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P. Morard · C. Fulcheri · M. Henry

Kinetics of mineral nutrient uptake by *Saponaria officinalis* L. suspension cell cultures in different media

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Abstract The uptake of mineral nutrients from two media with different mineral composition, a classical MSA medium and a modified MH2 medium, by *Saponaria officinalis* (soapwort) cells was studied over a growth cycle of 14 days, by continuous measurement of mineral consumption without opening the culture flasks. The mineral composition of the MH2 medium was found to be better suited to *S. officinalis* cells. Culture on MSA medium showed that copper is probably a factor limiting growth, that phosphate is rapidly exhausted from this medium, that its strong ammonium concentration is antagonistic to the absorption of potassium and, lastly, that sodium and chlorine may be considered as non-essential elements.

Key words Macronutrients · Micronutrients · Cell suspension cultures · In vitro mineral media · *Saponaria officinalis*

Introduction

Plant cell and tissue in vitro cultures depend on the development of culture media optimized for the interaction of three essential components (growth regulators, organic substances and mineral elements). Among the wide range of investigations which have led to culture media formulations, mineral composition appears to have been the least investigated whereas it has a decisive effect on the osmotic pressure and the pH of the medium and on the inorganic nutrition of plant cultures.

Empirical strategies are generally used to improve the mineral composition of in vitro culture media. The first consists in testing various media developed for different in vitro plant cultures (Fujita et al. 1981a; Khouri et al. 1986; Fernandes-Ferreira et al. 1992). The second approach involves varying the concentrations in each ion species in the medium (Murashige and Skoog 1962; Fujita et al. 1981b; De Eknamkul and Ellis 1985; Corchete et al. 1991). A third strategy, based upon calculation of the composition of the nutrient solution from the macronutrient consumption balance of the whole plant, has been recently developed (Morard 1995). This approach was used for increasing biomass and solamargine production by *Solanum paludosum* multiple-shoot cultures, using a new macronutrient composition called MH1 (El Badaoui et al. 1996).

The application of this method to the in vitro culture of *Saponaria officinalis* cells resulted in a biomass production and an intracellular accumulation of saponins twice as great as those of a classical in vitro culture medium (Fulcheri et al. 1998). The purpose of the present study was therefore to try to determine what causes the efficiency of the new macronutrient and micronutrient formulation of the medium called MH2. We monitored the mineral nutrition of *S. officinalis* cells cultured in the Murashige-and-Skoog-type medium (MSA) and that of cells cultivated in our improved medium (MH2).

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P. Morard (✉) · C. Fulcheri
Laboratoire d'Ingénierie Agronomique,
Ecole Nationale Supérieure Agronomique,
Avenue de l'Agrobiopole, B. P. 107, Auzeville Tolosane,
F-31326 Castanet Tolosan, France
e-mail: morard@ensat.fr
Fax: +33-5-62193901

M. Henry
Laboratoire de Botanique et Mycologie,
Faculté des Sciences Pharmaceutiques et Biologiques,
5, rue Albert Lebrun, B. P. 403, F-54001 Nancy cedex, France

Materials and methods

Plant cell cultures

Cell suspension cultures of *S. officinalis* L. (Caryophyllaceae) were established from primary calluses obtained from stem internodes of adult plants, in 1978, and were then stabilized for 1 year (Henry and Guignard 1982). Cell suspensions were regularly subcultured by add-

Table 1 Mineral formulation of MSA and MH2 media used for *Saponaria officinalis* cell culture: cations and anions

Cation (mM)	Anion (mM)					Total
	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-	I^-	
MSA medium						
K^+	18.8	1.25			0.005	20.055
Ca^{2+}				6		6
Mg^{2+}			3			3
NH_4^+	20.6					20.6
Total	39.4	1.25	3	6	0.005	49.655
MH2 medium						
K^+	3	1				4
Ca^{2+}	4.5					4.5
Mg^{2+}			1.5			1.5
NH_4^+	1					1
Total	8.5	1	1.5			11

Table 2 Mineral formulation of MSA and MH2 media used for *S. officinalis* cell culture: micronutrients and sources of Na^+ and Cl^-

	MSA	MH2
Micronutrient (μM ions)		
Fe	200	40
Mn	200	12
Zn	60	6.4
Cu	0.2	1.9
B	301	41.6
Mo	2.1	0.21
Co	0.01	
Na^+ sources	$EDTANa_2Fe$ NaOH	$EDTANa_2Fe$ NaOH
Total concentration (mM ion)	3.10	1.13
Cl^- sources	$CaCl_2$ $CoCl_2$ Gamborg's vitamins	Gamborg's vitamins
Total concentration (mM ion)	6.70	1.04

ing 20 ml of a 12-day-old culture to 500-ml Erlenmeyer flasks containing 166 ml of a Murashige and Skoog (1962) modified MSA medium or an improved medium MH2 (Fulcheri et al. 1998). Glucose is exhausted from the media at the end of the exponential phase of the growth cycle. The mineral composition of the MSA and MH2 media is given in mM of ions (mEq l^{-1}) in Tables 1 and 2: the MSA medium is characterized by a high total ionic concentration (100 mM of ions) due to high concentrations of K^+, NH_4^+ and NO_3^- and by a wide variety of elements (in particular, Cl^-, I^- and Co^{2+}); the MH2 medium differs in that its total ionic concentration is five fold weaker (22 mM of ions) and it has a simpler formulation containing only essential mineral nutrients. MSA and MH2 media also contain Gamborg's vitamins (Gamborg et al. 1968), 2,4-dichlorophenoxyacetic acid (0.1 mg l^{-1}), adenine (2 mg l^{-1}), kinetin (1 mg l^{-1}) and 30 g l^{-1} glucose. The pH of the media was adjusted to 5.7 with 0.5 M NaOH. The cells were maintained in suspension on a gyratory shaker at 80 rpm, in the dark, at 25 °C.

In vitro cell culture for mineral nutrition research

The culture of *S. officinalis* cells was carried out over a 14-day growth cycle, in 500-ml flasks containing 200 ml of MSA or MH2 medium and maintained in the dark at 25 °C under agitation (80 rpm). The mineral composition of the media was monitored continuously, without opening the flasks. For sample withdrawal, one tip of a sterile 1-ml glass pipette was put in the medium and the other end was connected with a rubber tubing fitted to a sterile syringe; every day, 1.5 ml of culture medium was sampled for pH and mineral concentration (mg l^{-1} of ions) measurements. The mineral analyses were performed using a Dionex DX-100 ion chromatograph fitted with a conductivity detector: anions (Cl^-, NO_3^-, HPO_4^{2-}, and SO_4^{2-}) were separated on an Ionpac AS4A-SC column (4×250 mm) (eluent 1.8 mM Na_2CO_3: 1.25 mM $NaHCO_3$) and cations (K^+, Mg^{2+}, Na^+, Ca^{2+} and NH_4^+) on an Ionpac CS12 A column (4×250 mm) (eluent 1.4 ml l^{-1} sulphonic methane acid). Micronutrients (Fe, Mn, Zn and Cu) were assayed by atomic absorption spectrophotometry. The "total consumed quantity" (mg/flask) of an element of the medium corresponds to the difference between the quantities measured in the flask at the beginning and end of culture, taking into account the quantities of elements sampled every day in the medium and the loss of volume by evaporation. The "total quantity of an element absorbed" (mg/flask) by the cells is the product of the content of the element in the cells (determined by analysis after mineralization) by the dry weight of cells at harvesting.

Six flasks were taken for each treatment. Each point on the curves of the residual mineral concentrations and media pH was subjected to variance analysis with total randomization according to ANOVA. The statistical analysis was complemented by the Newman and Keuls test at the 5% threshold.

Results and discussion

The comparison between the quantities of mineral nutrients consumed from the media and those absorbed by cells demonstrated, except for P in the MSA medium, the concordance of the results obtained using these two complementary methods (Table 3). These data proved, firstly, the validity of our continuous-measurement technique for the study of the mineral nutrition of *S. officinalis* cells. The correspondence of the results also showed that, unlike nutritive solutions used in soilless culture (Morard et al. 1987), in vitro culture media present little or no precipitation of insoluble salts in water: the constant agitation of in vitro culture flasks (80 rpm), to oxygenate cells maintained in suspension, probably prevents the formation of these insoluble compounds during culture.

The time course of the concentrations (mg l^{-1} of ions) of macronutrients and micronutrients (rarely studied in in vitro culture) of the MSA and MH2 media was monitored continuously during the culture cycle. At the end of the experiment, none of the elements introduced to the MH2 medium were totally exhausted (Figs. 1–3); on the other hand, from the 8th and 9th days of culture, the MSA medium contained no more Cu (Fig. 3) and no more P (Fig. 1), respectively. K^+ was used up slowly in the MSA medium (Fig. 2). The Na^+ and Cl^- contents in the cells of *S. officinalis* (Table 3) are related to the concentrations of these elements in the media (Table 2). Thus the absorption of these ions seems to be a passive process. These two nutrients are probably not essential for the growth of of *S. officinalis* cells similar to the situation in whole plants (Marschner 1995). These results also confirm the validity of the methodology we used for the mineral formulation of the MH2 medium to increase fresh weight biomass (348 ± 21 to 685 ± 71 g l^{-1})

Table 3 Comparison between total mineral quantities consumed from media and total absorbed mineral quantities by *S. officinalis* cells (values followed by different superscripts in two adjacent columns are significantly different at the 5% level)

Macronutrients	Quantities absorbed by cells (mg/flask)		Quantities consumed from medium (mg/flask)		Difference: medium–cells (mg/flask)	
	MSA medium	MH2 medium	MSA medium	MH2 medium	MSA	MH2
K	62.72±6.15[a]	28.94±3.97[b]	63.13±5.42[a]	29.06±4.07[b]	0.41	0.12
Ca	17.79±2.87[a]	16.34±1.41[a]	17.55±2.95[a]	16.88±1.55[a]	–0.24	0.54
Mg	4.78±0.78[a]	3.06±0.26[b]	4.84±0.84[a]	3.11±0.29[b]	0.06	0.05
N	122.63±12.42[a]	26.13±3.75[b]	123.86±12.71[a]	26.90±3.54[b]	1.23	0.77
Na	3.97±0.73[a]	2.34±0.28[b]	4.16±0.61[a]	2.38±0.31[b]	0.19	0.04
P	3.24±0.28[a]	5.00±0.44[b]	6.11±0.20[a]	5.16±0.49[b]	2.87	0.16
S	8.12±1.11[a]	4.65±0.45[b]	8.53±1.47[a]	4.75±0.51[b]	0.41	0.10
Cl	8.81±2.07[a]	3.51±0.53[b]	8.84±2.10[a]	3.61±0.45[b]	0.03	0.10
Micronutrients						
Fe	0.718±0.109[a]	0.1741±0.0222[b]	0.711±0.108[a]	0.1764±0.0229[b]	–0.007	0.0023
Mn	0.736±0.094[a]	0.0602±0.0138[b]	0.741±0.091[a]	0.0607±0.0144[b]	0.005	0.0005
Zn	0.238±0.025[a]	0.0317±0.0109[b]	0.235±0.029[a]	0.0342±0.0105[b]	–0.003	0.0025
Cu	no detectable	0.0110±0.0006	0.0011±0.0001[a]	0.0107±0.0006[b]		–0.0003

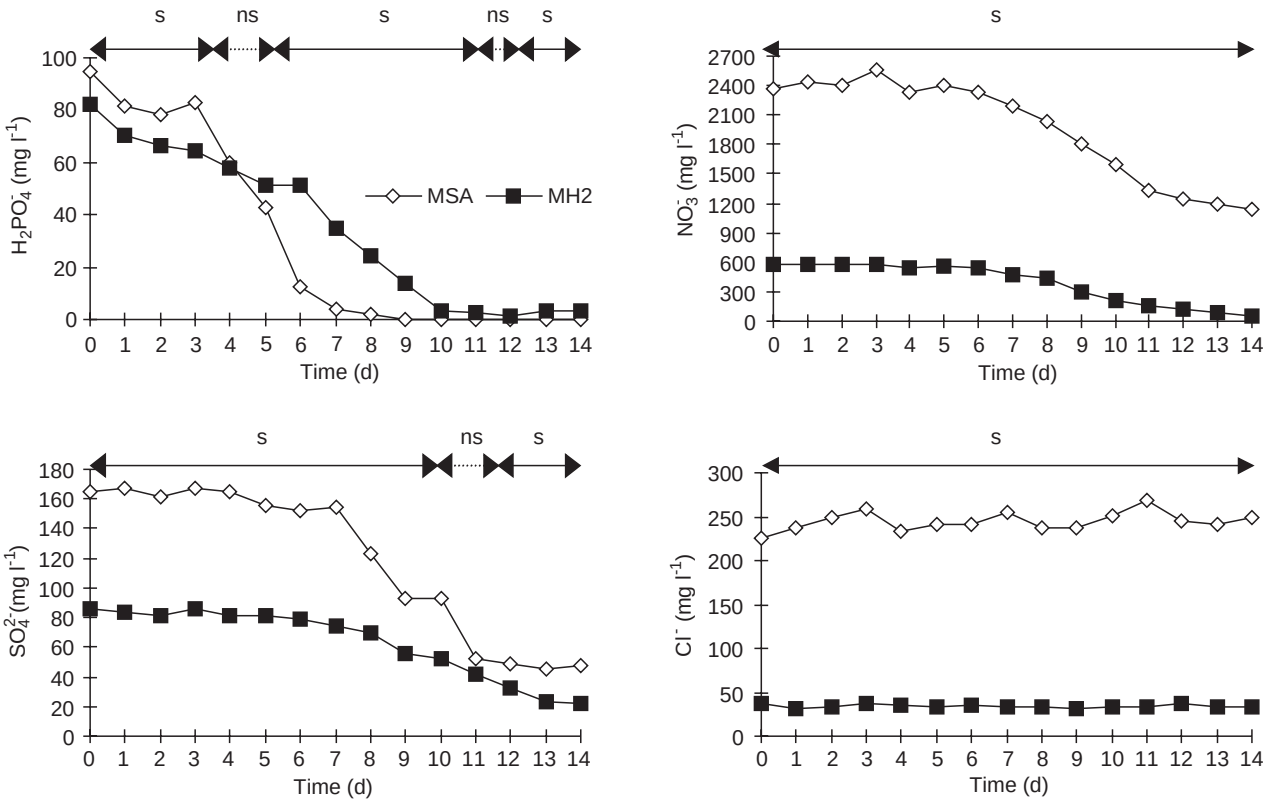

Fig. 1 Residual anion concentrations (mg l^{-1} ion) in MSA and MH2 media used for in vitro cell culture of *Saponaria officinalis* (*ns* non significant, *s* significant)

and the accumulation of saponins (2.47±0.07 to 4.36±0.18 mg g^{-1} fresh weight l^{-1}), without introducing, via salts, non-essential mineral nutrients such as Cl, Na, I and Co (Fulcheri et al. 1998). The efficiency of the MH2 medium was also confirmed by determining the quantities of dry matter at cell harvesting (3.75±0.29 g/flask) in comparison with the MSA medium (1.69±0.36 g/flask).

The following reasons may explain the less favourable effect of the MSA medium on biomass production and on the accumulation of saponins by *S. officinalis* cells.

(1) The insufficient initial concentration of copper in the MSA medium (Table 2), and its consumption tenfold lower than in MH2 medium (Table 3) demonstrate that this micronutrient is probably a limiting factor in the classical medium; as far as we know, it has never been demonstrated that copper might be a factor limiting in vitro

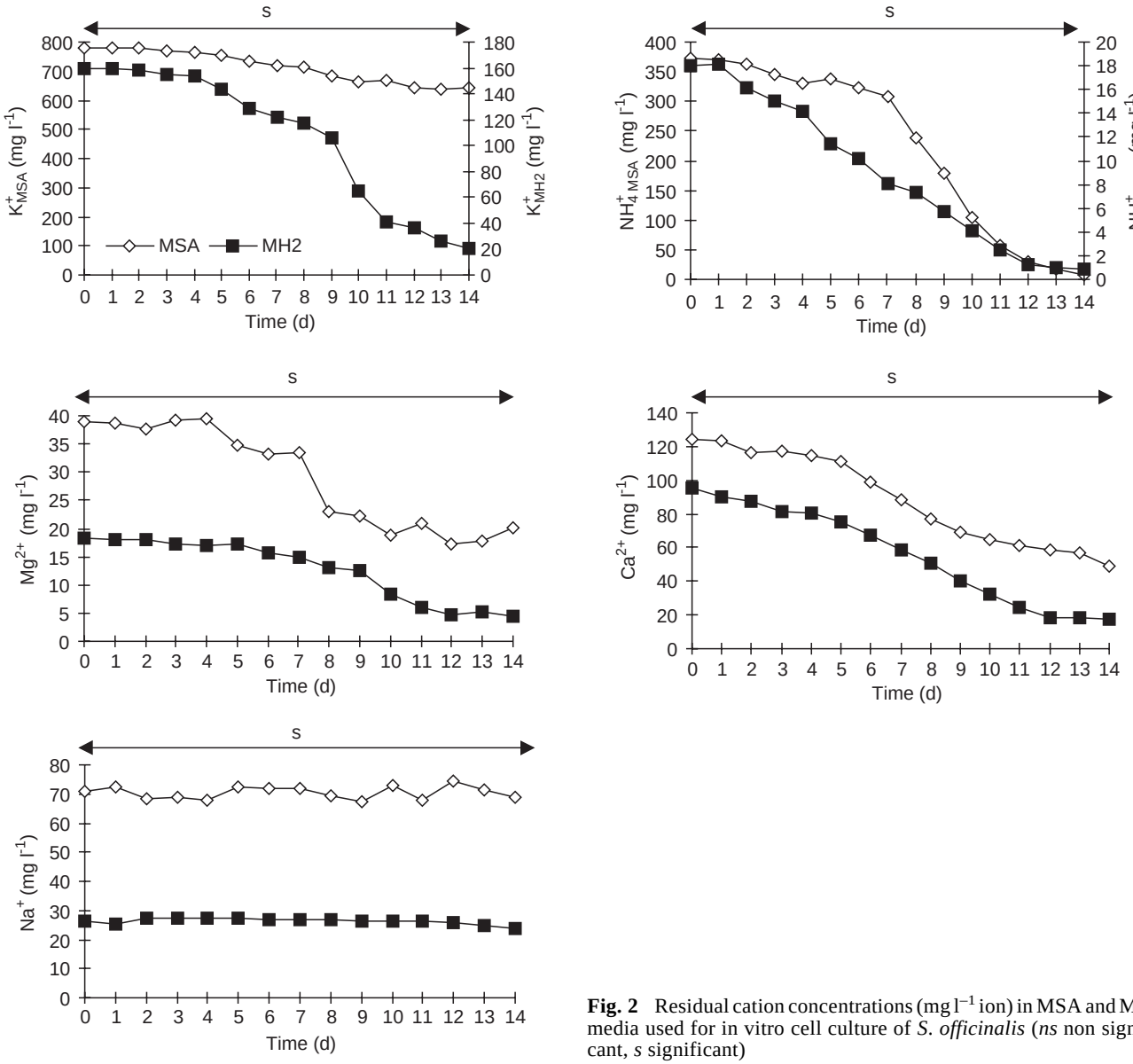

Fig. 2 Residual cation concentrations (mg l^{-1} ion) in MSA and MH2 media used for in vitro cell culture of *S. officinalis* (*ns* non significant, *s* significant)

growth. The adequate concentration of Cu in the MH2 medium (Table 1) also corresponds to an accumulation of saponins in *S. officinalis* cells which is twice as high (Fulcheri et al. 1998). The stimulating effect of copper has already been observed for the production of shikonin derivatives by *Lithospermum erythrorhizon* cells (Fujita et al. 1981b), of berberine by *Coptis japonica* cells (Morimoto et al. 1988) and of saponins by *Panax notoginseng* cells (Zhong and Wang 1996).

(2) A second explanation may be the rapid depletion of P in the MSA medium (Fig. 1). A similar observation has already been made for cultures of *Anchusa officinalis* cells (De Eknamkul and Ellis 1984), *Ginkgo biloba* cells (Carrier et al. 1990) and *P. notoginseng* cells (Zhong and Zhu 1995). However, the quantity of P absorbed by the cells at the end of culture on the MSA medium is lower than the quantity consumed from this medium (Table 3): this discrepancy can be explained if some of the P is deposited in the cells as an insoluble form which is not detected by analysis after mineralization.

(3) Another interpretation may be related to the presence of an initially high concentration of NH_4^+ (Table 1). In fact, this cation has an antagonistic effect on the absorption of other essential cations (Morard 1995): the depletion kinetics show that K^+ is consumed more slowly in relation to the other cations of the MSA medium and to its rate of absorption in MH2 medium (Fig. 2), where the initial concentration of NH_4^+ is 20-fold lower.

(4) A final explanation is provided by the high initial concentration of K^+ in the MSA medium (Table 1) which would saturate membrane carriers, also resulting in its slow absorption (Fig. 2) (Leifert et al. 1995).

The time course of the pH of the media (Fig. 4) shows a stronger alkalinization of the MH2 medium compared to

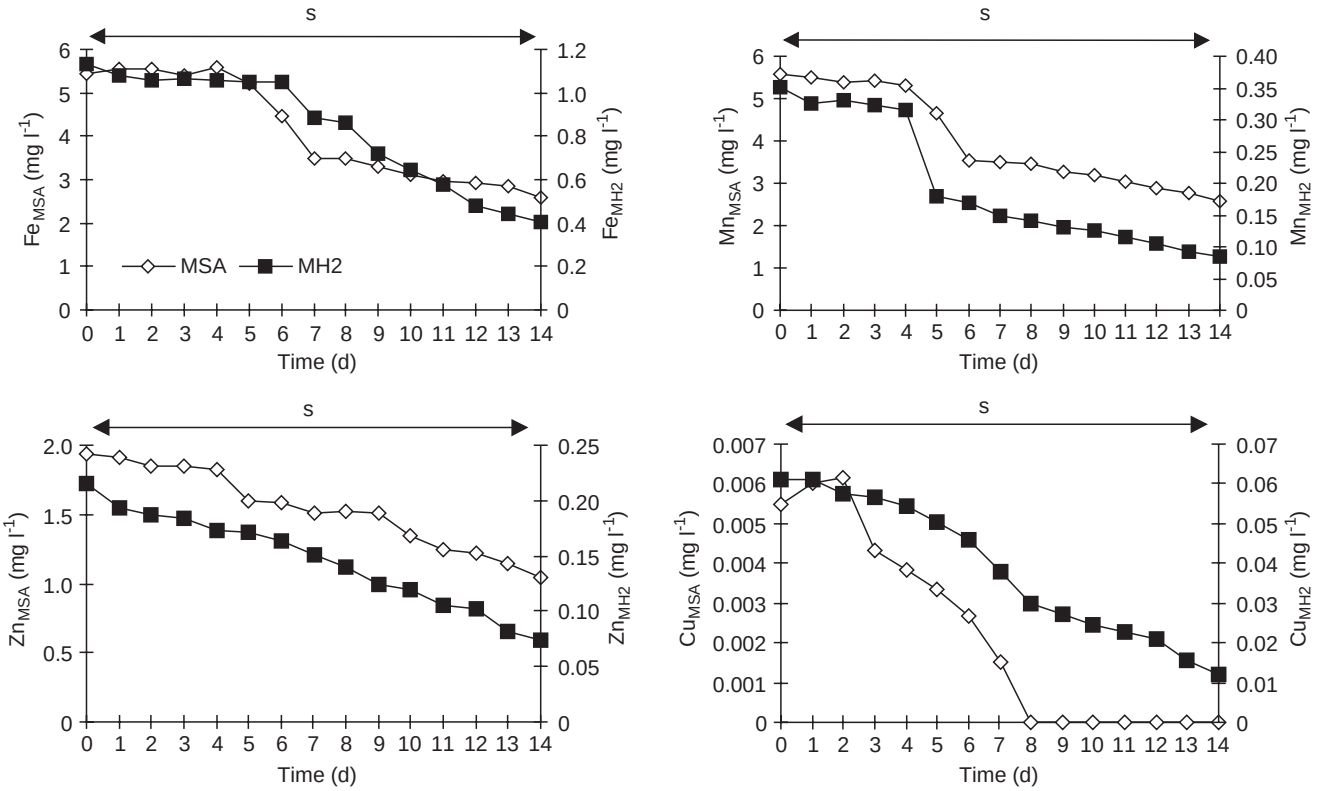

Fig. 3 Residual micronutrient concentrations (mg l^{-1} ion) in MSA and MH2 media used for in vitro cell culture of *S. officinalis* (*ns* non significant, *s* significant)

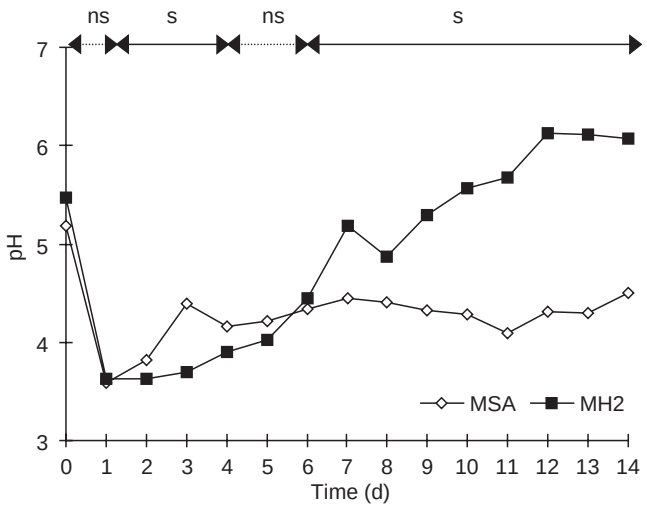

Fig. 4 Time course of pH of MSA and MH2 media during the growth of *S. officinalis* cells (*ns* non significant, *s* significant)

the MSA medium. In terms of the physiology of mineral nutrition of whole plants in soilless culture, it is generally accepted that the alkalinization of a nutritive solution is due to the active absorption of nitrate coupled with a passive efflux of OH^- ions produced during the reduction of nitrate (Morard 1995). It is probable that the nitrogen absorption of *S. officinalis* cells cultivated in vitro causes a similar phenomenon. The low initial concentration of NH_4^+ in the MH2 medium and the high NO_3^-/NH_4^+ ratio (9/1) (Table 1) explain the more marked alkalinization. The high concentration of NH_4^+ in the MSA medium and the low NO_3^-/NH_4^+ ratio (2/1) (Table 1) correspond to slow alkalinization due to the efflux of protons during the absorption of NH_4^+. The clear relationship between the nitrogenous form absorbed and the change in the pH of the medium could indicate the depletion of ammonium in the medium when the two nitrogen forms are present. For example, in a bioreactor, the acidification of the culture medium of transformed roots of *Datura* species (Hilton and Wilson 1995) would indicate a depletion of ammonium from the medium.

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