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Original article

Effects of gelling agents on growth, mineral composition and naphthoquinone content of *in vitro* explants of hybrid walnut tree (*Juglans regia* x *Juglans nigra*)

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Summary — Gelling agents affect growth of walnut *in vitro* cultured shoots. Gelrite promoted shoot elongation and bud production, whereas agar inhibited growth, induced mature leaf formation and necroses. The 2 gelling agents differed significantly in mineral content. They altered the chemical composition of the medium as well as that of the explants. A pronounced accumulation of Na and several microelements was observed in leaves after 16 d of culture on agar, probably due to a disturbance in the K selectivity mechanism and membrane permeability. Moreover, on agar, the level of hydrojuglone glucoside, a marker of juvenility in walnut, decreased drastically in the callus. Mineral element accumulation and decrease of hydrojuglone glucoside were evident after growth inhibition, indicating that they are a result rather than a cause of this inhibition. Lack of growth, mature foliar morphology, Na and microelement accumulation and hydrojuglone glucoside decline support the hypothesis that agar accelerates the ageing of *in vitro* propagated walnut trees.

Juglans / micropropagation / gelling agent / mineral composition

Résumé — Effets des agents de solidification du milieu de culture sur la croissance, la composition minérale et la teneur en hydrojuglone glucoside des explants de noyer hybride cultivés *in vitro*. *Les agents de solidification influent sur la croissance des pousses du noyer cultivées* in vitro *(fig 1). La gelrite a un effet bénéfique sur l'élongation des explants et la production de bourgeons, tandis que l'agar inhibe la croissance et provoque la maturation des feuilles ou encore des nécroses (tableau I). Les 2 agents de solidification présentent des différences importantes dans leurs teneurs en éléments minéraux (tableau II). Ils altèrent la composition minérale du milieu de culture, comme celle des explants (tableau III). Une accumulation importante de Na et de divers microéléments a été observée dans les feuilles après 16 h de culture sur l'agar (fig 2), probablement due aux perturbations du mécanisme de sélectivité de K et de la perméabilité membranaire. De plus sur*

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agar, la teneur du cal en hydrojuglone glucoside diminue (fig 5), alors qu'une teneur élevée de ce composé caractérise l'état juvénile chez le noyer. L'accumulation des éléments minéraux (figs 3 et 4) et la diminution de la teneur en hydrojuglone glucoside, interviennent après l'inhibition de la croissance, indiquant ainsi qu'il s'agit plutôt d'une conséquence que de la cause de cette inhibition. L'absence de croissance, la formation des feuilles matures, l'accumulation de Na et des microéléments supportent l'hypothèse que l'agar accélère le vieillissement des explants de noyer.

Juglans / micropropagation / gélifiant / composition minérale / polyphénol

INTRODUCTION

Although techniques for micropropagation of walnut species have been reported, mass propagation for fruit production and reforestation remains limited due to problems such as high transfer frequency, latent contamination, low multiplication and rooting rates (Driver and Kuniyuki, 1984; McGranahan *et al,* 1988; Cornu and Jay-Allemand, 1989; Revilla *et al,* 1989).

As a result of their physical and chemical properties, gelling agents influence growth (Lee *et al,* 1986; Cornu and Jay-Allemand, 1989) and organogenesis (Titel *et al,* 1987; Koda *et al,* 1988). It has been shown that agar gels and their aqueous extracts contain several cations (Kordan, 1988) which are available to plant tissues (Kordan, 1980, 1981). Organic impurities, absorbing in the same UV wavelength as phenols are also present (Scherer *et al,* 1988).

Walnut tissues contain a great amount of polyphenols. A major polyphenol of walnut, identified as the hydrojuglone glucoside, is found in significant quantities at the onset of growth in juvenile shoots and is therefore considered to be a biochemical marker of walnut juvenility and rejuvenation (Jay-Allemand *et al,* 1990). The level of this compound is also found to decrease with foliar ageing (Cline and Neely, 1984). Moreover, accumulation of phenolic compounds was associated with deficient mineral nutrition and stress (Di-Cosmo, 1984; Gershenzon, 1984) and could be used as an indicator of *in vitro* culture dysfunctioning.

The aim of this study was to compare the effects of 2 gelling agents, Difco Bacto Agar® (Difco) and Gelrite® (Kelco) on the growth of *in vitro* walnut explants, their mineral content and the typical naphthoquinone content associated with the abovementioned factors.

MATERIALS AND METHODS

Tissue culture and growth conditions

Walnut explants were obtained by micropropagation of an embryonic axis of hybrid walnut (*Juglans regia* x *Juglans nigra*) according to the technique described by Jay-Allemand and Cornu (1986). Shoots obtained from elongated buds of nodal segments were subcultured in 750-ml jars containing 125 ml of media solidified by Gelrite. The medium was the same as the DKW medium (Driver and Kuniyuki, 1984) except for the microelements which were (in μM): H_3BO_3, 200; $MnSO_4 \cdot H_2O$, 200; $ZnSO_4 \cdot 7H_2O$, 74; KI, 10; $Na_2MoO_4 \cdot 2H_2O$, 2, $CuSO_4 \cdot 5H_2O$, 2; $CoCl_2 \cdot 6H_2O$, 2.

The pH was adjusted to 6 prior to autoclaving. Each vessel contained 6 explants. Cultures were maintained in a growth chamber under a 16-h photoperiod with day and night temperatures of 25 ± 1 °C and 22 ± 1 °C respectively under cool-white fluorescent lamps at 75 μE m^{-2} s^{-1}. After excision of the callus, 8-month-old explants, subcultured on Gelrite every 14 d, were transferred to a medium which was solidified either by 0.6% (w:v) Difco Bacto agar (Difco) or by 0.2% (w:v) Gelrite (Kelco).

Determination of mineral content

A digestion method was used for inorganic cation analysis. Approximately 50 mg of dried leaves (at 80 °C, over 48 h) were mineralized by the consecutive addition of 1 ml concentrated nitric acid and 1 ml concentrated hyperchloric acid. Organic matter was totally digested by heating. The solution was evaporated to dryness and the ash was taken up in 10 ml hydrochloric acid (0.1 N). Analysis of Ca, K, Na, P, Fe, Mg, Mn, Zn, Cu, and B was performed by coupled plasma emission spectrometry. The same method was used on the gelling agents for the determination of their mineral content.

Analysis of polyphenols

Phenolic compounds were extracted and purified according to the method adapted to walnut by Jay-Allemand *et al* (1988). Twenty mg freeze-dried material of leaf, stem or callus were extracted with acetone/water (80/20, v/v) at 4 °C by sonication for 30 min. Supernatants were collected after centrifugation and solvents were evaporated *in vacuo* (Speed-vac). The phenolic compounds were separated by high performance liquid chromatography using a C18 reversed phase column: lichrospher 5 µm 100 CH-18/11 (Merck), 250 x 4.6 mm; solvent A was aqueous acetic acid (1%, v/v) and B methanol/acetronitrile (50/50, v/v); the elution gradient was 15–40% B in A for 20 min, 40–60% B in A for the next 5 min, then 60–100% B in A for 3 min and 100% B isocratically for 5 min; the flow rate was 1 ml min^{-1}. Peaks were recorded at 250 nm. The naphthoquinones hydrojuglone glucoside and juglone were characterized by their spectrum. Results were expressed in µmol g^{-1} DW of 6-methoxyflavone (internal standard). Quantitative variations due to the extraction and analysis method were determined from 6 replicates (extracts) of the same dry matter. The coefficient of variation did not exceed 6%.

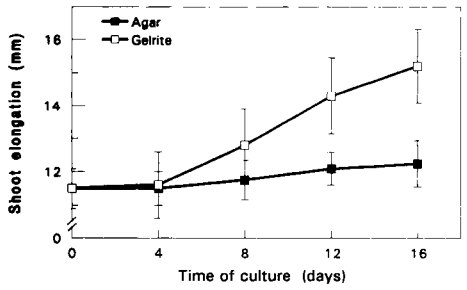

Fig 1. Effect of gelling agents on shoot elongation of walnut explants during 16 d of culture on agar (■) or on Gelrite (❑) solidified media. Each point represents the mean of 24 replicates. Vertical bars indicate SD.

RESULTS

Growth

Gelrite promoted shoot elongation whereas agar strongly inhibited elongation of explants (fig 1). Lack of elongation was apparent on agar, while explants cultured on Gelrite continue to grow until the end of the experiment (d 16). Morphological changes were also observed. Agar led to fully expanded leaves but the formation of new leaves was limited. On Gelrite solidified medium, the leaves were smaller, bright green in color and new leaves were regularly formed. After 2 subcultures (32 d), shoots cultured on Gelrite produced more buds than those on agar and the fresh weight of callus formed at the end of the stem was greater (table I). Leaf discolora-

Table I. Effects of gelling agents on bud production and callus weight of walnut explants after 2 subcultures (32 d).

	Buds (No per explant)	*Callus weight (mg FW)*
Agar	2^b	55^b
Gelrite	5.1^a	637^a

Results are average values of 48 replicates. Different letters correspond to values significantly different at the $\alpha = 0.05$ level.

tion, leaf abscission and episodic explant necroses resulted from repetitive use of agar.

Mineral content of gelling agents

The 2 gelling agents presented major differences in mineral content (table II). Gelrite contained a higher amount of Ca and Mg and K (4-fold) and Fe than agar. Agar contained 2-fold more Na than Gelrite. Since all the mineral elements are practically available in the capillaries of the gels (Debergh, 1983), it is expected that the 2 gelling agents alter the composition of the media in proportion to the quantities (0.6% agar and 0.2% Gelrite, w:v) required for medium solidification (table II). Thus, agar adds a greater amount of Na, P, Mn, Cu and B than Gelrite does. The latter adds a greater amount of Fe and K. The Na/K ratio is strongly modified: Na concentration was 3-fold higher in the medium solidified by agar than in the medium solidified by gelrite (table II) and K/Na ratio was 3-fold lower with agar than Gelrite (table III).

Mineral content of leaves

The mineral content of the leaves varied according to the gelling agent and the period of time in culture. A significant accumulation of Na was found in the leaves of the explants cultured on agar. After 16 d of culture the concentration of Na in leaves was 3-fold higher than in those explants cultured on Gelrite (fig 2). Explants cultured on Gelrite had a higher final (16th d) concentration of K and Mg, but only the increase in Mg was significant (fig 3). Differences in K and Na concentrations in the leaves led to a lower K/Na ratio in the leaves on agar than on Gelrite (table III). However, the amount of K+Na remained

Table II. Mineral content of gelling agents, expected amount of cations to be released into the medium by 0.6% agar or 0.2% Gelrite, and consequent medium composition.

	Gelling agent mineral content		*Amount of cations added to the medium by gelling agents*		*Medium composition as affected by gelling agents*	
	Agar	*Gelrite*	*Agar*	*Gelrite*	*Agar*	*Gelrite*
	(mmol g^{-1})		*(mmol l^{-1})*		*(mmol^{-1})*	
Macroelement						
Ca	0.58	1.6	0.35	0.32	9.4	9.4
P	0.78	0.94	0.47	0.19	2.36	2.08
K	1.02	4.56	0.61	0.92	20.4	20.7
Na	4.36	2.3	2.61	0.46	2.94	0.7
Mg	0.39	0.77	0.23	0.16	3.23	3.16
Microelement						
Fe	1.49	5.93	8.91	11.8	129	132
Mn	1.20	1.44	7.22	2.87	207	202
Cu	1.25	0.93	7.51	1.86	9.51	3.86
B	3.31	3.70	19.7	7.4	220	208

Table III. K and Na concentrations in media and leaves of walnut explants as affected by agar or Gelrite after 16 d of culture.

	Medium		*Leaves*	
	Agar	*Gelrite*	*Agar*	*Gelrite*
	(mmol l^{-1})		*(mmol g^{-1} DW)*	
K	20.41	20.72	0.84	0.99
Na	2.85	0.70	0.15	0.04
K/Na	7.16	29.60	5.54	22.76
K + Na	23.26	21.42	0.99	1.03

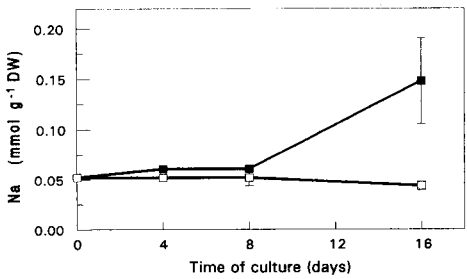

Fig 2. Changes of Na content in the leaves of walnut explants cultured on agar (■) or on Gelrite (❑) solidified media, during 16 d of culture. Each point represents the mean of 5 determinations. Vertical bars indicate SD.

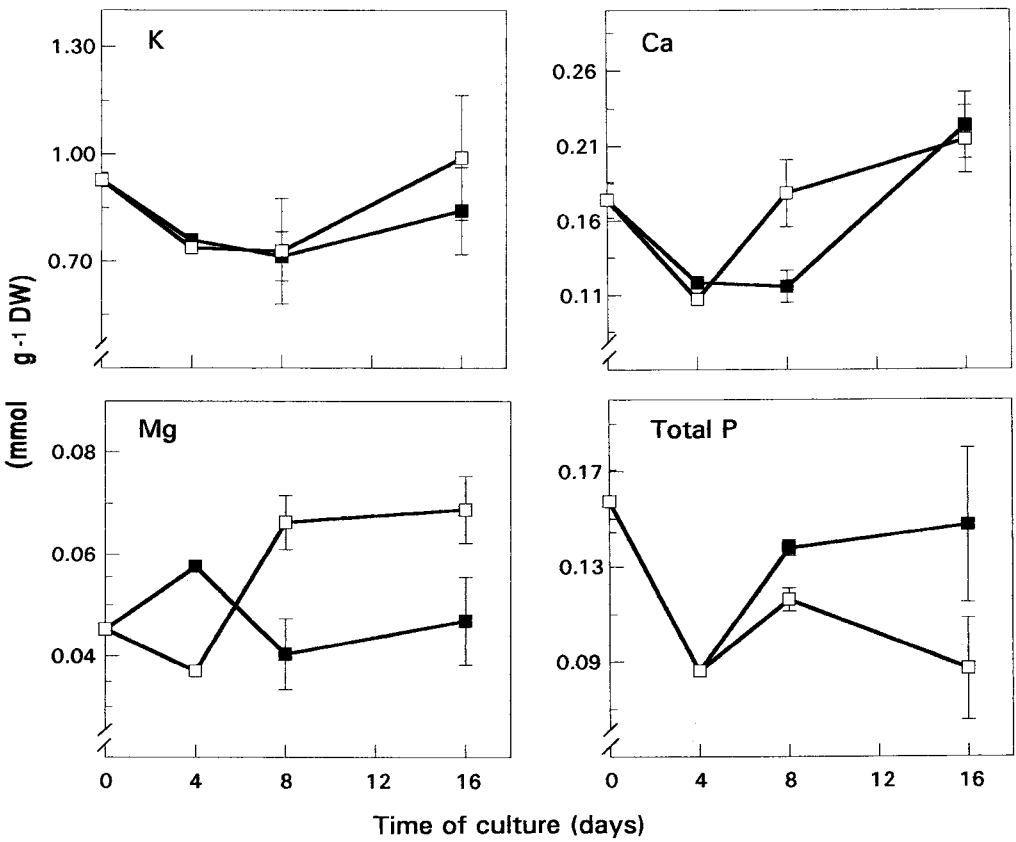

Fig 3. Changes of K, Ca, Mg and total P concentrations in the leaves of walnut explants on agar (■) or on Gelrite (❑) solidified media during 16 d of culture. Each point represents the mean of 5 determinations. Vertical bars indicate SD.

comparable in both gelling agents. Moreover, the K/Na ratio in the leaves was similar to that of the solidified media. Total P was the only macroelement with a foliar concentration which was significantly higher on agar than on Gelrite at the end of the subculture (fig 3).

Only minor differences were found in microelement concentrations in leaves until the 8th d of culture. However, at the end of 16 d of culture a much higher concentration of microelements Mn, Cu, Fe, B, Al and Zn was observed in leaves of explants growing on agar (fig 4).

Naphthoquinone content

Regarding the content of hydrojuglone glucoside and juglone in leaves, stem and callus, significant differences were found in leaves, stem or callus in shoots depending on the gelling agent. During the first 8 d of culture, hydrojuglone glucoside content in leaves of Gelrite-cultured explants was higher than that of agar (cultured explants) (fig 5a). In the callus of explants cultured in agar, the amount of this compound decreased to the lowest level determined (fig 5c). On the same gel, the amount of this polyphenol increased only in the explant stem (fig 5b). Juglone showed similar fluctuations in leaves and callus (figs 5d, f) on both gelling agents, while a significant accumulation of this compound was observed in the stem of the explants cultured in agar (fig 5e).

DISCUSSION

Considerable differences were observed in growth, mineral and phenolic content of explants growing on the same medium but solidified by 2 different gelling agents.

Na is considered an unnecessary element for glycophytes and even a toxic factor for fruit trees (Martin-Prevel *et al,* 1984). Natrophobic plants have effective mechanisms for blocking sodium transport to the upper parts of plants, in order to avoid its detrimental effect on the fine structure of chlorophyll. However, replacement of K by Na may occur in senescing leaves even in natrophobic plants (Marschner, 1986). Such a replacement probably occurred in walnut explants grown in agar since the K/Na ratio changed while the K + Na amount remained constant (table III). This suggests a substitution of K by Na, probably due to a disturbance in the K selectivity mechanisms. Van Steveninck (1978) showed that exogenously applied abscissic acid could induce Na selectivity even in K selective genotypes of beetroot slices. This stress-related regulator of growth has also been mentioned as a stimulator of membrane permeability (Penon, 1982; Marschner, 1986), and is involved in the senescing process. Altered membrane permeability could also explain the excessive accumulation of the microelements in the leaves of explants cultured in agar, which was observed after 16 d of culture.

It is unclear whether growth of walnut explants, which are Na-sensitive (Heller, 1981), was restricted by the accumulated amount of Na. It seems that accumulation of Na or microelements is not the primary cause of this inhibition because significant accumulation of these elements does not occur before growth inhibition takes place.

It has been shown that reduced growth is accompanied by decreasing amounts of the hydrojuglone glucoside in walnut annual shoots during senescence (Jay-Allemand *et al,* 1989). Growth decline accompanied by decrease in hydrojuglone glucoside content of callus was also observed on *in vitro* cultured explants as a result of agar used. The same compound was found to decrease drastically in callus of explants grown in Gelrite after 28 d of culture (data not shown). However, the

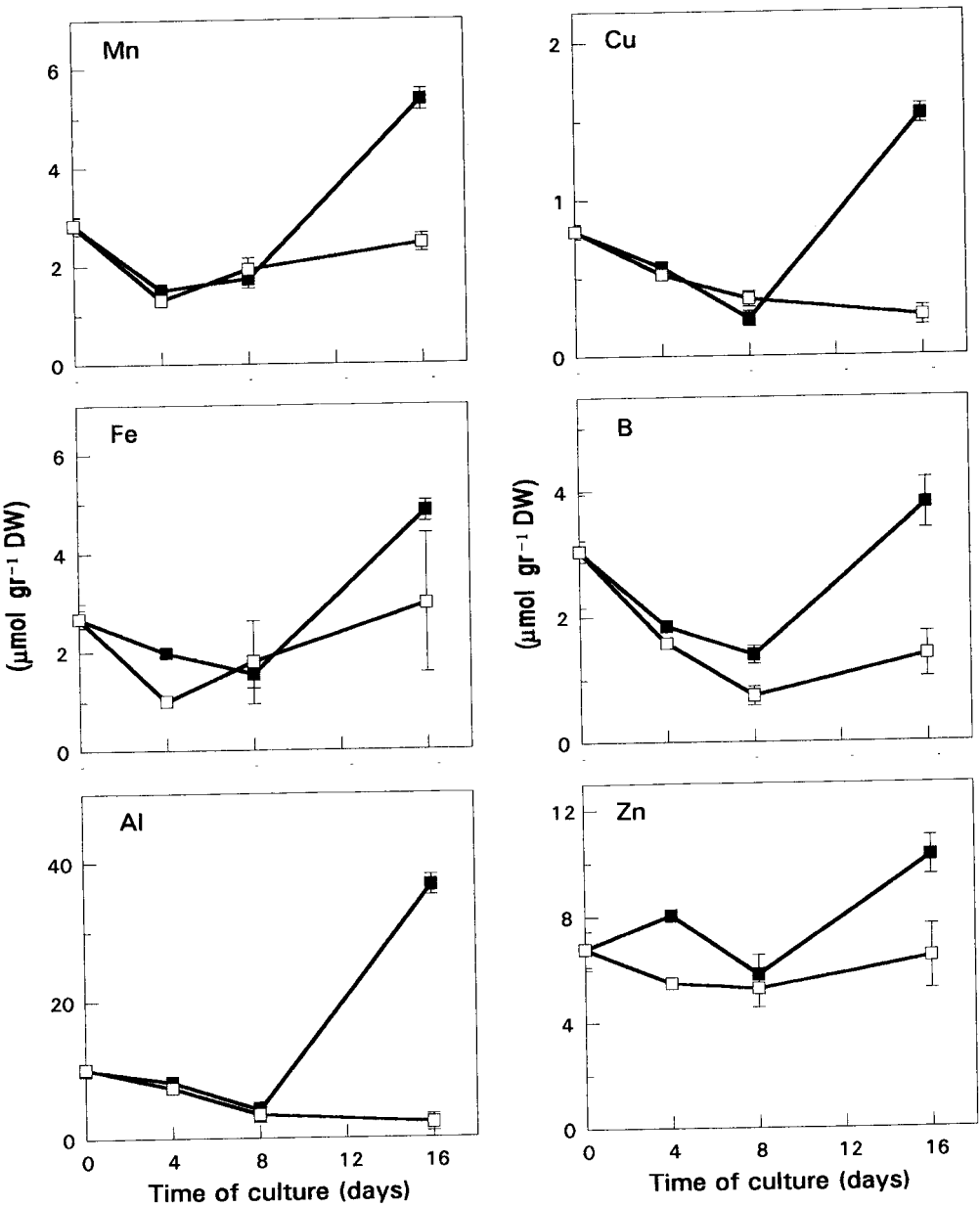

Fig 4. Changes of the Mn, Cu, Fe, B, Al and Zn concentrations in the leaves of walnut explants cultured on agar (■) or on Gelrite (❑) solidified media, during 16 d of culture. Each point represents the mean of 5 determinations.

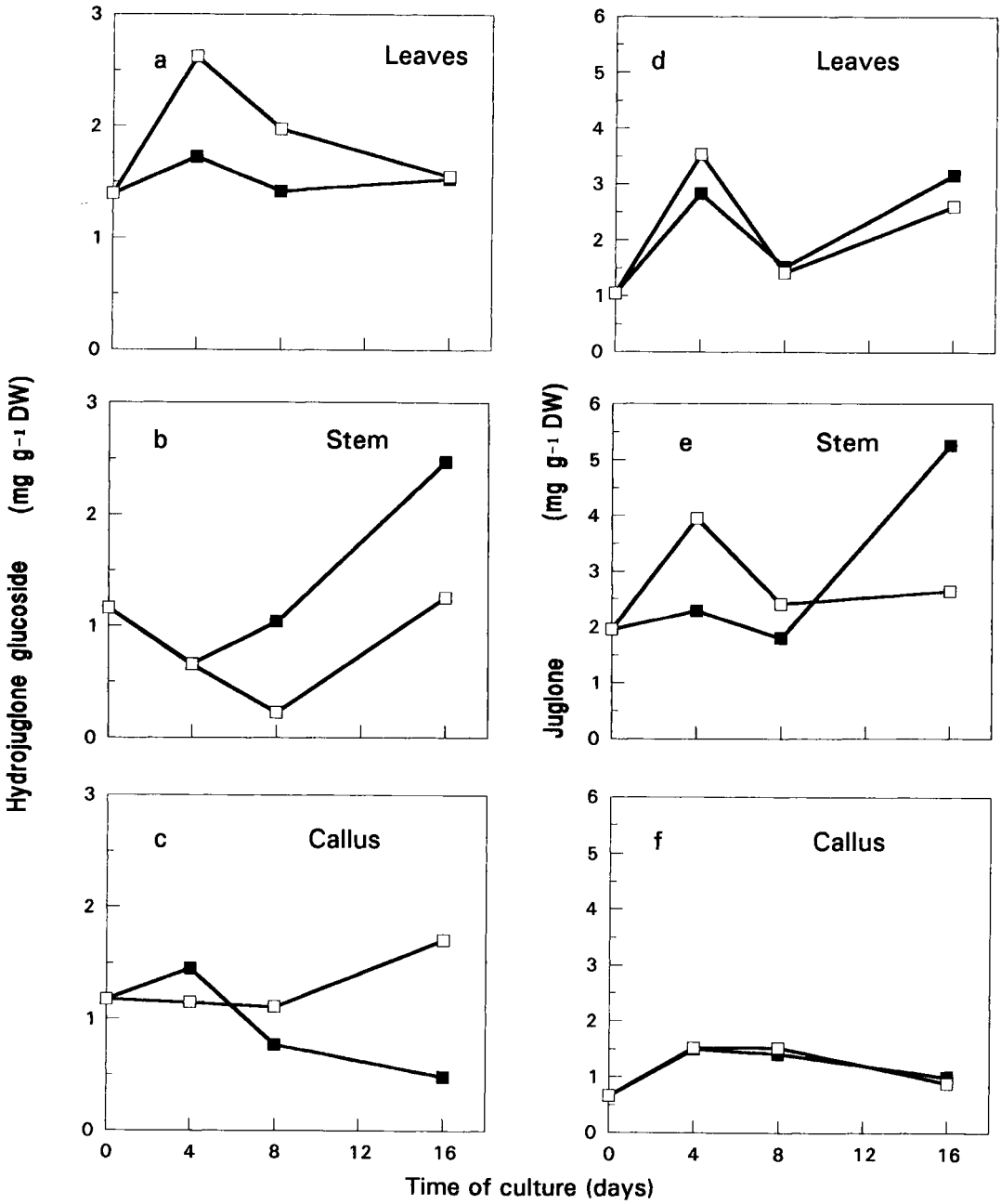

Fig 5. Changes of the hydrojuglone glucoside (a, b, c) and juglone (d, e, f) in leaves, stems and callus of walnut explants on agar (■) or on Gelrite (❑) solidified media during 16 d of *in vitro* culture. Each point represents an analysis of 6 explants.

reasons and the mechanisms of hydrojuglone glucoside decline are still unknown. It is possible that juglone was released from hydrojuglone glucoside after chemical or enzymatic hydrolysis. Juglone is an aglycone (oxidized form) which has been correlated with growth inhibition in other species (Ficher, 1978).

Most of the biochemical differences observed in tissues growing on the 2 gelling agents were evident after 8 d of culture, suggesting that they were a result rather than a cause of growth inhibition. Scherer *et al* (1988) pointed out that no significant differences exist between agar and Gelrite in water potential, osmolality and water activity, whereas significant differences are found in diffusion behavior of cationic dyes. So it is possible that growth inhibition could be related to events at the interface of the solidified medium and the basal part of the explants. These events could be a differential diffusion behavior of excreted substances in the 2 gelling agents, a retention of growth substances by the agar, or even a breakdown of callus cells related to one or both of the above events.

Inhibition of growth, formation of mature leaves, K substitution by Na, excessive accumulation of microelements and hydrojuglone glucoside decline combine to support the hypothesis that agar accelerates the ageing of *in vitro* propagated walnut explants.

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