^{-1}$ L^{-1} for *S. officinalis* cells and by 350−750 g L^{-1} and 10−30 mg (g of fresh weight)$^{-1}$ L^{-1} for *G. paniculata* root cultures.

Keywords: *Plant tissue cultures; mineral nutrition; new culture media; batch culture growth; plant secondary metabolite production*

INTRODUCTION

The improvement of biomass and secondary metabolite production by plant cell and organ cultures has been described by many authors. Among the improvement strategies, medium mineral constituents and their concentrations are of utmost importance. Two approaches have been employed to obtain increased growth and product formation: the first consists of testing various media developed for different in vitro plant cultures and selecting the most suitable medium (Fujita et al., 1981a; Rokem et al., 1985; Khouri et al., 1986); the second approach consists of changing, element by element, mineral concentrations of a well-known medium (Fujita et al., 1981b; De Eknamkul and Ellis, 1985; Rokem et al., 1985). A third approach was developed recently: the macronutrient concentrations of the in vitro medium were improved using the macronutrient requirement of the whole plant (Morard and Henry, 1998). This method was applied for the stimulation of growth and of glycoalkaloid production by multiple shoot cultures of *Solanum paludosum* (El Badaoui et al., 1996), using a new macronutrient composition called MH1 (media elaborated from hydroponic conditions).

The aim of our study was to improve the in vitro biomass production and the accumulation of the highly glycosylated pentacyclic triterpenoid saponin produced by two Caryophyllaceae species: *Saponaria officinalis* cells (Henry et al., 1981; Henry, 1988) and *Gypsophila paniculata* roots (Henry et al., 1991) subcultured in modified Murashige and Skoog and Gamborg liquid media, respectively, but present in lower quantities than in the whole plants. They are of interest for the pharmaceutical industries. Our approach targeted macronutrient composition but more specifically micronutrient concentrations that are not often studied (Fujita et al., 1981b; Morimoto et al., 1988; Ravishankar and Grewal, 1991; Zhong and Wang, 1996). This was carried out by determining water and mineral requirements of whole plants of the two species grown in soilless culture during a complete vegetative cycle. The data obtained were used in the calculation of two new mineral formulations used in in vitro culture, called MH2 for *S. officinalis* cells and MH3 for *G. paniculata* roots. The control of the new media efficiency was made on fresh weight and saponin accumulation.

EXPERIMENTAL PROCEDURES

In Vitro Cultures. Cell suspension cultures of *S. officinalis* L. (soapwort) were established from primary calluses obtained from stem internodes of adult plants, in 1978, and were then stabilized for 1 year for morphological expression (Henry and Guignard, 1982). Cell suspensions were subcultured every 12 days in 500 mL Erlenmeyer flasks containing 166 mL of a modified Murashige and Skoog's medium called MSA. The medium contains Murashige and Skoog's (MS) mineral nutrients (Murashige and Skoog, 1962) with slight modifications in some mineral concentrations: KI, 0.75 instead of 0.83 mg L^{-1}; $MnSO_4 \cdot 4H_2O$, 16.9 instead of 22.3 mg L^{-1}; Gamborg's vitamins (Gamborg et al., 1968), 2,4-dichlorophenoxyacetic acid (2,4-D) 0.1 mg L^{-1}, adenine (2 mg L^{-1}), kinetin (1 mg L^{-1}), and glucose (30 g L^{-1}). The pH of the medium

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was adjusted to 5.7. The cells were maintained in suspension on a gyratory shaker at 80 rpm, in darkness, at 25 °C.

Root cultures of *G. paniculata* were established in 1995: surface-sterilized seeds of *G. paniculata* L. (panicled gypsophila) were germinated, and sterile root explants were placed on solid culture medium of Gamborg B5 (Gamborg et al., 1968) with 6% (w/v) agar, without 2,4-D. One month later, the roots were transferred to 250 mL Erlenmeyer flasks containing 100 mL of liquid medium of Gamborg B5, and root cultures were then stabilized for 1 year. These cultures were maintained at 25 °C, in darkness, on a gyratory shaker at 80 rpm. They were subcultured, at the rate of 1 g of fresh weight per flask, every 3 weeks under the same conditions.

Plant Mineral Analysis. Seeds of *S. officinalis* and of *G. paniculata* were germinated, and seven plantlets of each species were cultivated, each one in an individual 6 L container. These plants were grown hydroponically on a usual complete standard nutrient solution, until flowering and maturity of seeds, in a greenhouse under natural lighting at a temperature of 15−25 °C and a relative humidity of 50−65%. The standard nutrient solution had the following composition (mg L^{-1}): KNO_3, 505.5; KH_2PO_4, 272; $Ca(NO_3)_2 \cdot 4H_2O$, 1181; $MgSO_4 \cdot 7H_2O$, 370; Fe(EDTA), 113; $MnSO_4 \cdot H_2O$, 1.5; $ZnSO_4 \cdot 7H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.25; H_3BO_3, 3; $Na_2MoO_4 \cdot 2H_2O$, 0.025. This solution was selected because it supplies all essential mineral nutrients for the development of most herbaceous plants. The pH of the nutrient solution was between 5.5 and 6.5, and the electrical conductivity was in the range of 1.8−2.0 mS cm^{-1}. Total water and mineral nutrient consumptions by the plants were measured during the culture. At the end of the culture, each plant was harvested, weighed, dried, and powdered. The mineral elements were determined after calcination at 550 °C. The cation (potassium, calcium, magnesium, iron, manganese, zinc, and copper) contents were measured using atomic absorption spectrophotometry. Phosphorus was determined spectrophotometrically at 800 nm using the phosphomolybdic complex. Boron was quantitatively analyzed by automatic absorption spectrophotometry at 410 nm. Nitrogen was determined after mineralization with sulfuric acid, whereas nitric acid was used in the case of sulfur. The analysis of the mineral content of whole plants was estimated as the percentage of dry weight. These results made it possible to calculate the quantities consumed, expressed in milligrams per plant, and then convert them to milliequivalents per 100 g of dry weight for macronutrients and to microequivalents per 100 g of dry weight for micronutrients (the unit scale is related to the mineral nutrient quantities in the plants).

Extraction, Purification, and Quantitative Determination of Triterpenoid Saponins. Three different Erlenmeyer flasks of the in vitro cultures taken at random were harvested at definite times during the growth cycle and worked up individually. Fresh samples were determined by weighing. All triterpenoid saponins were extracted three times from fresh samples, with 30 mL of MeOH/H_2O (30:70 v/v) for 10 g of fresh cells of *S. officinalis* and with 20 mL of EtOH/H_2O (10:90 v/v) for 10 g of fresh roots of *G. paniculata* (Henry, 1995). Fresh samples were used to avoid damaging saponins during biomass drying. The purification of triterpenoid saponins was performed with a Shandon Hypersep C_{18} silica gel column (500 mg of adsorbent): free sugars, not adsorbed on the column, were first totally eliminated with distilled water eluate; the saponins adsorbed on the column by their affinity to the C_{18} apolar chains were then eluted with 5 mL of MeOH/H_2O (75:25 v/v) or (80:20 v/v) for *S. officinalis* and *G. paniculata*, respectively. The quantitative determinations of triterpenoid saponins were accomplished by HPTLC using silica gel 60 plates (10 × 10 cm). The plates were developed in a Camag horizontal chamber with the following solvent system: $CHCl_3$/MeOH/H_2O (6:4.5:0.5 v/v/v). Triterpenoid saponin spots were detected at 110 °C after spraying of a 30% sulfuric acid solution in ethanol. The color intensity of the spots on the plates was measured with a Vernon densitometer at 256 nm. Overall triterpenoid saponins of *S. officinalis and G. paniculata* were conclusively detected by comparison of the migration with that of the reference saponin, "Pure White Saponin" (ref 7695, Merck), under identical conditions.

Table 1. Total Macronutrient Contents (Milliequivalents per 100 g of DW) and Total Micronutrient Contents (Microequivalents per 100 g of DW) in *S. officinalis* and *G. paniculata* Whole Plants and in *G. paniculata* Roots

	S. officinalis whole plant	*G. paniculata* whole plant	*G. paniculata* roots
macronutrients			
K	69	73	58
Ca	80	142	171
Mg	22	25	31
N	233	279	271
P	13	13	21
S	22	23	25
micronutrients			
Fe	866	1189	1753
Mn	259	370	762
Zn	139	147	282
Cu	41	48	85
B	899	1172	426

RESULTS

Water Consumption. The total water consumption required for plants cultivated in soilless culture was 8.21 L for *S. officinalis* for a total dry weight of 38 g and 8.03 L for *G. paniculata* for a total dry weight of 41.8 g. These values made it possible to determine the coefficient of transpiration—the total water quantity (milliliters) required for the elaboration of 1 g of dry weight—which was 216 for *S. officinalis* and 192 for *G. paniculata*.

Mineral Analysis. The amounts of macronutrients and micronutrients in *S. officinalis* and *G. paniculata* expressed, respectively, in milliequivalents per 100 g and in microequivalents per 100 g of dry weight, are given in Table 1. The ionic equivalents do not match the mineral nutrient forms in plants but correspond to their consumed ionic forms from nutrient solutions in soilless culture, as the salts dissolved in water occur as ions.

Formulation of New Mineral Solutions. The new mineral formulation was calculated using strategies used in soilless culture: in hydroponics, the approach developed is based upon the calculation of the composition of the nutrient solution from the macronutrient consumption balance of the whole plant (Morard, 1995), in order to have a new mineral solution adapted to the plant mineral requirements.

The mineral nutrient requirements (Table 1) of each species were divided by the corresponding water coefficient of transpiration.

The following results, expressed in milliequivalents per liter, were obtained for macronutrients: For *S. officinalis* whole plant, cation concentrations were potassium, 3.2, calcium, 3.7, and magnesium, 1.0; and anion concentrations were nitrate, 10.8, phosphate, 0.6, and sulfate, 1.0. For *G. paniculata* roots, cation concentrations were potassium, 3.0, calcium, 8.9, and magnesium, 1.6; and anion concentrations were nitrate, 14.1, phosphate, 1.1, and sulfate, 1.3.

These values could not be used directly for the preparation of a nutrient solution as the result of the absence of balance between the sum of the anions consumed (12.4 mequiv L^{-1} for *S. officinalis* and 16.5 mequiv L^{-1} for *G. paniculata*) and that of the cations (7.9 mequiv L^{-1} for *S. officinalis* and 13.5 mequiv L^{-1} for *G. paniculata*), in accordance with the data reported

Table 2. Formulation of the New Mineral Solution MH2 in Comparison with the Murashige and Skoog MSA Mineral Composition Used for *S. officinalis* Suspension Cell Cultures

Macronutrients (mequiv L^{-1})

	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-	I^-	cations
MH2						
K^+	3	1				4
Ca^{2+}	4.5					4.5
Mg^{2+}			1.5			1.5
NH_4^+	1					1
anions	8.5	1	1.5			11
MSA						
K^+	18.8	1.25			0.005	20.055
Ca^{2+}				6		6
Mg^{2+}			3			3
NH_4^+	20.6					20.6
anions	39.4	1.25	3	6	0.005	49.655

Micronutrients (μequiv L^{-1})

	MH2	MSA
Fe	40	200
Mn	12	200
Zn	6.4	60
Cu	1.9	0.2
B	41.6	301
Mo	0.21	2.1
Co	0	0.01

Table 3. Formulation of the New Mineral Solution MH3 in Comparison with the Gamborg Mineral Composition Used for *G. paniculata* Root Cultures

Macronutrients (mequiv L^{-1})

	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-	I^-	cations
MH3						
K^+	2.5	1.5				4
Ca^{2+}	10.5					10.5
Mg^{2+}			1.5			1.5
anions	13	1.5	1.5			16
Gamborg						
K^+	24.73				0.005	24.735
Ca^{2+}				2.04		2.04
Mg^{2+}			2.03			2.03
NH_4^+			2.03			2.03
Na^+		1.09				1.09
anions	24.73	1.09	4.06	2.04	0.005	31.925

Micronutrients (μequiv L^{-1})

	MH3	Gamborg
Fe	91.3	200
Mn	39.7	118
Zn	14.7	14.1
Cu	4.4	0.2
B	22.2	147
Mo	0.21	2.1
Co	0	0.01

in the literature. Various adjustments were therefore required for macronutrients. Although the method used may appear to be quite empirical, it takes into account the chemical and physiological constraints investigated in the case of the nutrient solutions used in soilless culture (Morard, 1995).

The micronutrient concentrations, expressed in microequivalents per liter, of the new mineral solutions were exactly based on the mineral plant analysis (Table 1). The related ions of each micronutrient ion associated as salts could be considered negligible as they are supplied in small quantities.

Table 2 gives the new mineral nutrient formulation of the MH2 solution in comparison with the Murashige and Skoog mineral composition, used for *S. officinalis* cells. Table 3 gives the new mineral nutrient formulation of the MH3 solution in comparison with that of Gamborg, used for *G. paniculata* roots. These values were then converted into the amounts (in milligrams per liter) of salts to be used in MH2 and MH3 solutions: The MH2 mineral solution was composed of KNO_3, 303; KH_2PO_4, 136; $Ca(NO_3)_2 \cdot 4H_2O$, 531; $MgSO_4 \cdot 7H_2O$, 185; NH_4NO_3, 80; Fe(EDTA), 8.45; $MnSO_4 \cdot H_2O$, 1.02; $ZnSO_4 \cdot 7H_2O$, 0.93; $CuSO_4 \cdot 5H_2O$, 0.22; H_3BO_3, 0.87; and $Na_2MoO_4 \cdot 2H_2O$, 0.025. The MH3 mineral solution was composed of KNO_3, 253; KH_2PO_4, 204; $Ca(NO_3)_2 \cdot 4H_2O$, 1240; $MgSO_4 \cdot 7H_2O$, 185; Fe(EDTA), 19.23; $MnSO_4 \cdot H_2O$, 3.35; $ZnSO_4 \cdot 7H_2O$, 2.11; $CuSO_4 \cdot 5H_2O$, 0.55; H_3BO_3, 0.48; and $Na_2MoO_4 \cdot 2\ H_2O$, 0.025.

The organic compounds of the respective modified liquid Murashige and Skoog and Gamborg media were added to these new MH2 and MH3 mineral solutions.

Comparison between the new MH2 and MH3 mineral solutions and their respective Murashige and Skoog and Gamborg media (Tables 2 and 3) revealed the following points. The MH2 mineral solution has a total salt concentration decreased by $\approx$4 times, and the MH3 solution has approximately half the total salt concentration. MH2 and MH3 solutions present a decrease of the concentrations of potassium, magnesium, nitrate, and sulfur. A decrease in ammonium, phosphate, and calcium concentrations is also observed for the MH2 solution, whereas an increase of the phosphate and calcium concentrations is noted in the MH3 solution, relative to the respective classical media (Tables 2 and 3). Finally, in the MH media, all mineral elements that are known as nonessential for higher plant physiology are systematically eliminated from mineral solutions, in particular, chloride, sodium, iodine, and cobalt. In all of our experiments, the lack of these elements had never involved any decrease of growth.

Compared with the corresponding media used in reference, MH2 (Table 2) and MH3 (Table 3) mineral solutions showed a decrease of all essential micronutrients (iron, manganese, zinc, boron, and molybdenum); only copper was increased by 10 and 20 times, respectively, in MH2 and MH3 media.

Fresh Weight and Saponin Accumulation. For *S. officinalis* cell cultures, a first mineral medium in which nitrogen was entirely supplied by nitrate did not give positive results: cells became dark without growing and died as early as the fourth day in batch culture. In this case, it was necessary to supply nitrogen with a part of ammonium in the MH2 medium (Table 2). The comparison of MH2 and Murashige and Skoog MSA media was first used for fresh weight production for *S. officinalis* suspension cell stabilized cultures, and the time courses of growth in batch culture (Erlenmeyer flasks) on both media are given in Figure 1. The growth curves show an exponential phase of 8 days for MH2 and MSA media, and the maximum biomass was reached at the same date for both media. However, the maximum biomass production was twice higher for the MH2 medium with 680 g L^{-1} of fresh cell weight, compared to only 350 g L^{-1} of fresh cell weight produced with the MSA medium. The doubling times measured during the exponential phase were, respectively, 59 and 52 h for MSA and MH2 media. The MH2 solution was therefore more satisfactory for the biomass production by *S. officinalis* cell cultures. The results observed for the cell growth were similar to those observed for the triterpenoid saponin accumulation (Figure 2) (data statistically different on the threshold of 5% with the

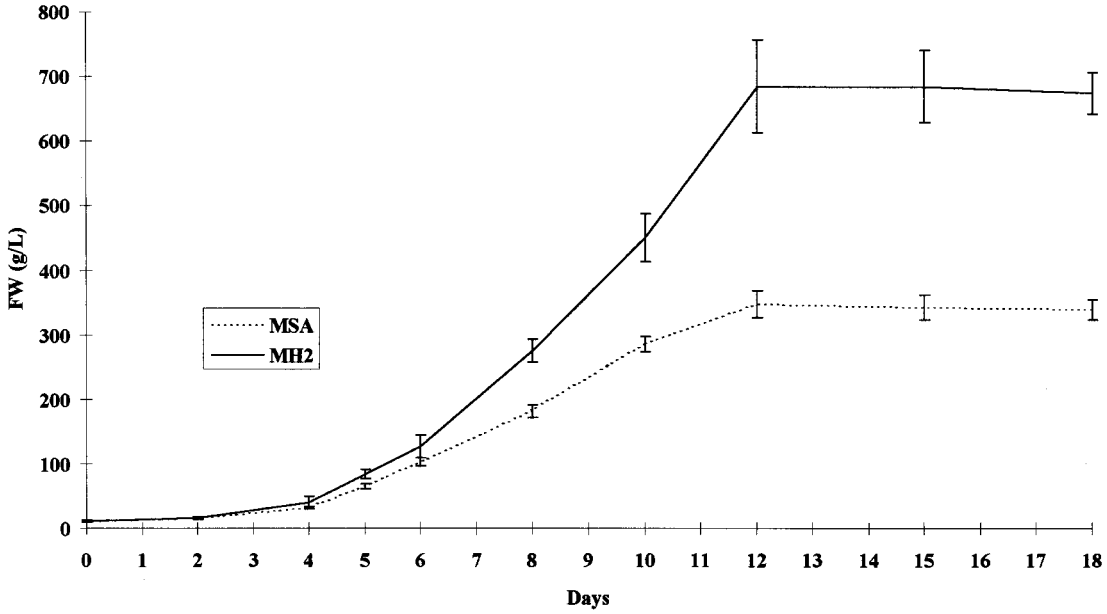

Figure 1. Batch growth of a suspension cell culture of *S. officinalis* cultured in modified Murashige and Skoog MSA medium and MH2 medium. (Three flasks were harvested for each point; maximum standard deviation was 70 g of FW L^{-1}.)

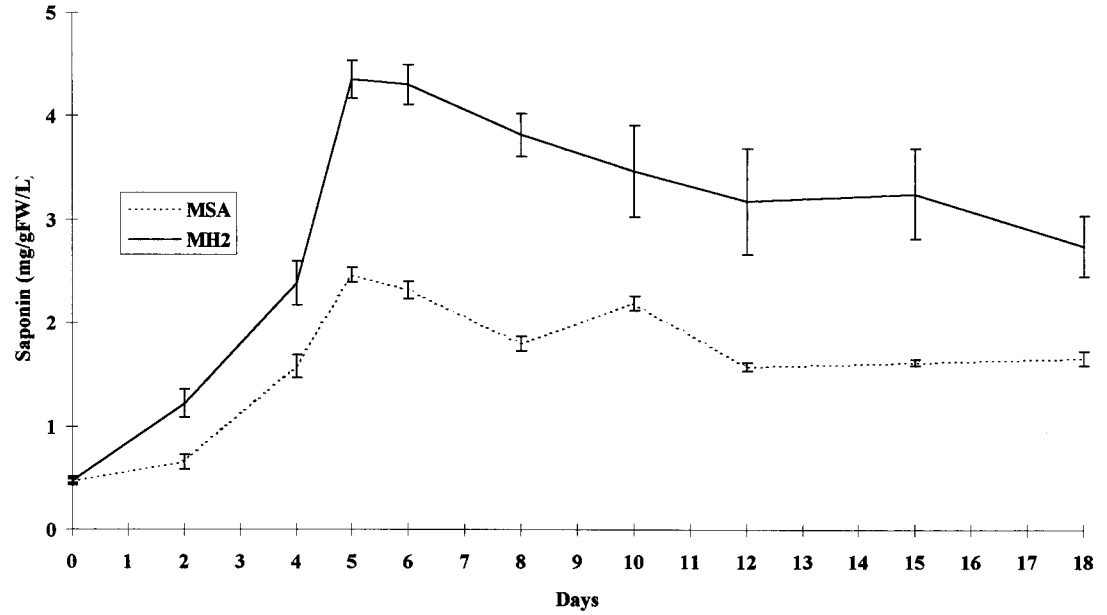

Figure 2. Triterpenoid saponin content [mg (g of FW)$^{-1}$ (L of culture medium)$^{-1}$] of the suspension cell culture of *S. officinalis* cultured on modified Murashige and Skoog MSA medium and MH2 medium. [Three flasks were used for extraction, purification, and quantitative determination of triterpenoid saponins; maximum standard deviation was 0.5 mg (g of FW)$^{-1}$ L^{-1}.]

Newman−Keuls test). The maximum triterpenoid saponin content was obtained on the fifth day of batch culture with 2.47 mg (g of fresh weight)$^{-1}$ L^{-1} for MSA medium and 4.36 mg (g of fresh weight)$^{-1}$ L^{-1} for MH2 medium, corresponding to triterpenoid saponin contents of 0.73 and 1.27% dry weight, respectively. Therefore, the intracellular saponin accumulation was enhanced 4 times with the MH2 medium.

A first mineral solution tested for *G. paniculata* root cultures was calculated by taking into account the whole plant mineral nutrient requirements (Table 1). This medium was not suitable for *G. paniculata* root growth: after 15 days of in vitro culture, the fresh weight was the same as the inoculum one, that is, 1 g/100 mL of liquid medium per flask. Another strategy was then used, by calculating the mineral composition of the MH3 medium only on *G. paniculata* root mineral requirements (Table 1) in accordance with the nature of the organ cultivated in vitro. The *G. paniculata* roots were then stabilized and cultivated on the new MH3 medium in comparison with Gamborg medium. The time courses of growth in batch culture (Erlenmeyer flasks) on MH3 and Gamborg media are given in Figure 3. The growth curves show an exponential phase of 12 days for the classical medium and 9 days for the MH3 medium. Moreover, the maximum biomass was much higher for the MH3 medium, with 760 g L^{-1} of fresh weight compared to only 340 g L^{-1} of fresh weight produced with Gamborg medium. The doubling times were, respectively, 67 and 55 h for Gamborg and MH3 media. The MH3 mineral solution was therefore more satisfactory for *G. paniculata* root biomass production. The results observed for the root growth were similar to those observed for the triterpenoid saponin accumula-

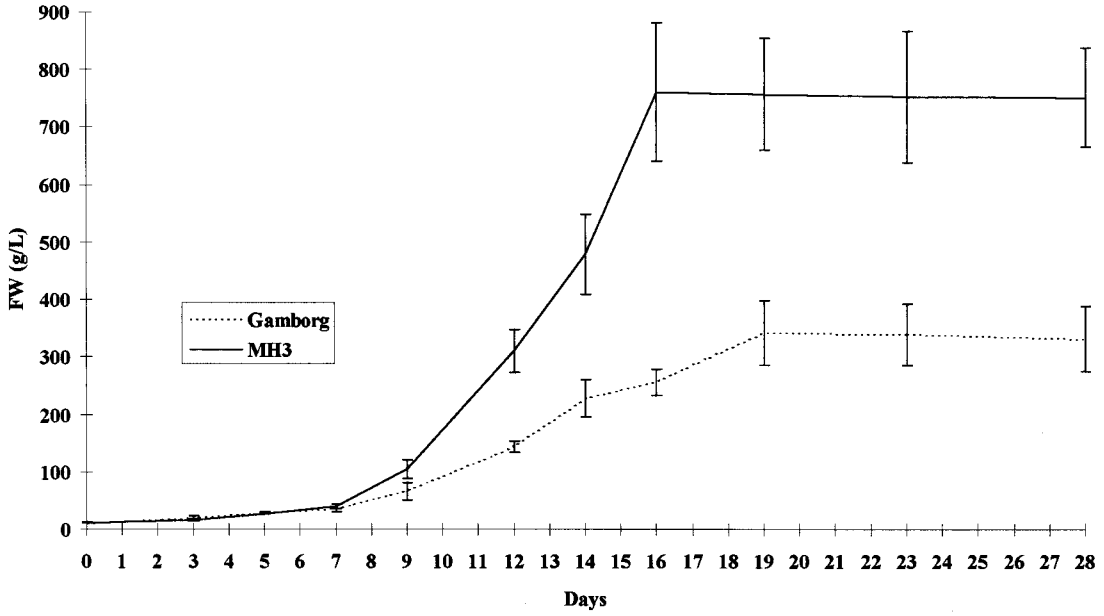

Figure 3. Batch growth of a root culture of *G. paniculata* cultured in Gamborg medium and MH3 medium. (Three flasks were harvested for each point; maximum standard deviation was 120 g of FW L^{-1}.)

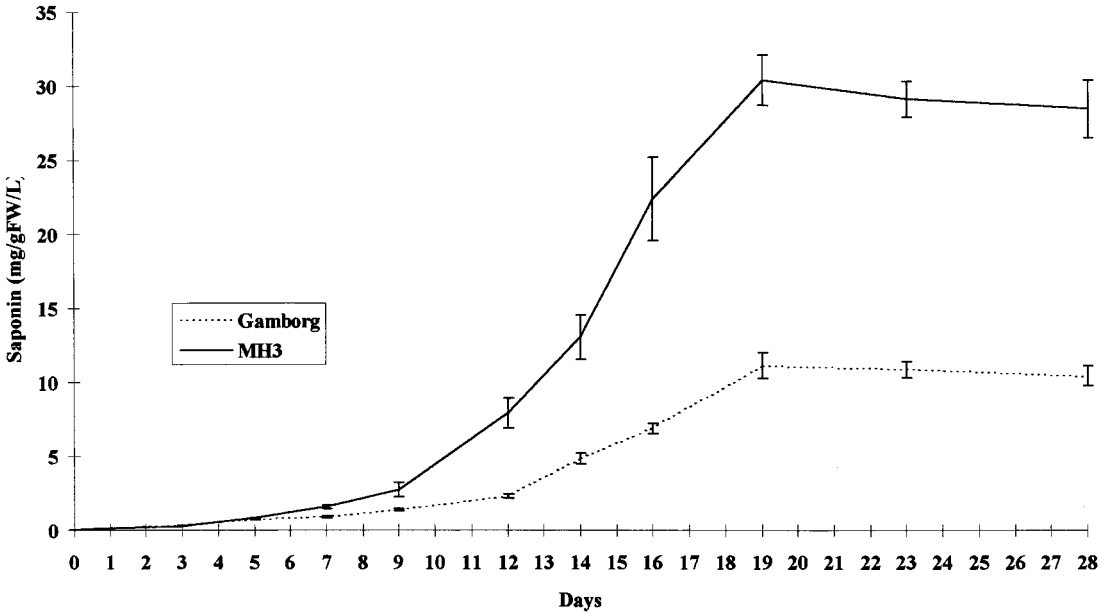

Figure 4. Triterpenoid saponin content [mg $(g^{-1}$ of FW$)^{-1}$ (L of culture medium$)^{-1}$] of the root culture of *G. paniculata* cultured on Gamborg medium and MH3 medium. [Three flasks were used for extraction, purification, and quantitative determination of triterpenoid saponins; maximum standard deviation was 2.8 mg (g of FW$)^{-1}$ L^{-1}.]

tion (Figure 4) (data statistically different on the threshold of 5% with the Newman−Keuls test). The maximum triterpenoid saponin content was obtained on the 19th day of batch culture with 11 mg (g of fresh weight$)^{-1}$ L^{-1} for Gamborg medium and 30 mg (g of fresh weight$)^{-1}$ L^{-1} for the MH3 medium, corresponding to triterpenoid saponin contents of 0.6 and 1.25% dry weight, respectively. Here, also, the saponin accumulation was ~6 times enhanced with the MH3 medium.

DISCUSSION

Macronutrient Formulations for *S. officinalis* Cell Cultures. Nitrogen nutrition is an important factor in the improvement of tissue growth and secondary metabolite production in batch cultures. Nitrate is the usual form of the nitrogenous nutrition of higher plants. For *S. officinalis* cell growth, a mineral medium in which nitrogen was entirely supplied by nitrate did not give positive results. In this case, it was necessary to supply nitrogen with a part of ammonium in a ratio of 10% for 90% of nitrate related to the total mineral nitrogen source (Table 2): this ratio is recommended for plant soilless culture (Morard, 1995) and also in some in vitro culture media, such as Gamborg medium (Gamborg et al., 1968). This formulation gave favorable results for biomass production and saponin accumulation by *S. officinalis* cell suspension cultures. These cells therefore required a reduced nitrogen form, like *Dioscorea deltoidea* cells (Rokem et al., 1985) or *Aralia cordata* cells (Sakamoto et al., 1993). Therefore, a higher ratio of NO_3^-/NH_4^+ (90:10) in the MH2 medium than in the MSA medium (66:34) is an important point for the improvement of *S. officinalis* cell cultures.

Growth and hyoscyamine production by *Hyoscyamus muticus* cultures were also improved with a high ratio of NO_3^-/NH_4^+ (Oksman-Caldentey et al., 1994). However, total nitrogen concentration in MH2 solution was decreased by >4 times for nitrates and by ~20 times for ammonium ions, in comparison to the Murashige and Skoog concentrations (Table 2).

Macronutrient and Micronutrient Formulations for *G. paniculata* Root Cultures. The first mineral solution tested for *G. paniculata* in vitro root cultures had taken into account the whole plant mineral requirements, but this medium was not suitable for root growth. Another mineral formulation was then calculated on *G. paniculata* root macronutrient and micronutrient requirements, in accordance with the nature of the organ cultivated in vitro: the MH3 mineral composition (Table 3) gave favorable results for biomass production and saponin accumulation. The establishment of a new mineral solution therefore has to take into consideration the nature of the organ cultivated in vitro.

Another important point is that no ammonium ion was supplied in the MH3 medium: *G. paniculata* root cultures required only nitrate ions as mineral nitrogen source. Such a result was also found by Vasseur and Dubois (1985) for small root explant cultures of *Cichorium intybus*, by Sakamoto et al. (1994) for anthocyanin production by *Aralia cordata* cells, by Zhang et al. (1996) for ginseng saponin production by *Panax notoginseng* cell cultures, and by El Badaoui et al. (1996) for solamargine production by *Solanum paludosum* multiple shoot cultures.

Finally, MH media are less concentrated than classical in vitro media (El Badaoui et al., 1996; Morard and Henry, 1998).

Stimulation of Growth and Triterpenoid Saponin Accumulation. Macronutrients and micronutrients in in vitro culture media have an influence on the growth and on the secondary metabolite production. Among the most important factors, we can see first of all that a 5-fold decrease of the potassium concentration in the MH2 medium (Table 2) and a 6-fold decrease of the concentration of this element in the MH3 medium (Table 3), in comparison with the respective classical media, partly enabled the stimulation of growth and saponin accumulation by, respectively, *S. officinalis* cell suspensions and *G. paniculata* root cultures. A similar observation was recorded for the production of solamargine by *S. paludosum* multiple shoot cultures with the MH1 medium (El Badaoui et al., 1996). With regard to calcium, there was also a decrease of its concentration in the MH2 medium in comparison with the modified Murashige and Skoog medium, but it was slower than that of the potassium concentration decrease (Table 2). A similar result was obtained for the production of rosmarinic acid by *Anchusa officinalis* cells when the concentration of calcium was a one-fourth that found in Gamborg medium (De Eknamkul and Ellis, 1985). On the other hand, the MH3 medium with a calcium concentration increased by >5 times in comparison with Gamborg medium (Table 3) was improved for *G. paniculata* root cultures. Vasseur and Dubois (1985), using *Cichorium intybus* small root explants, also showed that an increase of the calcium concentration in the culture medium stimulated the growth. The same result was found by El Badaoui et al. (1996) with *S. paludosum* multiple shoot cultures. In fact, if we refer to the mineral nutrition physiology of whole plants, we realize that the ratio of potassium and calcium ions is as important as their concentrations, because this ratio induces nutritional antagonism (Morard, 1995). It is conceivable that such a phenomenon also occurs at the level of in vitro cultures: in fact, MH media with a potassium/calcium ratio below 1 (respectively, 0.9 and 0.4 for MH2 and MH3 media) were improved in relation to the classical media, for which the potassium/calcium ratio was far higher than 1 (respectively, 3.3 and 12 for Murashige and Skoog and Gamborg media).

Another important factor for the stimulation of the saponin accumulation is certainly the decrease of the nitrate concentration in MH2 and MH3 media in comparison to the respective Murashige and Skoog and Gamborg media (Tables 2 and 3). Such a result was found with MH1 medium (Morard and Henry, 1998) for the stimulation of the production of solamargine by *S. paludosum* multiple shoot cultures (El Badaoui et al., 1996).

Finally, the great increase of the copper concentration in MH2 and MH3 media (Tables 2 and 3) is certainly an essential factor for cell and root primary metabolism, and, consequently, for secondary metabolite (saponin) accumulation. It seems that the copper content is a limiting nutritional factor in classical MS and Gamborg media (Fulcheri, 1998): 0.2 μequiv L^{-1} on MSA versus 1.9 μequiv L^{-1} on MH2 (Table 2) and 0.2 μequiv L^{-1} on Gamborg versus 4.4 μequiv L^{-1} on MH3 (Table 3). This result is consistent with the findings of Fujita et al. (1981b): copper was also found to stimulate strongly the production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon* when its concentration was 30 times as high as that in the classical medium. Such a result was also observed for the improvement of the production of berberine by *Coptis japonica* cells (Morimoto et al., 1988) or by *Thalictrum rugosum* cells (Kim et al., 1991), of diosgenin by *Dioscorea deltoidea* cells (Ravishankar and Grewal, 1991), and of ginseng saponin by *Panax notoginseng* cells (Zhong and Wang, 1996).

The increase of triterpenoid saponin content can therefore be interpreted as an effect of the improvement of the mineral nutrition of *S. officinalis* cells and *G. paniculata* roots on MH media directly connected with the improvement of the primary metabolism and of the growth. Saponin accumulation by cell suspension cultures of *S. officinalis* occurred as early as the fifth day of the growth cycle in batch culture, at the beginning of the exponential phase (Figures 1 and 2). Nevertheless, secondary metabolite production generally follows the same pattern as growth in batch culture. This was demonstrated for *G. paniculata* roots (Figures 3 and 4). A similar pattern was also reported for *G. paniculata* multiple shoot cultures for the production of pentacyclic triterpenoidal saponins (Pauthe-Dayde et al., 1990), for *Dioscorea caucasica* suspension cell cultures for the production of diosgenin (Deliu et al., 1992), for *Cinchona succirubra* cell cultures for an anthraquinone glucoside (Khouri et al., 1986), and for *S. paludosum* multiple shoot cultures for solamargine (El Badaoui et al., 1996). This time course is useful for the production of plant secondary metabolites since the harvest of the maximum biomass production coincides with the maximum secondary metabolite production.

CONCLUSION

New in vitro mineral formulations calculated on plant mineral nutrient requirements enabled a real improvement of biomass production and saponin accumulation by *S. officinalis* cell suspension cultures and by *G. paniculata* root cultures. This strategy is a simple procedure that also makes it possible to supply fewer salts in mineral solutions compared with classical in vitro media. Nevertheless, this strategy must be used cautiously and tested, as shown by the two examples above. In MH media modeled on nutrient solutions used for plant soilless culture, nitrogen is preferably supplied in nitrate form. However, it became obvious that ammonium ions were also necessary for *S. officinalis* cells. Similarly, the nature of the organ cultivated in vitro has to be taken into consideration: the MH3 mineral solution intended for *G. paniculata* root culture was best adapted when its mineral composition resembled organ mineral requirements rather than those of the whole plant.

The next step for these studies will concern the kinetics of consumption of the essential mineral ions by on-line measurements without opening of the culture flasks. The comparison of macronutrient and micronutrient depletion in classical media (MS and Gamborg) with the new media (MH2 and MH3) will allow elucidation of the relationship between in vitro mineral nutrition and the various phases of the growth cycle of *S. officinalis* cells and *G. paniculata* roots.

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